Lignification of Lesion Borders in *Rhizoctonia*-Infected Bean Hypocotyls

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ABSTRACT

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Lesions formed on bean (*Phaseolus vulgaris* L., 'Red Kidney') hypocotyls infected with *Rhizoctonia solani* are surrounded by autofluorescent cell walls resistant to maceration by cell wall-degrading enzymes. These lesion border cell walls stained positively for lignin and phenols. Lesion border walls were macerated by a mixture of cellulase and macerase after delignification, but not after extraction of calcium or fats and waxes. Peroxidase and polyphenoloxidase were active in young lesions in advance of fungal hyphae. Results suggest that lignification of cell walls may be an important factor in limiting lesion expansion in stem canker of beans.

Additional key word: resistance.

Lesions on bean hypocotyls caused by *Rhizoctonia solani* Kühn are rapidly contained and surrounded by cells with walls that are resistant to maceration by the fungal enzyme, endopolygalacturonase (endo-PG) (2). Young plants are susceptible to this pathogen, but by 3 wk become resistant to infection (3). Both lesion delimitation and resistance of older plants were suggested to result from failure of endo-PG to macerate calcium pectate in host cell walls. Stockwell and Hanchey (15) localized calcium at the ultrastructural level and did not find increased calcium in cell walls around lesions. An alternate explanation for lesion limitation is based on the observation that lesion border walls stained with toluidine blue, osmic acid, $KMnO_4$, and Schiff's reagent (9). These results suggest that increases in phenolic acids and/or lignin may render this area resistant to enzymatic degradation.

The purpose of the present research on *Rhizoctonia*-infected beans was to study changes in cell walls at the lesion border in situ and to evaluate the role of these changes in increased resistance to maceration and lesion containment.

MATERIALS AND METHODS

Plant material. *Phaseolus vulgaris* L., 'Red Kidney' seeds were planted in 10-cm^2 pots containing steamed soil (soil, peat, and perlite in a 2:1:1 mixture, respectively) and placed in a growth chamber under fluorescent and incandescent lights (25 ± 2 C, 14-hr photoperiod, 60 $\mu E\ m^{-2}\ s^{-1}$ at soil level). *R. solani*, isolate MAB, was provided by Dr. D. F. Bateman, North Carolina State University, and maintained in potato-dextrose agar slant tubes. Plants were inoculated with sclerotia as described previously (16). Lesions were classified as young (water soaking), intermediate (light tan color), or mature (brown and sunken) according to the descriptions of Van Etten et al (20). Each experiment described below was repeated at least three times and at least five samples were examined for each treatment. Results were consistently reproducible.

Autofluorescence. Fresh, freehand sections of inoculated and noninoculated tissues were examined by epifluorescence microscopy with a Leitz Dialux microscope equipped with a Ploemopak vertical illuminator, using 390–490 nm excitation with a BG 38 barrier filter. To observe the effects of calcium extraction, tissues were treated with 5 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) in 0.01 M PIPES buffer, pH 7.3, or PIPES buffer alone, for 6 hr at room temperature, rinsed with buffer, distilled water, and mounted on a glass slide in distilled water.

Phenol and lignin localization. Fresh, hand-sectioned tissues were treated with 1% (w/v) $FeCl_3$ in 0.1 N HCl, nitroso reagent, saturated aqueous phloroglucinol in 20% HCl, 0.025% (w/v) azure B in 0.1 M citrate buffer, pH 4.0 (8), 0.025% (w/v) toluidine blue in 0.1 M citrate buffer, pH 4.0 (11), or the Maule reagent (6). Sections from paraffin-embedded tissues were stained with either toluidine blue O or azure B.

Suberin localization. Fresh, hand-sectioned tissues were treated with 12 N KOH or saturated Sudan IV in 70% ethanol to stain lipophilic materials (6).

Light microscopy of polyphenoloxidase (PPO) activity. Segments of inoculated and noninoculated hypocotyls were placed in 0.5% L-DOPA (L-β-3,4-dihydroxyphenylalanine) in 0.67 M phosphate buffer, pH 7.0, or phosphate buffer alone (5). After a 1-hr incubation in the dark at room temperature, segments were rinsed and fixed in 3% glutaraldehyde in 0.05 M PIPES buffer, pH 7.0, for 6 hr. Tissues were slowly dehydrated in a graded ethanol and tertiary butyl alcohol series, infiltrated with heavy paraffin oil, transferred to paraffin, and infiltrated and embedded in Tissuemat. Eight-micron-thick serial sections were mounted on glass slides with Haupt's adhesive and counterstained with safranin-fast green (8). Reaction products appeared dark-brown without the safranin-fast green counterstain and rust-colored with it.

