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ELICITOR-INDUCED RESISTANCE IN TOMATO PLANTS AGAINST FUNGAL PATHOGENS: ULTRASTRUCTURE AND CYTOCHEMISTRY OF THE INDUCED RESPONSE

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Abstract

The introduction of gold cytochemistry in plant pathology has opened new avenues for accurately localizing molecules thought to play important biological functions in the plant metabolism. In an effort to understand the process associated with the induction of plant disease resistance, we investigated the effect of fungal elicitors on the cellular response of tomato root tissues during attack by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causal agent of tomato crown and root rot. Chitosan was found to induce marked morphological and ultrastructural changes in the fungus grown in pure culture, including cytoplasm aggregation and abnormal deposition of an amorphous chitin-rich material. Examination of colonized root tissues at sites of fungal penetration revealed that a pre-treatment with chitosan (as well as with other elicitors) was associated with the expression of typical defense reactions. In the outer cortex, invading fungal cells suffered from serious damage and were frequently encircled by an electron-dense material. In the non-colonized inner cortex, strong host reactions were detected that were mainly associated with the deposition of two types of material that differed by their electron-density. Gold cytochemistry with purified β-1,3-glucanase and laccase showed that the more electron-dense material was of phenolic nature whereas the other material, occurring as deposits inserted between the phenolic aggregates, was made of callose. Application of various gold-complexed probes revealed that callose and phenolic-like compounds (likely lignin) were also the main components of newly-formed wall appositions. Results of these studies provide evidence that cytochemical approaches have the potential to significantly improve our knowledge of how plants defend themselves and how plant disease resistance can be directly enhanced by elicitors.

Key Words: Chitosan, laminarin, β-1,3-glucans, plant defense reactions, gold cytochemistry, callose, phenolic compounds, structural barriers.

Introduction

In the past decade, increasing knowledge and growing concern about the harmful impact of repeated fungicide applications have sensitized research scientists and industries to search for biorational strategies which, in addition to provide safe and reliable means for improving crop protection, could also move beyond system descriptions and towards mechanistic studies (Chet, 1987). The discovery of more and more biocontrol agents and the increasing knowledge of the mechanisms underlying the expression of plant genes in response to pathogen attack have led to suggest that plant resistance to disease was one of the aspects where the best prospects were set for the generation of commercially available crops in the near future (Chet, 1993).

Since the first conclusive demonstration that tobacco plants reacting hypersensitively to tobacco mosaic virus (TMV) were protected against an array of viral pathogens (Ross, 1961), the process of plant "immunization" or induced resistance to disease has received increasing attention and has been abundantly documented (Kùc, 1982, 1987; Uknes *et al.*, 1992). The rationale for such an interest was that not only these studies could lead to a better understanding of how physiological resistance to disease proceeds in terms of hierarchical expression of defense genes but also they could open new avenues for improving crop protection to microbial infections (Lamb *et al.*, 1992). Today, increasing expectations are emerging in the area of plant disease management for new strategies that have the potential to be efficient, reliable, and safe for the environment. Among the avenues being explored, induced resistance, triggered by either avirulent microorganisms (Kùc, 1987) or by biotic and abiotic agents (Ward *et al.*, 1991), is probably one of the biocontrol approach that offers the best prospects for protecting plants against microbial diseases and reducing environmental pollution.

In the last ten years, major advances have been made in understanding the sequential events taking place in the regulation and expression of plant disease resistance. Progress in characterizing the mechanisms of cell-

to-cell signalling and identifying the cascade of biochemical events leading to resistance establishment has led to the consideration that manipulating plant defense genes could provide a conceptual basis to engineer enhanced protection against microbial attack (Collinge and Slusarenko, 1987). However, recent studies have convincingly shown that, in many cases, levels of effective protection could not be reached by overexpressing a single gene (Lamb *et al.*, 1992), thus, confirming the idea that the nature and spatio-temporal coordination of the events involved in the resistance process are crucial in defining the outcome of the interaction. Alternatively, the possibility of stimulating defense gene transcription following perception of an external signal, or in other words, the possibility of sensitizing a plant to respond more rapidly to pathogen attack by prior application of appropriate inducing agents has become an exciting and promising means for enhancing natural disease resistance mechanisms in a coordinated manner (Roby *et al.*, 1988; Chen and Klessig, 1991; Metraux *et al.*, 1991). Compelling evidence indicates that enhanced tolerance to a wide variety of pathogens can be conferred to plants following treatment with either biologically-active oligoglucosides, salicylic acid or with certain chemicals such as β-aminobutyric acid (Cohen *et al.*, 1994).

In spite of the increasing attention focused on how resistance mechanisms are regulated and coordinated in plants challenged by pathogens, the spatio-temporal localization of induced defense molecules has not been investigated in depth. However, it has become more and more apparent that measuring molecular responses at the cellular level was an essential complement to biochemical and molecular analyses (Graham and Graham, 1991). The visualization of induced defense reactions is, indeed, of particular interest when studying the mode of action of specific molecules in relation to pathogen restriction *in planta* (Benhamou, 1993; Benhamou and Asselin, 1993). Innovative developments in plant cyto- and immunocytochemistry appear with increasing frequency and it is expected that improvements in both tissue processing and probe specificity will extend the applicability of this approach to more and more research areas in plant disease resistance.

The past few years have witnessed the implementation of new methods for *in situ* localization of plant molecules and several reviews have been published that address the wide range of applications of immunocytochemistry in plant biology (Herman, 1988; Vandenbosh, 1991; Benhamou and Asselin, 1993). In this paper, we will review some of the recent findings associated with the spatio-temporal localization of molecules involved in plant induced resistance with special emphasis on how an elicitation stimulus may confer increased plant resistance to pathogenic attack. To put this information in context, we will concentrate on a host-pathogen system that has been the focus of much interest as a model in both basic and applied research. For more than two decades, the tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) interaction has received increasing attention mainly because losses from the disease could be considerable in some greenhouse districts and also because chemical control proved unsuccessful for controlling the pathogen population (Jarvis, 1988). Therefore, attention has been recently focused on the development of alternative control strategies that could be efficient, reliable and safe for the environment. In this review, results obtained upon treatment of tomato plants with various biotic elicitors will be described in relation to the cytologically visible consequences of the elicitation stimulus on the infection process *in planta*.

Elicitor-Mediated Induced Resistance

Among the promising approaches for minimizing damage from plant pathogens, an exciting strategy that is receiving increasing attention concerns the potential value of signaling molecules, called "elicitors", in promoting plant disease resistance (Ward *et al.*, 1991; Yoshikawa *et al.*, 1993). The term biotic elicitor usually refers to macromolecules, originating either from the host plant (endogenous elicitors) or from the plant pathogens (exogenous elicitors), which are capable of inducing structural and/or biochemical responses associated with expression of plant disease resistance (Dixon *et al.*, 1994). A wide range of compounds including defined oligosaccharides (Ryan and Farmer, 1991), glycoproteins and peptides (DeWit and Spikman, 1982), as well as smaller molecules such as arachidonic acid (Bloch *et al.*, 1984), jasmonic acid (Creelman *et al.*, 1992), and salicylic acid (Yalpani *et al.*, 1991) have been suggested to play a key role in mediating the induction of plant defense reactions. Among these molecules, oligosaccharides have been the most extensively studied (Ryan and Farmer, 1991). A growing body of evidence from recent studies indicates that some of the characterized oligosaccharides have the potential of becoming a new class of biocontrol agents in agriculture (Hadwiger *et al.*, 1988; El Ghaouth *et al.*, 1994; Lafontaine and Benhamou, 1995) and post-harvest storage (El Ghaouth *et al.*, 1992a, 1992b).

Evidence supporting the role of structurally defined pectic fragments as regulators of plant defense reactions has been provided from several studies dealing with the biochemical analysis of induced molecules upon plant treatment with polygalacturonic acids (Nothnagel *et al.*, 1983; Broekaert and Peumans, 1988). Since the first demonstration that linear oligosaccharides of δ-1,4-linked galactosyluronic acid residues could elicit phytoalexin

synthesis in soybean (Hahn *et al.*, 1981), oligogalacturonides with a degree of polymerization (DP) between 10 and 13 carbons have been shown to be active elicitors of lignification (Robertsen, 1986), accumulation of hydroxyproline-rich glycoproteins (Roby *et al.*, 1985), synthesis of protease inhibitors (Bishop *et al.*, 1984), and production of chitinase (Broekaert and Peumans, 1988).

In recent years, microbial endopolygalacturonases (endo-PGs) have received particular attention for their biological significance in plant pathogenesis. The rationale for such an interest was that such enzymes could display a dual function, being on the one hand involved in the degradation of host cell walls and on the other hand responsible for the release of pectic fragments capable of signalling the plant to defend itself through the transcriptional activation of defense genes (Collmer and Keen, 1986). The finding that short pectic fragments with a DP < 8 were weak elicitors raised the question of the mechanism involved in the regulation of endo-PG activity. The isolation of wall-bound proteins and their identification as polygalacturonase-inhibiting proteins (PGIPs) has led to the concept that such host proteins were of key importance in the regulation of endo-PG activity during host-pathogen interactions (Cervone *et al.*, 1990). However, in spite of the increasing research directed towards understanding how oligogalacturonides, released from the host cell wall through the action of microbial endo-PGs, interact with membrane receptors to influence cell regulation, practical applications for the biological properties of pectic fragments in agriculture are still limited mainly because of the difficulty in obtaining large amounts of these elicitor-active molecules at a low cost.

The potential of microbial oligosaccharides in stimulating a general resistance response in plants has also been the focus of extensive research. The first and probably the best characterized elicitor of phytoalexins isolated from fungal cell walls is a branched hepta-β-glucoside. Sharp *et al.* (1984) demonstrated that the smallest elicitor-active fungal oligosaccharide was a molecule of seven glucosyl residues, being composed of a linear chain of five β-1,6-linked with two branched β-1,3-linked residues in position two and four (Fig. 1). Evidence was provided that similar hepta-β-glucopyranosides with differently positioned β-1,3-linked residues did not exhibit an eliciting activity.

