European Journal of Plant Pathology **103:** 125–136, 1997.

Early vascular defense reactions of cotton roots infected with a defoliating mutant strain of *Verticillium dahliae*

F. Daayf, M. Nicole*, B. Boher, A. Pando and J.P. Geiger
*ORSTOM, Laboratoire de Phytopathologie, BP 5045, 34032 Montpellier, France; *Author for correspondence (Fax: 33 04 67 54 78 00)*

Accepted 9 September 1996

Key words: callose, cytochemistry, phenolics, terpenoids, ultrastructure, vascular wilt

Abstract

Susceptible and resistant cotton lines were cytologically and histochemically investigated for their defense reactions to a highly aggressive and defoliating strain of *Verticillium dahliae*, a fungus responsible for vascular wilt. Cytochemistry showed that early responses consisted of reinforcement in structural barriers with polysaccharides, including callose and cellulose. Ultrastructural modifications of parenchyma cells of the vascular tissues were associated with strong production of terpenoids and phenolics. These defense reactions were detected early in roots of the resistant line, one to four days after inoculation, while they were seen later in roots of the susceptible line.

Introduction

Vascular wilt of cotton (*Gossypium* sp.) caused by the soilborne fungus *Verticillium dahliae* Klebahn (abbreviation: VD) is an economically important disease in most cotton growing areas of the world (Bell, 1992; Hillocks, 1992). Infection of cotton plants by VD results either from direct penetration of young roots or infection through root or stem wounds. In both susceptible and resistant lines, spores are detected in the xylem vessels, one to three days after inoculation (Harrison and Beckman, 1982) although they are more abundant in susceptible and partially resistant lines than in totally resistant ones. Symptoms exhibited by susceptible lines are plant stunting and extensive defoliation, the severity of which varies according to temperature and VD virulence (Daayf et al., 1995). When environmental conditions favour the disease, symptoms progress rapidly and plants die prematurely.

Work on integrated crop management, including epidemiology, host resistance, genetic potential and pathotypes, has resulted in the development of effective and economical control measures of VD wilt of cotton (El-Zik, 1985). In the field, cultivar resistance to VD wilt is expressed as a delay in the onset of visible symptoms and a decrease in the rate of the disease progress (Bell, 1992). Investigations on cotton-VD relationships revealed that resistance appears to depend on active plant responses that occur early during the interaction. Harrison and Beckman (1982) indicated that a 2-step system may explain processes by which VD resistance operates in cotton lines. The initial determinant appears to be the physical walling-off of infected vessels immediately above the trapping sites, after which the production of terpenoid aldehydes and their release into the infected vessel lumen restricts the vascular ingress of the fungus.

Histochemical and biochemical studies that focused on cotton defense to VD demonstrated that phytoalexins play a key role in resistance (Bell, 1969). Isolation of gossypol-related terpenoids and their histochemical localization showed that stems and roots of resistant lines contained significantly more terpenoids than susceptible varieties (Mace et al., 1974; Mace et al., 1976). Also, the histological detection of desoxyhemigossypol, the precursor of hemigossypol, in the paravascular parenchyma cells and over the vascular fungal spores suggests that cotton phytoalexins may be fungitoxic molecules for wilt pathogens (Mace et al., 1985, 1989; Zhang et al., 1993). High concentrations of polyphenols and tannins have also been found correlated with host resistance to VD, although they

are thought to be probably of minor importance in the initial defense of vascular cells (Bell, 1969). Similarly, the contribution of tyloses in occlusion of stem vessels has been reported to prevent the distribution of secondary conidia released at the primary infection sites (Beckman, 1987; Mace, 1978). Although these studies provided histochemical evidence for correlations between cotton resistance and phytoalexin synthesis, little information is available about the fine cellular aspects of the production of toxic compounds against VD. Recently, Mueller and Morgham (1993) briefly reported on the ultrastructural reorganization of vessel contact cells indicating that lipoidal substances produced by these cells may coat the pathogen within vessels.

The present ultrastructural and cytochemical study is based upon the postulate of Harrison and Beckman (1982) on a 2-step system to explain VD resistance in cotton lines. In contrast to previous works that have focused on defense reactions in stems, we have investigated the early responses of susceptible and resistant cotton lines at the root level, 1 to 6 days after infection with a highly aggressive and defoliating strain of VD using a hyaline mutant pathotype, known for its pathogenic stability (Daayf et al., 1995). Particular attention has been paid (1) to cytolocalization of host cell wall polymers involved in paramural deposits or cell coatings and of newly synthesized molecules by vascular parenchyma cells, and (2) to ultrastructural effects *in planta* of such compounds on the fungal spore and on mycelium ultrastructure.

Materials and methods

Plant material and inoculum. Fifteen-day-old seedlings of two upland cotton lines were used in this experiment. The susceptible line ISA 205 from *G. hirsutum* and the resistant line Ashmouni from *G. barbadense* were kindly provided by IRCT (CIRAD, Montpellier, France). A highly aggressive strain (V 7.7) from a VD hyaline mutant known for its virulence stability was used at a concentration of 10^6 spores per ml (Daayf et al., 1995). Young plants were infected by dipping roots 20 min in the inoculum and then strictly maintained at 24 °C (day) and 18 °C (night). Five roots were sampled at 1, 2, 4, 6 and 8 days after inoculation (d.a.i.). Observations of spores and necrosis in infected roots and stems were performed under a light microscope (LM) on transverse sections made with blades. Three to five fragments per healthy or infected root were examined for histochemistry or ultrastructure.

Tissue processing for electron microscopy. For conventional ultrastructural observations, root fragments were fixed for 2 h in 2.5% glutaraldehyde, rinsed in cacodylate buffer 0.1M pH 7.2 and postfixed for 1 h in 1% osmium tetroxide. After dehydration in ethanol, samples were embbeded in Epon (José Delville manufacturer, France). For immunocytochemistry, samples were fixed for 4 h in 1% glutaraldehyde – 4% paraformaldehyde, rinsed in cacodylate buffer 0.1M pH 7.2 and dehydrated in alcohol. Fragments were embbeded in LR white (José Delville manufacturer, France). Impregnation of root fragments in resins and resin polymerization were processed according to the company's instructions. After being sectioned, samples were stained and examined with a Jeol 100X transmission electron microscope.

Preparation of the conjugated probes. β-1,4-glucans were localized using a purified exoglucanase kindly provided by C. Breuil (University of British Columbia, Canada). This enzyme was complexed to gold at pH 9 according to the methodology described by Benhamou et al. (1987). Labelling of sections was performed 30 min at 25 °C on a drop of the probe (pH 6.5), before rinsing and staining. Specificity of labelling was assessed by incubating sections with the gold-complexed protein saturated with an excess of β-1,4-glucans from barley.

