Hypersensitive Resistance: Determination of Lignin in Leaves with a Localized Virus Infection

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ABSTRACT

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The amount of lignin in dwarf bean leaves infected with the necrotic lesion-inducing tobacco necrosis virus was determined every 24 hr for 120 hr following inoculation and compared with the lignin levels in abraded leaves and untreated leaves. The results from examining changes in absorption due to ionization, and the yields of lignothioglycolic acid and phenolic aldehyde derivatives showed that between 24 and 120 hr more lignin was present in virus-infected and abraded leaves than in untreated controls. The relative yields of phenolic aldehydes indicated that the lignin recovered from the three treatments was similar in structure. The amount of lignin in dwarf bean leaves with localized infections caused either by tobacco necrosis, tobacco mosaic, or southern bean mosaic viruses reached higher levels than in abraded tissue and was correlated with the size and number of necrotic lesions. Therefore in addition to abrasion, virus-induced necrosis also was associated with increased lignin and may have resulted from secondary injury to the tissue as infection progressed. It is concluded that lignin is a constituent of the secondary cell wall thickening induced by wounding and postulated to be a barrier to spread of the virus.

Additional key words: localization, hypersensitivity, resistance to plant viruses.

Esau (2) suggested that wound healing is a normal part of the symptomology of plant virus infections and it has recently been proposed that the healing process may produce a zone of tissue which inhibits cell-to-cell spread of virus in localized infections (10, 14). The nature of the barrier to virus spread has not been determined, but may be related to the secondary cell wall thickening that has been observed at the periphery of local lesions (7).

From histochemical studies there is evidence for callose (3, 7, 15), lignin (3, 14), and suberin (3) in the cell walls of tissue bordering local lesions. However, staining reactions in these virus-infected tissues are not always satisfactory (3) and we have found this particularly to be true for lignin.

In this investigation, in order to obtain more reliable data for lignification in leaves with a localized virus infection, a lignin-containing fraction was isolated and its lignin content estimated by ionization difference spectra and gas-liquid chromatography of the nitrobenzene oxidation products.

MATERIALS AND METHODS

Plants and viruses.—Conditions used for germination and growth of *Phaseolus vulgaris* L. 'Prince' and 'Pinto' were the same as described previously (8). The preparation of tobacco necrosis (TNV), tobacco mosaic (TMV), and southern bean mosaic (SBMV) virus inocula and the inoculation procedures also have been reported (9, 11).

The primary leaves were deribbed and weighed. For a dry weight determination 10 leaves were weighed and oven-dried (90 C, 48 hr). The remaining leaves were extracted with hot 80% ethanol until pigment free, then macerated in a Waring Blendor with hot distilled water for 5 min. The homogenate was boiled in distilled water for 15 min, centrifuged, and the pellet was washed three times with hot water and re-extracted with ammonium oxalate (0.5% at 6-70 C for 12 hr). The residue collected by centrifugation was washed with water and extracted with boiling benzene/95% ethanol (1:1, v/v) for 1 hr. The insoluble fraction collected by filtration was washed with ethanol, acetone, and diethyl ether, then dried in a rotary evaporator to give a finely divided white powder of extractant-free lignin-containing material (LCM).

Preparation of lignothioglycolic acids.—These were prepared by the procedure of Freudenberg et al. (4). A 0.5-g portion of each LCM sample was treated with 15 ml of anhydrous thioglycolic acid containing 0.9 ml boron trifluoride for 3 days at 20 C, then for 16 hr at 70 C. The precipitate obtained by acidifying the reaction mixture was freeze-dried and weighed.

Spectrophotometric assay in dimethylformamide.—Lignothioglycolic preparations were leached with N, N-dimethylformamide. Any undissolved material was collected by centrifugation, dried, and weighed. Each supernatant solution (0.1 ml) was diluted to 5.0 ml with dimethylformamide and the absorbance at 278 nm was determined.