In addition to β-linked glucans, other microbial compounds including chitin and chitosan, mannose-rich glycopeptides, toxins (the so-called elicitins), and lipids have been shown to be signalling molecules potentially involved in the development of plant disease resistance (Yoshikawa *et al.*, 1993). Pierce and Ride (1982) reported that treatment of wheat plants with fungal chitin, a polymer of β-1,4-*N*-acetyl-D-glucosamine, resulted in a rapid induction of cell wall lignification, a structural

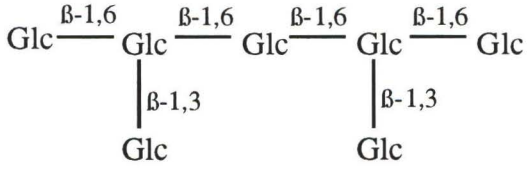


Figure 1. Schematic representation of an elicitor-active hepta-β-glucoside isolated from fungal cell walls.

process considered to be a defense reaction designed to prevent pathogen penetration. In a subsequent report, Barber *et al.* (1989) convincingly showed that only chitin oligomers with a DP between four and six possessed significant lignification-eliciting activity. Similarly, chitosan, the partially deacetylated derivative of chitin, was found to play a major signaling role in plant-fungus interactions (Hadwiger and Beckman, 1980). However, in that case also, it was reported that the degree of polymerization determined the ability of the molecule to elicit plant defense reactions (Kauss *et al.*, 1989). In a recent study, Hadwiger *et al.* (1994) confirmed the key importance of the oligomer size and reported that octameric oligomers of chitosan were the most active elicitors of phytoalexins in pea.

Recent advances in our understanding of the biological significance of elicitors in the induction of plant defense reactions have strengthened the idea that sensitizing a plant to respond more rapidly to infection could confer increased protection against virulent pathogens (Kùc, 1987). However, in spite of the large research effort expended, little has been reported about the cellular biochemistry of host-pathogen interactions following elicitor treatment. Research progress in this area has led to the exploration of useful tools that could be directly applied on intact tissue sections and provide a detailed picture of the cellular and molecular events *in planta* (Graham and Graham, 1991). It has become more and more apparent that measuring molecular responses at the cellular level was an essential complement to biochemical and molecular analyses. The visualization of induced defense reactions is of particular interest when studying their mode of action in relation to pathogen restriction *in planta*. Plant pathogens have evolved special mechanisms to penetrate the barrier imposed by the cuticle and by the cellulose-containing cell walls (Collmer and Keen, 1986). It is, therefore, not surprising that, in turn, plants have developed the potential to prevent effective pathogen penetration by producing an array of substances that contribute to rapid modifications of the cell walls. In an effort to bring new insights into the ultrastructural response of plants to injury, we became interested in investigating the effect of fungal elicitors on

the cellular response of tomato root tissues upon attack by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), the causal agent of tomato crown and root rot (Benhamou and Thériault, 1992; Benhamou *et al.*, 1994). Particular emphasis was given to chitosan not only because this compound exhibited specific biological properties but also because, unlike other elicitors, it could be easily obtained from crustacean shell wastes. Before illustrating some of the results obtained through our ultrastructural and cytochemical investigations of infected plant tissues, the principles of cytochemical labeling are briefly reviewed.

Cytochemical Labeling of Fungi and Plant Tissues: Principles and General Practice

Cytochemical approaches are based on the affinity properties existing between macromolecules. Several compounds, including enzymes, lectins and antibodies, once tagged to an appropriate marker, enable the ultrastructural localization of their target molecules in intracellular compartments. In this paper, it is not our purpose to review the wide range of cytochemical probes that have been developed over the past few years. Instead, our objective is to briefly describe the cytochemical methods that are currently used in plant pathology.

Tissue processing

For lectin- and enzyme-gold cytochemistry, tissue processing is usually performed according to standard procedures. Fixation of fungal or plant fragments is usually performed using low concentrations (1-3%) of either glutaraldehyde or paraformaldehyde. Post-fixation of the tissues with osmium tetroxide (OsO_4) is recommended, since this excellent membrane stabilizer and contrasting agent does not alter accessibility of both the lectins and the enzymes to their corresponding receptors (Benhamou, 1989). Following dehydration in a graded series of ethanol, embedding is usually performed in Epon 812. Several types of resin are available and have been successfully used for cytochemical purposes. Epoxy resins such as Epon, Spurr and Araldites as well as hydrophilic methacrylates offer the advantage to yield good ultrastructural preservation and high beam stability and are widely used for cytochemical studies of plant tissues (Benhamou, 1993).

Section preparation

Grids of gold or other unoxidizable metals such as nickel are used to collect ultrathin tissue sections (70 nm in thickness). Copper grids should be avoided because copper is known to interact with buffer solutions during cytochemical procedures. In order to enhance stability of the sections, previous grid coating with Formvar or Collodion is recommended.

Lectin cytochemistry

Lectins can be defined as a group of carbohydrate-binding proteins (usually glycoproteins) of non-immune origin that occur predominantly in plants (mainly Leguminosae) and invertebrates (Molluscs). Because of their specific binding properties and their ability to recognize subtle differences in complex carbohydrate structures, lectins have provided versatile tools for topochemistry (Benhamou, 1988). In recent years, lectins have found wide application in the study of intracellular carbohydrate-containing molecules (Benhamou, 1989). The list of available lectins has rapidly increased providing a large spectrum of accurate probes for sugar localization. The currently used lectins in plant pathology are:

(1) **Wheat-germ agglutinin (WGA)**, specific for N- acetylglucosamine residues, and used for the localization of fungal chitin in fungus-infected plant tissues (Benhamou, 1989; Hajlaoui *et al.*, 1992).

(2) ***Helix pomatia* agglutinin (HpA)**, specific for N-acetylgalactosamine residues (Benhamou, 1988).

(3) ***Ricinus communis* agglutinin I (RcA)**, specific for galactose residues (Benhamou *et al.*, 1988a).

(4) ***Aplysia* gonad lectin (AGL)**, a lectin isolated from the sea mollusc *Aplysia depilans* and found to specifically bind to polygalacturonic acids (pectic molecules) (Benhamou *et al.*, 1988b).

(5) **Concanavalin A (Con A)**, specific for terminal glucopyranosyl and mannopyranosyl residues (Benhamou, 1988).

Unfortunately, to date no lectins are known for the identification of polysaccharides with linear β-1-4-linkages which are, by far, the most important components in plant cells walls. However, such polysaccharides can be easily visualized with a purified β-1,4-exoglucanase.

Since lectins are not electron-opaque, they cannot be visualized at the electron-microscope level without an electron-dense marker. The introduction of colloidal gold as a particulate marker of high electron-density has contributed to the extensive use of lectin-gold cytochemistry in a variety of biological systems including plant-pathogen interactions (Benhamou *et al.*, 1988a; Benhamou, 1989; Chamberland *et al.*, 1989). Colloidal gold is formed by reducing tetrachloroauric acid with organic agents such as white phosphorus, formaldehyde, tannic acid, ascorbic acid and sodium citrate. However, the most popular approach is the sodium citrate method (Frens, 1973) to produce mono-disperse gold particles ranging from 5-40 nm in diameter.

Lectins with high molecular weight (MW, > 15 KDa) can be directly complexed to colloidal gold and applied to tissue sections in a single-step procedure (Benhamou, 1989). By contrast, lectins with low MW

Table 1. Optimal conditions for preparation of some lectin-gold complexes.

Lectin	Sugar specificity	pH of colloidal gold	Optimal pH for labeling
Wheat germ agglutinin	*N*-Acetyl-D-glucosamine	–	–
Ovomucoid	–	5.3	6.0
Helix pomatia agglutinin	*N*-Acetyl-D-galactosamine	7.4	7.4
Ricinus communis agglutinin	D-galactose	8.0	8.0
Aplysia gonad lectin	Polygalacturonic acids	9.5	8.0
Concanavalin A	D-mannose and D-glucose	8.0	8.0

Table 2. Optimal conditions for preparation of some enzyme-gold complexes

Enzyme	Source	Substrate specificity	pH of colloidal gold	Optimal pH of labeling
β-1,4-exoglucanase	*Trichoderma*	β-1,4-glucans	9.0	6.0
β-glucosidase	Almonds	β-D-glucosides	9.3	6.0
Phospholipase A_2	Bee venom	Phospholipids	4.5	7.0
β-1,3-glucanase	Tobacco	β-1,3-glucans	5.5	6.0
Laccase	*Rigidoporus lignosus*	Phenolics	4.0	6.0

(< 15 KDa) are not easy to conjugate to colloidal gold. In such cases, an indirect (two-step) labeling in which the marker is complexed to a secondary reagent that has affinity for the lectin is used (Benhamou, 1989). Secondary reagents include glycoproteins such as ovomucoid (specific for WGA) or polysaccharides with appropriate sugar-binding sites.

Cytochemical labeling: Both direct and indirect labeling with lectins can be applied to ultrathin tissue sections for localizing specific sugar residues (Benhamou, 1989). As a general rule, all experiments are performed in a moist chamber to avoid desiccation.

For direct labeling, the protocol is:

(1) Sections are floated on a drop of phosphate buffered saline (PBS) containing 0.01 % (weight/volume, w/v) polyethylene glycol (PEG 20000) for 5 minutes. The pH is adjusted according to the pH of optimal activity of the lectin (Table 1).

(2) Sections are transferred onto a drop containing gold-complexed lectin at the appropriate dilution in PBS-PEG for 30-60 minutes.

(3) Sections are thoroughly washed with PBS, rinsed with distilled water, and air-dried.

(4) Sections are contrasted with uranyl acetate and lead citrate.

For indirect labeling, the protocol is:

(1) Sections are pre-incubated on a drop of PBS, pH 7.2.

(2) Sections are transferred onto a drop of the uncomplexed lectin at the appropriate dilution in PBS for 30 minutes.

(3) Sections are washed with PBS, pH 7.2. The excess of buffer is removed with filter paper.

(4) Sections are incubated on the gold-complexed secondary reagent at the appropriate dilution.

(5) Sections are washed with PBS, rinsed with distilled water and stained with uranyl acetate and lead citrate.

Specificity of the labeling obtained with lectin-gold complexes has to be assessed through several control tests. For direct labeling procedures, these controls include: (a) incubation with the lectin-gold complex to which was previously added its corresponding sugar; (b) incubation with the uncomplexed lectin, followed by incubation with the gold-complexed lectin. For indirect labeling procedures, the controls include: (c) incubation with the lectin previously absorbed with its corresponding sugar, followed by incubation with the secondary reagent complexed to gold; and (d) incubation with the gold-complexed secondary reagent alone.