Electron microscopy of PPO activity. Segments from inoculated and noninoculated bean hypocotyls were fixed in 2.5% glutaraldehyde in 0.067 M PIPES buffer, pH 7.0, at 4 C for 1 hr and rinsed with cold PIPES buffer, followed by cold 0.067 M phosphate buffer, pH 7.0, containing 0.5% L-DOPA for 18 hr at 4 C. After infiltration with L-DOPA, tissues were transferred to freshly prepared L-DOPA substrate solution and placed in a 37 C oven for 1 hr. After incubation, tissues were rinsed with phosphate buffer, followed by PIPES buffer and postfixed in 1% OsO_4 in 0.067 M PIPES buffer, pH 7.0, for 1 hr at room temperature (5). Tissues were dehydrated in a graded ethanol series, embedded in Spurr's standard medium (14), sectioned with a diamond knife, and examined with an AEI-6B electron microscope without poststaining. Tissues were poststained with Reynold's lead citrate for 1 min to increase contrast for photomicroscopy. Controls consisted of tissues boiled for 10 min after glutaraldehyde fixation and tissues treated by the above procedure with phosphate buffer substituted for the L-DOPA solution.

Peroxidase localization. Inoculated and noninoculated bean hypocotyl tissue was fixed in 3% glutaraldehyde in 0.05 M Tris-HCl buffer, pH 7.2, for 1 hr at 4 C. After fixation, tissues were

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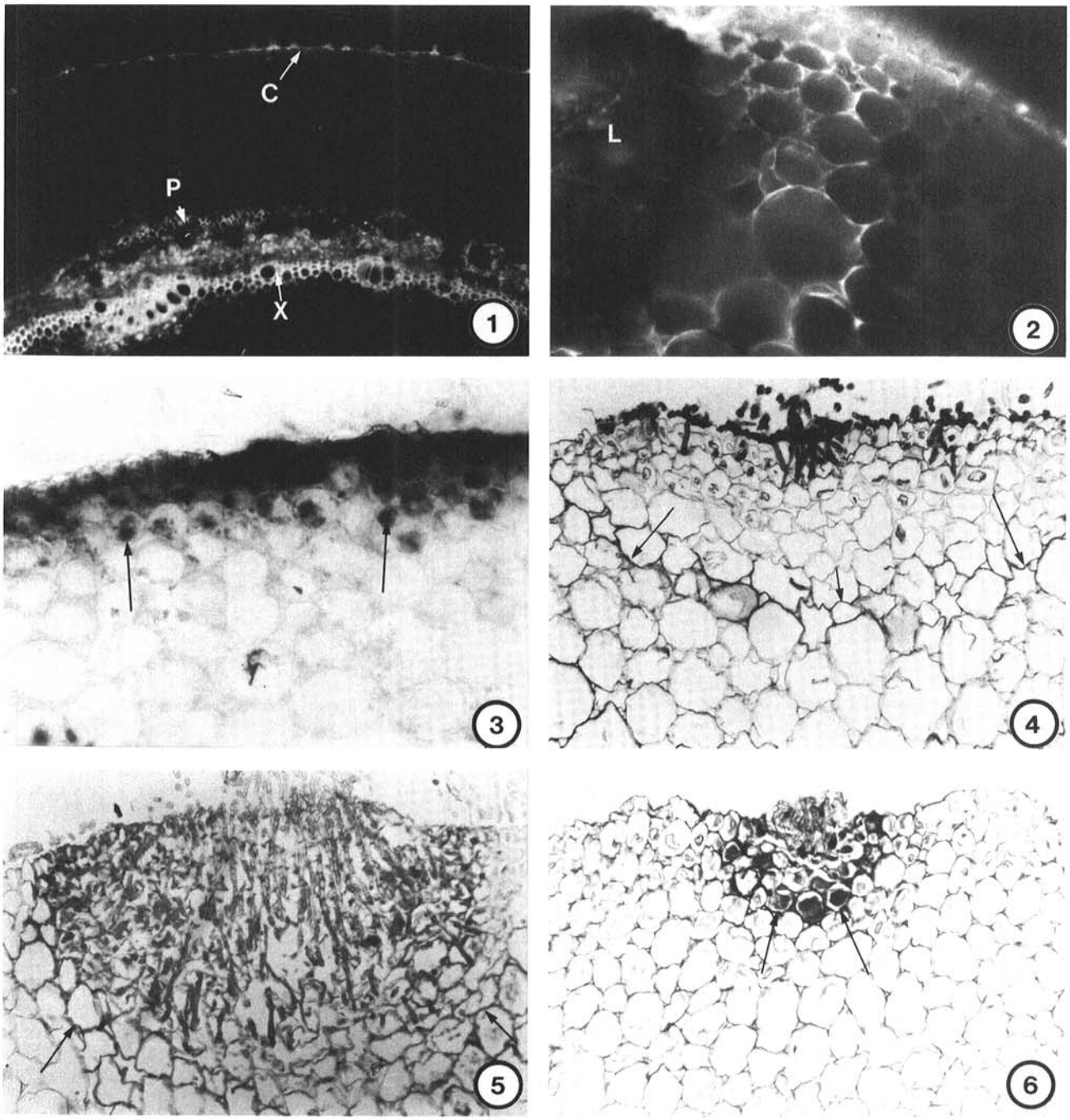
thoroughly rinsed with buffer overnight in a refrigerator. Tissues were then placed in a freshly prepared solution of 0.0005% diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer, pH 7.2, with 0.05% H_2O_2 and incubated in the dark for 37 C for 30 min. After incubation, tissues were rinsed three times with buffer and observed with a light microscope for the presence of a brown precipitate indicating oxidation of DAB. Controls consisted of boiling tissues in distilled water for 10 min after fixation, omitting H_2O_2 from the DAB incubation solution, or the addition of 20 mM 3-amino-1,2,4-triazole to the incubation solution to inhibit catalase (7).