Ionization difference spectra.—Samples of LCM (0.5

g) were extracted with 30 ml of 2% NaOH for 48 hr at room temperature, centrifuged, and re-extracted with 40 ml of 4% NaOH at 70 C overnight. The NaOH extracts and washes were pooled and acidified to pH 1.5 with 5 N HCl. The precipitates collected by centrifugation were dissolved in aqueous pyridine (10%), freeze-dried, and the differences in their spectral properties at pH 7.0 and 12.0 were determined (5). Samples were dissolved in 1 ml of 2% NaOH, adjusted to pH 8.5 - 9.0 with phosphoric acid and diluted to 5 ml with phosphate buffer (0.05 M, pH 7.0). Aliquots (2.4 ml) were taken from these solutions and diluted to 10 ml with either 0.05 N NaOH (to give a pH of 12.0) or 0.05 M phosphate buffer (pH 7.0). The difference spectra of these solutions were recorded from 230 to 450 nm by reading the pH 7.0 samples directly against those at pH 12.0.

Preparation and estimation of the phenolic aldehydes.—The LCM samples were oxidized with alkaline nitrobenzene according to a modification of the Stone and Blundell method (12). To 0.2 g of LCM in a stainless steel combustion chamber, were added 0.8 ml of redistilled nitrobenzene and 10 ml of 2 N NaOH. This was heated (with shaking) for 2-5 hr at 175 C. The bomb then was cooled rapidly and the reaction mixture was rinsed out with distilled water. Insoluble material was removed by centrifugation and the supernatant was cooled to 4 C to allow excess nitrobenzene and azobenzene to separate. The resulting clear solution was acidified with HCl to pH 1.5 and extracted continuously with diethyl ether until all phenolic substances had been removed. Ether extracts were pooled and evaporated to dryness.

The aldehyde products were estimated by gas chromatography using a flame ionization detector and a glass column (93.75 cm long, 2.0 mm internal diameter) of 246/177-μm (60/80-mesh), 2, 6-diphenyl-p-phenylene (Tenax-GC; Applied Science Laboratories, Inc., State College, PA 16801. USA). A discontinuous temperature program was used (120 to 210 C at 10 C/min; 210 to 240 C at 5 C/min; 240 to 300 C at 10 C/minute) with a carrier gas (N_2) flow rate of 15 ml/min.

RESULTS

Lignin content in abraded and virus-inoculated dwarf bean leaves.—The primary leaves of 11-day-old Prince plants were treated in one of the following ways: (i) inoculated with TNV at 350 μg nucleoprotein/ml which produced an average of 224 lesions per leaf, (ii) abraded (virus omitted from the inoculum), and (iii) left noninoculated (control plants). There were 360 plants in each treatment. Samples of 120 primary leaves were harvested at 24-hr intervals for 120 hr from the time of treatment. The ionization difference spectra of extracts from control and abraded plants (Fig. 1) show two major peaks, one at approximately 255 nm and the other at 300 nm, which are characteristic of the phenolate moiety of simple substituted aromatic hydroxyl compounds (1). In samples from the virus-infected leaves at 72 hr, a third maximum was detected between 350-400 nm (Fig. 1-B). This also was present in samples from control and abraded leaves by 96 hr, although to a lesser extent, and probably is due to phenolic hydroxyl groups associated with carbonyl groups, carbon-carbon double bonds, or biphenyl groups. The presence of α-dicarbonyl groups may be the cause of reduction in the 300-nm difference curve maximum observed in the virus treatments at 96 and 120 hr.

The higher absorbtivity differences between 350-400 nm in the virus treatments, suggest that increased synthesis of a conjugated phenolic polymer (such as lignin) had occurred earlier and to a greater extent than in leaves with abrasion wounds.

