

Wound-Induced Lignin Formation and Resistance to Cellulase in Oat Leaves

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ABSTRACT

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In previous papers we described the development of resistance to cellulolytic digestion of oat mesophyll cell walls following wounding and the role of wound ethylene in the development of resistance. This paper demonstrates that resistance is due to wound-induced lignification of the mesophyll cell walls. Peeling the lower epidermis from oat leaves induced the appearance of autofluorescence in the walls of mesophyll cells adjacent to the lesion. The walls remained autofluorescent after incubation in polar or nonpolar solvents, but were less autofluorescent after incubation in NaOH (1 N for 24 hr), NaOCl (5.25% for 24 hr), or chlorine/sodium sulfite solution (24 hr). An autofluorescent compound with similar solubility characteristics was present in fresh or wounded xylem and guard cell walls. These results are consistent with the hypothesis that peeling induces lignification of the mesophyll cell walls. Peeling induced an increase in

phenylalanine ammonia lyase (PAL) activity within 2 hr. Aminooxyacetic acid (AOA) and aminooxyphenylpropionic acid (AOPP) inhibited PAL activity and the development of resistance to cellulolytic digestion; ferulic acid, *p*-coumaric acid, or coniferyl alcohol partially reversed the latter inhibition. Peeling also induced an increase in peroxidase activity and induced the formation of new peroxidase isozymes; inhibition of ethylene synthesis or activity inhibited these changes. Compounds that inhibited PAL activity or ethylene synthesis or activity inhibited the appearance of autofluorescence and the development of resistance to cellulase. These results suggest that peeling stimulates PAL and peroxidase activity, the latter being caused by wound-induced ethylene synthesis, and that subsequent lignification of the cell walls confers resistance to cellulolytic digestion.

Additional key words: *Avena sativa*.

Lignification occurs in walls of specific plant cells, most notably in xylem and guard cell walls, during normal development (5). Lignin can also be formed in response to external stimuli; eg, wounding induces lignification in cucumber (6), sweet potato (20), and swede root (14) and exogenously supplied ethylene induces lignification in swede root (14).

Attack of leaf tissue by fungi can also induce lignification (21). *Peronospora parasitica*, a foliar pathogen, induced lignin

formation in the cell walls of radish root slices (3). Papillae induced on reed canarygrass (*Phalaris arundinacea*), by an inappropriate pathogen, *Helminthosporium avenae*, contained lignin (18,23). Lignified papillae formed when *Botrytis cinerea* attempted penetration of wheat leaves (15). The break in a host's barrier caused by the fungus could stimulate a wound response that leads to lignification (3), and therefore resistance to degradation (4). Since many pathogens enter plants through wounds or cause wounds during infection, studying responses of plants to wounding will add to understanding of the initial events in disease resistance.

The experiments reported here were undertaken to understand the nature and the cause of resistance to cellulolytic digestion induced by peeling the lower epidermis from oat leaves (8).

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MATERIALS AND METHODS

Methods for growing plant material, wounding, and quantifying resistance to cellulase (Cellulysin; Calbiochem-Behring, La Jolla, CA 92037) are described in reference (8). Oat (*Avena sativa* 'Victory') plants were grown on vermiculite in growth chambers for 2 wk at 24 C. Plants were grown on 24-hr photoperiods: 16 hr in the light (16,700 ergs/cm²/sec) and 8 hr in the dark. Peeled leaf segments (1 cm each) of the first leaf were floated 24 hr on test solutions and then were placed on 0.5% cellulase at pH 5.6 at 31 C for 2 hr. Digestion was quantified by measuring the change in absorbance at 760 nm due to the increased light scattering by