Tissue extractions. Fresh transverse sections of inoculated and noninoculated tissues were placed in distilled water in a 50 C oven for 1 hr. Tissues were then stored in distilled water, 0.01 M PIPES buffer, pH 7.3, or treated by one or more of the following methods before incubation in cellulase and pectinase: 1) Removal of fats and waxes: Sections were immersed in chloroform for 10 min, then rinsed with distilled water. 2) Calcium chelation: Tissues were treated with 5 mM EGTA in 0.1 M PIPES buffer, pH 7.3, for 6 hr at room temperature, then rinsed with PIPES buffer (15). 3) Delignification: Sections were extracted for 1 hr with 5% sodium hypochlorite, rinsed for 15 min with distilled water, and treated with 1% sodium bisulfite for 5 min. Extraction was repeated twice (19).

Tissue maceration. After treatment of tissues by one or a combination of the above methods, tissues were placed in 0.1 M



Figs. 1–6. 1, Autofluorescence of noninoculated bean hypocotyl. The cuticle (C), xylem (X), and phloem fibers (P) fluoresced yellow. ×70. **2,** Autofluorescence of young lesion. Cells and cell walls around lesion (L) fluoresced yellow. ×150. **3,** Cross section of area of penetration beneath an infection cushion. Ferric chloride-positive compounds are present within cells (arrows). ×150. **4,** Toluidine blue O staining of intermediate lesion. Hyphae penetrated into second layer of cortex. Cell walls in advance of the pathogen were toluidine blue O positive (arrows). ×150. **5,** Toluidine blue O staining of intermediate lesion. Hyphae were not observed beyond the border of toluidine blue-positive cells (arrows). ×150. **6,** Cross section of young lesion incubated in L-DOPA and counter-stained with safranin-fast green. Hyphae penetrated into the first layer of cortical cells. Reaction product is present in cells in advance of hyphae (arrows). ×150.

citrate buffer, pH 5.0, for 30 min. Sections were incubated for up to 72 hr in either 5% macerase and 5% cellulysin (Calbiochem-Behring Corp., La Jolla, CA) in 0.1 M citrate buffer, pH 5.0, or in citrate buffer alone. After incubation, tissues were placed on glass slides and observed by epifluorescence, polarized light microscopy, or stained with 0.025% toluidine blue O and observed by light microscopy.