Cytolocalization of phenol-like compounds was performed with a purified laccase (EC 1.10.3.2) (Geiger et al., 1986) conjugated to colloidal gold; 100 μg of the purified protein was added to stabilize 10 ml of the gold solution at pH 5.02 (Benhamou et al., 1994). Labelling of sections was performed 30 min at 25 °C on a drop of the probe (pH 6.0). Specificity of labelling was assessed by incubating sections with the gold-complexed laccase previously incubated with an excess of 1% guaïacol, 0.25M ferulic acid or 0.15M chlorogenic acid.

Immunocytochemistry. Polyclonal antibodies raised against β-1,3-glucans (Cambridge Research Biochemicals, England) were used for immunolocalizing callose in plants (Northcote et al., 1989). Briefly, sections were first incubated for 30 min at 25 °C on a drop of primary antibodies (1/2000 in PBS 0.1M pH 7.2 – BSA 1% – Tween 0.05%) before being incubated

on gold-labelled goat anti-rabbit antibodies (1/20) conjugated to colloidal gold (GAR-15, Biocell, England).

A monoclonal antibody (JIM5, kindly provided by K. Roberts, J. Innes Center, England) raised against un-esterified epitopes of pectin was used to visualize galacturonic acid-containing molecules. Immunogold localization of pectin was performed as previously described by Knox et al. (1990). Briefly, sections were first incubated on a drop of primary antibodies for 2 h at 37 °C and then on a drop of a gold-labelled goat anti-rat antibodies (GAT 15, Biocell, England) for 30 min at 37 °C.

Specificity of labelling was assessed through the following control experiments performed on sections from healthy and infected roots, (i) incubation with the antiserum previously adsorbed with the antigen, laminarin and galacturonic acids, respectively (ii) incubation with pre-immune rabbit or rat serum instead of the primary antiserum, and (iii) omission of the primary antibody incubation step.

Histochemistry. Cross sections (20–50 μm thick) of fresh infected or healthy roots were cut with a freezing microtome.

Detection of phenolic compounds was performed using methods described by Daï et al. (1995). Flavonols and coumarins (scopoletin and aesculin) were detected based on their fluorescence and using Neu's reagent; sections were immersed in 1% of 2-amino-ethyldiphenyl-borinate in absolute methanol for 2–5 min and examined with an epifluorescence microscope (Leitz G filter; Diaplan, Leitz). The vanillin-HCl reagent was used for staining flavan compounds (catechins and condensed tannins); root sections were immersed for 5 min in 10% w/v vanillin in 1 vol of absolute ethanol mixed with 1 vol of concentrated HCl and examined with a light microscope (Diaplan, Leitz). Lignin was visualized with light microscope after staining the sections for 5 min with phloroglucinol-HCl. Suberin was stained using Sudan IV in alcoholic solution. Autofluorescence was also monitored under UV illumination using Leitz filter A2 (270–380 nm).

A saturated solution of antimony trichlorid ($SbCl_3$) in 60% $HClO_4$ was used to histochemically localize terpenoids. Sections were incubated for 3 min in the reagent and observed with a light microscope as described by (Mace et al., 1989).

Thin sections of healthy and infected roots were stained with Sudan black B as described by Shi et al. (1991) for detection of lipids.

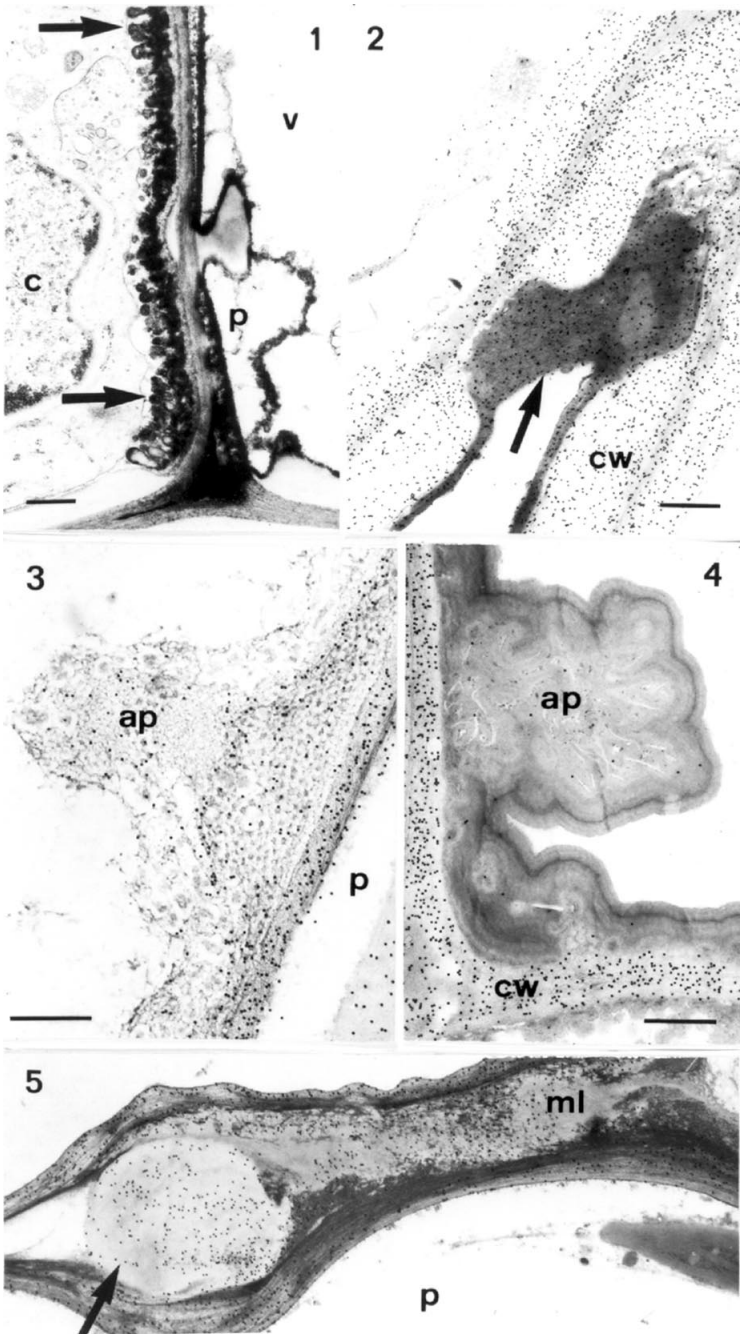
Results

Development of infection in susceptible and resistant lines. In our system, the pattern of early vascular colonization was similar in both the susceptible and the resistant lines as presence of spores and early root necrosis were observed 1 and 2 d.a.i., respectively (Table 1). In the susceptible line, the first stem necrosis was detected 4 d.a.i. and the first foliar symptoms 5 d.a.i. By contrast, stem necrosis and foliar symptoms were delayed in the resistant plants, not appearing until 7 and 9 d.a.i., respectively. Fifteen d.a.i., considerable variation in plant mortality was observed ranging from 20 to 30 percent for the resistant line to 90 to 100 percent for the susceptible line.