The formation of lignothioglycolic acid derivatives is a good indication for both the presence and the amount of lignin in herbaceous plants. The yield of lignin (as lignothioglycolic acid) from leaves of 11-day-old control plants was 0.75% of the dry weight and increased to 4.69% by 16 days (Table 1). Where wounding had occurred (abraded leaves), lignification was greater at 24 hr than in the control leaves, and at 120 hr the yield was 7.9% of the dry weight, higher by a factor of 1.68 than that from nonabraded tissue. In the virus-infected leaves at 48 hr (prior to lesion formation), the yield had increased to 3.34%. Between 48 and 72 hr, when lesions had developed, the lignin content had more than doubled to 8.58% and increased further to 19.59% at 120 hr; this was 4.17 times that of control plants and 2.48 times that of the abraded ones.

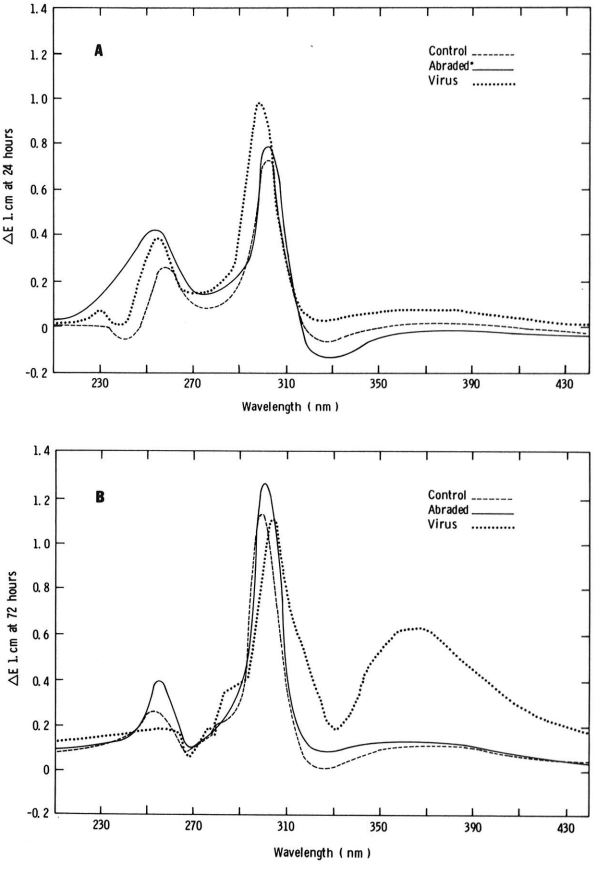

Fig. 1-(A, B). The ionization difference spectra for **A**) 24-hr and **B**) 72 hr extracts from control, abraded, and TNV-infected leaves of *Phaseolus vulgaris* cultivar Prince.

Similar results were obtained from the absorbance in N, N-dimethylformamide which at 278 nm is proportional to the amount of lignin thioglycolic derivative present (Table 1). At 120 hr the yield from abraded plant leaves was 2.14 times that from the control leaves. Virus-infected leaves contained 6.49 and 3.04 times the amount from control and abraded leaves, respectively.

The yield of phenolic aldehydes from the nitrobenzene oxidation provided more conclusive evidence of lignification. In samples from control plants all three aldehydes increased in amount during the experiment (Table 2), but the increase was much larger in samples from the abraded and virus extracts. In extracts from abraded leaves, yields of the aldehydes increased 24 hr after wounding and a similar increase was found in the virus-treated plants. Between 24 and 120 hr yields of aldehydes from the virus-infected plants exceeded those

TABLE 1. Determination of lignin in extracts from control, abraded, and TNV-inoculated Prince bean leaves

