Evidence of Pectinase Activity Between Cronartium ribicola and Pinus monticola

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ABSTRACT

This report contains the first in vivo evidence of pectinase activity during the pathogenesis of a rust disease. Comparisons between noninfected bark of *Pinus monticola*, and bark infected by *Cronartium ribicola*, show a significant decrease (44%) in the amount of extractable pectic substances from infected bark. Data are provided that indicate significant increases in infected bark of materials soluble in petroleum ether and in 95% ethanol.

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Pectinase activity is involved in the development of several different kinds of diseases: soft- and dry rots, blights, wilts, and leaf spots (1, 2, 13). However, the authors have found no reports of in vivo evidence of pectinase involvement in the development of a rust disease. One report does present in vitro evidence that germinating urediospores of the wheat stem rust organism, *Puccinia graminis* var. *tritici*, can be induced to secrete pectinases into properly treated agar (14). This observation does not prove that pectinases are implicated in the disease, or that pectinases produced outside the host are the same as those produced in vivo (1, 2, 13). In vitro studies only give inferences as to whether pectinases are potentially available (1, 2, 13). For that reason, blister rust-infected trees from natural stands were used for this study.

The objective of this study was to determine if *Pinus monticola* Dougl. bark infected with *Cronartium ribicola* J. C. Fisch. ex Rabenh. contained less extractable pectic substances than noninfected bark. A reduction would indicate, by inference, that pectinase activity occurred in the infected bark (2, 13).

MATERIALS AND METHODS.—Forty 10- to 15-yr-old western white pines, each supporting a single bole canker (Fig. 1) were selected in October 1971 from a natural stand of mixed conifers on the East Fork of Potlatch Creek, Bovill, Idaho. Cankers were checked at that time, and again just before sampling, to insure that no secondary canker-invading organisms were present. In June 1972, 11 of the trees were harvested. The portion of stem containing the bole canker and a similar length of noninfected stem about 30.5 cm above the canker were cut into workable lengths (11 infected and 11 noninfected stem segments). Stem segments were quick-frozen on site and transferred to laboratory freezers (—5 C).

When needed, stem segments were washed in tap water to thaw the bark. Only infected bark located between the pycnia scars and 1 cm beyond the discolored canker margin was used (Fig. 1). Bark samples (11 infected, and 11 noninfected) were cut into small pieces, submerged in liquid nitrogen, and pulverized to a fine powder in an electric mortar and pestle. Pulverized samples were stored

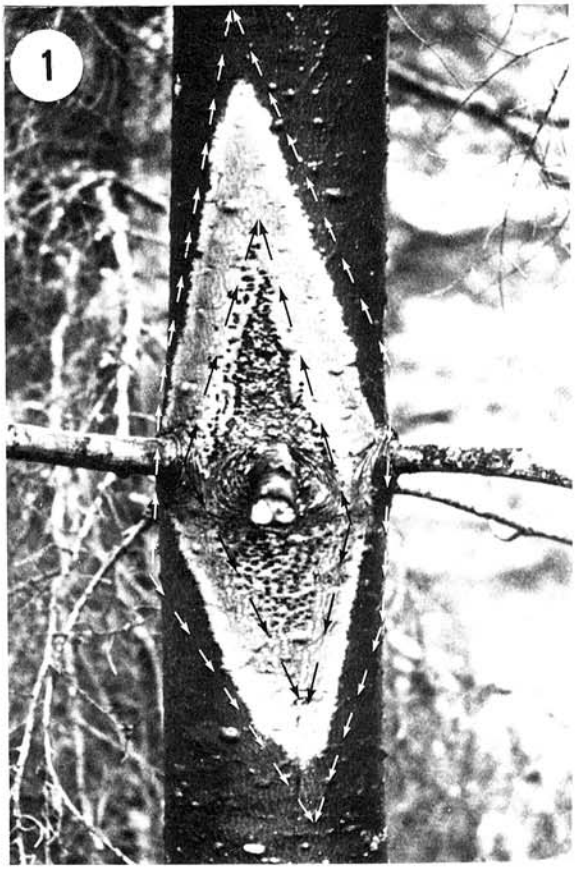

Fig. 1. A blister rust canker on western white pine. The arrows outline the portion of bark removed and extracted for pectic substances.

in prechilled petri dishes in laboratory freezers until freeze-dried (96 h).