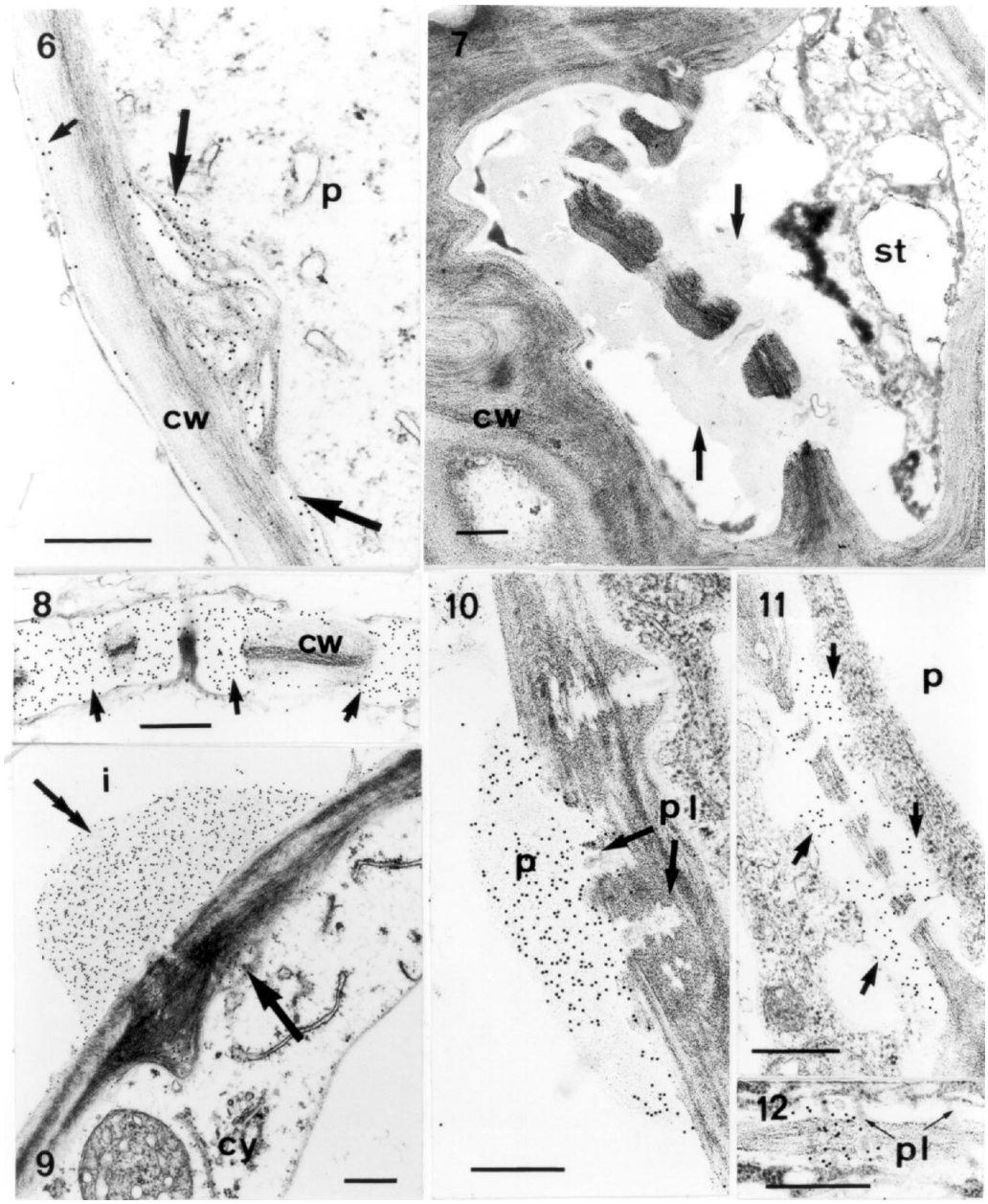
Cytochemistry and histochemistry of host cell wall modifications, apposition layers and vessel coatings observed 1 and 2 d.a.i. Numerous modifications were recorded in root cell walls of both the susceptible and the resistant line, including paramural deposits – layers or papillae – and cell wall coatings; but tyloses were never observed. Apposition layers were more commonly present in paramural areas of phloem and xylem parenchyma cells in the roots of the resistant line (Figure 1) than in the susceptible line (Table 2).

Cytolocalization of β-1,4-glucans by means of an exoglucanase conjugated to colloidal gold indicated that gold particles could be seen over paramural deposits of both young vessels (Figure 2) and xylem-associated parenchyma cells close to pit areas (Figure 3). In phloem cells, apposition layers deposited along walls were devoid of any significant labelling (Figure 4). Hemispherical protuberances localized between phloem cells also contained β-1,4-glucans (Figure 5).

Immunocytolocalization of β-1,3-glucans after treatment of sections with anti-β-1,3-glucans polyclonal antibodies followed by GAR-gold antibodies revealed that labelling occurred over paramural deposits in phloem paramural areas (Figure 6). Sieve plates were also seen to be occluded by an electron-lucent material (Figure 7) similar in its aspects to that labelled in normal sieve tubes (Figure 8). Gold labelling was also detected over wall material located in phloem intercellular areas (Figure 9) and over material deposited close to plasmodesmata (Figures 10 and 11). In healthy roots of both lines a weaker labelling was seen over plasmodesmatal areas (Figure 12). Pre-incubation of the antibodies with laminarin to assess labelling specificity yielded negative results (data not shown).



Figures 1–5. (1) Transmission electron micrograph of *Verticillium*-infected cotton roots two days after root infection of a resistant cultivar. Wall appositions (arrows) are seen in the paramural area of a vessel-associated parenchyma cell. They are electron-dense and located close to a pit area (p) of an infected vessel (v). Bar = 0,5 μm. (2–5) Transmission electron micrographs of *Verticillium*-infected cotton roots two days after root infection of a resistant cultivar. Cytolocalization of β-1,4-glucans by means of an exoglucanase conjugated to colloidal gold. (2) The electron-dense layer coating the secondary cell wall (cw) of a young vessel (v) is evenly decorated with gold particles (arrow). Bar = 0,5 μm. (3) Gold particles are seen over apposition layers (ap) in the paramural area of a vessel-associated parenchyma cell close to a pit area (p). Bar = 0,5 μm. (4) Layers of material (ap) coating the primary wall (cw) of a phloem cell are devoid of significant labelling as compared to the primary cell wall (cw). Bar = 0,5 μm. (5) Gold particles are located over the primary cell walls of two phloem cells (p) and over a hemispherical protuberance occurring in the intercellular space (arrow). Note that the middle lamella (ml) shows disrupted areas that are slightly labelled. Bar = 0,5 μm.



