Staining Reactions of the Tissue Bordering Lesions Induced by Wounding, Tobacco Mosaic Virus, and Tobacco Necrosis Virus in Bean

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ABSTRACT

Necrotic lesions were produced on the primary leaves of *Phaseolus vulgaris* 'Pinto' and 'Prince' by inoculation with tobacco mosaic and tobacco necrosis viruses, and through chemically and mechanically induced wounding. During lesion development histochemical tests were made to determine whether changes in the cell wall composition occurred in the associated tissue. Positive reactions were noted for lignin, suberin, and callose in cells at the border of mechanical lesions and at later periods for virus lesions. While changes in cell wall composition were recorded in tissue adjacent to localized virus infections, the staining reactions were difficult to interpret and to relate to any resistance mechanism.

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The spread of plant viruses in hypersensitive hosts is limited by a resistant zone of cells which develop at the periphery of local lesions (12, 21, 26). Secondary cell wall thickening which has been observed to occur within the resistant zone may be a factor in localizing the virus (11, 22, 24, 25, 26). Kimmins and Brown (3, 16) have reported an increased synthesis of cellulose in the primary leaves of Pinto bean infected with tobacco mosaic (TMV) or tobacco necrosis virus (TNV); however, the nature of the matrix material associated with the cellulose in the secondary thickening is uncertain.

Weintraub and Ragetli (23) observed that the staining reaction of cells at the lesion's periphery in *Nicotiana glutinosa* infected with TMV indicated that there may be some abnormality in the cellulose composition of the walls. Wu et al. (25), Wu and Dimitman (26), and Hiruki and Tu (11) have used the fluorochrome aniline blue to study the marginal tissue of virus lesions and concluded that the β-1,3-glucan callose, is present. Wu (24) has also obtained evidence for lignins in the resistant zone around lesions resulting from infection by a very mild strain of tobacco mosaic virus (TMV-VM) on *Nicotiana glutinosa*.

Histochemical studies of the lesion and adjacent tissue carried out in our laboratory over the past several years have emphasized the very difficult nature of interpreting the staining reaction of this tissue and identifying the cell wall matrix materials. The following paper is a summary of our results from histochemical studies of the lesions of TMV on Pinto and TNV on Prince.

MATERIALS AND METHODS.—The germination and growing conditions for *Phaseolus vulgaris* 'Pinto' and 'Prince' were identical to those previously described (14). The preparation of inocula and the method of inoculation onto the primary leaves of bean with either TNV or TMV have also been reported (14, 15). Mechanical lesions were produced by puncturing the upper surface of a primary leaf with a dissecting needle. Chemical lesions were obtained by spraying the primary leaves with a 1% (w/v) solution of hydrated copper sulfate.

Histochemistry.—Primary leaves were harvested at 24-hour intervals up to 144 hours post-treatment. The excised leaves were deribbed and then decolorized by placing in boiling 95% ethanol for 3-5 minutes. Divided leaves were briefly immersed in 50% ethanol and either transferred to water for observation (controls) or stained.

1)—Aniline blue (visible light).—Five to ten drops of 0.5% (w/v) aniline blue (C.I. No. 42755) in 50% ethanol were placed on the leaf sample for 4-8 hours and rinsed with 50% ethanol. Callose deposits were stained blue (13).

2)—Aniline blue (ultraviolet light).—The leaf samples were flooded with 0.01% (w/v) aniline blue in 1/15 M K_3PO_4, pH 9.5, for 2-3 hours at 20 C and rinsed with the phosphate buffer. Callose deposits in the plant tissue are believed to be detected as bright yellow fluorescence under ultraviolet light (5, 6, 8).

3)—Phloroglucinol.—The tissue was treated with up to 1 ml of 1% (w/v) phloroglucinol in 70% ethanol for 3-6 minutes, followed with 11 N HCl. After 2-5 minutes, lignin containing aromatic aldehydes stain red-violet. However, this reaction may be weak or absent with lignins containing high amounts of syringyl propane units. Suberin may also react with this stain to produce a pink color (9).

4)—Chlorine-sulfite.—Tissue samples were rinsed with distilled water and flooded with a fresh saturated solution of $Ca(ClO)_2$ (acidified with 11 N HCl) followed by 1% Na_2CO_3. In the presence of lignins, a bright red color develops, gradually fading to a brownish hue in 30-45 minutes (19).

5)—Mäule stain.—Leaf tissue was rinsed with water, flooded with 1% (w/v) $KMnO_4$ for 15 minutes, rinsed twice with water and flooded with 2% (w/v) HCl for 5 minutes. Following a further water wash, 2N NH_4Cl was added. Syringyl-lignins give a purple-red to brown response (2).

