

## Detection of a necrosis-inducing factor of nonhost plant leaves produced by *Pseudomonas syringae* pv. *tomato*

YOAV BASHAN,<sup>1</sup> YAACOV OKON, AND YIGAL HENIS

Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76 100, Israel

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A necrosis-inducing factor (NIF) was extracted from two virulent strains of *Pseudomonas syringae* pv. *tomato* sonicated cells. The factor had an approximate molecular weight of 108 000, contained 90% protein and 10% polysaccharide, and was partially purified by affinity chromatography, gel chromatography, gel electrophoresis, and electrophoresis on cellulose acetate strips. The factor caused loss of electrolytes within 48 h and necrosis on bean, pepper, and cucumber leaves but not on tomato leaves of different cultivars. Similar fractions extracted from cells of *P. fluorescens* did not show any activity. Activity was destroyed by pronase or by hydrolysis with HCl indicating that the activity is due to the protein component. The factor did not show any pectolytic or cellulolytic activity. The factor was produced at a maximum after 24 h of growth of *P. syringae* pv. *tomato* and was more abundant when extracted from cells grown in rich medium.

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Un facteur inducteur de la nécrose (NIF) a été extrait de cellules traitées par la sonication appartenant à deux souches virulentes de *Pseudomonas syringae* pv. *tomato*. Ce facteur a un poids moléculaire d'environ 108 000, contient 90% de protéines et 10% de polysaccharides et il a été partiellement purifié par chromatographie d'affinité, chromatographie sur gel, électrophorèse sur gel et électrophorèse sur bandes d'acétate de cellulose. Le facteur provoque la perte d'électrolytes en moins de 48 h et la nécrose chez des feuilles de haricot, de piment et de concombre, mais non chez les feuilles de différents cultivars de tomate. Des fractions semblables extraites des cellules de *P. fluorescens* ne montrent aucune activité. Cette activité est détruite par le pronase ou par l'hydrolyse au HCl et est donc due à la composante protéique du facteur. Le facteur ne présente aucune activité pectolytique ou cellulolytique. Sa production est maximale après 24 h de croissance du *P. syringae* pv. *tomato* et elle est plus abondante chez les cellules croissant dans un milieu riche.

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### Introduction

The relationships between phytopathogenic bacteria and nonhost plants are problematical and controversial. On the one hand it seems that the hypersensitive reaction (HR) to incompatible bacteria in plant tissue is a general phytopathological event (13, 18), and some modes of action have been suggested for this phenomenon (14, 27, 28). On the other hand there are, however, only few reports available dealing with the isolation of substances from bacterial cells which induced cell collapse of nonhost tissues (9) or which inhibit the HR and cause slight chlorosis (21). Although many studies reported on HR-inducing substances, there is no adequate evidence, so far, of the existence of a bacterial component responsible directly for the HR in plants (12, 15, 26). Many extracellular toxins from pathogenic bacteria have been extensively studied and documented (29), but very little information is available regarding isolation of endobacterial components which affect plant tissue.

<sup>1</sup>Present address: Division of Plant Pathology, The Volcani Center, Agricultural Research Organization, Bet-Dagan, P.O. Box 6, 50250, Israel.

The purpose of this study was to explore a substance originating from *P. syringae* pv. *tomato* cells, the causal agent of bacterial speck of tomato, which had an effect on nonhost plant tissue.

### Materials and methods

#### Organisms and growth conditions

*Pseudomonas syringae* pv. *tomato* (Okabe, 1933) Young, Dye & Wilkie, 1978 (ATCC 10852), a local *P. syringae* pv. *tomato* isolate (WT-1) which attacks tomato plants severely in Israel and an isolate of the saprophyte *P. fluorescens* were used. Bacteria were grown on several liquid media: yeast-peptone (YP) (25), sucrose-nutrient (AJ) (1), nutrient broth (Difco) and glucose-salts (GS) (23).

Bean plants (*Phaseolus vulgaris*) cv. Brittle wax, pepper plants (*Capsicum annuum*) cv. Ma'or, cucumber plants (*Cucumis sativus*) cv. Delila and tomato plants (*Lycopersicon esculentum*) cv. VF-198 (highly susceptible to bacterial speck) and cv. Rehovot 13 (resistant to bacterial speck) were used.

Growth conditions for both bacteria and plants and inoculum preparation were described by Bashan et al. (3). Plants were examined for their capacity to respond by necrosis to the presence of the factor fractions of *P. syringae* pv. *tomato* as described later.

### Bioassay

Activity of the necrosis-inducing factor was tested on leaves of each plant species using the prick assay method or hypodermic injection. Fifty microlitres of the solution were placed on the upper side of the leaf. The liquid was introduced into the leaf by puncturing the leaf through the droplet with a hypodermic needle. After the droplets had dried or water soaking disappeared, plants were returned to an air-conditioned greenhouse ( $25 \pm 2^\circ\text{C}$ ; relative humidity,  $<60\%$ ). Leaves were examined for the appearance of necrosis 5–7 days after treatment. The relationship between the factor concentration and the development of a necrotic area was determined by recording leaf necrosis greater than 1 mm in diameter across. Living bacteria were introduced into entire leaves by means of a hypodermic syringe. Quantitation of the activity was measured by dilution to end-point titer of activity.

### Preparation of crude NIF

*Pseudomonas syringae* pv. *tomato* necrosis-inducing factor was isolated by a modification of the method of Crosthwaite and Patil (9). Cultures of *P. syringae* pv. *tomato* (WT-1 and ATCC 10852) and *P. fluorescens* were grown in yeast-peptone liquid medium (5 L for each bacterium) in 250-mL Erlenmeyer flasks in a rotary shaker (100 strokes/min) at  $30^\circ\text{C}$ . Bacteria were collected by centrifugation at  $10\,000 \times g$  for 10 min. The supernatant was collected separately, and the pellets were resuspended and washed five times in 0.06 M potassium phosphate buffer (pH 7.0). The final concentration was adjusted to 5 g cells / 20 mL buffer.

