Production of a Host-Specific, Wilt-Inducing Toxin in Apple Cell Suspension Cultures Inoculated with Erwinia amylovora

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ABSTRACT

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A wilt-inducing toxin was detected in filtrates prepared from apple cell suspension cultures (ACSC) which had been inoculated with *Erwinia amylovora*. The toxin induced rapid wilting of susceptible host (apple) but not of nonhost plants (tomato and tobacco). The toxin was produced by a virulent strain but not by an avirulent strain of the pathogen. Growth of the virulent bacteria paralleled production of the toxin in the suspension cultures. The virulent strain of the bacterium produced high amounts of toxin in living ACSC, but little or none was produced in heat-killed ACSC or extracts of ACSC.

Erwinia amylovora (Burrill) Winslow et al., the causal organism of fire blight of apple and pear, produces a toxic polysaccharide on infected host tissue (2, 3). The toxin, which is called amylovorin or fire blight toxin, induces wilting in many hosts but not in nonhost plants (3). Because of its host specificity, the toxin may be used to evaluate varietal resistance of apple and pear to the fire blight bacterium. The toxin has not yet been produced in an artificial medium. Preparation of the toxin in our laboratory usually requires immature apple or pear fruits which are available only at certain times of the year. Even these green fruits become less effective in yielding toxin as they ripen. If the toxin is produced as a result of interaction between the host tissues and the pathogen, it should be possible to employ tissue culture techniques for toxin production. The purpose of this study was to determine whether the fire blight toxin could be produced in apple cell suspension cultures (ACSC) inoculated with *E. amylovora*.

MATERIALS AND METHODS

Apple callus tissue and cell suspension cultures.—Attempts were made to grow stem and petiole tissues of several cultivars (Jonathan, Red Delicious, Malling-26) of apple (*Pyrus malus*) on various tissue culture media. Calli were induced but these grew poorly on all the media tested. Callus tissues of the fire-blight-susceptible cultivar Antonovka (obtained from the School of Forestry, University of Missouri, Columbia) however, were found to grow well on Schenk and Hildebrandt's medium (SH medium) (6) and were used throughout the study. Callus tissues were grown on SH agar medium in 160-ml prescription bottles at 26 ± 2 C and were subcultured at 1-mo intervals. The ACSC were initiated from pieces of callus tissue placed in SH liquid medium and incubated at 26 C on a horizontal, rotary shaker at a speed of 110 rpm. The ACSC were initially grown in 500-ml Erlenmeyer flasks containing 100 ml of SH liquid medium and these were used as stock cultures for all experimental suspension cultures. Most of the experimental suspension cultures were grown in 250-ml flasks and were started by transferring 10 ml of 14-day-old stock suspension cultures into flasks which contained 50 ml of fresh SH liquid medium. Stock suspension cultures were transferred at 14-day intervals.

Bacterial cultures and toxin production.—Virulent (E9) and avirulent (E8) strains of *E. amylovora* were used (4) in these studies. The isolate E8 is a mutant of E9; however, in its inability to produce toxin and cause symptom development it is similar to several other avirulent isolates in our collection and a number of avirulent isolates obtained from Dr. Mortimer Starr, University of California, Davis, 95616 (1, 5). Suspensions of the bacterial cells were prepared from 24-hr cultures grown at 28 C on Difco nutrient agar supplemented with 0.5% yeast extract and 1% dextrose, and were adjusted to 10^8 cells/ml. The ACSC were grown for 14 days in SH liquid medium in 250-ml flasks and then were inoculated with 1 ml of bacterial suspension. Culture filtrates were collected at various times after inoculation by filtration through Whatman No. 1 filter papers, centrifuging at 5,000 *g* for 30 min, and finally by passage through a Millipore filter (0.45 μm). The filtrates were tested for toxin activity (wilt induction) by the excised apple shoot (5 cm in length) bioassay procedure (3). The length of the apple shoot used was crucial to the sensitivity of the bioassay. Stoffl (7) found that the apical 5 cm of Jonathan apple shoots 5, 10, and 20 cm in length wilted in 1.5, 4.5, and 10.0 hr, respectively, when exposed to 100 μg/ml amylovorin.

It was found that excised apple leaves with petioles attached also could be used to determine toxin activity.

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The two or three youngest, fully expanded leaves on a shoot appeared equally sensitive to the toxin and these were usually the 4th, 5th, and 6th from the apex. The bioassay was performed precisely as indicated with 5-cm-long shoots (7). Nonhost cultivars used were Samsun N-N tobacco and Bonny Best tomato. Shoots of these, 5 cm in length, as well as apple leaves and shoots were excised under water and placed in vials containing the test solutions.