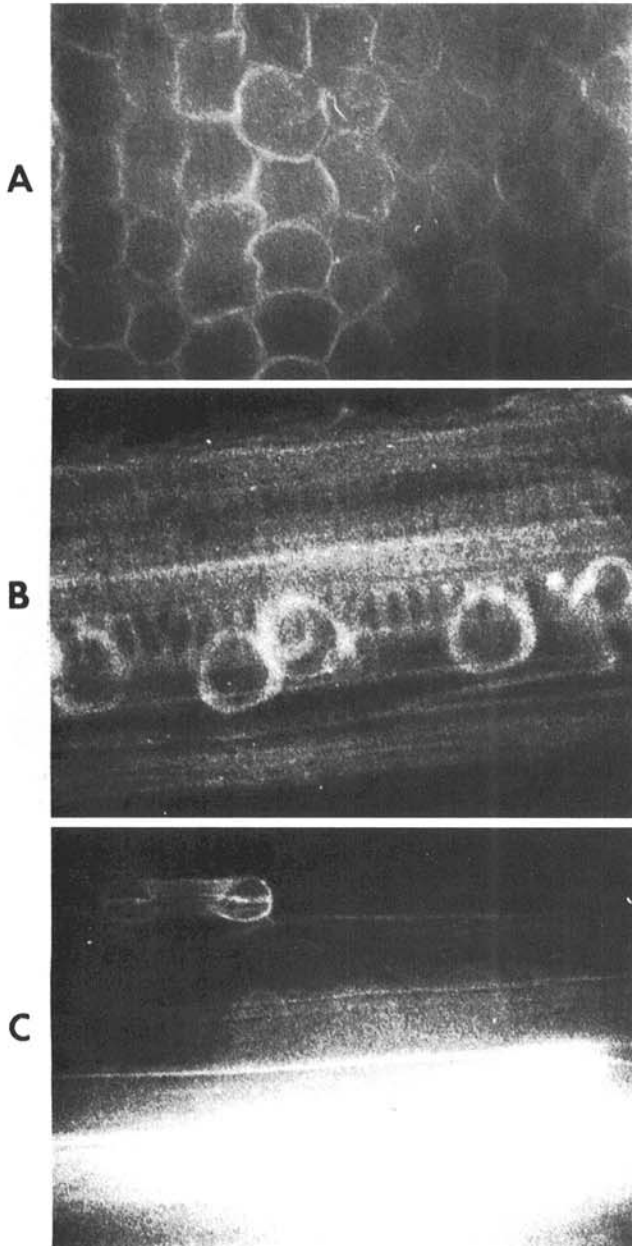


Fig. 1. Fluorescence micrographs of unstained, cleared (MeOH for 30 min) oat leaf tissue. (Excitation = 360 nm, final magnification $\times 400$.) **A**, An oat leaf was partially peeled (left side of photomicrograph) and floated overnight on water. Then the other half (right side) was peeled and the tissue was cleared and observed. The peeled and aged (wounded, left side) tissue contains a compound localized to the cell wall that autofluoresces blue (white in photograph). The unpeeled tissue (right side) contains little autofluorescent material in the cell walls. **B**, Autofluorescent xylem walls. Xylem from wounded (this picture) and unpeeled (not shown) tissue appear the same. **C**, Autofluorescent guard cell walls. Guard cell walls from wounded (this picture) and unpeeled (not shown) tissue appear the same. A bright, autofluorescing hair is visible in the lower, right quadrant.

protoplasts released by the cellulase (8). Results are reported as percent of freshly peeled, untreated tissue. Coniferyl alcohol was dissolved in MeOH containing 0.004% (w/v) of an emulsion stabilizer (19) (Pluronic F-68; BASF Wyandotte Corp., Wyandotte, MI 48192). This stock solution was diluted with water/Pluronic F-68, and controls were run on water/Pluronic F-68. There was no effect of the Pluronic F-68 on the resistance to digestion by cellulase.