RESULTS

Autofluorescence. In fresh cross sections of noninoculated bean hypocotyls, vessel walls, fibers, and the cuticle fluoresced yellow (Fig. 1). In inoculated tissues, yellow autofluorescence occurred in walls as well as in the cytoplasm and intercellular spaces near penetrating hyphae. Autofluorescence extended two to three cells from the hyphae (Fig. 2). Treatment with a calcium chelator, EGTA, did not affect the pattern or intensity of autofluorescence in either inoculated or noninoculated tissue.

Phenol and lignin localization. All stains used for identification of lignin and phenolic compounds reacted positively with vessels. Epidermal and cortical cells in noninoculated tissues did not react.

As hyphae penetrated the epidermal cells, cytoplasm and cell walls in the area of ingress reacted with the nitroso reagent and were ferric chloride-positive (Fig. 3). Cortex cells adjacent to the invaded area did not react with these stains.

Hyphal penetration of the cortex coincided with the water soaking stage. At this stage, walls several cells distant from the pathogen stained blue-green with toluidine blue O, red with phloroglucinol, and purple-red with the Maule reagent (Fig. 4, Table 1). Reactivity to lignin stains coincided with the appearance of autofluorescence in those cell walls. Fungal hyphae were not observed in tissues beyond the zone of lignin positive cell walls (Fig. 5).

Suberin localization. Treatment of tissues with KOH or Sudan IV resulted in staining of the cuticle of inoculated and noninoculated tissues. Cell walls within and around lesions did not react with these stains.

Light microscopy of PPO activity. Reaction product was not observed in cell walls and cytoplasm of noninoculated tissues treated with L-DOPA or in those incubated in phosphate buffer alone.

In inoculated tissues incubated in L-DOPA before fixation, cytoplasm of cortical cells within the lesion contained a densely fibrillar material (Fig. 6). The reaction product also occurred in cell walls and intercellular spaces in and adjacent to the lesion. Reaction products did not occur in the absence of L-DOPA or in tissue boiled before L-DOPA incubation.

Electron microscopy of PPO activity. Epidermal and cortex cell walls within noninoculated tissues treated with L-DOPA appeared electron translucent. Reaction product was not evident in the cytoplasm of these cells or in tissues boiled before incubation in L-DOPA. Occasionally granules were present in intercellular spaces.

In inoculated tissue treated with L-DOPA, walls at or near lesion borders had increased electron density compared with lesion border walls fixed without incubation in this substrate (Figs. 7 and 8). Thus, increased electron density of cell walls at lesion borders was indicative of increased PPO activity rather than merely reflecting increased osmiophilic reactivity of these cell walls (9). Fungal hyphae had good cytoplasmic preservation; however, L-DOPA reaction product was not apparent. Cell walls beyond the lesion border did not show increased PPO activity. This method was satisfactory in localizing PPO activity near the lesion border in tissue where osmiophilic reactions normally change during lesion development (9) and confirmed results obtained from light microscopic localization of PPO.

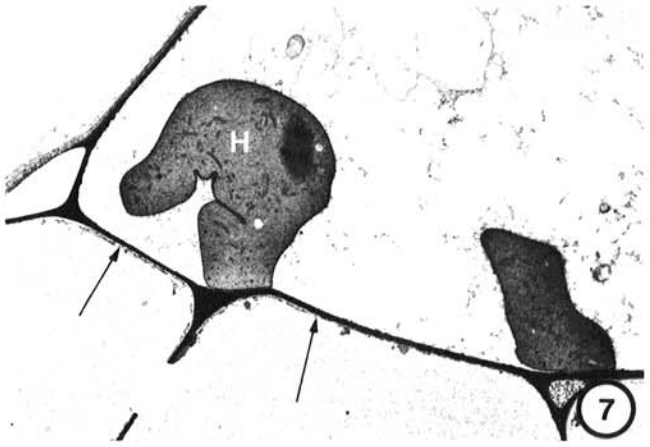
Peroxidase localization. The pattern of peroxidase localization was similar to that of PPO. In noninoculated tissues, fine brown

