Partial Purification and Properties of Chlorosis Inducing Toxins of Pseudomonas phaseolicola and Pseudomonas glycinea

Harry A. J. Hoitink and S. L. Sinden

Assistant Professor, Department of Plant Pathology, the Ohio Agricultural Research and Development Center and The Ohio State University, Wooster 44691; and Plant Physiologist, Crops Research Division, ARS, USDA, Beltsville, Maryland 20705, respectively.

Cooperative investigations of the Ohio Agricultural Research and Development Center and The Ohio State University, Wooster; and Crops Research Division, ARS, USDA, Beltsville, Maryland.

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ABSTRACT

A chemically defined medium was used to produce high titers of exotoxins from *Pseudomonas phaseolicola* and *P. glycinia*. The exotoxins were isolated and partially purified from culture filtrates by methanol extraction, charcoal adsorption, and thin-layer chromatography. R_F values of the toxins in three different solvent systems indicated that the toxins are probably the same compound. The toxin was also isolated from soybean leaves. Results of gel filtration experiments indicated that the toxin produced in vivo is the same compound as the toxin produced in culture. This toxin caused systemic as well as localized chlorosis on soybean plants. The development of chlorosis was not light-sensitive. This toxin differs from the wildfire toxin in both chemical characteristics and biological activity. Phytopathology 60:1236-1237.

Additional key words: bean, soybean, exotoxin.

Five species of plant pathogenic pseudomonads that cause chlorotic symptoms on leaves also produce exotoxins in culture (2). From *Pseudomonas tabaci* and *P. coronafaciens*, identical toxins have been purified (8, 10). Although several reports are published on the properties of exotoxins of *P. phaseolicola* (2, 3, 7, 8, 9), *P. glycinea* (2), and *P. tomato* (2), only the exotoxin of *P. phaseolicola* has been purified and partially characterized (6). The toxin of *P. phaseolicola* is a heat-stable, dialyzable polysaccharide, possibly related to the wildfire toxin (3, 6, 7). Garber & Shaeffer (2) reported different R_F values for the exotoxins of all five pseudomonads. But only *P. tabaci* and *P. tomato* produced high toxin titers in the culture medium.

This report gives a procedure for obtaining high titers of exotoxins of *P. phaseolicola* and *P. glycinea* and for partial purification of these toxins from culture filtrates and infected soybeans.

MATERIALS AND METHODS.—Isolates P 56 and P 33 of *P. phaseolicola*, which differ in the amount of toxin produced in culture (3), and isolates Is 40 and Is 80 of *P. glycinea* (selected from several hundred field isolates by A. F. Schmitthenner) were used in the studies. Isolate Is 40 caused systemic chlorosis in soybean, whereas isolate Is 80 caused chlorosis on infected leaves only. The *P. phaseolicola* isolates caused a hypersensitive reaction on soybean; those of *P. glycinea* caused hypersensitivity on Red Kidney bean.

Inoculum was grown for 24 hr at 25 C in a yeast-glucose broth culture [5 g yeast extract, 5 g glucose, 0.2 g $MgSO_4$, 4.1 g KH_2PO_4, 3.6 g K_2HPO_4/liter (pH 6.8)]. Two ml inoculum were added to 500 ml chemically defined medium of 10 g glucose, 1 mg biotin, 1.0 g NH_4Cl, 0.2 g $MgSO_4$, 4.1 g KH_2PO_4 and 3.6 g K_2HPO_4/liter (pH 6.8). This latter medium was sterilized by filtration. After 4 days' incubation on a rotary shaker at 17 C, cells (in late log-phase) were removed by centrifugation and supernatant portions were sterilized by Millipore (0.45 μ) filtration. Toxicity of culture filtrates was tested with 20-μliter dilution droplets as described previously (3). All filtrates caused chlorosis on *Nicotiana tabacum* L. 'White Burley', *Glycine max* (L.) Merr. 'Harosoy 63', *Phaseolus vulgaris* L. 'Red Kidney', *Brassica oleracea* var. *botrytis* L. 'Snowdrift', *B. oleracea* var. *capitata* L. 'Badger Ballhead', *Capsicum frutescens* L. (*C. annuum* L.) 'Yolo Wonder', and *Cucurbita maxima* Duchesne 'Acorn Table Queen', but little or no reaction on *Zea mays* L. and *Vicia faba* L. These data support previous findings that the toxin lacked host specificity (3, 7). The dilution end points with 20-μliters droplets of culture filtrates of Is 40, Is 80, P 56, and P 33 on tobacco were the 80, 20, 20 and 2X dilutions, respectively. Chlorotic symptoms produced by the filtrates were similar.