Identification of lignin. Incident light fluorescence microscopy was used to study endogenous, autofluorescing compounds in fresh and wounded oat leaf segments. At hourly intervals after peeling, chlorophyll was extracted in MeOH, and the segments were observed in the fluorescence microscope to determine the timing of appearance of autofluorescence in the cell walls. Tissue was observed with a Leitz fluorescence microscope (excitation 360 nm). Fresh and wounded segments were cleared in MeOH and stained in Azure B (12), Cl/Na sulfite (15), Maule reagent (15), phloroglucinol/HCl (12), or toluidine blue O (12) and observed in a light microscope.

The solubility of this autofluorescing compound was studied by incubating segments for 24 hr in solvents in screw-capped vials.

Phenylalanine ammonia lyase. Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) activity was assayed *in situ* by using the procedure of Amrhein et al (2) based on the assay of Mitra et al (13) for enzymes that increase the lability of carbon-hydrogen bonds. Thirteen 1-cm-long peeled leaf segments were floated on 2 ml of distilled H₂O in a plastic petri dish (35 mm in diameter). Phenylalanine labeled with tritium (19 Ci/mmol; New England Nuclear, Boston, MA 02118) at carbons 2 and 3 was added to the dish (10 μ l, 2.5 μ Ci/dish) at indicated times. As the incubation progressed, samples (20 μ l) of the incubation medium were removed and pipetted onto filter paper disks (5.2 mm [0.25 inches] in diameter, Schleicher and Schuell, Keene, NH 03431) pinned inside plastic caps of glass mini-scintillation vials (5 ml). The vials were capped and placed in holes in a Styrofoam tray in a box containing dry ice and ethanol. The bottoms of the vials were in contact with the liquid. Heat was applied to the upper part of the vials with an incandescent lamp (300 W, 15 cm from the vials). Volatile molecules, including water, evaporated from the warm filter paper and condensed on the cold vial walls. After 1 hr (recovery of tritiated water = 85%) the caps with the filter paper disks were discarded, and 2.5 ml Econofluor (New England Nuclear) containing BBS-3 (5% v/v, Bio-Solv.; Beckman, Fullerton, CA 92632) was added to each vial. Radioactivity was counted in a Beckman LS 1006 liquid scintillation counter using the tritium wide-window channel isoset. Approximately 2% of the label at time 0 was labile; these counts were subtracted from all subsequent values. The results are the average of three experiments, each with two replicates.

Peroxidase. Total soluble peroxidase (EC 1.11.1.7) was extracted by grinding peeled oat leaf segments (10 cm) in potassium phosphate buffer (1.5 ml, 50 mM, pH 6.5, 4 C). After centrifuging (27,000 g, 20 min) and decanting, the supernatant fraction (10 μ l) was assayed spectrophotometrically (470 nm) with guaiacol (15 mM, Merck & Co., Rahway, NJ 07065) and H₂O₂ (15 mM) in potassium phosphate buffer (2 ml, 50 mM, pH 5.6, 24.5 C). To determine changes in isozymes caused by peeling, samples (45 μ l) were run on starch gels (10% w/v, 23 mM Na-borate buffer, pH 8.3), then stained with guaiacol (15 mM)/H₂O₂ (15 mM) at pH 5.6. The activities reported are the average of three trials; the isozyme patterns shown are from the clearest gels.

RESULTS AND DISCUSSION

Wound-induced lignin formation. The cell walls of unpeeled mesophyll cells did not autofluoresce, but those of peeled and aged tissue did (Fig. 1A). Wound-induced autofluorescence appeared between the third and fourth hours after peeling (7). This autofluorescence was light blue, which is indicative of lignin (17) and similar to that of xylem walls (Fig. 1B) or guard cells (Fig. 1C). In an alkaline mounting medium the mesophyll cell walls of both

fresh and wounded tissue autofluoresced yellow-green (7), which is consistent with the hypothesis that unwounded and wounded oat cell walls contain a low-molecular-weight phenolic compound (possibly ferulic acid) that autofluoresces yellow-green at basic pH (11). Neither water (24, 31, or 70 C for 24 hr) nor a variety of organic solvents (MeOH, EtOH, acetone, chloroform, DMSO, formamide, xylene, benzene, or toluene at 70 C) affected induced autofluorescence. However, treatment with Cl/Na sulfite or 5.25% sodium hypochlorite (24 C) caused a loss of induced autofluorescence. Stains known to stain lignin stained wounded cell walls, xylem, and guard cell walls, but not unwounded, mesophyll cell walls (Table 1).