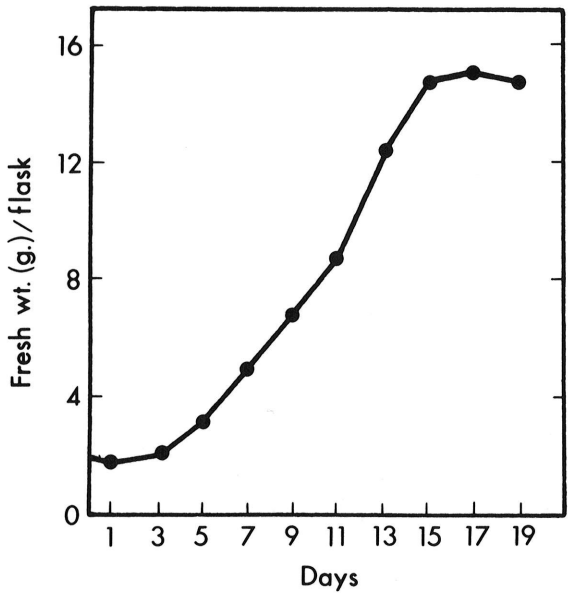

Fig. 1. Fresh weight change of Antonovka apple cells in Schenk and Hildebrandt's (4) liquid medium.

TABLE 1. Wilt-induction[a] activity in filtrates from apple cell suspension cultures (ACSC) or culture medium[b] 3 days after inoculation with the virulent (strain E9) or avirulent (strain E8) strains of *Erwinia amylovora*

Filtrate from:	Shoots wilted after placement in filtrate for: 1 hr (%)	3 hr (%)	6 hr (%)	12 hr (%)
ACSC in SH-medium inoculated with virulent strain (E9)	80	100		
ACSC in SH-medium inoculated with avirulent strain (E8)	0	0	0	0
ACSC in SH-medium control suspension culture	0	0	0	0
SH-medium inoculated with strain E9	0	0	0	0
SH-medium inoculated with strain E8	0	0	0	0
Control SH-medium	0	0	0	0

[a]Succulent shoots of Jonathan apple 5 cm in length were used in the bioassay.

[b]Schenk and Hildebrandt's medium (SH-medium) used for cultivation of apple cells.

RESULTS

Growth of apple cells in SH liquid medium.—To determine the growth rate of the ACSC, the cells were harvested for fresh weight determination at 0 and 24 hr and thereafter at 2-day intervals during a 20-day period. The cells were collected on pre-weighed nylon fabric disks, washed with distilled water and drained under vacuum. The weight of cells from three flasks was averaged for each determination.

There was no increase in cell fresh weight during the first 3 days (Fig. 1). After this lag phase, the cells began to grow rapidly and continued to increase for 12 days. The cultures generally reached the stationary phase after 15 days of incubation.

Toxin production in suspension cultures.—Filtrates from ACSC obtained 3 days after inoculation with *E. amylovora* were assayed for toxin activity on excised Jonathan shoots. Wilt-inducing activity was detected only in filtrates from the suspension cultures inoculated with the virulent strain (E9) of the bacterium (Table 1). The toxin-containing filtrate induced wilting of Jonathan apple shoots in 1-3 hr. Filtrates from the cell cultures inoculated with the avirulent strain (E8) of the pathogen or from control suspension cultures did not cause wilting even after 12 hr. Toxin activity was not detected either in culture medium (SH medium) inoculated with strain E8 or E9, or in control culture medium.

Host specificity of the partially purified toxin (7, 8) was tested on shoots of host and nonhost cultivars. Shoots from Jonathan apple (a susceptible cultivar) showed wilting within 30 min to 1 hr, whereas those from Red Delicious apple (a resistant cultivar) did not wilt until after 6 to 10 hr. Wilting was not observed when tomato or tobacco cuttings were exposed to a toxin-containing filtrate for a 24-hr period.

Time-course of toxin accumulation in suspension cultures.—The time-course of toxin accumulation was followed by collecting filtrates from duplicate ACSC at 0, 1, 2, 3, 5, and 7 days after inoculation with the E9 strain of the pathogen. Filtrates were tested for toxin activity by the excised Jonathan apple leaf bioassay. Bacterial populations in the inoculated ACSC also were determined at each sampling period by the dilution plate method.

Toxin activity was detectable 2 days after inoculation with the bacterium and increased over a period of several days, reaching a maximum within 5-7 days (Fig. 2). The bacterial population in the suspension cultures increased rapidly and also reached a maximum at 5 days after inoculation. Bacterial growth paralleled toxin production from day 2 to termination of the experiment. Parenthetically, the growth of the avirulent strain E8 in ACSC paralleled that of E9. However, the former was nontoxigenic.