To purify the toxin, 1-liter quantities of sterile culture filtrate were concd to a thick syrup (about 10 ml) under vacuum at 50 C, and the toxin was extracted with 100 ml of methanol. The filtered methanol extract was then concd to dryness and the residue dissolved in 10-ml water. After adsorbing cations from the preparation on a Dowex 50W × 8 column (1.5 cm × 20 cm) in the H^+ form, the toxin was eluted from the column with 100 ml distilled water. No detectable amount of toxin was adsorbed to the ion exchange column. This result agrees with a previous report on the properties of the *P. phaseolicola* toxin (3), and clearly differentiates it and the soybean toxin from the tobacco wildfire toxin which is adsorbed, or inactivated, by Dowex 50 cation exchange resin (10). The column eluate was concd to dryness, redissolved in 10 ml water, and neutralized with NaOH. Five g Norit A were added and the toxin adsorbed to the charcoal (4 C for 1 hr, continuous stirring). Charcoal was removed by filtration and the filter was washed with 50 ml water. Toxin was eluted from the charcoal with 100 ml acetone (4 C for 1 hr, continuous stirring). After filtration and concn to dryness, the toxin preparations were suitable for chromatography. To determine R_F values for toxins,

partially purified toxin preparations from isolate P 56 and Is 40 were chromatographed in four different solvent systems on thin-layer silica gel plates (5). For detection of the toxin activity bands, the gel was scraped from the plates in 5-mm bands from the origin to the solvent front, and toxin was eluted from the gel with 25 ml of 80% methanol. After filtration to remove the gel and concn to dryness, gel eluate fractions were dissolved in 25 μliters of water. Aliquots of ten μliters were bioassayed on tobacco and bean leaves.

RESULTS AND DISCUSSION.—In addition to localized chlorosis caused by culture filtrates and partially purified toxin preparations of isolates P 56 and Is 40 on soybean primary leaves when punctured through the midrib, systemic chlorosis was observed frequently in upper trifoliolate leaves that had not been treated. Typical halos developed when toxic filtrates were applied to tobacco, soybean, and bean plants which had been in the dark for 24 hr and were maintained in the dark for 48 hr. This indicates that unlike the wildfire toxin (8), light is not essential for development of chlorosis caused by these toxins. Efforts to obtain a more quantitative toxin bioassay using inhibition of microorganisms as a measure of potency showed that neither *Escherichia coli* (Mig.) Castell. & Chalmers nor *Geotrichum candidum* Lk. ex Pers. was inhibited by toxic culture filtrates of the isolates. These findings indicate that the toxins of *P. phaseolicola* and *P. glycinea* are toxins specific to plants rather than general protoplasmic poisons such as the wildfire toxin of *P. tabaci* (1).

Thin-layer chromatography of toxins indicated that the toxins from isolate P 56 and Is 40 are probably the same toxic compound (Table 1). In two of the chromatographic solvent systems used, two peaks of toxin activity could be detected when toxin preparations from *P. phaseolicola* were chromatographed. The faster-moving toxin produced the same type of chlorotic lesion on tobacco and bean, but the possibility of artifacts in the chromatography experiments is not ruled out, as the toxins were not completely purified.

Toxin was extracted from primary soybean leaves infected with Is 40 (4). Six days after inoculation, 200 g water-soaked leaves were washed in 900 ml distilled water on a shaker at 4 C for 10 min. The washing solution was centrifuged at 12,000 *g* for 15 min (4 C) and sterilized by Millipore filtration (0.45 μ). Toxin was isolated from this leaf extract with the technique described for toxic culture filtrates. An extract was obtained that caused typical chlorosis. The toxin was heat-stable (121.5 C for 15 min). Extracts from noninoculated plants did not contain this chlorosis-inducing activity.

TABLE 1. R_F values of toxins from *Pseudomonas glycinea* and *P. phaseolicola*

	Solvent systems[a] R_F value[b]			
Toxin	1	2	3	4
P. glycinea	0.61	0.28	0.39	0.68
P. phaseolicola	0.61	0.28, 0.50	0.39, 0.98	0.68

[a] 1 = *n*-propanol:water (64:36, w/w); 2 = chloroform:96% acetic acid (95:5, v/v); 3 = benzene:methanol:acetic acid (45:8:4, v/v); 4 = *n*-butanol:acetic acid:water (60:20:20, w/w).

[b] Thin-layer Silica Gel G plates, solvent front 15 cm from origin.

An approximate molecular wt of the toxin was determined using a Sephadex G-10 (1.7 × 50 cm) column. Toxin from culture filtrates of isolates P 56, Is 40, and Is 80 and from the toxic soybean leaf extract eluted as a single peak from this column at twice the void volume (2 Vo), which indicates that the molecular wt of the toxin is less than 700. (After this paper was submitted, Rudolph [6] published a report on the purification and partial characterization of a phytotoxic polysaccharide isolated from cultures of *P. phaseolicola*. This toxin had a molecular wt of ca. 2,200.)

Results of these experiments indicate that toxins produced in culture by the isolates of *P. glycinea* and *P. phaseolicola* are identical. This toxin was also isolated from infected soybean leaves. It caused systemic as well as localized chlorosis. Final classification of identity must await chemical characterization.

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