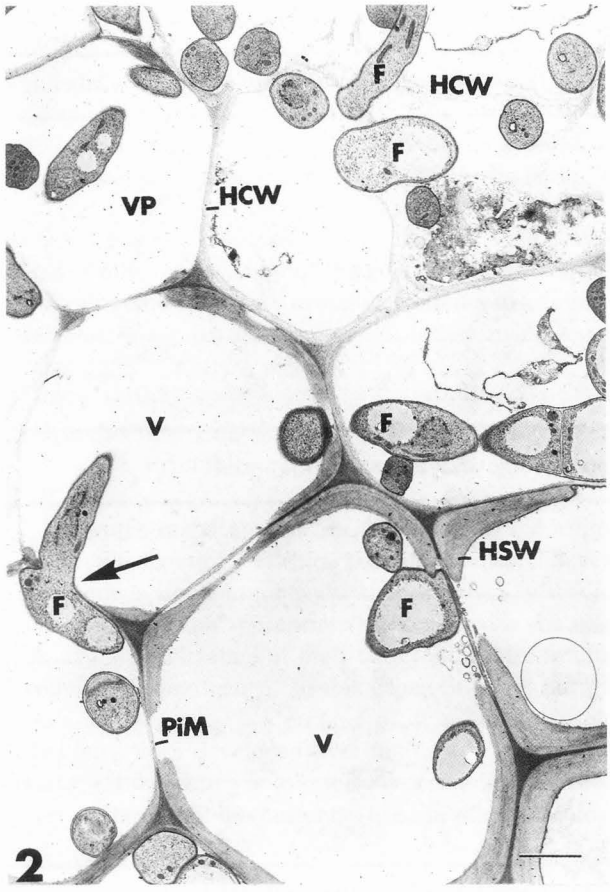


Figure 2. Transmission electron micrograph of tomato root tissue infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and sampled four days after inoculation. Transverse section of an infected root, at a low magnification, showing massive colonization of the vascular parenchyma (VP) and the xylem vessels (V) by several hyphae (F) of the pathogen. Host cell walls (HCW) in the vascular parenchyma show some damage. Colonization of the xylem vessels proceeds via the penetration of the pit membranes (PiM) (arrow). Host secondary walls are not apparently altered. Bar = 2 μm.

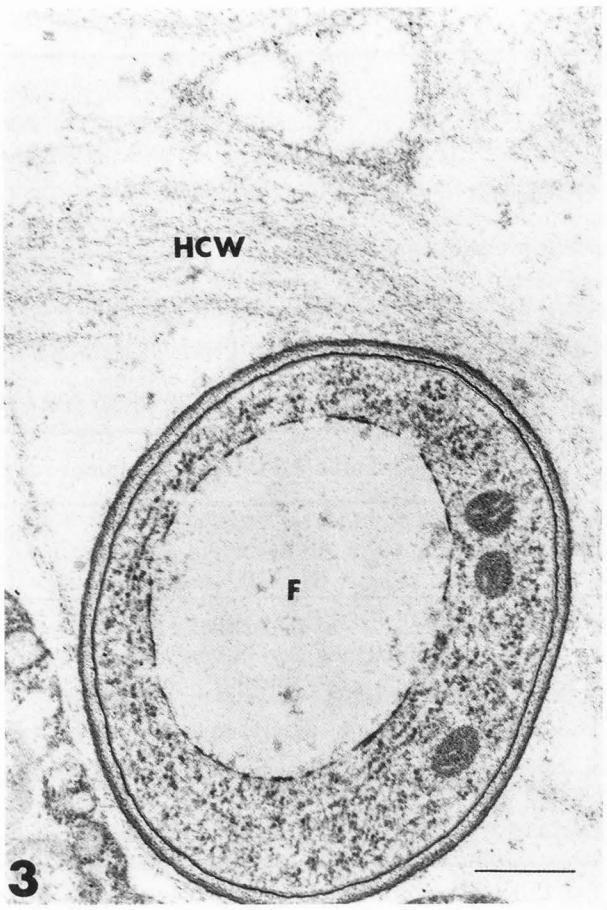


Figure 3. Transmission electron micrograph of tomato root tissues infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* and sampled four days after inoculation. Labeling with the *Aplysia*-gold complex for the localization of pectin. The highly degraded host cell wall (HCW) is nearly unlabeled. Bar = 0.5 μm.

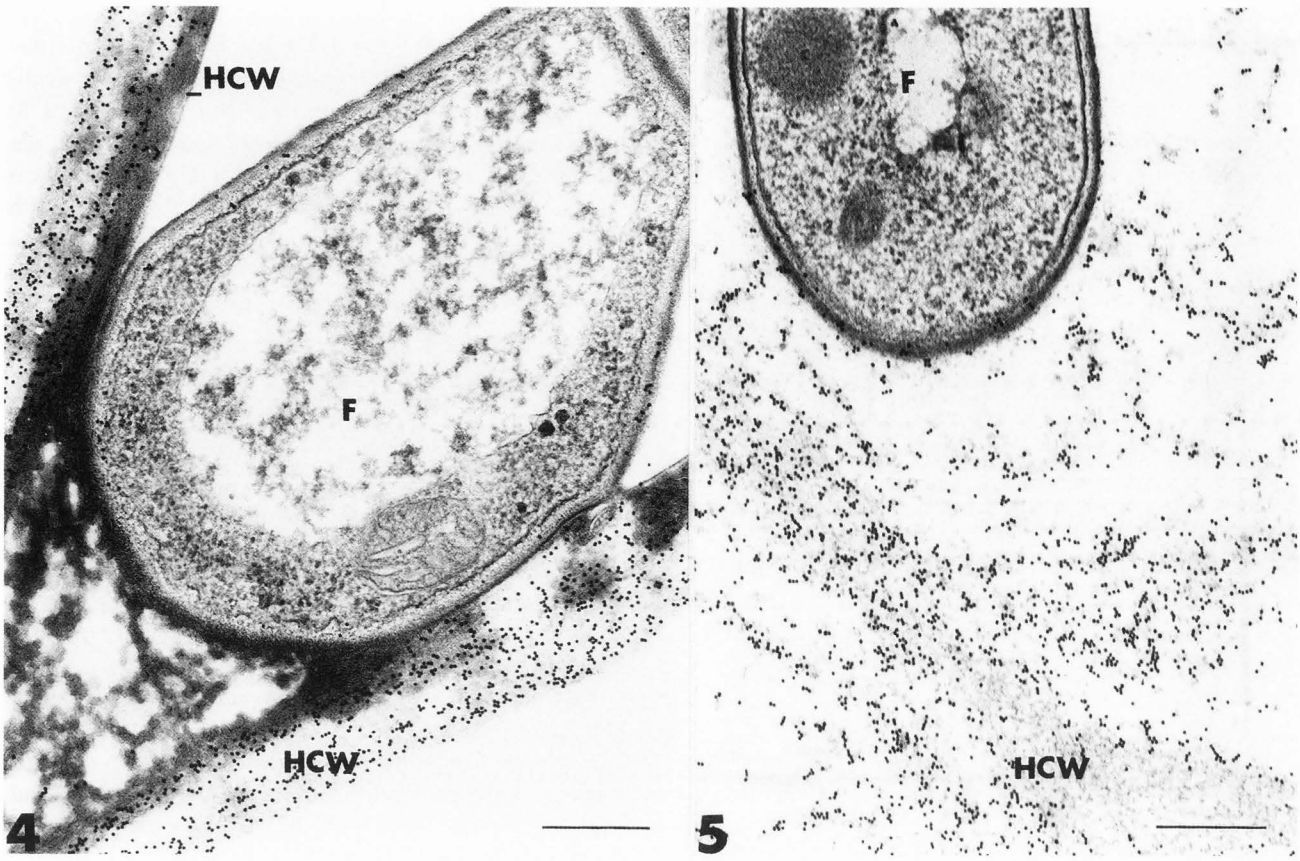
Enzyme cytochemistry

Since the first demonstration that enzymes could be valuable biological probes for revealing nucleic acids (Bendayan, 1981), the enzyme-gold approach has acquired increasing applicability and relevance in both animal and plant biology (Benhamou *et al.*, 1987; Benhamou, 1989). Based on the specific affinity of enzymes for their corresponding substrate molecules, the enzyme-gold method is similar in principle to the lectin-gold approach.

The potential value of the enzyme-gold technique relies in the use of highly purified enzymes. Parameters such as minimal amount of enzyme required for full stabilization of the colloidal gold solution, optimal pH for the absorption of the enzyme to the gold particles and optimal pH of labeling are of crucial importance for both reducing non-specific binding and preserving the biological activity of the enzyme under study (Bendayan, 1984). Nearly all enzymes can be directly complexed to colloidal gold under appropriate conditions (Table 2) and applied to tissue sections for localizing the corresponding substrate molecules. The most commonly used enzymes in plant biology and pathology are:

(1) **Exoglucanase**, a β-1,4-glucan cellobiohydrolase, specific for cellulosic β-1,4-glucans (Benhamou *et al.*, 1987).

(2) **β-glucosidase**, a β-D-glucoside glucanohydrolase, specific for β-D-glucosides (Bendayan and Benhamou, 1987).



Figures 4 and 5. Transmission electron micrographs of tomato root tissues infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* and sampled four days after inoculation. Labeling with the exoglucanase-gold complex for the localization of cellulosic subunits. **Figure 4.** Pathogen (F) ingress towards the vascular stele is accompanied by a gradual alteration of the host cell wall (HCW) as judged by the general decrease in electron density. However, labeling is present over the altered host cell wall. **Figure 5.** Gold particles are distributed over isolated fibrils from completely disorganized host cell walls (HCW). Bars = 0.5 μm.

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(3) **Phospholipase A_2**, an enzyme specific for phospholipids (Coulombe *et al.*, 1988).

(4) **β-1,3-glucanase**, an enzyme purified from tobacco plants reacting hypersensitively to tobacco mosaic virus (Benhamou, 1992a).

(5) **Laccase**, an enzyme produced by the fungus *Rigidoporus lignosus* and specific for phenolic compounds (Benhamou *et al.*, 1994).

Cytochemical labeling with enzyme-gold complexes is identical to direct labeling with gold-complexed lectins. Cytochemical controls performed to assess specificity of the labeling include: (a) addition of the corresponding substrate to each enzyme-gold complex for a competition experiment: β-1,4-glucans from barley (1 mg ml^{-1}) for the β-1,4-exoglucanase-gold complex; Salicin (1 mg ml^{-1}) for the β-glucosidase-gold complex; L-δ-phosphalidylcholine for the phospholipase A_2, laminarin (1 mg ml^{-1}) for the β-1,3-glucanase, and *p*-coumaric acid, ferulic acid or sinapinic acid (1 mg ml^{-1}) for the laccase; (b) digestion of tissue sections with the uncomplexed enzyme prior to incubation with the enzyme-gold complex; (c) substitution of the enzyme-gold complex under study by bovine serum albumin (BSA)-gold complex to assess the non-specific adsorption of the protein-gold complex to the tissue sections; (d) incubation of the tissue sections with the enzyme-gold complex under non-optimal conditions for biological activity; (e) incubation of the tissue sections with colloidal gold alone to assess non-specific adsorption of the gold particles to the tissue sections.