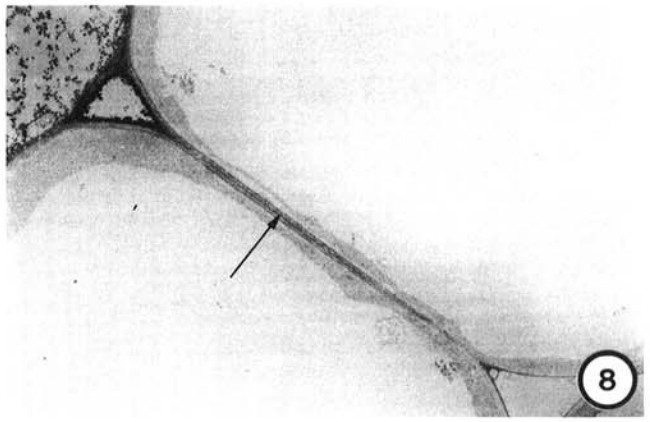
TABLE 1. Staining of inoculated tissues for phenolic compounds and lignin

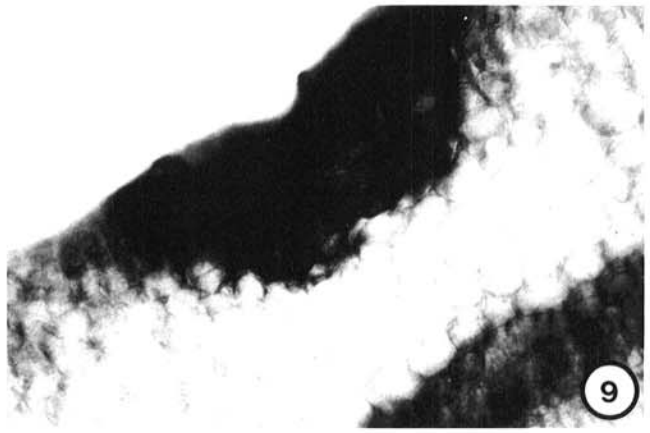
Stain	Stage of lesion development			
	Penetration	Young	Intermediate	Mature
Nitroso	+[a]	++	+++	+++
$FeCl_3$	+	++	+++	+++
Toluidine blue O	−[b]	++	+++	+++
Azure B	−	++	+++	+++
Phloroglucinol	−	+	++	++
Maule test	−	+	++	+++

[a] + indicates intensity of staining. + = few cells or cell walls stained with a positive reaction. +++ = all cells or cell walls within an area stained positively.

[b] − denotes that the stain reacted positively for lignified cell walls of the xylem vessels, but not for lesion or lesion border cell walls.