Figures 6–12. (6) Transmission electron micrograph of *Verticillium*-infected cotton roots two days after root infection of a resistant cultivar. Immunocytolocalization of β-1,3-glucans with an anti-β-1,3-glucan polyclonal antibody. Gold particles are seen over cell wall portions (arrows) in the paramural area of a parenchyma phloem cell (p). A weak labelling is also seen at the edge of the wall of the adjacent cell (small arrow). Bar = 0,5 μm. (7) Transmission electron micrograph of *Verticillium*-infected cotton roots two days after root infection of a resistant cultivar. A pad of electron-lucent material (arrows) is seen occluding the pores of a sieve tube plate (st) (cw: sieve tube cell wall). Bar = 0,5 μm. (8–12) Transmission electron micrographs of *Verticillium*-infected cotton roots two days after root infection of a resistant cultivar. Immunocytolocalization of β-1,3-glucans with an anti-β-1,3-glucan polyclonal antibody. (8) In a healthy plant, a strong labelling is seen over the material that occludes pores of a sieve tube (arrows). Gold particles are also observed at the edge of the cell wall (cw). Bar = 0,5 μm. (9) A papilla (double arrow) located in the intercellular area (i) of the infected phloem is evenly labelled. Gold particles are also seen over portions of the adjacent cell wall (arrow). Bar = 0,5 μm. (10–12) Strongly labelled electron-lucent material (p) for β-1,3-glucan localization occludes plasmodesmatal regions (pl) in the infected phloem (Figure 10). Plasmodesmatal areas in infected cells generally displayed a more extend gold decoration (Figure 11) as compared to the weaker labelling seen at such sites in the phloem of healthy roots (Figure 12). Bar = 0,5 μm.

Table 1. Development of symptoms in susceptible and resistant cotton lines infected with an aggressive strain of *Verticillium dahliae*

Time after infection (days)	S	R
1	First observation of spores	First observation of spores
2	First root necrosis	First root necrosis
4	First stem necrosis	
5	First foliar symptoms	
7		First stem necrosis
9		First foliar symptoms
15	Dead plants (90 to 100%)*	Dead plants (20 to 30%)*

S, R: susceptible and resistant cultivars, respectively
*: average percentage

Table 2. Fluorescence, histochemical and cytochemical identification of paramural depositions in parenchyma cells and cell wall coatings in vessels of cotton roots, 1-2 days after inoculation with *Verticillium dahliae*

	Paramural depositions		Cell wall coatings	
	S	R	S	R
Relative occurrence	+	+++	++	++
Cellulose	+	++	+	++
Pectin	+	+	0	0
Callose	+	+++	0	0
Lignin	0	0	+	+
Suberin	0	0	0	0
Autofluorescence	+	+	+	+

S, R: susceptible and resistant cultivars respectively
0, +: semi-quantitative estimation ranging from 0 (no or non significant response) to +++ (strong response)

Table 3. Histochemistry and fluorescence microscopy of terpenoids and phenolics in vascular bundle parenchyma cells of cotton roots infected by *Verticillium dahliae*

	Day after infection	S	R
Terpenoids (a)	1	+	++
	2	+	+++
	4	+++	+++
Flavans (b)	1	+	+
	2	+	++
	4	++	++
Flavonols	1	+	++
	2	+	++
	4	++	++
Coumarins	1	+	+
	2	+	++
	4	++	++

S, R: susceptible and resistant cultivars respectively
0, +: semi-quantitative estimation ranging from 0 (no response) to +++ (strong response)
(a) including naphtol and aldehyde terpenoids
(b) including condensed tannins

A weak labelling was observed over paramural deposits in phloem cells after incubation of section with JIM 5 antipectin monoclonal antibody followed by GAT-gold antibodies (not illustrated). No gold particles were seen over papillae and wall coatings. Controls including incubation of sections with the antibody to which the corresponding antigenic molecule was previously added yielded negative results.

Cytochemistry and fluorescence microscopy of terpenoids and phenolics. Infected and healthy tissues, portions of which were used for electron microscopy observations, were investigated for terpenoids and phenolics histochemistry (Table 3). Cotton terpenoids, visualized after the use of antimony chloride, were produced early in infected resistant roots. They accumulated abundantly 2 and 4 d.a.i. in roots of the resistant and the susceptible plants, respectively. They were mainly seen to be produced in parenchyma cells adjacent to vessels and in phloem parenchyma cells. Phenolic compounds, including flavonoids and coumarins, were also seen early in the resistant line. The same histochemical test indicated that they were produced abundantly in xylem cells, including vessels, of the resistant line from 2 d.a.i. while in the susceptible line they reacted strongly and positively only at 4 d.a.i. Lignin, identified by the phloroglucinol-HCl test, occurred only in limited amount in vessel coatings of resistant and susceptible plants (Table 2).

Attempts to cytochemically identify phenolic compounds with a laccase conjugated to gold revealed

that gold particles decorated globules and electron-dense compounds located in cortical parenchyma cells in infected roots (Figure 13). Structures or compounds in xylem and phloem cells, including cell walls, were generally unlabelled by the probe (not shown). Incubation of the enzyme-gold conjugate with guaïacol, ferulic acid or chlorogenic acid prior to section treatment resulted in the presence of very low gold decoration.

Ultrastructure of early accumulation of electron-dense compounds in vascular parenchyma cells. In the resistant line, electron-dense compounds (EDC) were seen as early as the first d.a.i. while this took 2 days more in the susceptible line. Such EDC were seen first in xylem parenchyma cells and then in phloem cells.They were produced both in the cytoplasm where they were associated with different organelles, including the endoplasmic reticulum, the membrane envelope of mitochondria and other globular vesicles (Figure 14). They were also seen in paramural areas, between the plasma membrane and the cell wall (Figure 15). Within the cytoplasm, these EDC varied in size, probably after becoming fused with each other, but not in electron-density. Four to six d.a.i., the amount of osmiophilic material of these EDC producing-cells increased to the point that good ultrastructural definition was difficult to obtain (Figure 16); the cytoplasm of such cells sometimes showed disruption of the plasma membrane thus favoring close associations of EDC with the host cell wall. When contacts between EDC and the host cell wall occurred, the presence of dense areas within the cell wall was also observed. Small electron-dense droplets were observed associated with loosened cell wall microfibrils in contact with EDC and with cell walls (Figure 17). At this stage, membranes broke, resulting in loss of organellar and cellular integrity.