Thus, peeling induced formation of a compound, probably lignin, similar in autofluorescence (light blue), staining, and solubility properties to a compound in the cell walls of xylem and guard cells of oats.

PAL activity in wounded tissue. Changes in PAL activity were measured in two ways (Fig. 2A and B). After a 1- to 2-hr lag, PAL activity increased linearly for about 4 hr, then decreased to a rate similar to that of the initial lag period (Fig. 2A). Neither actinomycin D (AD, 20 $\mu\text{g}/\text{ml}$) nor cycloheximide (CH, 1 $\mu\text{g}/\text{ml}$) altered this response, suggesting that these activity changes were not dependent on RNA or protein synthesis. Figure 2B shows that wounding induced an increase in PAL activity for about 5 hr, after which the rate returned to that found immediately after wounding. Thus, the lag seen in Fig. 2A is probably not due to slow uptake; otherwise, the rate during the first hour of each sample in Fig. 2B would be equal to the rate of first sample. Both AOA and AOPP, two inhibitors of PAL activity in buckwheat hypocotyls (1), inhibited PAL activity in oat leaves whether present from time 0 or hour 3 (Fig. 2A). These results support the premise that the activity being measured by this assay is due to PAL, aminovinylglycine (AVG), CoCl_2 , or AgNO_3 , inhibitors of wound-induced ethylene synthesis or activity (9), blocked PAL activity by only 25% over a 24-hr period (7). This suggests that ethylene is not the dominant factor controlling PAL activity following peeling. Increase of PAL activity probably increased the concentration of the phenolic precursors required for lignification.

Peroxidase activity in fresh and wounded tissue. Soluble peroxidase activity in wounded tissue (W) after 24 hr was twice as great as in unwounded tissue (F), probably due to the appearance of isozymes C3, C4, and A1 (see Fig. 3 for isozyme patterns). Since detaching the leaf without peeling (U) had no effect on peroxidase activity, this increase is due to wounding, not senescence. Peeled segments floated on AVG (50 μM), CoCl_2 (0.1 mM), or AgNO_3 (0.1 mM) for 24 hr had less activity than fresh tissue (0.5, 0.8, and 0.3 that of fresh tissue, respectively), suggesting that ethylene synthesis and activity is required for wound peroxidase increases. AOA (1 mM), but not AOPP (0.1 mM), blocked wound-induced peroxidase increases (1.2 and 1.9 that of fresh tissue, respectively). AOA probably acts by blocking ethylene synthesis (9). AD (20 $\mu\text{g}/\text{ml}$) and CH (1 $\mu\text{g}/\text{ml}$) blocked peroxidase increases (1.0 and 0.7 that of fresh tissue, respectively), suggesting that RNA and protein synthesis is required for wound-induced peroxidase induction. Ionically and covalently bound fractions had $\sim 10\%$ total activity compared to the phosphate-soluble fraction and were not investigated in detail. Isozyme A3 leaked into the incubation medium immediately after peeling (7).

Wound-induced peroxidase activity changes were apparently

dependent on ethylene synthesis and action. AVG, CoCl_2 , AOA, and AgNO_3 blocked wound-induced ethylene (9) and wound-induced peroxidase activity. AOPP, which is chemically similar to AOA (both are inhibitors of PAL, Fig. 2A), did not affect ethylene biosynthesis (9) or peroxidase activity (Fig. 3).