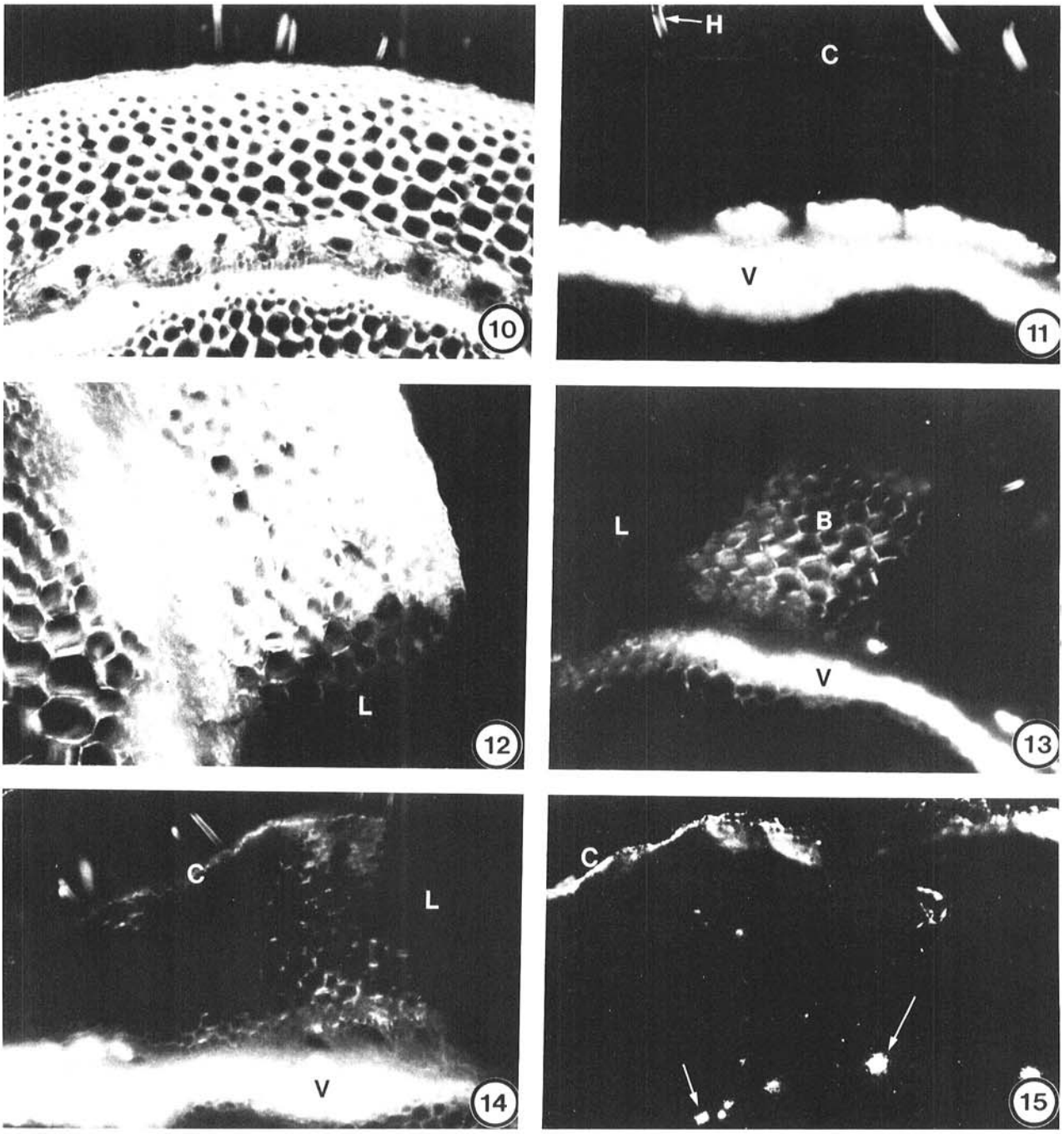


Figs. 7–9. 7, Electron micrograph of lesion border of intermediate lesion incubated in L-DOPA. Hyphae (H) are appressed to an electron-dense cell wall (arrows). ×2,400. **8,** Lesion border of intermediate lesion fixed without the addition of L-DOPA. Cell walls are electron dense (arrows) but the intensity is less than that of L-DOPA treated tissue. ×2,800. **9,** Freehand cross section of an intermediate lesion densely stained after incubation in DAB and H_2O_2. ×150.

precipitates were detected occasionally in the vascular tissue, but not in the pith, cortex, or epidermis. Peroxidase activity was detected in all stages of lesion development. Brown precipitates were observed in cell walls and cells around hyphae penetrating from infection cushions. As lesions developed and matured, the intensity of staining increased. The area demonstrating visible peroxidase activity consisted of the vascular tissue and lesion and extended three to five cells from the lesion border (Fig. 9). The addition of a catalase inhibitor, 3-amino-1,2,4-triazole, to the DAB incubation solution did not alter the pattern of peroxidase localization. The intensity of reaction product in tissues was similar to untreated tissues. The brown reaction product was not observed in tissues when H_2O_2 was omitted from the DAB incubation solution, or when inoculated or noninoculated tissues were boiled before incubation.

Tissue maceration. In noninoculated tissues, cell walls of the epidermis, cortex, vascular tissue, and pith were birefringent (Fig. 10). Incubation in macerase and cellulysin resulted in a loss of birefringence and concomitant maceration within 12–24 hr. The vessels, fibers, and the cuticle remained birefringent (Fig. 11).

Birefringence was not observed within lesions (Fig. 12). Treatment of inoculated tissues with macerase and cellulysin resulted in a loss of birefringence in cell walls of the pith, cortex, and epidermal cells surrounding lesions. However, lesion border cell walls as well as vessels and fibers in the vascular tissue remained birefringent and undigested (Fig. 13).



Figs. 10–15. Birefringence patterns of freehand cross sections of fresh material. **10,** Noninoculated bean hypocotyl tissue. ×70. **11,** Noninoculated tissue after incubation in macerase and cellulysin. Vascular tissue (V), cuticle (C), and epidermal hairs (H) remained intact. ×100. **12,** Lesion (L) and surrounding tissue. ×75. **13,** Infected tissue after incubation in macerase and cellulysin. Cells surrounding lesion (B) and vascular tissue (V) remain undigested. ×70. **14,** Infected tissue pretreated with EGTA before incubation in macerase and cellulysin. Cuticle (C), vascular tissue (V), and lesion border cells (B) remained intact. ×80. **15,** Infected tissue pretreated with sodium hypochlorite and sodium bisulfite before incubation in macerase and cellulysin. Only the cuticle (C) and scattered vessels (arrows) remain intact. ×75.