6)—Potassium permanganate.—Samples of leaf tissue were flooded with a solution of 1% $KMnO_4$ for 15 minutes and rinsed with water. Precipitated $KMnO_4$ appears very dark and is claimed to be quite specific for lignin (10).

7)—Potassium hydroxide.—Leaf samples were rinsed with distilled water for 3-5 minutes, covered with a cover slip and up to 1 ml of 12 N KOH allowed to diffuse under the cover slip. A color in the yellow to red range will

develop in 5-10 minutes in the presence of suberin. A dull brown staining reaction may occur with lignin material (9).

8)—Sudan IV.—Several drops of a saturated solution of Sudan IV (C.I. No. 26105) in 70% ethanol were added to the tissue for 15 minutes following by quick rinses in three changes of 50% ethanol. Lipid containing material such as suberin, and cutin, stain pink to orange (13).

9)—Gentian violet.—Samples of the leaf tissue were flooded for 5 minutes with a solution of 1% (w/v) gentian violet (C.I. No. 42555) in 70% ethanol plus 1 or 2 drops of concentrated ammonium hydroxide. The tissues were rinsed with 9% HCl for 2 minutes or until clear. The leaf samples were then flooded with 95% ethanol for 10 minutes and rinsed with ethanol and water. Suberized areas stain blue or purple (20).

10)—Iodide-sulfuric.—The tissue was treated with up to 1 ml of potassium triiodide solution for 15 minutes. A cover slip was added and a drop of 65% H_2SO_4 introduced and allowed to diffuse under the glass. Cellulose or hemicellulose stains dark blue; lignin stains orange to yellow (13).

11)—Zinc-chloro-iodide.—The leaf sample was treated with a few drops of zinc chloro-iodide (50 g of zinc chloride plus 16 g of potassium iodide in 17 ml of water). Large amounts of cellulose or hemicellulose stain blue. Lignin, cutin, suberin, or chitin stain yellow to orange and may mask cellulose (17).

12)—Ferric chloride.—A few drops of 10% (w/v) ferric chloride solution were added to the leaf tissue for up to 2 hours. A blue-green precipitate will develop in the presence of phenolic compounds and tannins (23).

Microscopy.—Observations on the stained tissue were made with a Zeiss Photomicroscope II (6.3/0.16 and 16/0.35 objective lenses). Photographic records were made using Kodak Ektachrome X (ASA 64) color film and Panatomic X (ASA 32) black/white film. For fluorescence microscopy, a Zeiss Universal microscope was used with an HBO 100-W, high - pressure mercury vapor lamp as the illuminator. The preparations were illuminated with reflected light passing through exciter filter BG 3/4 (transmission range 270-480 nm, peak transmission 320-444 nm) and barrier filter 47 or 53 (transmission of 10% at 470 nm and 530 nm, respectively). The objective lens was a Neofluar 10/0.30. Photographic records were made using Kodak Ektachrome high speed 135 (ASA 160) (3,200K) color film and Kodak Tri-X (ASA 400) black/white film.

RESULTS AND DISCUSSION.—*General lesion characteristics.*—Local lesions on primary leaves of bean, induced by TMV and TNV infections, were detected microscopically 40-50 hours after inoculation. These lesions appeared as small clusters of necrotic cells in the upper half of the leaf encircled by normal appearing tissue. Developing TMV-lesions on Pinto increased in diameter up to 96 hours, whereas TNV-lesions on Prince extended into adjacent healthy tissue in a less regular pattern and continued to enlarge for 144 hours or longer (Fig. 1).

The mechanical lesions, produced by puncturing the leaf surface, measured 0.2 mm in diameter. A necrotic band of tissue had formed around the puncture by 1-2 hours. Between 72-96 hours, a zone of wound periderm 0.05-0.10 mm wide was observed at the periphery of the

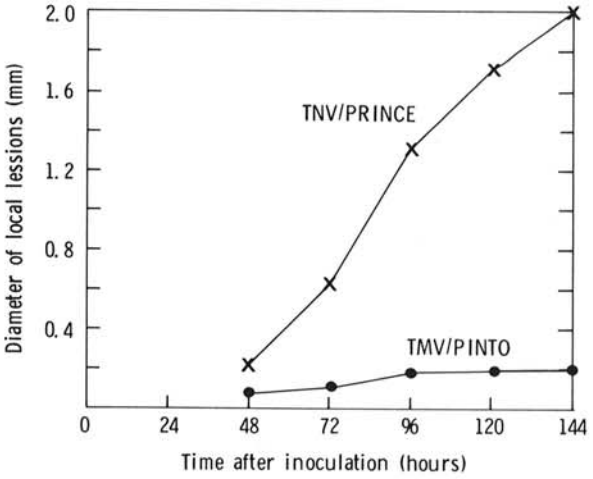

Fig. 1. The average size for lesions of tobacco necrosis and tobacco mosaic viruses on Prince and Pinto bean. Each point is derived from the measurement of seven lesions. Inoculations were done on the primary leaves of 10-day-old plants grown with a 16-hour light (9,000 lux)/8-hour dark cycle at 25 C.

necrotic cells. The cells in the periderm were enlarged mesophyll cell, relatively free of chloroplasts and subdivided by two or three transverse cell walls. A similar occurrence of periderm at a lesion border was reported by Wu (24) for mechanical and TMV-VM lesions on *N. glutinosa.* We did not, however, find a periderm associated with the chemical or virus induced lesions on Pinto or Prince.

