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Host-Specific Phytotoxic Polysaccharide from Apple Tissue Infected by Erwinia amylovora

Abstract. *A toxin isolated from apple fruit tissue infected by* Erwinia amylovora *is 98 percent galactose in polymeric form, 0.375 percent protein, and has an average molecular weight of approximately 165,000. Young shoots of rosaceous, but not nonrosaceous, species wilt in a manner characteristic of the disease when placed in toxin solutions with concentrations as low as 10 micrograms per milliliter. Varieties of apple and pear susceptible to* Erwinia amylovora *wilt in 1 to 3 hours, whereas resistant varieties display symptoms 12 to 24 hours after treatment.*

The enterobacterium *Erwinia amylovora* (Burrill) Winslow *et al.* is pathogenic to many plant species of Rosaceae (*1*); however, it is most important economically on apple and pear. It is responsible for limiting commercial cultivation of favored susceptible pear varieties—for example, limiting Bartlett to the northwestern United States—and has annually reduced apple and pear crops in the United States and elsewhere (*2*).

The biochemical basis for pathogenesis has received significant attention (*3*); however, the precise manner by which host cell damage occurs has remained unknown. Degradation of host cell walls by cellulases and pectinases has been suspected but has never been established, and protease production by the pathogen in vitro (*4*) has not been causally linked with pathogenesis.

Many researchers have sought to relate pathogenicity to toxigenicity, and this too has not yielded positive results (*5*). Nevertheless, Hildebrand (*6*) did report that a component of the bacterial "ooze," which exudes from infected tissue, could reproduce a part of the disease syndrome. He observed that pear shoots wilted within 2 hours when their cut ends were immersed in sterile ooze diluted to 50 times its volume. The ooze or bacterial capsule has also been shown to maintain viability of the pathogen under adverse conditions (*7*).

We report here the nature of the toxigenic agent and its relationship to pathogenesis in the disease called fire blight caused by *E. amylovora*.

A bacterial suspension of a virulent strain of *E. amylovora*, at a concentration of 2×10^9 cells per milliliter, was prepared from a 24-hour culture grown on nutrient agar fortified with 0.5 percent yeast extract and 1 percent glucose. One-half milliliter of the suspension was pipetted onto the surface of slices of aseptically sectioned green Jonathan apple fruits. Inoculated apple slices were kept in a moist chamber at 28°C for 3 to 4 days. Bacteria-laden ooze produced on the surface of inoculated apple slices was collected by capillary pipette and diluted to 50 times its volume with distilled water. The diluted ooze solution was centrifuged at 20,000*g* three times for 30 minutes, and the supernatant fluid was filtered through a 0.45-μm Millipore membrane. Characteristically, the filtered ooze solution has a *p*H of 7.45 and 2.38 mg of dry matter per milliliter. One milligram of the dry matter contains 0.966 mg of carbohydrate, 0.012 mg of amino acids, and traces of protein and lipid (*8, 9*). The polysaccharide-containing solution was passed through columns of Dowex 1 (Cl^- form, 2.5 by 25 cm) and Dowex 50 (H^+ form, 2.5 by 15 cm). The effluent was evaporated under reduced pressure to one-tenth the original volume. Polysaccharide was precipitated with three volumes of 95 percent ethyl alcohol, washed with absolute ethyl alcohol, dried under vacuum, and finally dissolved in a small amount of water and lyophilized.

One milligram of the lyophilized

Table 1. Specific activities of the phytotoxic polysaccharide against rosaceous species. The activity is expressed as the length of time in hours required for a plant cutting to show wilting when its base is placed in toxin solution at a concentration of 100 μg/ml. The following nonrosaceous species were exposed to toxin solution at 500 μg/ml for 24 hours and showed no wilting: *Lonicera tartarica, Forsythia suspensa, Medicago sativa, Melilotus altissima, Lespedeza stipulaceae*, and *Lycopersicum esculentum*.

Plant tested	Specific activity (hours)	Susceptibility to *Erwinia amylovora*	References
Pyrus malus			
Jonathan	1	Susceptible	(*17*)
Malling-26	1	Susceptible	(*18*)
Red Delicious	18	Resistant	(*19*)
Pyrus communis			
Bartlett	2	Susceptible	(*17, 20, 21*)
Starkrimson	3	Susceptible	(*19*)
Magness	12	Resistant	(*21*)
Moonglow	12	Resistant	(*21*)
Kieffer	12	Resistant	(*17, 20, 21*)
Starking Delicious	14	Resistant	(*20*)
Old Home	15	Resistant	(*20*)
Cydonia oblonga			
EM-A	8	Moderately resistant	(*19*)
Province	12	Resistant	(*19*)
Polish 1	12	Resistant	(*19*)
Polish 2	24	Very resistant	(*19*)
Polish 3	18	Resistant	(*19*)
Crataegus crusgalli	1	Susceptible	(*22*)
Spiraea corymbosa	1	Susceptible	(*22*)
Cotoneaster pyracantha	6	Susceptible	(*22*)
Sorbus americana	6	Susceptible	(*22*)