Accumulation of electron-dense compounds in infected vessels. Observation of infected roots 3 and 4 d.a.i. revealed the presence of damaged or dead hyphae in cells of both the resistant and the susceptible line. The cytoplasm of *V. dahliae* hyphae displayed various stages of cell disorganization such as the occurrence of numerous vesicles, the coagulation of cytoplasmic material and withdrawal of the plasmalemma from the cell wall, as compared to seemingly healthy hyphae (Figures 18 to 20). Fungal spores and hyphae were seen to be trapped by electron-dense layers resembling the vessel coating (Figure 21). Germinating hyphae that penetrated pit membranes were also coated by such dense compounds (Figure 21). Labelling of chitin (not shown) or β-1,3-glucans (Figure 23), two major compounds from fungal cell walls, revealed that the fungal cell walls were not apparently altered, as indicated by the even decoration of fungal cell walls by gold particles.

Discussion

Our work has focused on the early responses of cotton after infection with a highly aggressive and defoliating mutant strain of VD. The most thorough studies on defense responses in cotton against vascular diseases caused by fungi dealt primarily with biochemical and histochemical evaluation of the role of terpenoids as phytoalexins (Bell, 1981, 1992). In contrast, the objectives of our work were to cytochemically investigate whether early ultrastructural changes of xylem and phloem parenchyma cells in roots may contribute to resistance.

Production of antifungal compounds

According to Bell (1969), wilt resistance in cotton species is not due to simple quantitative differences in the amount of phytoalexins produced during infection, since constitutive terpenoids occurring in healthy roots may play a role as preformed fungitoxins (Mace et al., 1974; Mace et al., 1985). In our experimental system, terpenoids identified by histochemical tests and confirmed by biochemical experiments (data not shown) were actively produced by resistant cotton roots during the first two d.a.i. Although several lines of experimental evidence demonstrated that terpenoids have a fungicidal action in VD-infected cotton stems (Mace et al., 1976; Mace et al., 1989), few data exist on the early production of such compounds in infected roots. It was demonstrated histochemically that terpenoids initially occur within vessel contact cells of the infected stele of the stem (Mace et al., 1989). We were able to show that paravascular and phloem parenchyma cells produce terpenoids in roots. The ultrastructural modifications and the positive reaction of such cells with terpenoid stains suggested that the electron-dense compounds produced by paravascular and phloem parenchyma cells are terpenoids. These are seemingly synthesized in uninfected cells. Previous ultrastructural studies on *Fusarium*-infected cotton pointed out that osmiophilic lipoidal droplets developed in adjacent vascular parenchyma cells might

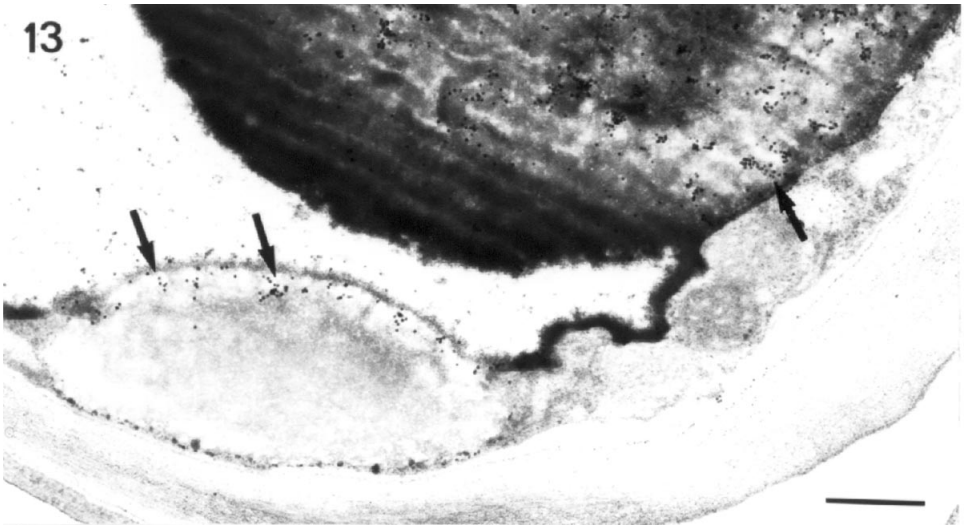


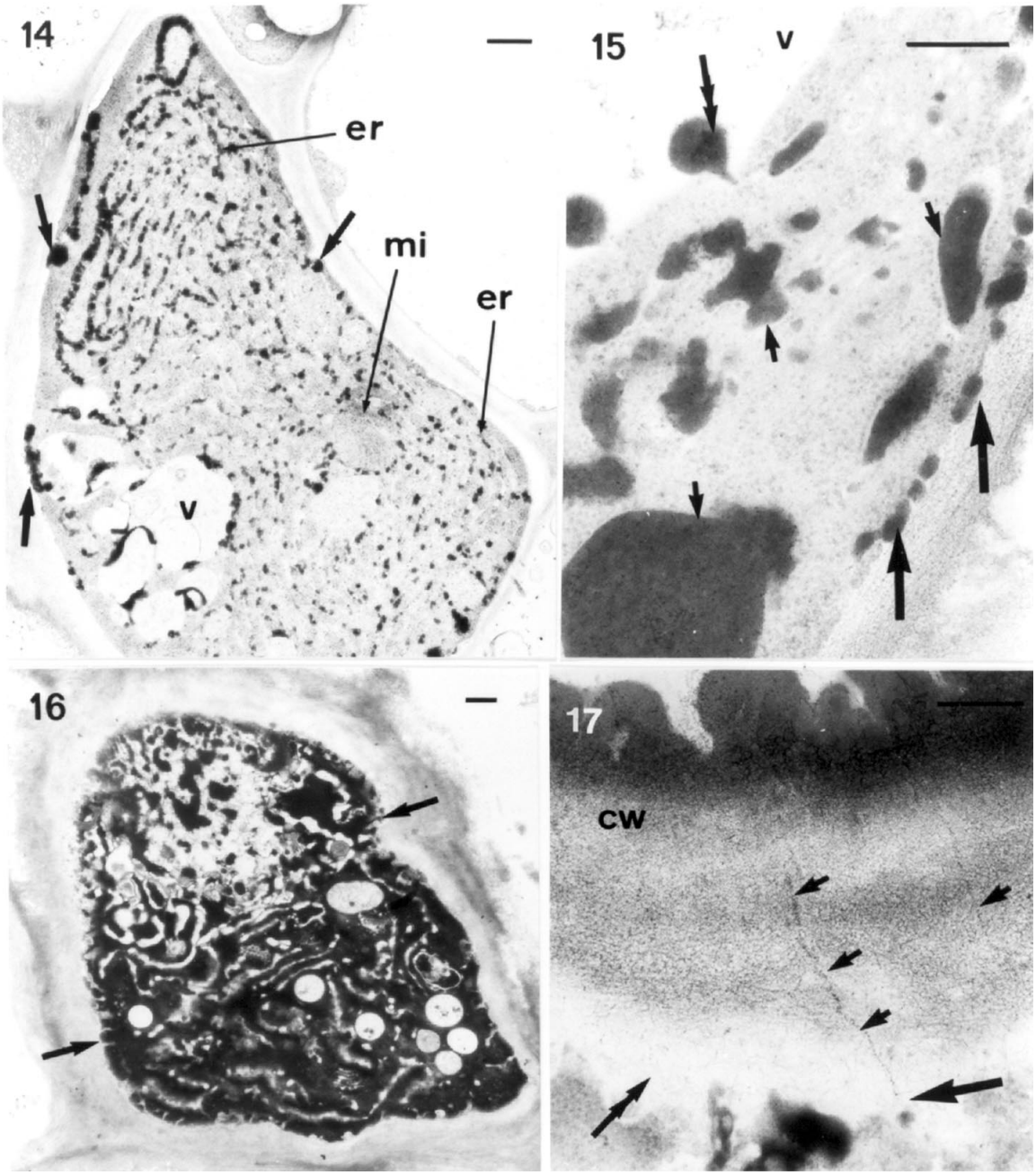
Figure 13. Transmission electron micrograph of *Verticillium*-infected cotton roots three days after root infection of a resistant cultivar. Cytolocalization of phenol-like molecules with a laccase conjugated to colloidal gold. Labelling is observed over the osmiophilic material located within the vacuole (double arrow) of a cortical cell and over a cytoplasm area bordering the tonoplast (arrows). Bar = 0,5 μm.