Treatment and postinoculation time (hr)		Yield of lignin-containing material (%, dry wt basis)	Yield of lignothioglycolic acid (%, dry wt basis)	Absorbance[a] of lignothioglycolic acid in N, N-dimethylformamide
Control	0	31.80	0.75[b]	0.20[b]
	24	32.01	0.86	0.30
	48	32.06	1.83	0.72
	72	32.87	2.13	0.87
	96	33.18	2.66	1.23
	120	34.47	4.69	2.72
Abraded	0	31.13	0.78	0.21
	24	38.53	1.54	0.59
	48	40.66	2.47	1.04
	72	42.61	3.03	1.48
	96	43.47	4.20	2.42
	120	44.85	7.90	5.81
TNV[c]	24	42.24	1.30	0.46
	48	43.76	3.34	2.62
	72	49.90	8.58	8.06
	96	55.24	14.18	14.88
	120	65.21	19.59	17.65

[a]Absorbance at 278 nm of lignothioglycolic acid preparation in N, N-dimethylformamide. Expressed as absorbance units per milligram dry weight of plant material.

[b]Results are the mean from duplicate lignin-containing samples derived from 60 plants except for those at 0 hr and 72 hr which are the mean of four determinations from lignin-containing material obtained from two separate experiments.

[c]The TNV inoculum contained 350 μg nucleoprotein/ml and at 120 hr had produced an average of 224 lesions (mean diameter, 1.8 mm) per leaf.

TABLE 2. Yield of nitrobenzene oxidation products from control, abraded, and TNV-inoculated Prince bean leaves

Treatment and postinoculation time (hr)		Yield[a] (mg/g dry weight) of:		
		p-hydroxybenzaldehyde	vanillin	syringaldehyde
Control	0	0.10	0.54	0.17
	24	0.21	0.38	0.28
	48	0.13	0.53	0.23
	72	0.32	0.35	0.48
	96	0.33	0.66	0.32
	120	0.54	0.51	1.10
Abraded	24	0.28	0.65	0.18
	48	0.39	0.67	0.45
	72	0.41	0.47	0.69
	96	0.54	1.14	0.51
	120	0.65	0.70	1.62
TNV-inoculated	24	0.20	0.70	0.24
	48	0.42	0.86	0.29
	72	0.62	0.92	0.54
	96	1.21	1.30	0.58
	120	1.32	1.17	2.21

[a]Yields are averaged from two determinations except for those of the control 24 hr group which were based on a single analysis.

TABLE 3. Determination of lignothioglycolic derivatives in extracts from southern bean mosaic virus (SBMV)- and tobacco mosaic virus (TMV)- inoculated Pinto bean leaves

Treatment and postinoculation time (hr)		Yield of lignin-containing material (%, dry wt basis)	Yield of lignothio-glycolic acid (%, dry wt basis)
SBMV[b]	96	50.24	7.92[a]
TMV[b]	96	47.99	5.38
Abraded	96	44.08	3.73
Control	96	32.17	2.24
Control	0	28.93	0.88

[a]Yields are an average from duplicate lignin-containing material prepared from 55- to 70-gram (fresh weight) samples of primary leaves.

[b]The SBMV inoculum contained 120 μg nucleoprotein/ml and produced an average of 209 lesions/leaf with a mean diameter of 0.7 mm. The TMV inoculum contained 900 μg nucleoprotein/ml and produced an average of 186 lesions/leaf with a mean diameter of 0.2 mm.

TABLE 4. Effect of the degree of infection of dwarf bean leaves with tobacco necrosis virus (TNV) on the yield of lignin

TNV-inoculum concentration (μg/ml)	Lesions/leaf (avg. no.)	Yield[a] of lignothio-glycolic acid (%, dry wt basis)
350	209	13.60
35	85	8.59
3.5	47	7.72
2.8	18	5.70
1.75	13	5.62
Control-(Abraded)	0	4.53
Control-(Healthy)	0	2.41

[a]Yields are an average from duplicate lignin-containing samples prepared 96 hr after treatment from 30 plants per treatment. Average lesion diameter was 1.4 mm.

from abraded leaves.