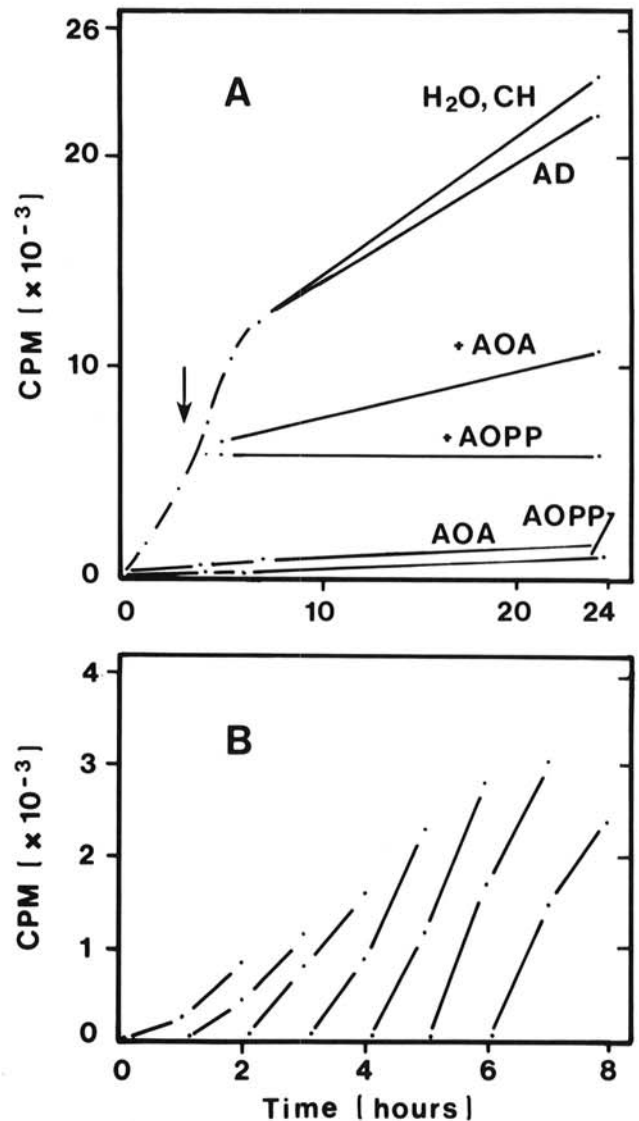


Fig. 2. Wound-induced changes in PAL activity in oat leaf segments. **A**, Oat leaf segments were peeled and immediately floated on ^3H -phenylalanine (labeled on C 2 and 3). At the indicated times aliquots were removed and PAL activity was assayed (see text). AOA and AOPP were added at time 0 or 3 hr after wounding (arrow). Actinomycin D (AD, 20 $\mu\text{g}/\text{ml}$) or cycloheximide (CH, 1 $\mu\text{g}/\text{ml}$) were added at time 0; H_2O and CH experiments gave similar results. **B**, Oat leaf segments were peeled and floated on water. At 0, 1, 2, 3, 4, 5, and 6 hr after wounding, ^3H -phenylalanine (labeled on C 2 and 3) was added. Aliquots were assayed at these times and at 1 and 2 hr later. Each set of three points was from a separate petri dish.

TABLE 1. Staining properties of unwounded and wounded mesophyll cell walls and xylem and guard cell walls of oat leaves

Stain	Xylem	Guard cell walls	Mesophyll walls	
			Unwounded	Wounded
Azure B	Blue	Blue	N.C.	Blue
Cl-Sodium sulfite	Faint pink	N.C. ^a	Grey	Faint pink
Maule reagent	Orange	Orange	Grey	Orange
Phloroglucinol/HCl	Pink	N.C.	N.C.	Faint pink
Toluidine Blue O	Blue-green	Blue-green	Purple	Blue-green

^aN.C. = no color.