be terpenoid in origin (Shi et al., 1991, 1992). The production of these lipoidal droplets was demonstrated to be associated with a reorganization of the cytoplasm. Similar conclusions were reached by Mueller and Morgham (1993) who described the occurrence of such lipoidal droplets in VD-infected cotton stems. These authors also reported the direct secretion of this osmiophilic material into the vessel lumina where it accumulated to form aggregates. We never observed such a direct secretion from EDC-producing cells towards the adjacent cells, but rather a diffusion throughout the cell wall in which dense droplets were seen to be associated with cellulose microfibrils. Diffusion of water-soluble cotton terpenoids – gossypol-related molecules – in cell walls has already been reported (Mace et al., 1989). The dense material present within the vessel lumina was seen to coat both the secondary cell walls and the fungal hyphae and spores. The alteration of fungal cells, including membranes, associated with the presence of material that physically interacts with the fungal cell wall is consistent with the possible fungitoxicity of plant molecules. This observation suggests that plant molecules may act on the fungal membrane integrity by causing protein and phospholipid alteration. Diverse phytoalexins have been shown to have comparable effects on the fungal plasma membrane (Bailey and Mansfield, 1982; Pezet and Pont, 1990). Although histochemical tests identified terpenoids and various phenolics, including flavans and coumarines in infected vessels and vascular parenchyma cells, the use of cytochemical probes – e.g. laccase conjugated to gold – was not successful to discriminate phenolics targeted by this enzyme at the ultrastructural level. In addition to cytology, biochemical studies carried out on VD-infected roots (unpublished data), showed that cotton phytoalexins, such as hemigossypol and and hemigossypol methyl ester terpenoids are effective fungitoxic molecules *in vitro*. In light of these cytochemical observations, it can be suggested that the dense material encircling VD hyphae in root vessels is constituted of phytoalexins enriched with phenolics responsible for the cytoplasmic disorganization of the pathogen.

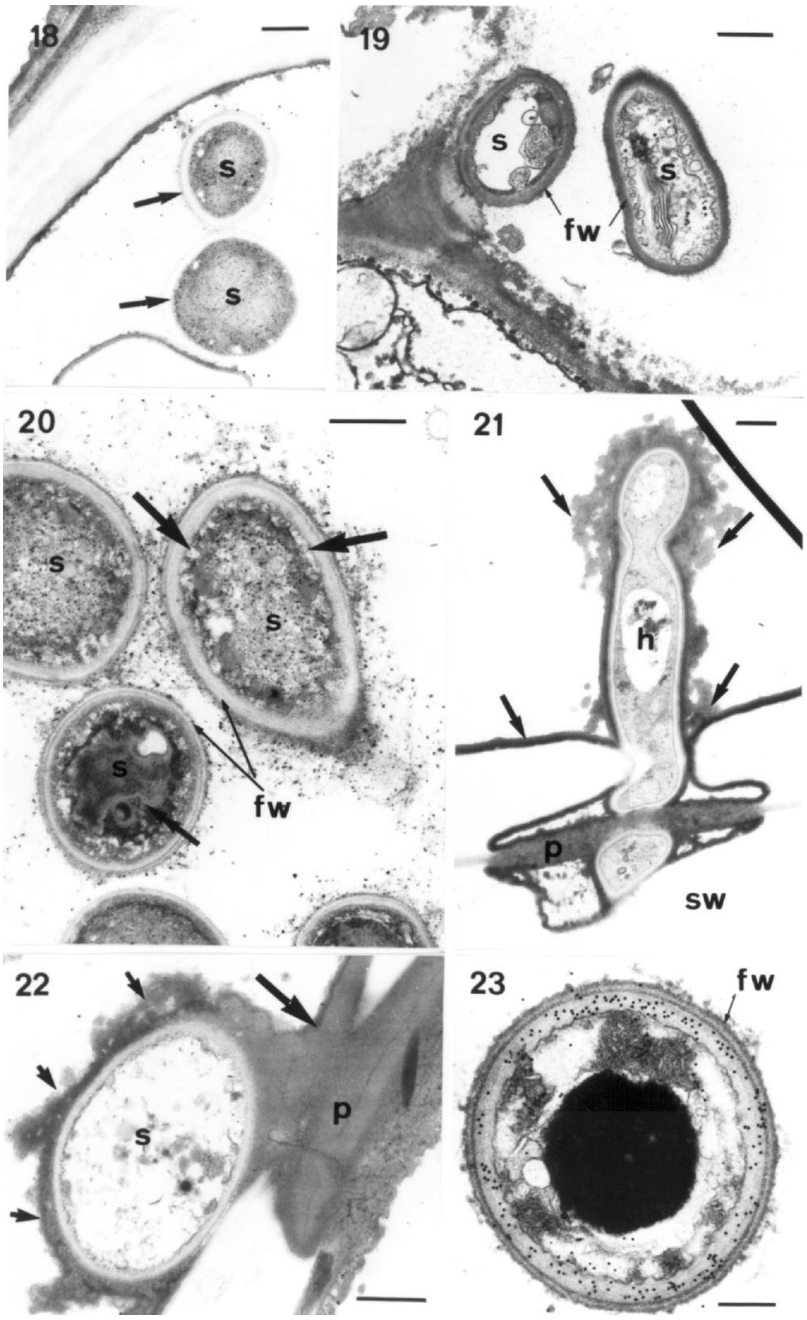
Apparent integrity of the fungal cell wall as judged by the unaltered pattern of chitin and glucan labelling, suggests that chitinases and glucanases are not produced early by the plant in response to the infection. On the other hand, Liu et al. (1995) recently reported the synthesis of ten new pathogenesis-related proteins, including a chitinase, in cotton roots and leaves 1–2 weeks after infection with a highly virulent type of VD. However they did not associate these proteins with hydrolytic activity on fungal cells.