Fusarium Crown and Root Rot of Tomatoes

Fusarium crown and root rot causes widespread, heavy economic losses in commercially grown greenhouse tomato plants. In hydroponic production systems,

the disease is devastating because of the ease with which the pathogens can spread from one plant to another. Various strategies for controlling FORL have been introduced over the years (i.e., soil disinfestation, cultural practices, fungicide treatments, and allelopathy) but serious losses still occur, largely because the effectiveness of these approaches is most often short-lived (Jarvis, 1988). As a result, research efforts have been directed towards developing effective and environmentally safe means of controlling *Fusarium* crown and root rot of tomato. Several microorganisms, such as, *Trichoderma* spp., *Gliocladium* spp., and *Pseudomonas* spp. have been identified as potential biocontrol agents for use against a number of soil-borne pathogens (Chet, 1987). However, commercial applications of these antagonists have been limited so far, mainly because their efficiency not only requires an excessively large amount of inoculum but also varies with environmental conditions.

In the past decade, considerable progress has been made in our understanding of the cytology of infection of root tissues from susceptible and resistant tomato cultivars (Brammall and Higgins, 1988). It has been shown that in susceptible tomato plants, the pathogen ramified rapidly through much of the root tissues causing extensive host cell damage (Fig. 2). Fungal ingress towards the vascular stele coincided always with marked cell wall damage involving loosening of the fibrillar layers (Fig. 3), disruption of the primary walls and middle lamella matrices, and, in some cases, complete wall breakdown leading to tissue maceration.

Incubation of sections from invaded tomato root tissues (fixed with glutaraldehyde and osmium tetroxide) with the gold-complexed *Aplysia* gonad lectin (AGL) resulted in a near absence of labeling over the altered host cell walls, indicating the hydrolysis of wall-bound pectin (Fig. 3). By contrast, labeling of cellulose subunits with the exoglucanase-gold complex was not totally abolished, even over highly degraded cell walls reduced to isolated fibrils (Figs. 4 and 5). Typical features of host reactions such as formation of wall appositions, intercellular space plugging and vascular occlusion (i.e., gelation, tyloses, phenolic deposition) were not detected in the invaded tissues. This massive fungal colonization and alteration of the root tissues correlate usually with the presence of numerous dark brown lesions on the root system and the expression of typical symptoms including leaf chlorosis and wilting.

Chitosan: Biological Properties

Chitosan, a mostly deacetylated β-1,4-linked-D-glucosamine polymer, is a structural component of the cell wall of several plant pathogenic fungi, including *Fusarium* spp. (Bartnicki-Garcia, 1968). It is obtained from

Figures 6-8 (on the facing page). Transmission electron micrographs of *Fusarium oxysporum* f.sp. *radicis-lycopersici* grown on potato dextrose agar (PDA) medium, unamended (Fig. 6) or amended (Figs. 7 and 8) with chitosan. **Figure 6.** Control. Cells of FORL are delimited by a thin cell wall and contain a dense cytoplasm. Bar = 2 μm. **Figure 7.** Cells treated with chitosan at 1 mg/ml. An increased vacuolation (Va) is observed. Bar = 2 μm. **Figure 8.** Cells treated with chitosan at 3 mg/ml. Hyphae are severely damaged. The cytoplasm (Cy) is reduced to strands of aggregated material where lipid bodies (L) are the only recognizable structures. An unusual deposition of a wall-like, amorphous material (AM) occurs between the cell wall (FCW) and the retracted cytoplasm. Bars = 0.5 μm.

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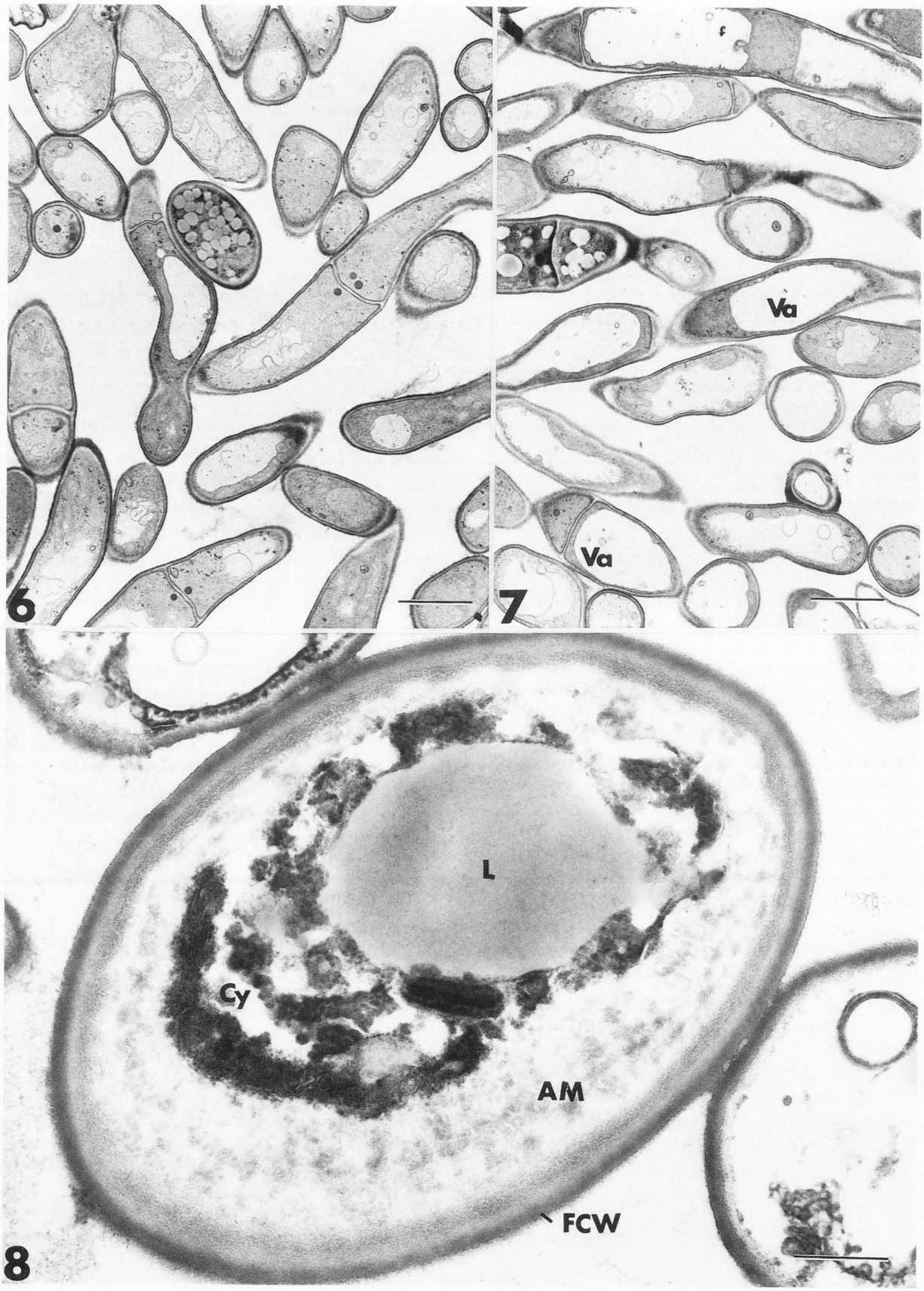
the chitin of either fungal walls, arthropod exoskeleton, or crustacean shells by fragmentation and deacetylation using strong alkali or enzymatic hydrolysis. Due to its high amine content and its unique polycationic nature, chitosan is considered to be a non-toxic biopolymer with great potential for industrial and biomedical use (Sudarshan *et al.*, 1992). In agriculture, chitosan is still incompletely exploited although its specific properties such as biodegradability, antimicrobial potential, and eliciting activity meet with the criteria of a promising biocontrol agent.

Results from a number of studies have convincingly shown that chitosan could inhibit the growth of several pathogenic fungi, with the exception of zygomycetes (fungi containing chitosan as a major cell wall component) (Allan and Hadwiger, 1979; Stössel and Leuba, 1984). Ultra-violet (UV)-absorption analyses revealed that chitosan caused marked leakage of proteinaceous material from *Pythium paroecandrum* at pH 5.8, thus, indicating that fungal cell permeability was altered (Leuba and Stössel, 1986). Considering its polycationic nature, it has been suggested that chitosan interfered with the negatively charged residues of macromolecules exposed at the fungal cell surface, causing alteration in the permeability of the plasma membrane by altering the fluidity properties (Benhamou, 1992b).

In addition to its antimicrobial potential, chitosan has also been shown to be a potent elicitor of plant defense reactions including the accumulation of chitinases (Mauch *et al.*, 1984), the production of phytoalexins (Kendra and Hadwiger, 1984), the synthesis of protease inhibitors (Walker-Simmons and Ryan, 1984), and the induction of structural compounds such as callose (Kauss *et al.*, 1989) and lignin (Pierce and Read, 1982). However, the mechanisms by which chitosan may on the one hand inhibit pathogen growth and on the other hand induce defense gene expression in plants are not fully elucidated. In spite of this gap in our understanding of the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exact mode(s) of action of chitosan, it has become increasingly clear that the unique properties of chitosan could be exploited as a powerful strategy of biological control against damaging pathogens such as FORL.

Among the known elicitors, chitosan is probably one of the compounds which offers the best prospects as a biocontrol agent due to its commercial availability from the chitin of crustacean shell wastes. However, before chitosan-induced resistance can be implemented in plant disease management, efforts need to be directed towards understanding the mechanisms by which this component may affect cell regulation and gene expression in plants challenged by virulent pathogens.

Antimicrobial potential of chitosan

Recent ultrastructural and cytochemical studies conducted on FORL grown *in vitro* revealed that chitosan was not only effective in halting growth of the pathogen but also induced marked morphological changes, structural alterations, and molecular disorganization of the fungal cells (Benhamou, 1992b). Under chitosan-free conditions (absence of chitosan in the agar medium), hyphae of the pathogen showed a normal ultrastructure characterized by a thin cell wall and a dense cytoplasm (Fig. 6). Addition of chitosan to the agar medium led to gradual alterations ranging from increased vacuolation (chitosan at 1 mg/ml) (Fig. 7) to complete cellular disorganization (chitosan at 3 mg/ml) (Fig. 8). In addition to plasmalemma retraction and cytoplasm aggregation, an interesting feature of these damaged fungal cells was the apparent deposition of an amorphous material between the retracted plasmalemma and the cell wall (Fig. 8).