Chemical lesions induced by copper sulfate developed within 3-4 hours, varied in size (0.1-0.3 mm) and were comprised of necrotic epidermal and palisade cells that appeared highly desiccated.

Histochemistry.—The staining reactions for virus and mechanical lesions are recorded in Tables 1, 2. The chemical lesions (24-144 hours) fluoresced strongly with aniline-blue and ultraviolet (UV) light but did not react with any of the other stains.

Whole mount preparations from virus, mechanical and chemical lesions treated with aniline-blue and observed with visible light did not at anytime give the blue staining reaction that indicates the presence of callose; however, all these preparations when viewed with UV light showed a strong yellow to white fluorescence, typical of callose (8), on the cell walls throughout the lesions area. At high magnifications, this fluorescence appeared to be continuous along cell wall segments of spongy- and palisade-parenchyma cells within the central zone of lesions and also in some healthy upper epidermal cells on the lesion border. This bright fluorescence was seen less and in fewer lesions at later postinoculation periods (96-144 hours).

The presence of callose-induced fluorescence was correlated with the timing of necrosis, appearing at 24 hours in mechanical and chemical lesions, but not until 40-50 hours in virus lesions. This suggests that callose deposition may be induced by the events of necrosis (26) and not by the wound produced to establish infection. This is a possibility which is supported by the study of

TABLE 1. Cell wall staining reactions for tobacco mosaic virus (TMV) and mechanical (M) lesions on *Phaseolus vulgaris* 'Pinto'

	Post-treatment time (hours)													
	24		48		72		96		120		144		168	
Stain	TMV	M	TMV	M	TMV	M	TMV	M	TMV	M	TMV	M	TMV	M
Aniline blue (visible)	[a]	–[b]	–	–	–	–	–	–	–	–	–	–	–	–
Aniline blue (U.V.)		+[c]	+	+	+	+	+	+	+	+	±[d]	±	±	±
Phloroglucinol		–	–	–	–	+	–	+	–	+	±	+	±	+
Chlorine-sulfite		–	±	–	±	–	±	–	–	–	–	–	–	–
Mäule		–	–	–	–	–	–	–	–	–	–	–	–	–
Potassium permanganate		–	–	–	+	–	+	–	+	+	±	+	±	+
Potassium hydroxide		–	±	–	±	–	±	–	±	–	±	–	±	–
Sudan IV		–	–	–	+	±	+	±	+	+	±	+	±	+
Gentian violet		–	–	–	+	–	+	–	+	+	±	+	±	+
IKI-H_2SO_4		–	–	–	–	–	–	–	–	–	–	–	–	–
Zinc-chloro-iodide		∓[e]	–	–	–	–	–	–	∓	∓	∓	∓	∓	∓
Ferric chloride		–	–	–	–	–	–	–	–	–	–	–	–	–

[a]Blank = no observation.
[b]– = staining reaction absent or identical for lesion and healthy tissue.
[c]+ = highly reproducible staining reaction of the cell wall.
[d]± = as for above (+), but not observed in all lesions.
[e]∓ = significant staining, but not associated with cell wall.

TABLE 2. Cell wall staining reactions for tobacco necrosis virus (TNV) and mechanical (M) lesions on *Phaseolus vulgaris* 'Prince'

	Post-treatment time (hours)											
	24		48		72		96		120		144	
Stain	TNV	M	TNV	M	TNV	M	TNV	M	TNV	M	TNV	M
Aniline-blue (visible)	[a]	–[b]	–	–	–	–	–	–	–	–		–
Aniline-blue (U.V.)		+[c]	+	+	+	+	+	+	+	+		+
Phloroglucinol		–	–	–	–	+	–	+	–	+		–
Chlorine-sulfite		–	±[d]	–	±	–	±	–	–	–		–
Mäule		–	–	–	–	–	–	–	–	–		–
Potassium permanganate		–	–	–	–	+	–	+	–	+		+
Potassium hydroxide		–	±	–	±	–	±	–	–	–		–
Sudan IV		–	–	–	–	+	–	+	–	+		+
Gentian violet		–	–	–	–	+	–	+	–	+		+
IKI-H_2SO_4		–	–	–	–	–	–	–	–	–		–
Zinc-chloro-iodide		–	–	–	∓[e]	–	∓	–	∓	∓		∓
Ferric chloride		–	–	–	–	–	–	–	–	–		–

[a]Blank = no observation. For TNV lesions at 144 hours, desiccation of the tissue made interpretation difficult.
[b]– = staining reactions absent or identical for lesion and healthy tissue.
[c]+ = highly reproducible staining reaction of the cell wall.
[d]± = as for above (+) but not observed in all lesions.
[e]∓ = significant staining, but not associated with cell wall.