Reinforcement of structural barriers

In parallel, cytochemistry of plant cell walls provided evidence that material deposited in cell paramural areas of infected tissues contained sugar molecules.



Figures 14–17. (14 and 15) Transmission electron micrographs of *Verticillium*-infected cotton roots. Ultrastructural observations of electron-dense compounds one day after infection of a resistant cultivar. A phloem parenchyma cell (14) displays numerous electron-dense compounds located close to mitochondria (mi), in the endoplasmic reticulum (er) or in the paramural area (arrows). Such compounds are also located in the vacuoles (v). In vessel-associated parenchyma cells (15), electron-dense compounds are located in large cytoplasmic vesicles (small arrows), in the paramural area (arrows) or in the main vacuole (v; double arrow). Figure 14: Bar = 0,5 μm; Figure 15: Bar = 0,25 μm. (16 and 17) Transmission electron micrographs of *Verticillium*-infected cotton roots. Ultrastructural observations of electron-dense compounds six days after infection of a resistant cultivar. The cytoplasm of cells producing electron-dense compounds (16) can be highly electron-dense. Disruption of the plasma membrane (arrows) favours close contacts between the electron-dense compounds and the cell wall. The cell wall (cw) adjacent to the paramural area of cell producing electron-dense compounds is loosened (double arrow) (17). Microfibrils of cellulose are associated with such dense material (large arrows); some of them are seen to be decorated with droplet-like structures (small arrows). Bar = 0,5 μm.



Figures 18–23. (18) Transmission electron micrographs of *Verticillium*-infected cotton roots, two days after inoculation of a susceptible cultivar. Spores of *Verticillium* (s) are located within a xylem vessel. No apparent aleration of the cytoplasm is observed within these fungal cells. Bar = 0,25 μm. (19–22) Transmission electron micrographs of *Verticillium*-infected cotton roots, three days after root inoculation of a resistant cultivar. Ultrastructural aspects of VD spores and mycelium in infected vessels. (19 and 20) The fungal elements (s) display various aspects of cell modifications such as membrane disorganization (19), coagulation of the cytoplasm (20, double arrow) and the retraction of the plasma membrane from the cell wall (20, arrows). Note that the fungal cell wall (fw) is apparently structurally intact. Bar = 0,5 μm. (21) A dense material coats both the secondary vessel cell wall (sw) and the fungal cell wall (arrows) of a germinating hyphae (h) (p: pit area). Bar = 0,5 μm. (22) The electron-dense material coating the vessel secondary wall (arrow) is also encircling (small arrows) a spore (s) located close to the pit area (p). Note the alteration of the fungal cytoplasm. Bar = 0,5 μm. (23) Transmission electron micrograph of *Verticillium*-infected cotton roots, three days after root inoculation of a resistant cultivar. Immunocytolocalization of β-1,3-glucans with an anti-β-1,3-glucan polyclonal antibody. The fungal cell wall (fw) is evenly decorated by gold particles. No apparent degradation of the fungal cell wall is observed, while the cytoplasm displays cellular modifications. Bar = 0,25 μm.

Vascular parenchyma cells have been shown to synthesize polysaccharides, including cellulose, pectin (not illustrated) and callose, contributing to the reinforcement of host cell walls (Kauss, 1994). Accumulation of callose in apposition layers of vessel contact cells of cotton plants in response to vascular fungi has already been described (Mueller et al., 1994). In addition, our study shows that callose accumulated in papillae differentiated in phloem intercellular and plasmodesmatal areas. Synthesis of high amounts of callose in plasmodesmatal regions may be involved in blocking the molecular exchanges between phloem cells, including movement of VD toxins. β-1,3-glucan synthase, known to be located in the plasma membrane, possesses a high activity in developing cotton fibers (Maltaby et al., 1979). One may suggest that after wounding or infection by pathogens, enhancement of the callose synthase activity is sufficient to explain the important accumulation of callose in roots two d.a.i. by VD.

Callose-containing material failed to be labelled by the laccase-gold probe, suggesting that phenolic compounds normally oxidized by this enzyme did not occur at these sites. In contrast, in egg-plants infected by *V. albo-atrum*, phenolics and proteins were involved in the composition of the deposited material (Benhamou, 1995) based on laccase gold labelling. Similarly, extensive deposition of suberin during the formation of a wound periderm was observed on potatoes infected by VD (Vaughn and Lulai, 1991). Suberin was also chemically identified in vessel coatings of tomato plants attacked by VD (Robb et al., 1989, 1991), but coating as a defense reaction was shown to be overcome by the pathogen (Gold and Robb, 1995). In our experimental conditions, suberin deposits were not detected in roots suggesting that this response is not involved in early defense reaction of cotton roots to VD infection.

Histochemical detection of lignin indicated that this polymer occurred in root vessel coatings, in agreement with a recent study on cotton that demonstrated the induction of the phenylalanine ammonia-lyase (PAL) activity by a VD phytotoxin (Dubery and Smit, 1994; Meyer et al., 1994). But in light of our data, there is no evidence of a role for lignin in cotton resistance at the root level.

In conclusion, the present paper shows that in cotton roots infected by VD, parenchyma cells in phloem and those adjacent to vessels were involved in early activation of plant defense. In infected resistant plants, reinforcement of host cell wall barriers, including deposition of polysaccharidic material in paramural areas, associated with terpenoid and phenolic synthesis were histochemically detected and ultrastructurally observed 2 d.a.i.; the collapse of VD spores and hyphae were seen since 3 d.a.i. Delay in reactions in the susceptible line, consistent with the observation reported by Harrison and Beckman (1982), (1) indicates that recognition and elicitation of defense mechanisms also occurred, but are modulated differently and (2) suggests that the resistant and the susceptible cotton plants have the capacity to localize vascular infection, but defense genes are triggered earlier in the resistant line.

These early structural responses did not prevent contamination of xylem vessels, but likely limited the lateral ingress of VD hyphae within the vascular tissues of resistant plants, accounting for the restriction of vascular colonization by the pathogen as also demonstrated on VD-infected tomatoes (Gold and Robb, 1995). Blocking the fungus in these reactive areas may favor contacts between the pathogen and plant fungitoxic molecules, including phytoalexins, thus increasing the efficacy of such local defense responses.