There was a higher proportion of vanillin during the first 48 hr in both control and abraded extracts (11- to 12-day-old plants) which in the virus-infected plants persisted to 96 hr. By 120 hr, the proportions of hydroxybenzaldehyde, vanillin, and syringaldehyde were approximately 1:1:2 in all three treatments.

To compare these results with those of other virus-induced lesion and host associations, lignin content (as lignothioglycolic acid) was determined in Pinto bean leaves 96 hr after inoculation with either TMV or SBMV (Table 3). At this time, the TMV-induced lesions had stopped growing but the SBMV lesions were still enlarging. In the TMV-infected and SBMV-infected leaves, lignin content increased to 5.38% and 7.92%, respectively. For the TMV treatment this was 2.40 and 1.44 times, and for the SBMV treatment, 3.34 and 2.12 times that of control and abraded treatments, respectively.

Relationship between number of lesions and lignin content.—Primary leaves of Prince bean were inoculated with one of a series of TNV concentrations and the yield of lignothioglycolic acid was determined in leaves harvested 96 hr after inoculation (Table 4). With an average of 13 and 209 lesions per leaf, lignin material was 1.24 times and 3.00 times that of abraded tissue, respectively, showing that lignothioglycolic acid yields were related to the amount of necrotic tissue.

DISCUSSION

Results from difference spectra, absorbance measurements in N, N-dimethylformamide, lignothioglycolic acid isolation, and estimation of phenolic aldehydes formed by nitrobenzene oxidation show that lignin was isolated from all three groups of Prince plants, (Tables 1, 2; Fig. 1). Greater amounts were detected in abraded leaves than in leaves from untreated plants, showing that lignin was more rapidly synthesized in response to wounding. In TNV-infected leaves, although the amount of lignin at 24 hr was similar to that in leaves of abraded plants, all the techniques used for lignin estimation indicated that after 48 hr lignin levels in the virus-infected plants were higher than those resulting from the wounding effect of inoculation alone. Lesions induced by TNV increased in diameter from 0.2 mm at 48 hr to 1.8 mm at 120 hr; it is possible that, the increased disruption of the tissue by virus infection became significant enough to cause additional wound or injury-induced lignin synthesis. This suggestion is consistent with the correlation between lignothioglycolic acid yields and the number of TNV-induced lesions (Table 4) and also may explain the smaller yields obtained from SBMV- and TMV-infected Pinto (Table 3) in which the average lesion diameters were 0.7 mm and 0.2 mm, respectively, compared to 1.4 mm for TNV-induced lesions at 96 hr.

The nitrobenzene oxidation products were present in similar proportions in each of the treatments. This suggests that the newly synthesized lignin in abraded and TNV-infected leaves was structurally similar to the lignin present in the control leaves. One difference in the relative proportion of the aldehydes in the three treatments was the higher proportion of vanillin which persisted for a longer time in virus-infected plants. This is interesting because it has been suggested (6, 13) that a higher yield of vanillin is characteristic of an early phase of lignification. If this hypothesis is correct, the yield of vanillin from infected plant leaves correlates well with the more extensive wounding that is likely to occur in this treatment.

Comparison of the results for lignin assay by the

lignothioglycolic acid method with those reported previously that were obtained by histochemical methods (3), shows that the staining techniques were less-sensitive and in certain instances, unreliable. The increased lignification indicated by the yields of phenolic aldehydes obtained in this study were not detected in the same experimental system when Maule and phloroglucinol stains were used (3). A further inconsistency is noted with chlorine-sulfite and potassium hydroxide stains which both failed to indicate the presence of lignin before 48 hr and after 96 hr in lesions induced by TNV in bean.

In conclusion, the results indicate that lignification is associated with injury from the inoculation procedure and also with virus-induced necrosis. These observations support the view that a barrier to spread of the virus may be caused by the induction of secondary cell wall thickening which is induced by the inoculation wound (10, 11): they also are consistent with previous reports that the matrix material associated with the wall thickening contains lignin (3, 14).

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