Comparison of toxin production in suspension cultures and suspension culture extracts.—In order to determine whether living ACSC were necessary for toxin production, live 2-wk-old ACSC and heat-killed ACSC (cultures placed in boiling water bath for 30 min) were

inoculated with the virulent E9 strain of the pathogen. Three days after inoculation, filtrates of these suspension cultures were prepared as previously described. Filtrates also were prepared from 2-wk-old nontreated ACSC and from homogenized ACSC. Then these filtrates were inoculated with the virulent E9 isolate of *E. amylovora* and after 3 days they were freed of bacteria by centrifugation and passage through 0.45 μm Millipore filters. Each of the four filtrates was tested for toxin activity by the Jonathan apple shoot bioassay procedure.

Only living ACSC which had been inoculated with bacteria produced significant toxin activity. Table 2 indicates that slight toxin activity was detected in both the E9-inoculated, heat-killed ACSC and the E9-inoculated, homogenized ACSC filtrate. No wilting was caused by filtrates of nontreated ACSC that were subsequently inoculated with the E9 strain of *E. amylovora*.

DISCUSSION

The fire blight toxin was produced prior to this study only in the infected host tissue (3). The experiments reported herein revealed that the toxic polysaccharide could be produced in ACSC inoculated with virulent *E. amylovora*. The host specificity of the toxin produced from the suspension cultures appeared to be similar to that obtained from the immature fruits (3). As noted in our initial study (3), the apple cultivar that was susceptible to the pathogen was more sensitive to the toxin than the resistant cultivar; and nonhost plants were not affected by the toxin. The toxin was produced by a virulent *E. amylovora* strain but not by an avirulent strain (Table 1). One of us (RNG) has repeated the experiments initially reported (3) and obtained essentially the same results. In addition, other apple cultivars were tested for sensitivity to the toxin; e.g., Red Rome, Lodi, Ida Red, Holly, Golden Delicious, and Jonared. With one explicable exception, each revealed a sensitivity to the toxin that paralleled its observed sensitivity to the pathogen in the field (R. N. Goodman, *unpublished*).

Whether toxin synthesis reflects the metabolic activity of both pathogen and ACSC suspension cultures or green fruit cells remains unknown. However, it would seem that living host cells are necessary for production of significant amounts of the toxin, since slight or no activity of the toxin was observed in heat-killed ACSC or in filtrates of either a homogenate of ACSC or filtrates of ACSC that had been inoculated with the bacterium (Table 2). Furthermore, the toxin did not appear to be a staling product of bacterial growth, but was produced during the logarithmic phase growth of the bacterium in the suspension cultures (Fig. 2).

The present study has demonstrated the feasibility of employing ACSC systems for production of the toxin. The toxin can now be obtained easily and at all times in the laboratory. By using a fermentor or larger culture vessels we have found it possible to increase the production of crude toxin (Hsu and Goodman, *unpublished*). The crude preparation from this source also is being used for the further purification and structural characterization of the toxic compound. Whether the toxin produced from ACSC and that produced from immature apple fruits are identical is currently being studied.

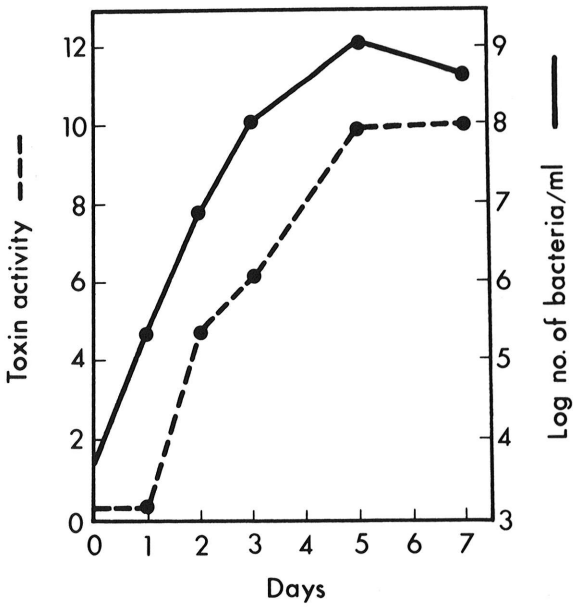

Fig. 2. Growth of *Erwinia amylovora* (virulent strain E9) and production of the fire blight toxin in apple suspension cultures following inoculation with the bacterium. Toxin activity was expressed as the reciprocal of dilution end points of serial 2-fold series of the filtrates which would induce wilting in 5 hr.

TABLE 2. Production of a host-specific toxin in various apple cell suspension cultures or media 3 days after inoculation with virulent *Erwinia amylovora* (strain E9)

Culture or medium	Apple shoots wilted after placement in the filtrates from various cultures or media for: 1 hr (%)	2 hr (%)	3 hr (%)	6 hr (%)
Living apple cell suspension culture	60	100		
Heat-killed apple cell suspension culture	0	0	40[a]	80[a]
Filtrate of homogenized apple cell suspension culture	0	0	0	40[a]
Filtrate of apple cell suspension culture	0	0	0	0

[a]Shoots were only partially wilted.

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