Acknowledgements

The authors kindly acknowledge Drs. D. Fernandez, J.F. Daniel (ORSTOM), and Dr. R. Bélanger (Université Laval, Canada) for critically revising the manuscript. Electron microscope observations were realized at the Laboratoire de Phytovirologie des Régions Chaudes (LPRC, CIRAD, Montpellier, France).

References

Bailey JA and Mansfield JW (1982) Phytoalexins. Blackie, Glasgow and London

Beckman CH (1987) The Nature of Wilt Diseases of Plants. APS Press, St Paul, Minnesota

Bell AA (1969) Phytoalexin production and *Verticillium* wilt resistance in cotton. Phytopathology 59: 1119–1127

Bell AA (1981) Biochemical mechanisms of disease resistance. Annual Review of Plant Physiology 32: 21–81

Bell AA (1992) *Verticillium* wilt. In: Hillocks JH (ed) Cotton Diseases (pp. 87–126) CAB International, Wallingford UK

Benhamou N (1995) Ultrastructural and cytochemical aspects of the response of eggplant parenchyma cells in direct contact with *Verticillium*-infected xylem vessels. Physiological and Molecular Plant Pathology 46: 321–338

Benhamou N, Lafontaine PJ and Nicole M (1994) Seed treatment with chitosan induces systemic resistance to *Fusarium* crown and root rot in tomato plants. Phytopathology 45: 1432–1444

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

Daayf F, Nicole M and Geiger JP (1995) Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. European Journal of Plant Pathology 101: 69–79

Daï GH, Andary C, Mondolot-Cosson L and Boubals D (1995) Histochemical responses of leaves of *in vitro* plantlets of *Vitis* spp. to infection with *Plasmopara viticola*. Phytopathology 85: 149–154

Dubery IA and Smit F (1994) Phenylalanine ammonia-lyase from cotton hypocotyls: Properties of the enzyme induced by a *Verticillium dahliae* phytotoxin. Biochemica and Biophysica Acta – Protein Structure and Molecular Enzymology 12: 24–30

El-Zik KM (1985) Integrated control of *Verticillium* wilt of cotton. Plant Disease 69: 1025–1032

Geiger JP, Rio B, Nandris D and Nicole M (1986) Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. I. Purification and some physico-chemical properties. Applied Biochemistry Biotechnology 12: 121–133

Gold J and Robb J (1995) The role of the coating response in Craigella tomatoes infected with *Verticillium dahliae*, races 1 and 2. Physiological and Molecular Plant Pathology 47: 141–157

Harrison NA and Beckman CH (1982) Time/space relationships of colonization and host response in wilt-resistant and wilt-susceptible cotton (Gossypium) cultivars inoculated with *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. Physiological Plant Pathology 21: 193–207

Hillocks RJ (1992) Hillocks JH (ed) Cotton Diseases. CAB International, Wallingford

Kauss H (1994) Callose synthesis. In: Smallwood M, Knox P and Bowles DJ (eds) Membrane-Specialized Functions in Plant Cells (pp. 609–663) JAI Press

Knox JP, Linstead PJ, King J, Cooper C and Roberts K (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta 181: 512–521

Liu RJ, Li HF and Chiu WF (1995) Detection of pathogenesis-related proteins in cotton plants. Physiological and Molecular Plant Pathology 47: 357–363

Mace ME (1978) Contribution of tyloses and terpenoid aldehyde phytoalexins to *Verticillium* wilt resistance in cotton. Physiological Plant Pathology 12: 1–11

Mace ME, Bell AA and Beckman CH (1976) Histochemistry and identification of disease-induced terpenoid aldehydes in *Verticillium*-wilt resistant and -susceptible cottons. Canadian Journal of Botany 54: 2095–2099

Mace ME, Bell AA and Stipanovic RD (1974) Histochemistry and isolation of gossypol and related terpenoids in root cotton seedlings. Phytopathology 64: 1297–1302

Mace ME, Stipanovic RD and Bell AA (1985) Toxicity and role of terpenoid phytoalexins in *Verticillium* wilt resistance in cotton. Physiological Plant Pathology 26: 209–218

Mace ME, Stipanovic RD and Bell AA (1989) Histochemical localization of desoxyhemigossypol a phytoalexin in *Verticillium dahliae*-infected cotton stems. New Phytologist 111: 229–232

Maltaby D, Carpita NC, Montezinos D, Kulow C and Delmer DP (1979) β-1,3-glucan in developing cotton fibers. Plant Physiology 63: 1158–1164

Meyer R, Slater V and Dubery IA (1994) A phytotoxic protein-lipopolysaccharide complex produced by *Verticillium dahliae*. Phytochemistry 6: 1449–1453

Mueller WC and Morgham AT (1993) Ultrastructure of the vascular responses of cotton to *Verticillium dahliae*. Canadian Journal of Botany 71: 32–36

Mueller WC, Morgham AT and Roberts EM (1994) Immunocytochemical localization of callose in the vascular tissue of tomato and cotton plants infected with *Fusarium oxysporum*. Canadian Journal of Botany 72: 505–509

Northcote DH, Davey R and Lay J (1989) Use of antisera to localize callose, xylan, and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. Planta 178: 353–366

Pezet R and Pont V (1990) Ultrastructural observations of pterostilbene fungitoxicity in dormant conidia of *Botrytis cinerea* Pers. Journal of Phytopathology 129: 19–30

Robb J, Poweel DA and Street PFS (1989) Vascular coating: A barrier to colonization by the pathogen in *Verticillium* wilt of tomato. Canadian Journal of Botany 67: 600–607

Robb J, Lee SW, Mohan R and Kolattukudy PE (1991) Chemical characterization of stress-induced vascular coating in tomato. Plant Physiology 97: 528–536

Shi J, Mueller WC and Beckman CH (1991) Ultrastructure and histochemistry of lipoidal droplets in vessel contact cells and adjacent parenchyma cells in cotton plants infected by *Fusarium oxysporum* f. sp. *vasinfectum*. Physiological and Molecular Plant Pathology 39: 201–211

Shi J, Mueller WC and Beckman CH (1992) Vessel occlusion and secretory activities of vessel contact cells in resistant or susceptible cotton plants infected with *Fusarium oxysporum* f. sp. *vasinfectum*. Physiological and Molecular Plant Pathology 40: 133–147

Vaughn SF and Lulai CL (1991) The involvement of mechanical barriers in the resistance response of a field-resistant and field-susceptible potato cultivar to *Verticillium dahliae*. Physiological and Molecular Plant Pathology 38: 455–465

Zhang J, Mace ME Stipanovic RD and Bell AA (1993) Production and fungitoxicity of the terpenoid phytoalexins in cotton inoculated with *Fusarium oxysporum* f. sp. *vasinfectum*. Journal of Phytopathology 139: 247–252