Effect of selective extractions on autofluorescence, toluidine blue staining, and maceration. To determine the cell wall changes responsible for autofluorescence, toluidine blue staining, and failure of maceration, segments were extracted with chloroform, EGTA, or sodium hypochlorite and sodium bisulfate. Removal of fats and waxes with chloroform resulted in a slight decrease in cuticular autofluorescence, but vascular tissues and lesion border cell walls remained autofluorescent. After incubation of these tissues in cellulysin and macerase for 72 hr, lesion border cell walls remained birefringent and toluidine blue positive.

Treatment of lesions with EGTA did not alter birefringence, autofluorescence, or toluidine blue staining of cell walls around lesions. Lesion border cell walls in EGTA-treated tissues remained birefringent and undigested after incubation in cellulysin and macerase for 72 hr (Fig. 14).

After extraction of lignin from lesion tissues, walls around lesions remained birefringent, but these walls were toluidine blue and nitroso negative, and autofluorescence was absent. After incubation in macerase and cellulysin for 12–24 hr, delignified tissues showed loss of birefringence and complete cell wall degradation (Fig. 15).

DISCUSSION

Stem canker of beans is cited frequently as an example of a disease in which calcium is important in resistance. A dialyzable, heat-stable inhibitor of endo-PG was found in lesions and was precipitated with oxalate (2). Calcium was also shown to prevent hydrolysis of sodium polypectate or potato tubers by endo-PG. ^{45}Ca accumulated in and around lesions on bean hypocotyls. Older beans, not normally susceptible to *R. solani* or to digestion by its purified enzyme, endo-PG, had a higher calcium content than young, susceptible plants (3,4). These results formed the basis for the hypothesis that infection and/or ingress by the pathogen was facilitated by endo-PG and occurred only through walls low in calcium pectate (3).

Stockwell and Hanchey (15) tested this hypothesis by localizing calcium in situ in lesion border walls and in cell walls of older plants. Their results confirmed the increased calcium content in cell walls of older plants, but not in lesion borders of young plants. In the present study, EGTA pretreatment to remove calcium from cell walls did not permit digestion of these walls with the macerase-cellulysin mixture. Stockwell and Hanchey (16,17) also showed that removal of the cuticle of older plants permitted lesion development despite increased cell wall calcification. Thus, on the basis of the present and previous results, there is no direct evidence for cell wall calcification as a resistance mechanism in this disease.

The results show that cell walls surrounding lesions of *R. solani*-infected bean hypocotyls become impregnated with phenolics and lignin-like materials. Although walls within the lesion were digested, lesion border walls remained intact, and the pathogen was confined to the lesion. Cell walls outside the lesion, with the exception of vessels and fibers in the vascular system, were digested with a mixture of cellulysin and macerase, but the lesion border remained intact, autofluorescent, and reactive with stains for lignin and phenolics. Extraction of "lignins" permitted digestion of lesion border walls. The possibility that the extraction technique altered other cell wall polysaccharaides was not examined. These results suggest that lignin and/or phenols prevent digestion of the lesion border walls and are impermeable to enzymes such as the endo-PG produced by the pathogen.

In another study, increases in phenol oxidase, catalase, and peroxidase were not found in extracts of young lesions caused by *R. solani* on beans, although they did increase in intermediate and mature lesions (10). This suggested that these enzymes were not significant in limitation of lesion expansion. However, the in situ localizations of these enzymes show increases only in a narrow band of cells near the lesion border. This change may not have been detectable in previous studies of tissue extracts.

Morphological barriers are commonly suggested as factors in disease resistance or limitation of spread of pathogens within plants. Types of barriers include papillae, callose depositions, suberin, silicon, lignin, and calcium (1,12,18). Although these structures can be demonstrated to occur in infected tissue, evidence that a barrier alone is sufficient to prevent ingress is usually lacking. Other factors such as the accumulation of phytoalexins or the failure of the pathogen to produce toxins or enzymes necessary for disease development, may also be involved (4,13).

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