Application of the WGA-ovomucoid-gold complex for the localization of N-actylglucosamine residues (chitin) showed a regular distribution of the gold particles over the thin cell walls of hyphae grown in the absence of chitosan (Fig. 9). An unusual pattern of gold labeling was observed over hyphae grown in the presence of chitosan (Fig. 10). The amorphous wall-like material was densely labeled while gold particles appeared to be less numerous than normal over the cell wall. Upon incubation with the HPA-gold complex for the localization of N-acetylgalactosamine residues, the cell wall of hyphae grown in the absence of chitosan was specifically labeled (Fig. 11) while the wall-like material formed in cells grown in the presence of chitosan was nearly unlabeled (Fig. 12). Application of the RcA and ConA-gold complexes to sections did not result in significant labeling, thus, suggesting the absence of galactose and mannose in the fungal cell walls and in the amorphous wall-like material.

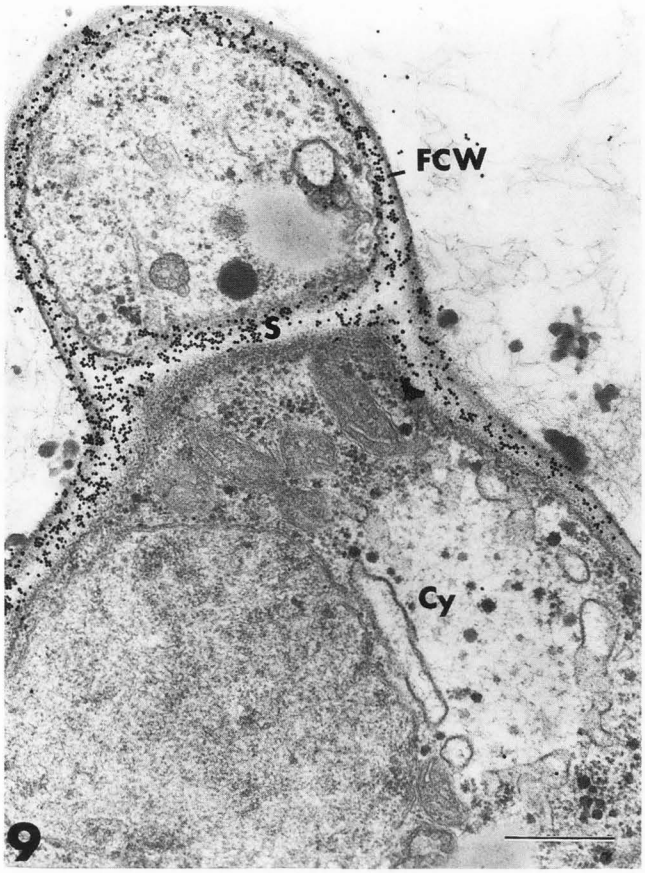
Considering the polycationic nature of chitosan, it is likely that chitosan-induced alterations in the permeability of the plasma membrane have promoted internal osmotic imbalances, leading to cytoplasm aggregation and deregulation in membrane-bound enzymes. Disturbance in the regulation of chitin synthase, the enzyme involved in the synthesis of chitin, may explain the accumulation of chitin at sites where deposition of wall polymers normally does not occur (Benhamou, 1992b). However, the possibility that this unusual accumulation results from a release of pre-existing wall polymers cannot be ruled out, although the absence of N-acetylgalactosamine in the new material makes this process unlikely. The inhibitory effect of chitosan is probably associated with more than one mechanism and further studies are needed to elucidate this complex phenomenon.

Figures 9-12 (on the facing page). Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* grown on potato dextrose agar (PDA) medium, unamended (Figs. 9 and 11) or amended (1 mg/ml) (Figs. 10 and 12) with chitosan. **Figures 9 and 10.** Labeling with the WGA/ovomucoid-gold complex for the localization of chitin. The cell wall (FCW) as well as the septum (S) of a control cell are regularly labeled (Fig. 9; bar = 0.5 μm). The amorphous material (AM) deposited in a hyphal cell grown in the presence of chitosan (3 mg/ml) is intensely labeled (Fig. 10; bar = 0.5 μm). **Figures 11 and 12.** Labeling with the HPA-gold complex for the localization of N-acetylgalactosamine residues. Gold particles are distributed over the cell wall (FCW) and occur also over some electron-dense vesicles in the cytoplasm (Cy) of a control cell (Fig. 11; bar = 0.2 μm). The amorphous material formed in cells grown in the presence of chitosan is nearly unlabeled (Fig. 12; bar = 0.4 μm).

Chitosan and other elicitors as inducers of plant disease resistance

We have recently demonstrated that in addition to inhibit FORL growth *in vitro*, chitosan displayed the ability to induce defense reactions in susceptible tomato plants when applied as root coating, leaf spraying, and seed coating (Benhamou and Thériault, 1992; Benhamou *et al.*, 1994).

Host cell reactions induced by chitosan: Time-course studies of fungal colonization in root tissues from tomato seedlings (cv. Bonny Best, highly susceptible to *Fusarium*) treated with chitosan by leaf spray and/or root coating, or produced by chitosan-treated seeds revealed that pathogen growth was restricted to the epidermis and the outermost cortical cell layers. In all examined sections, fungal cells were never detected in inner tissues, including the endodermis and the vascular parenchyma.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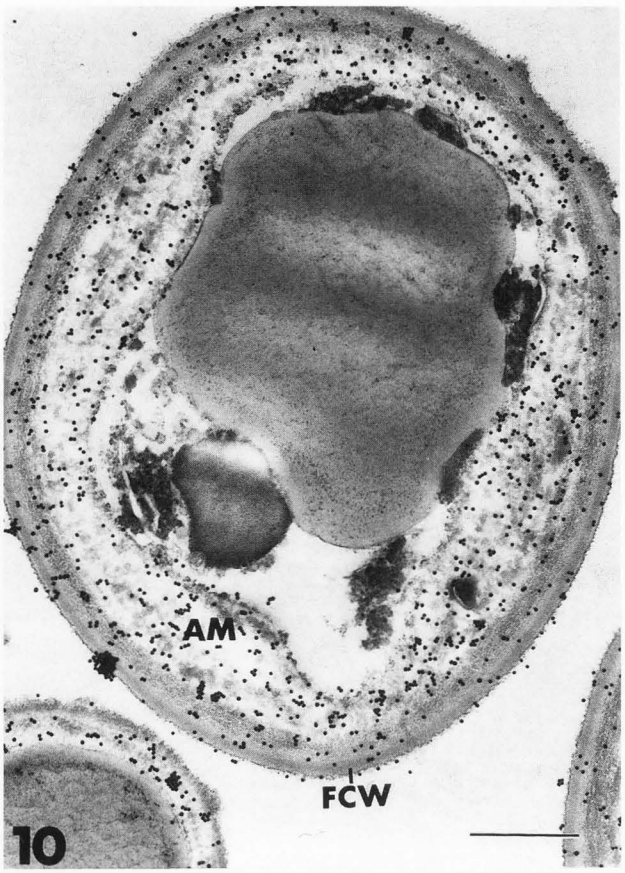
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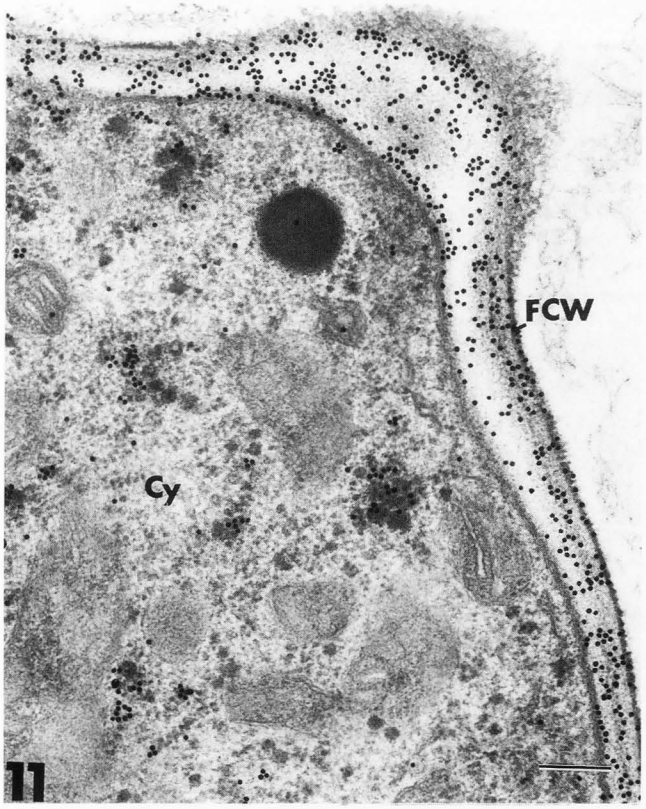
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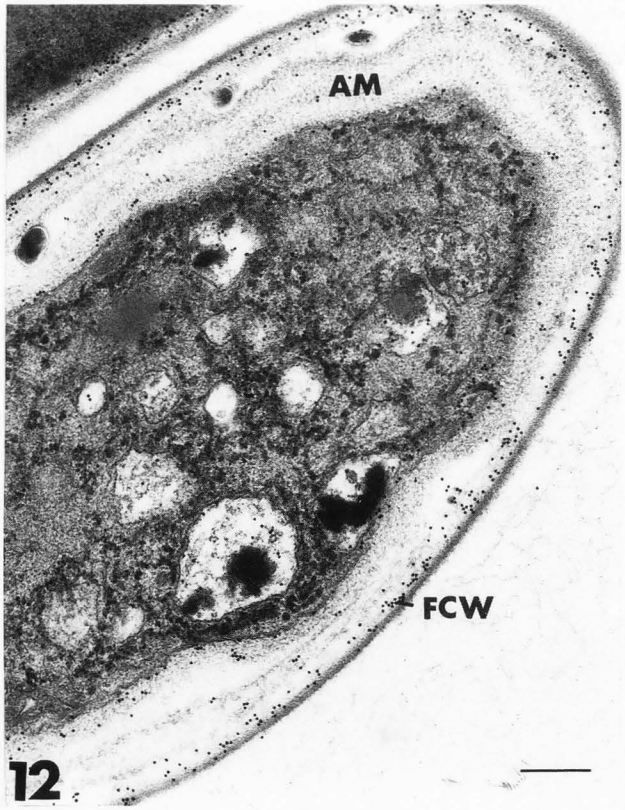
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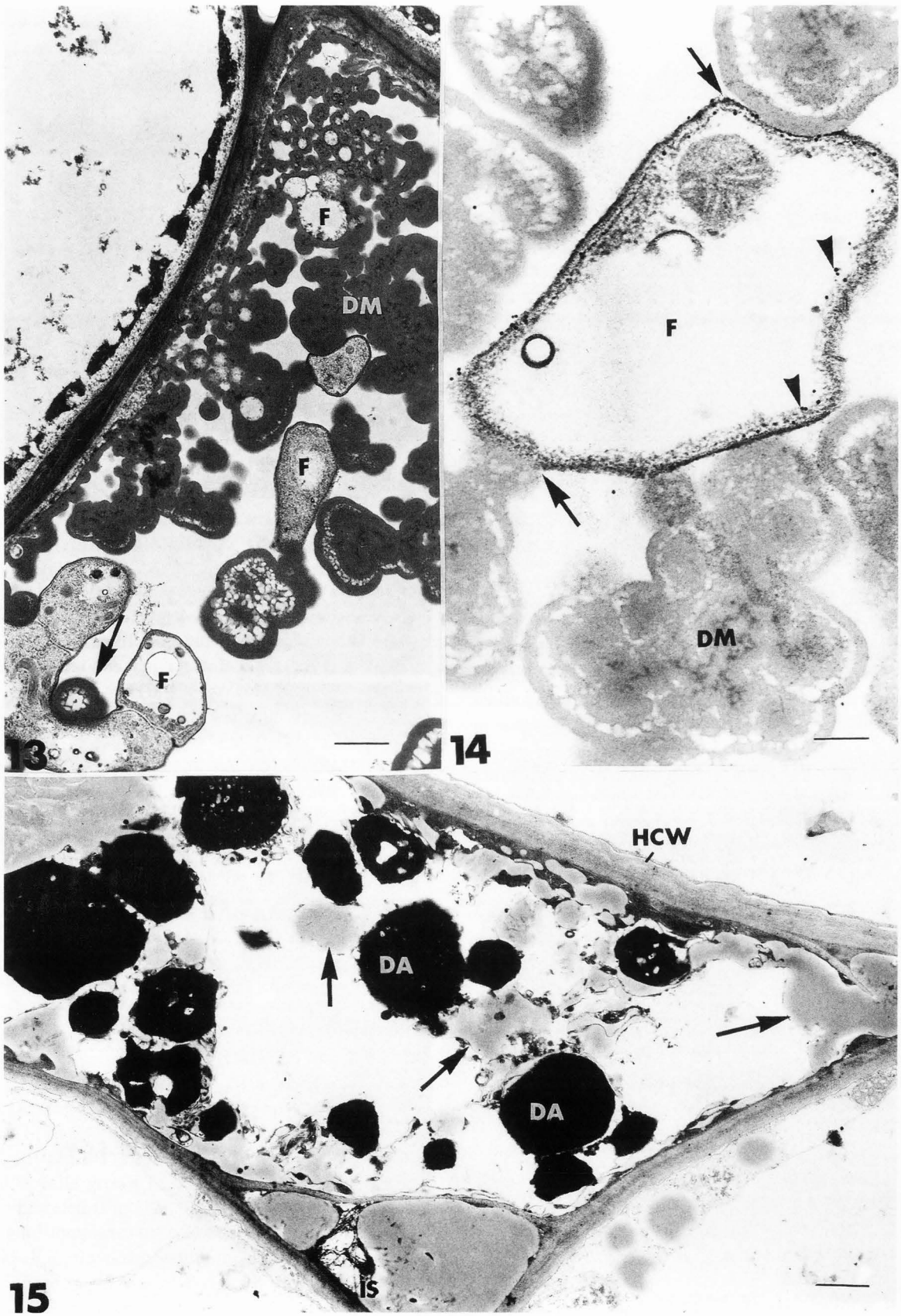
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Figures 13-15 (on the facing page). Transmission electron micrographs of root tissues from tomato plants treated with chitosan (seed coating) and infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici*, four days after inoculation. **Figures 13 and 14.** In the outer root cortex, pathogen invasion is accompanied by marked host cell changes including an abnormal accumulation of a dense material (DM) which often encases fungal cells (F) (arrow) (Fig. 13; bar = 1 μm). Invading hyphae (F) suffer from severe damage leading to complete loss of protoplast. Incubation with the WGA/ovomucoid-gold complex results in a reduced and uneven distribution of gold particles (arrowheads) (Fig. 14; bar = 0.2 μm). A close interaction between the dense material (DM) and the fungal cell wall is visible (arrow). **Figure 15.** In the uninvaded inner cortex, considerable changes characterized by the accumulation of two types of material are detected: (1) large aggregates of very high electron-density (DA), and (2) polymorphic intracellular deposits of lower electron-density (arrows). Intercellular spaces (IS) are also filled with an amorphous material resembling the intracellular deposits of low electron-density. Bar = 1 μm.