Five grams of freeze-dried bark powder from each sample were placed in individual Soxhlet extraction thimbles (35 × 50 mm). The Soxhlet extracting schedule for each sample was as follows: (i) petroleum ether (60-110 C) for 8 h; (ii) 95% ethanol for 16 h; and (iii) glass-distilled water for 16 h. After each extraction, the materials in the thimbles were oven-dried (80 C) for 24 h, 24 h, and 48 h, respectively.

After the Soxhlet extracting schedule was completed, apparatuses consisting of Liebig condensers, stopcocks, Soxhlet extracting chambers, and 1.0-liter boiling flasks, were built to extract pectic substances soluble in 0.01 N HCl or 0.01 N NaOH. Two burners, placed beneath a coil of copper tubing provided the hot water (85 C) that was circulated through the Liebig condensers. The Soxhlet extracting chambers and stopcocks were insulated by wrapping them in linen towels. Extraction temp were 55 C.

The procedure for using these apparatuses was as follows: a liter of extracting solvent (0.01 N HCl) drained slowly (30-40 ml/min) from the internal chambers of the heated condensers into the extracting chambers; the extracting solvent was passed through twice more and then the apparatuses were flushed with 250 ml of fresh solvent. The thimbles were oven-dried (80 C) for 48 h. The same procedure was followed when NaOH was used.

The water, HCl-, and NaOH-soluble fractions of each bark sample were concd by reduced pressure evaporation to about 100, 200, and 300 ml, respectively. Pectic substances within these three fractions were precipitated by adding 95% ethanol until the solutions were 70% ethanol (4). Precipitated pectic substances were removed by centrifugation at 10 C (20,000 *g* for 10 min). The precipitants of the three fractions from each bark sample were placed in separate petri dishes and dried at room temp. Dried precipitants were stored at −5 C.

Precipitated pectic substances were prepared for galacturonic acid analysis by first wetting them with 95% ethanol. Four 25-ml aliquants of extracting solvent (water or 0.01 N HCl or 0.01 N NaOH) was used to redissolve the dried, precipitated pectic substances. Boiling the solutions facilitated solvation. Galacturonic acid content of the three fractions from each bark sample was measured by a modified carbazole method (3), heating periods were doubled (9), and the absorbance measured at 520 nm (7).

After completing the galacturonic acid determinations, the water-, acid-, and base-soluble pectic materials from the infected and noninfected bark of one tree were concd to 25 ml. A two-ml quantity of each concd bark sample extract was mixed with 1.0 ml of 3.0 N HCl, placed in airtight vials, and autoclaved (124 C) for 50 min. Powdered NaOH was used to adjust the pH of the autoclaved solutions to about 6.0. The hydrolyzed extracts were spotted along with a galacturonic acid standard onto Whatman No. 1 chromatography paper. Chromatograms were developed in butanol-acetic acid-water (4-1-5, v/v; upper layer) solvent for 40 h (4). Galacturonic acid spots were localized by means of an aniline-diphenylamine reagent (10). The findings of paper chromatography were verified by thin-layer chromatography. Silica gel (glass) chromatographic plates (20 × 20 cm) were spotted, developed twice in a chloroform-acetic acid-water (50-35-5, v/v) solvent, and air-dried between developments. The same analine-diphenylamine reagent was used to localize galacturonic acid spots.

To insure that the extracting scheme, previously outlined, was effectively removing pectic substances, 0.1-g samples of residue material were suspended in 3 ml of 1.0 N HCl, then hydrolyzed and chromatographically examined for galacturonic acid, as previously described.

RESULTS AND DISCUSSION.—The quantities of bark solutes extractable in the five solvents used are listed in Table 1. Percent moisture and wt of residue after extraction are also included. The method of comparing sample means with paired observations (8) was used to calculate "*t*" values.