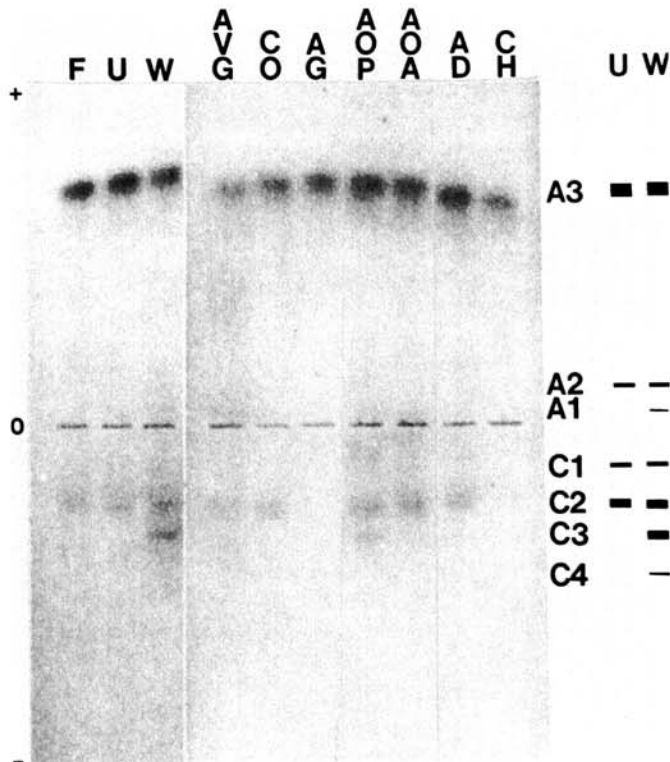


Fig. 3. Wound-induced changes in peroxidase isozymes in oat leaf segments. Twenty-four hours after peeling and incubation, oat leaf tissue was extracted in phosphate buffer. The starch gel was developed with guaiacol/H₂O₂. F = freshly peeled, untreated tissue; U = detached but unpeeled tissue; W = wounded tissue; AVG = aminovinylglycine, 50 μ M; CO = cobaltous chloride, 0.1 mM; AG = silver nitrate, 0.1 mM; AOA = aminooxyacetic acid, 1 mM; AOP = aminooxyphenylpropionic acid, 0.1 mM; AD = actinomycin D, 20 μ g/ml; and CH = cycloheximide, 1 μ g/ml.

Lignin and resistance to cellulolytic digestion. Three hours after oat leaf segments were peeled, resistance to cellulolytic digestion developed in the mesophyll cell walls (8). Compounds that inhibited ethylene synthesis and the development of resistance (9) also inhibited the development of autofluorescence in the cell walls (Table 2). AOPP inhibited PAL activity (Fig. 2A and B), as well as lignification and the development of resistance to cellulase (Table 2). AD (20 μ g/ml) and CH (1 μ g/ml) blocked increased peroxidase activity (Fig. 3), wound-induced lignification (Table 2), and wound-induced resistance to cellulase (8).

Wound-induced resistance was inhibited 50% by AOPP (0.1 mM, 7). If ferulic acid, *p*-coumaric acid, or coniferyl alcohol was added with AOPP, resistance was partially restored after 24 hr (Fig. 4). Similar results were found with AOA (1.0 mM, 7). At high phenolic concentrations (10 mM) the tissue was flaccid and digestion was extensive. Probably the phenolic compounds at the higher concentration disrupted the metabolism of the tissue and therefore its ability to respond to wounding. However, the phenolics did not inhibit the activity of cellulysin. At 0.1 mM phenolic, digestion was about 20% that of fresh tissue, compared with expected 50% with AOPP alone (Fig. 4). This increase in resistance was possibly due to the incorporation of these phenolics into lignin.