This localized colonization at infection sites was consistently associated with strong host defense reactions.

Occurrence of fungal cells in the epidermis was recorded between 2 and 3 days after challenge inoculation, thus, later than in control (untreated plants). The low number of detected fungal cells indicated that the frequency of penetration was reduced as compared to non-treated, control plants. In the outer cortex, pathogen penetration was accompanied by drastic host cell reactions (Fig. 13). Most fungal cells suffered from severe damage and were often reduced to empty shells (Fig. 14). Host reactions in this tissue were mainly characterized by the accumulation of an electron-dense material along the cell walls (Fig. 13). The polymorphic deposits, made of an amorphous material, often extended towards the inside of the cell and encircled invading hyphae through an apparent physical interaction (Figs. 13 and 14, arrows). Trapped hyphae were distorted and exhibited marked changes including cytoplasm disintegration and cell wall loosening (Fig. 14). A reduced and uneven distribution of gold particles associated with chitin was observed over the altered cell walls of these trapped hyphae (Fig. 14, arrowheads).

Fungal cells did not penetrate the innermost cortical cells. However, such cells exhibited marked changes characterized by the accumulation of deposits varying in size, shape and texture (Fig. 15). Two types of intracellular material could be easily distinguished. The first one displayed a very high electron-density and was always encountered as large aggregates in the cell lumen (Fig. 15). The second one was less electron-dense, amorphous and of smaller size. It occurred as opaque deposits inserted between the electron-dense aggregates (Fig. 15, arrows). A large number of intercellular spaces were also found to be partially or completely plugged by opaque substances resembling those found inside the cells (Fig. 15). Section labeling with the gold-complexed exoglucanase for localizing cellulosic β-1,4-glucans resulted in a specific deposition of gold particles over the structurally preserved host cell walls (Fig. 16). However, the electron-dense and opaque aggregates accumulating in the reacting cortical cells were free of labeling (Fig. 16). A similar labeling distribution was observed upon incubation with the AGL-gold complex used for localizing pectic subunits. Gold particles were predominantly associated with the host cell walls and seldom occurred over the electron-dense and opaque deposits induced in response to infection in root tissues of seedlings derived from chitosan-treated seeds (not shown).

A β-1,3-glucanase, purified from tobacco plants reacting hypersensitively to TMV infection, was used for localizing callose, a polymer of β-1,3-glucans, in infected root tissues from tomato seedlings obtained by chitosan-coated seeds. Incubation with the gold-complexed enzyme resulted in an intense and specific accumulation of gold particles over the opaque deposits plugging the intercellular spaces and occurring in most reacting cortical cells (Fig. 17). By contrast, the electron-dense material neighboring the opaque deposits in cortical cells was never labeled.

A purified laccase, produced by the white rot fungus *Rigidoporus lignosus*, was used for localizing lignin-like compounds and polymerized phenols in the reacting tomato root tissues. Upon incubation with the gold-complexed enzyme, labeling was found to be predominantly associated with the electron-dense aggregates accumulating in the reacting cortical cells and to a lesser extent with the host cell walls (Fig. 18). The opaque material plugging the intercellular spaces was unlabeled (not shown).

These results demonstrated that pretreatment with chitosan enhanced resistance response of tomato seedlings by a marked reduction in fungal biomass and a restriction of pathogen growth in the outer root tissues. Although indirect evidence for the production of chitinases was provided by the altered pattern of chitin distribution over the cell walls of the invading hyphae, the observation that chitin molecules still occurred over the walls of highly disorganized fungal cells was taken as an indication that production of chitinases was probably preceded by another defense mechanism. The heavy accumulation of densely-stained deposits, encircling pathogen hyphae in the colonized cells and also accumulating

in the non-infected inner cortex, appeared to be an important feature of the host defense strategy. The strong electron-density of this material suggested that it could be enriched with phenolic compounds. This hypothesis was confirmed by the labeling pattern obtained after incubation of the sections with the gold-complexed laccase. The labeling distribution obtained with this gold-complexed enzyme suggested that chitosan treatment triggered the *de novo* synthesis of phenolic compounds and/or the polymerization of pre-existing free, soluble phenols. The observation that these compounds often interacted physically with the cell walls of hyphae apparently suffering from severe damage suggested a direct fungitoxic activity. Interestingly, laccase-binding molecules were also detected in the host cell walls, thus, indicating the infusion of phenolic or lignin-like compounds. Since a similar wall labeling was not seen in non-treated infected plants, one may assume that the deposition of these molecules in the cell walls of elicitor-treated plants is involved in the resistance process, probably by strengthening the wall architecture, thus, contributing to the formation of a stronger barrier to fungal penetration. Phenolic compounds may, thus, play a dual function characterized by both a direct and an indirect effect on the pathogen.

Structural barriers induced by chitosan: Formation of structural barriers at sites of attempted fungal penetration were also important features of host reaction to chitosan treatment (Benhamou and Lafontaine, 1995). A large number of wall appositions were detected in the invaded epidermis and outer cortex (Figs. 19-21). These newly-formed appositions varied in their appearance from multi-textured (Fig. 19) to multi-layered structures (not shown), from elongated deposits along a large portion of the host cell wall (Fig. 19) to small, hemispherical protuberances (Fig. 21).

Incubation of sections with the tobacco β-1,3-glucanase resulted in the deposition of a considerable number of gold particles over all wall appositions (Figs. 19 and 20). Upon incubation of sections with the gold-complexed laccase a significant deposition of gold particles was observed over both the wall appositions (Fig. 21) and the material filling some intercellular spaces. β-glucosides, visualized by the β-glucosidase-gold complex, were found to be closely associated with callose in wall appositions.