Percent moisture was not significantly higher in infected bark (65.5 vs. 64.5). Infected bark, however, was significantly higher in materials soluble in petroleum ether (0.707 g vs. 0.611 g) and in 95% ethanol (0.972 g vs. 0.775 g). Water- and 0.01 N HCl-extractable materials were not significantly decreased in the infected bark; there was, however, a significant reduction in the amount of

TABLE 1. Comparisons of the quantity of materials extractable in five solvents from noninfected *Pinus monticola* bark and bark infected with *Cronartium ribicola*

Treatment	Material extracted from 5 g of freeze-dried tissue (g)			
	Noninfected bark[a]	Infected bark[a]	$\bar{d}$[b]	*t*
Petroleum ether	0.611	0.707	0.096	5.80*[c]
95% ethanol	0.775	0.972	0.197	6.64*
Water	0.416	0.380	0.036	0.89
0.01 N HCl	0.318	0.257	0.061	1.66
0.01 N NaOH	1.278	0.898	0.380	4.57*
Residue	1.599	1.757	0.142	1.26
Moisture (%)	64.5	65.5	1.06	1.67

[a]A mean of 11 samples.
[b]d = the mean difference.
[c]Asterisk denotes a significant difference ($P = 0.05$).

TABLE 2. Galacturonic acid content of noninfected *Pinus monticola* bark and bark infected with *Cronartium ribicola*

Fraction	Noninfected bark[a] (mg/g)	Infected bark[a] (mg/g)	$\bar{d}$[b]	t
Water	17.6	9.7	7.9	3.98*[c]
0.01 N HCl	18.4	10.2	7.4	4.70*
0.01 N NaOH	5.7	4.1	1.7	5.07*
Total	41.7	24.0	17.8	12.06*

[a]Means based on 11 samples.
[b]d = the mean difference.
[c]Asterisk denotes a significant difference ($P = 0.05$).

0.01 N NaOH-extractable materials in infected bark (0.898 g vs. 1.278 g).

Chromatographic examination of the acid hydrolysates of the water-, HCl-, and NaOH-soluble materials precipitated by ethanol revealed large, greenish spots corresponding in R_f to the greenish galacturonic acid spots of the standards. Two unidentified bluish spots were nearer the solvent front. No spots having the migration characteristic of galacturonic acid were found in the acid hydrolysates of the residue material. Therefore, it was concluded that the extracting scheme outlined in this study effectively removed all pectic substances from bark samples.

Concentrations of galacturonic acid within infected and noninfected bark among the water-, HCl-, and NaOH-soluble pectic substances are listed in Table 2. In all cases, pectic substances were significantly lower in infected bark. Thus, by inference, pectinases are actively present in diseased tissue.

Because three different types of pectic substances, high-methoxyl pectins (water-soluble), protopectin (HCl-soluble), and low-methoxyl pectins and pectates (NaOH-soluble), are being degraded within infected bark, the class of pectinases responsible cannot be specified (13).

These data suggest that a wide variety of pectic materials are being degraded. However, degradation of these materials is not so intense as to cause maceration of host tissue. These findings support the histological data reported by Wicker and Woo (12).

Histological studies of infected bark suggest that the middle lamella is degraded (6). Pectinase activity could so soften host tissue that the rust mycelium could mechanically force its way between host cells. However, scanning electron microscopy of infected bark from the periphery of a canker revealed little evidence of mechanical separation or splitting apart of host cells by the expanding mycelium (11). Preliminary results indicate that in this particular region of the canker, the rust mycelium was "passively" growing through large intercellular spaces in host bark. Therefore, it cannot be assumed that pectinase activity plays a major role in the invasion processes of *C. ribicola* in the periphery of the canker.

Pectinase activity may be involved in the penetration of the host cell wall by rust haustoria; pectinase may be removing pectic substances that cover and protect certain cell wall components from enzymatic degradation (5). If so, removal of this pectic barrier would enable the rust to digest exposed components and to force its way into the cell interior. Pectinase could also soften the cell wall to a point where the pathogen could thrust itself into the cell.

Results of this study indicate, by inference, that pectinases are actively present in infected bark. But, at present, no specific role has been assigned to these enzymes in the invasion processes of the blister rust organism.

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