Thus, peeling the epidermis induced an increase in PAL activity and ethylene biosynthesis, which apparently led to increased peroxidase activity. Lignin formation in the mesophyll cell walls occurred at the wound site, resulting in inhibition of cellulolytic digestion. Ride and Pearce (15,16) found that papillae formed in reaction to attempted penetration of leaves by a non-pathogenic fungus were resistant to cellulolytic digestion and were lignified. Similarly, Vance and Sherwood (18,22,23) reported that an inappropriate pathogen induced peroxidase activity increase and lignification in reed canarygrass. Hargreaves (10) detected deposition of a fluorescent compound(s), but did not detect lignin histochemically in papillae formed by oat leaves in response to

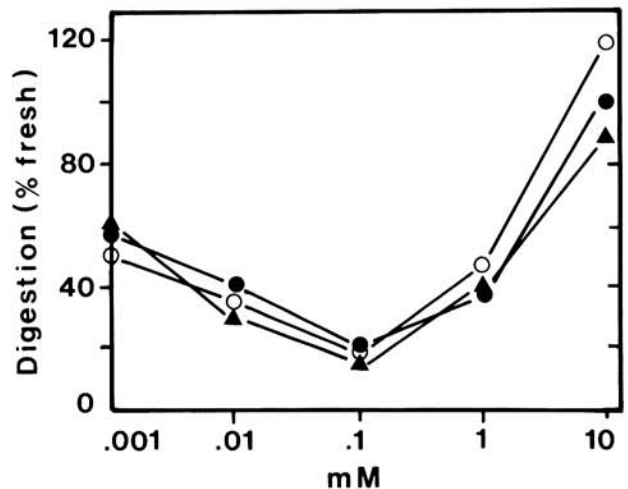


Fig. 4. Reversal of AOPP (0.1 mM) inhibition of the development of resistance of oat leaf tissue to cellulase. Peeled leaf segments were floated for 24 hr on AOPP at a concentration that caused 50% inhibition of development of resistance, and ferulic acid (\blacktriangle), *p*-coumaric acid (\bullet), or coniferyl alcohol (\circ). Digestion by cellulysin is the change in absorbance at 760 nm as a percent of that of freshly peeled, untreated segments.

TABLE 2. Relationship between presence of autofluorescence in oat leaf cell walls and resistance to digestion by cellulase

Treatment	Autofluorescence of cell walls ^a	Digestion by cellulase ^b
Fresh	—	100
Aged, then peeled	—	90
Peeled and aged	+	0
AD (20 μ g/ml)	—	95
CH (1 μ g/ml)	—	99
Ethylene synthesis inhibitors		
AVG (0.1 mm)	—	100
CoCl ₂ (0.1 mm)	—	97
AgNO ₃ (1 mm)	—	99
AOA (1 mm)	—	50
PAL inhibitors		
AOA (1 mm)	—	50
AOPP (0.1 mm)	—	50
AOPP + ferulic acid (0.1 mm)	+	23
AOPP + coumaric acid (0.1 mm)	+	19
AOPP + coniferyl alcohol (0.1 mm)	+	21

^a+ autofluorescence present; — no autofluorescence.

^bDigestion of freshly peeled tissue set equal to 100%.

attempted penetration by *Pyrenophora teres*. In this report, peeling induced lignification of oat cell walls, suggesting that the initial response of plants to fungal attack are nonspecific responses to wounding during penetration (21).

LITERATURE CITED

1. Amrhein, N., Gödeke, K.-H., and Kefeli, V. I. 1976. The estimation of relative intracellular phenylalanine ammonia-lyase (PAL)-activities and their modulation *in vivo* and *in vitro* by competitive inhibitors. *Ber. Deutsch. Bot. Ges.* 89:247-258.
2. Amrhein, N., Gödeke, K.-H., and Kefeli, V. I. 1976. The estimation of phenylalanine ammonia-lyase (PAL)-activity in intact cells of higher plant tissue. *Planta* 131:33-40.
3. Asada, Y., Ohguchi, T., and Matsumoto, I. 1979. Induction of lignification in response to fungal infection. Pages 99-112 in: *Biochemical Aspects of Plant-Parasite Relationships*. J. Friend and D. R. Threlfall, eds. Academic Press, New York.
4. Crawford, R. L. 1981. *Lignin Biodegradation and Transformation*. John Wiley & Sons, New York. 154 pp.
5. Esau, K. 1977. *Anatomy of Seed Plants*. John Wiley & Sons, New York. 550 pp.