In control, untreated plants, the labeling patterns of pectin and cellulose provided evidence that the fungus was able to produce cell wall-degrading enzymes that diffused at a distance from the areas of penetration and were responsible for considerable alterations of primary walls and middle lamella matrices (see Figs. 3 and 4). In that context, success of a plant in warding off invading pathogens relies on its early capability of elaborating

Figures 16-18 (on the facing page). Transmission electron micrographs of root tissues from tomato plants treated with chitosan at 1 mg/ml (seed coating) and infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici*, four days after inoculation. **Figure 16.** Labeling with the exoglucanase-gold complex for the localization of cellulosic subunits. An intense labeling occurs over the host cell walls (HCW). The dense aggregates (DA) and the opaque material formed in response to infection are unlabeled. **Figure 17.** Labeling of β-1,3-glucans with a purified tobacco β-1,3 glucanase complexed to gold. The opaque material (arrows)) filling the intercellular space and occurring in most reacting cortical cells is specifically and intensely labeled. Scattered gold particles are seen over the host cell wall (HCW). **Figure 18.** Labeling of phenolic and lignin-like compounds with a purified laccase complexed to gold. The dense aggregates (DA) are heavily labeled. The host cell wall (HCW) is decorated by a significant number of gold particles. Bars = 0.5 μm.

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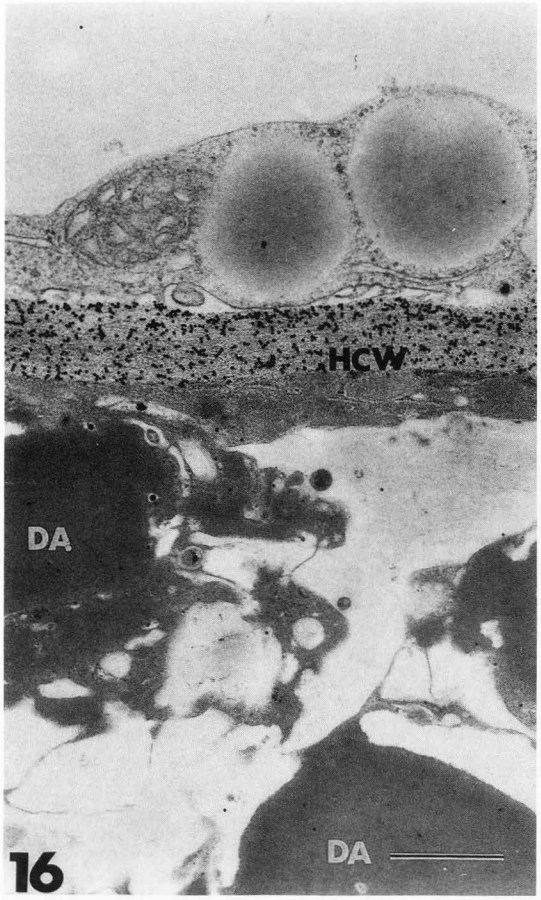
rapidly a first defensive line for protecting the cell walls against both penetration and enzymatic degradation. Thus, structural changes of the host cell walls as well as deposition of newly-formed appositions are of key importance in the resistance process.

The labeling pattern obtained with the gold-complexed laccase showed that phenolics were widely distributed in the reacting host cells as illustrated by their association with the host cell wall and the wall appositions. Conceivably, enrichment of both the host cell walls and the wall appositions with phenolics is likely to contribute to the elaboration of permeability barriers preventing pathogen spread and enzymatic degradation. Several studies have convincingly shown that phenolic structures could confer strong rigidity to the host cell walls through peroxidase-mediated cross-linking with preexisting wall carbohydrates such as hemicellulose, pectin, and callose (Fry, 1986). In line with these studies, we found that elicitor treatment initiated a marked increase in phenylalanine ammonialyase (PAL) and peroxidase activities (unpublished observations). The time-course of PAL and peroxidase levels suggested a coordinated action of these enzymes. Induction of PAL and peroxidase activities was chronologically related to phenolic accumulation and/or polymerization in elicitor-treated plants. Such a relationship was not observed in control plants although some stimulation of the two enzymes could be recorded by 1 or 2 days after inoculation.

Callose also occurred in the newly-formed wall appositions. According to our observations, the wall appositions formed in tomato root tissues upon chitosan treatment and fungal challenge are made of a poly-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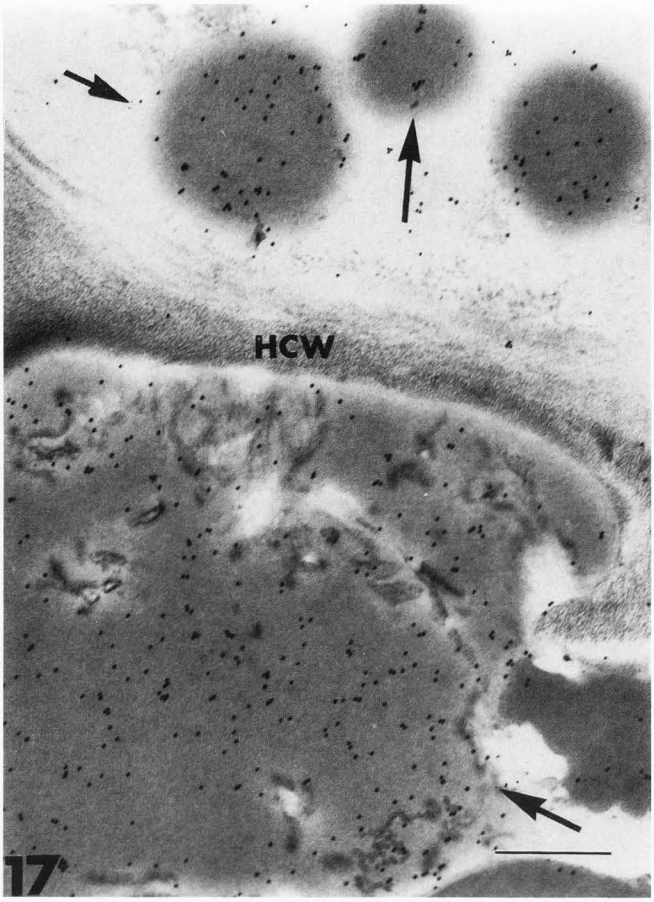
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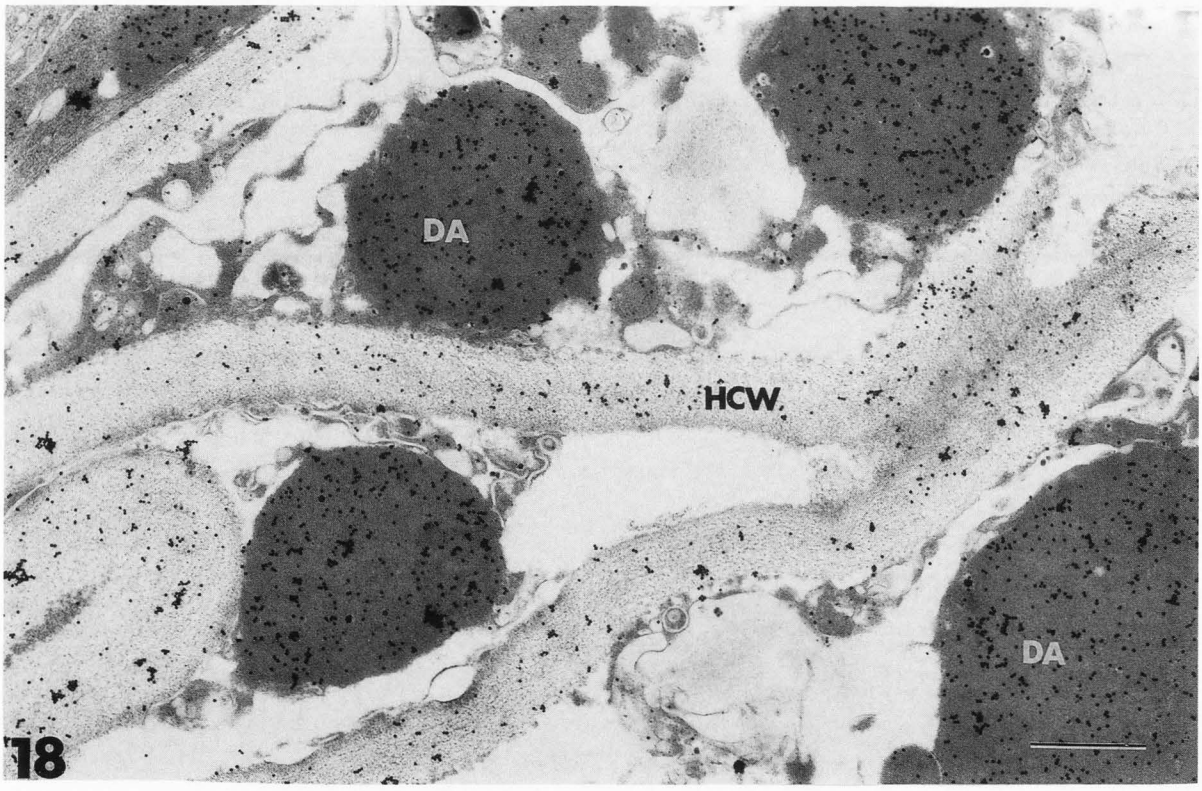
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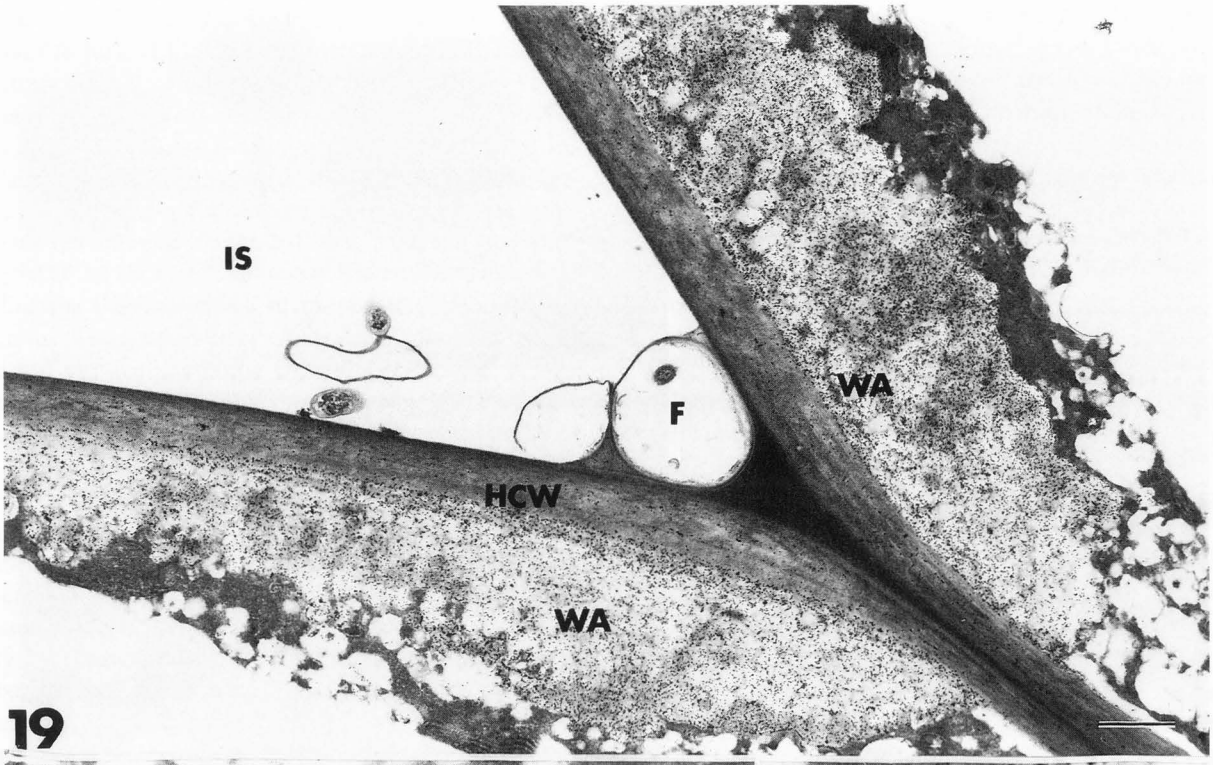