polysaccharide consists of 98 percent carbohydrate, 0.375 percent protein (*10*), and traces of unidentified substances. To identify its components more specifically, it was hydrolyzed with 1*N* H_2SO_4 at 100°C for 3 hours. After neutralization with excess $BaCO_3$, the hydrolyzate was chromatographed by the descending method on Whatman No. 1 paper with two solvent systems: (i) ethyl acetate, pyridine, and water (8 : 2 : 1, by volume) and (ii) *n*-propanol, acetic acid, and water (3 : 1 : 1, by volume). Galactose was the only sugar residue detected after the chromatogram was developed by the method of Trevelyan *et al.* (*11*). The polysaccharide preparation migrated as a single peak during ultracentrifugation with a sedimentation coefficient at 20°C in water, $s_{20,w}$, of 3.89 in a Spinco model E instrument. It also eluted as a single peak through a Sephadex G-200 column, and its average molecular weight was estimated to be 165,000 by gel filtration (*12*). Using a sedimentation equilibrium method, we calculated the molecular weight as 168,000 (the partial specific volume was assumed to be 0.6). Electron microscopic examination of a 0.5 percent aqueous solution of the polysaccharide that was negatively stained with 2 percent uranyl acetate revealed filaments with a dimension of 7.0 nm.

The toxic effect of the compound was assayed on Jonathan apple shoots, which usually wilted in solutions with concentrations of 10, 50, and 100 μg/ml within 4, 3, and 1 hour, respectively. No wilting effect was observed within 24 hours when similar shoots were placed in dextran (type 200 C, average molecular weight 204,000, Sigma Chemical Co.) solution (1000 μg/ml).

The toxigenicity of the substance is retained even after it is heated at 100°C for 1 hour. However, hydrolysis of the toxin in boiling 1*N* H_2SO_4 for 3 hours completely destroyed the biological activity of the toxin. Shoots exposed to galactose at 1000 μg/ml exhibited no toxic effects.

We were unable to detect the toxin in 48-hour cultures of *E. amylovora* grown in defined liquid medium (*13*) or in the small quantities of ooze produced on apple slices by an avirulent strain of the pathogen.

The specific activities of the toxin to various plant species were determined by the time taken for young shoots 5 to 7 cm long to wilt when their bases were placed in a solution of 100 μg/ml. It was found that host plants from seven species of the family Rosaceae wilt in the toxin solution, whereas nonrosaceous species remain unaffected. The fire blight pathogen, *E. amylovora*, attacks only rosaceous species. Tip cuttings (shoots) from susceptible varieties of apple and pear showed severe wilting within 1 to 3 hours, whereas those from resistant varieties showed no visible symptom until 12 to 24 hours after they were placed in toxin solution. Similarly, uptake of toxin-containing solution was correlated with the intensity and rapidity with which wilting occurred. The controls, resistant varieties and species, took up three to five times as much liquid as the susceptible ones. Table 1 summarizes these results.

Because of its high host specificity, it should be possible to use the toxin to evaluate resistance in the progenies of apple and pear breeding programs. The effects of the host-specific toxins victorin, produced by *Helminthosporium victoriae*, and helminthosporoside, produced by *Helminthosporium sacchari*, and a phytotoxic glycopeptide produced by *Corynebacterium insidiosum* on oat, sugar cane, and alfalfa, respectively, have been successfully used in this manner (*14*). From the data in Table 1, it appears that sensitivity to the toxin is directly correlated with susceptibility of the variety or culture to the pathogen under field conditions. It seems likely that a standardized dosage-time response curve could be developed to quantitate resistance to the pathogen. By using the toxin, mass screening of seedlings could be accomplished in a matter of hours in the laboratory and with limited greenhouse space. This would otherwise take several years and many acres of plantings. In addition, since only the apical shoot of the seedling is exposed to the toxin, the entire plant need not be destroyed.

From preliminary observations it would appear that the subcellular disorientation caused by the toxin (plasmolysis followed by cytoplasmic aggregation) is analogous to that caused by the pathogen per se (*15*).

This report describes for the first time a host-specific toxin produced by a plant pathogen bacterium. Although the ooze is produced copiously on the surface of apple slices, it is 80 percent polysaccharide matrix and 20 percent bacterial cells (*16*). Since the toxic polysaccharide is not produced in vitro, nor is it produced in vivo by avirulent strains of the pathogen, the toxin is at present considered the product of the interaction between susceptible host and virulent pathogen. We propose to call the toxin amylovorin.

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23. At the Second International Congress of Plant Pathology, St. Paul, Minnesota, 5 to 12 September 1973, where we presented this research, we became aware of a thesis by S. Eden-Green, East Malling Research Station, Maidstone, Kent, England, describing a chemical analysis of the bacterial ooze of *E. amylovora* produced in apple, pear, and hawthorne stem tissue. The main sugar residue was galactose, but other sugars were also detected. We thank D. F. Millikan and M. S. Feather for their suggestions. Supported in part by NSF grant GB-17729. Missouri Agricultural Experiment Station Journal Series No. 6910.

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