6. Friend, J. 1976. Lignification in infected tissue. Pages 290-303 in: *Biochemical Aspects of Plant-Parasite Relationships*. J. Friend and D. R. Threlfall, eds. Academic Press, New York.
7. Geballe, G. T. 1981. Wound-induced resistance to cellulase in oat leaves. Ph.D. thesis. Yale University, New Haven, CT. 157 pp.
8. Geballe, G. T., and Galston, A. W. 1982. Wound-induced resistance to cellulase in oat leaves. *Plant Physiol.* 70:781-787.
9. Geballe, G. T., and Galston, A. W. 1982. Ethylene as an effector of wound-induced resistance to cellulase in oat leaves. *Plant Physiol.* 70:788-790.
10. Hargreaves, J. A. 1982. The nature of the resistance of oat leaves to infection by *Pyrenophora teres*. *Physiol. Plant Pathol.* 20:165-171.
11. Harris, P. J., and Hartley, R. D. 1976. Detection of bound ferulic acid in the cell walls of the Gramineae by U.V. fluorescence microscopy. *Nature* 259:508-510.
12. Kutscha, N. R., and Gray, J. R. 1972. The suitability of certain stains for studying lignification in balsam fir. *Abies balsamea* (L.) Mill. Tech. Bull. of the Life Sci. and Agric. Exp. Stn., Univ. of Maine at Orono. 50 pp.
13. Mitra, R., Gross, R. D., and Varner, J. E. 1975. An intact assay for enzymes that labilize C-H bonds. *Anal. Biochem.* 64:102-109.
14. Rhodes, M. J. C., and Wooldorton, L. S. C. 1973. Stimulation of phenolic acid and lignin biosynthesis in swede root tissue by ethylene. *Phytochemistry* 12:107-118.
15. Ride, J. P. 1975. Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. *Physiol. Plant Pathol.* 5:125-134.
16. Ride, J. P., and Pearce, R. B. 1979. Lignification and papilla formation at sites of attempted penetration of wheat leaves by non-pathogenic fungi. *Physiol. Plant Pathol.* 15:79-92.
17. Sarkanen, K. V., and Ludwig, C. H. 1971. Definition and nomenclature. Page 6 in: *Lignins: Occurrence, Structure and Reactions*. K. V. Sarkanen and C. H. Ludwig, eds. John Wiley & Sons, New York.
18. Sherwood, R. T., and Vance, C. P. 1976. Histochemistry of papillae formed in reed canarygrass leaves in response to noninfecting fungi. *Phytopathology* 66:503-510.
19. Stowe, B. B. 1960. Growth promotion in pea stem sections. I. Stimulation of auxin and gibberellin action by alkyl lipids. *Plant Physiol.* 35:262-269.
20. Uritani, I., and Oba, K. 1978. The tissue slice system as a model for studies of host-parasite relationships. Pages 287-308 in: *Biochemistry of Wounded Plants*. G. Kahl, ed. Walter de Gruyter, New York.
21. Vance, C. P., Kirk, T. K., and Sherwood, R. T. 1980. Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopathol.* 18:259-288.
22. Vance, C. P., and Sherwood, R. T. 1976. Soluble and cell wall peroxidases in reed canarygrass in relation to disease resistance and localized lignin formation. *Plant Physiol.* 57:920-922.
23. Vance, C. P., and Sherwood, R. T. 1977. Lignified papillae formation as a mechanism for protection in reed canarygrass. *Physiol. Plant Pathol.* 10:247-256.