Sonication was carried out with an MSE ultrasonic disintegrator for 10 min under an ice bath. Cell debris were removed by three successive centrifugations at  $30\,000 \times g$  for 1 h each and the supernatant was filtered through a  $0.45 \mu\text{m}$  Millipore filter. This sonicate preparation was dialyzed at  $4^\circ\text{C}$  against 0.06 M phosphate buffer pH 7.0, which was replaced three times during the dialysis. The dialysate was lyophilized and redissolved in 5 mL of the buffer solution.

A 1-mL sample was subjected to gel filtration on a column of Sephadex G-25 ( $1.25 \times 61$  cm), previously equilibrated with the same buffer, at a flow rate of 0.2 mL/min. Six fractions which showed necrotic activity were combined and fractionated on Sephadex G-200.

The factor was isolated by affinity chromatography on a  $1 \times 30$  cm concanavalin A (Con A) – Sepharose column (Pharmacia). After applying a sample, the column was washed with 10 mM potassium phosphate buffer, pH 6.9. NIF was eluted with 100 mM methyl- $\alpha$ -D-manopyranoside in the same buffer. This substance was later removed by ultrafiltration and subjected to another gel chromatography of Sephadex G-200.

To determine whether *P. syringae* pv. *tomato* produced any extracellular high-molecular-weight toxic substances, 5 L of the culture medium were evaporated under reduced pressure at  $38^\circ\text{C}$  to 200 mL according to the method of El-Banouby and Rudolph (11). Six-hundred millilitres of absolute ethanol was then added at  $4^\circ\text{C}$  and the mixture was incubated at  $4^\circ\text{C}$  for 20 h. After three centrifugations at  $15\,000 \times g$  for 10 min each, the pellet (1.8 g fresh weight) was resuspended in the buffer and checked for biological activity.

### Gel chromatography and estimation of molecular weight

Gel chromatography and estimation of the factor molecular

weight was done with the aid of a  $1.5 \times 61$  cm column of Sephadex G-200 (which has an exclusion limit of 600 000) in the 0.06 M buffer at the same flow rate as Sephadex G-25, using the following proteins as markers: trypsin, molecular weight 24 000; galactose oxidase, 42 000; malate dehydrogenase, 70 000; glutaminase, 110 000; polyphenol oxidase, 125 000; and glucose oxidase, 150 000 (Sigma).

### Chromatography of CM-cellulose (6)

CM-cellulose chromatography was carried out in 0.001 M Tris-maleate buffer, pH 6.4 ( $40 \times 2.5$  cm column). Samples were dissolved in the buffer and eluted at  $30^\circ\text{C}$  successively, with (a) 200 mL of the starting buffer and (b) a linear gradient of NaCl (0–0.375 M) in starting buffer. The rate of flow was 30 mL/h and 15-mL fractions were collected. The reservoir contained 450 mL of 0.001 M Tris-maleate buffer, pH 6.4, which was 0.75 M NaCl, while the mixing flask contained the same 450 mL buffer without NaCl. Fractions containing protein were determined by absorbancy at 280 nm, combined, dialyzed at  $4^\circ\text{C}$  for 48 h against potassium phosphate buffer, 0.006 M, pH 7.0, lyophilized to dryness, and subjected to electrophoresis.

### Polyacrylamide gel electrophoresis

The factor was further electrophoretically analyzed on 5% polyacrylamide gels or on the same gels supplemented during preparation with 1.87% *N,N'*-diallyltartardiamine (DATD) in tube apparatus (10) for 2.5 h at 100 V, 10 mA. Gels were stained with Coomassie brilliant blue. Biological activity of the band was determined by preparing a gel from slices of gels obtained from the parallel of 10 band sites in the unstained gels and the factor was obtained from this later-prepared gel by reverse electrophoresis (7). Biological activity of the DATD gels was determined as follows. Slices from unstained gels were cut from the parallel place of band appearance and put in 0.1% periodic acid at  $25^\circ\text{C}$  for 24 h (2) and dialyzed at  $4^\circ\text{C}$  against distilled water for 24 h. Samples from the two types of gels were lyophilized to dryness, dissolved in 100  $\mu\text{L}$  or 50  $\mu\text{L}$  distilled water, respectively, and injected into pepper plants. Controls were prepared from slices of stained gels which did not show any staining.

### Electrophoresis on cellulose acetate strips (19)

Four samples, 300  $\mu\text{g}$  each, of the factor were run on a cellulose acetate strip in a Beckman model R-100 microzone electrophoresis system Rm-T-B-010 in 2,4,6-collidine buffer pH 7.5 for 20 min at 400 V, 4 mA. The strip was cut in the middle, and two samples were stained with Ponceaus color and washed with 5% acetic acid. The parallel band sites in the unstained strip were cut and vigorously shaken for 2 h in 10 mL 0.006 M phosphate buffer at pH 7.0. The solution was further treated as already described for gel bands. However, plants were injected only with 20- $\mu\text{L}$  aliquots.

### Electrolyte leakage from treated pepper, bean, or cucumber leaf tissues (8)

Discs (1 cm diameter) of plant leaves were taken with a cork borer at various intervals after inoculation, weighed, and placed in 20 mL of sterile double-distilled water and incubated at  $27^\circ\text{C}$  for 6 h. Water conductivity of 1-mL aliquots was then measured with a Radiometer CDM2b conductivity bridge.