Cohen and Loebenstein (4) where TMV infection of cucumber, was localized without concomitant necrosis and in the absence of any evidence for callose deposition.

A less conspicuous yellow band of autofluorescence was observed between 72-96 hours in the cell walls of non-necrotic, upper epidermal cells at the periphery of TMV and TNV lesions. Subsequent staining with aniline blue usually enhanced the fluorescence in these cells. In lesions viewed at later periods (96-144 hours), the autofluorescence in the epidermal cells was less evident and sometimes absent. A similar distribution of fluorescence in epidermal cells bordering virus lesions has been noted for TMV-U1 in Pinto (25) and PVM on Red Kidney bean (11). However, these authors only detected the fluorescent band after staining with aniline-blue. A new distribution of autofluorescence was observed in TMV lesions and mechanical lesions after 144 and 72 hours, respectively. Discrete yellow autofluorescence was noted in the cell walls of palisade and spongy parenchyma cells often extending more than ten cell diameters from the lesion edge. The intensity and quality of the yellow autofluorescence described above was similar to the autofluorescence we observed in xylem tissue. Currier (5) suggests autofluorescence of this type may be due to lignin.

Further evidence that the observed autofluorescence may be attributed to lignin was provided by the staining reactions of phloroglucinol and potassium permanganate. A small percentage of the TMV lesions examined responded to both stains in a narrow zone of

cells around the lesion 144-168 hours after inoculation. A more frequently observed but similar zone of cells was also stained around mechanical lesions after 72 hours. The staining reaction was often most noticeable in the radial walls of the cells. Unlike callose, lignification did not always accompany necrosis. A ring of cells bordering scar tissue was always found to react strongly with both phloroglucinol and potassium permanganate by 72 hours after the abrasion associated with inoculation.

The tests with Mäule's stain for lignin were negative in all the tissue examined. Chlorine-sulfite and potassium hydroxide also failed to stain the tissues of chemical and mechanical lesions but did give a weak positive reaction in a few cells at the borders of virus lesions between 48-96 hours.

The presence of suberin around TMV lesions was supported by positive staining reactions with Sudan IV and gentian violet. Time of occurrence and distribution of suberin material corresponded closely to the staining reactions observed for lignin. In addition to staining the cell walls, Sudan IV was sometimes taken up by the protoplasts of virus (TMV) lesion cells between 96-144 hours.

Zinc-chloro-iodide and iodide-sulfuric were used to identify sites of cellulose deposition but provided inconclusive results. Zinc-chloro-iodide produced a pale blue coloration in a band of cells surrounding mature virus (TMV, TNV) lesions and a few mechanical lesions (24 hours). However, the reaction appeared to be localized within the chloroplasts and not the cell wall region. Surrounding the band of blue-stained cells in virus lesions, a zone 10-30 cells wide was stained brown to grey by both staining reagents. In this instance also, the reaction appeared to be associated with the chloroplasts and not the cell wall. The successful identification of cellulose with iodine reagents depends on the composition of the cell wall. In the presence of suberin the reaction is absent or very weak (18), and this could be the explanation of our results.

Based on the ferric chloride reaction, phenolic compounds and tannins were not present in the cell wall region of any lesion tissue. This is contrary to the suggestion made by Bawden (1) but agrees with a previous observation made on *N. glutinosa* infected with TMV (23).

We have found that the cell walls of cells bordering both viral and nonviral lesions produced a similar response to the stains tested. There were indications for callose, lignin and suberin in both viral as well as mechanical lesions, although the timing of the response differed; deposition of callose, lignin and suberin being detected at an earlier stage in nonviral lesions.

It has been suggested (7) that modification of the cell walls by these matrix materials occurs as a general response to injury. Our observations indicate that they also accompany necrotic lesion formation in virus infected tissue and are associated with virus infection per se rather than the injury from inoculation alone, as we have previously reported (16). While this study provided information on the occurrence and nature of cell wall modifications around areas of localized virus infections, the complexity of these changes were possibly responsible for the unsatisfactory responses of the tissue to many of the stains. In view of this, whether the observed cell wall changes are responsible for virus localization, will require further investigation.

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