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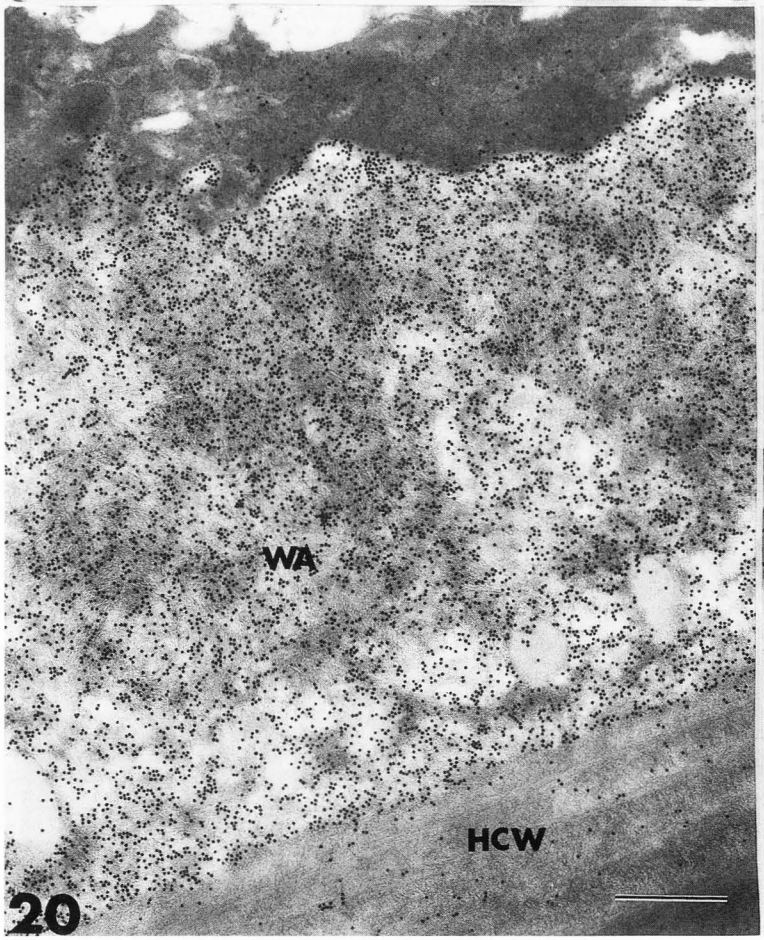

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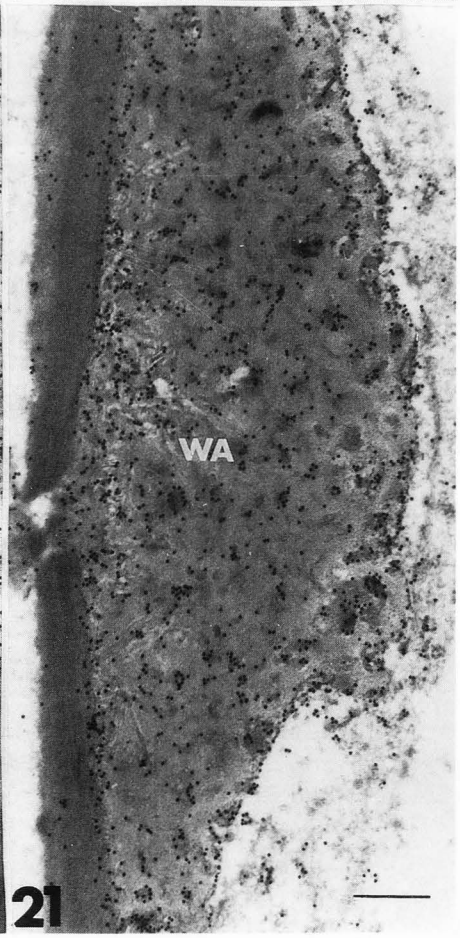

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Figures 19-21 (on the facing page). Transmission electron micrographs of root tissues from tomato plants treated with chitosan at 1 mg/ml (seed coating) and infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici*, four days after inoculation. Formation of physical barriers. **Figure 19.** Wall appositions (WA) are formed on both sides of an invaded intercellular space (IS). Fungal cells (F) show pronounced alteration characterized by complete loss of protoplast. Bar = 1 μm. **Figure 20.** Labeling of β-1,3-glucans with a purified tobacco β-1,3 glucanase complexed to gold. A heavy labeling occurs over the wall apposition (WA) while scattered gold particles are present over the host cell wall (HCW). Bar = 0.5 μm. **Figure 21.** Labeling of phenolic and lignin-like compounds with a purified laccase complexed to gold. An electron-opaque, dome-like wall apposition (WA) is specifically labeled. Bar = 0.25 μm.

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saccharidic matrix mainly composed of callose on which phenolic compounds (likely lignin) were sequentially deposited, probably to build a more impervious composite. Recently, published studies agreed on the key role of lignification and phenolic deposition in resistance to disease and speculated on the secondary importance of callose and other polysaccharides in defense (Ride and Barber, 1987; Cohen *et al.*, 1990). It is obvious that callose, unlike phenolics, does not contribute to the creation of a fungitoxic environment. However, it may contribute to the establishment of lignin-like compounds by providing potential binding sites.

In summary, evidence has been presented in this review that exogenous applications of biotic elicitors sensitize susceptible tomato plants to react more rapidly and more efficiently to FORL attack through the accumulation of biopolymers at sites of attempted pathogen penetration. However, our results suggest that expression of these resistance mechanisms relies on an alarm signal given by the pathogen itself, thus, reinforcing the idea that a synergistic action of elicitor molecules is required for defense gene expression. Whether the alarm signal originates from the pathogen itself or derives from the plant following cell wall degradation by microbial enzymes remains to be investigated further.

Host Defense Induced by Other Biotic Elicitors

The potential of other elicitors including β-1,3-glucans from *Phytophthora megasperma* f. sp. *glycinea* and *Saccharomyces cerevisiae* or laminarin, a commercial polymer of β-1,3-glucans to induce tomato plant defense reactions against FORL attack was also tested (Benhamou and Lafontaine, 1995). Application of these elicitors to tomato plants induced cellular and structural responses similar to those observed upon chitosan treatment. In all cases, growth and ingress of the invading hyphae were prevented by the deposition of phenolic compounds and the rapid elaboration of thick wall appositions at attempted sites of host wall penetration. Callose and phenolic compounds were found to be the key factors involved in the structural response.

Conclusions and Future Prospects

In experiments summarized here, we have shown that tomato plants treated by biotic elicitors express increased resistance to infection by the soil-borne pathogen, FORL than are untreated, control plants. The resistance of these plants was found to be associated with drastic host metabolic changes including the *de novo* synthesis of fungitoxic compounds (phenolic compounds) and the accumulation of structural substances such as lignin and callose. Evidence is provided in this review that natural host defense mechanisms may be manipulated to produce fungal-resistant plants.

These studies further pointed out the interest of using ultrastructure and gold cytochemistry for a better understanding of the cellular and molecular events occurring *in planta*. Exciting progress has been made in the understanding of the cellular events induced in response to infection, mainly because of the introduction of new biological probes (i.e., laccase and β-1,3-glucanase-gold complexes). In conjunction with biochemistry and molecular biology, cyto-and immunocytochemistry of plant tissues have proved useful for elucidating some aspects of the natural defense system that plants elaborate upon microbial attack. Thus, it is clear that both molecular and traditional approaches will continue to benefit from novel findings derived from the *in situ* localization of an increasing number of plant induced molecules.

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Discussion with Reviewers

M. St. Arnaud: You suggest that chitosan induces, at the site of attempted fungal penetration, the formation of wall appositions which appeared to be "multi-textured to multi-layered structures". You also postulate that callose and phenolic compounds were sequentially deposited during the formation of these wall appositions. Figures 19-21 illustrate the presence of callose and phenolics in the wall appositions but the multi-layered or sequential formation is not obvious. Could you comment on this and give more evidence of postulated sequential deposition of the constitutive molecules as opposed to a simultaneous deposition.
Author: Although not illustrated in this paper, this has been shown in Benhamou and Lafontaine (1995).

R.L. Grayson: Considering that chitosan's solubility is limited, how does one explain the movement of externally supplied chitosan molecules **through plant cell walls to the plasma membrane receptor sites**? What are your thoughts on the possible movement of chitosan molecules to the transcription level of the plants?
Author: In the solution that was exogeneously applied, the chitosan fragments varied in sizes. Some of them were small enough (7-10 subunits) to go easily through the plant cell wall and reach the plasma membrane. By contrast, bigger fragments remained outside. The idea now is to develop a fragmentation method allowing the formation of more fragments with the appropriate size. This will allow the use of reduced concentrations of chitosan since less elicitor fragments will be lost.

R.L. Grayson: Relative to the accumulation of structural substances, such as lignin and callose, do you think the difference between resistant and susceptible varieties is basically how fast they can form these structures?
Author: Yes, of course.

R.L. Grayson: When one applies biotic elicitors to sensitize susceptible plants to react more rapidly, are the reactions localized or systemic or vary according to the elicitor used?
Author: Following elicitor treatment, the plant response is usually non-specific to the elicitor. It is systemic since reactions can be detected at a large distance from the area submitted to treatment. For example, soil amendment with chitosan generates resistance to foliar pathogens such as *Botrytis cinerea* in the aerial parts of the plant (unpublished results).