

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

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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.

[Traduit par le journal]

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.

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.

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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°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°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 (1.25×61 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 1×30 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- α -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°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°C and the mixture was incubated at 4°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 1.5×61 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 (40×2.5 cm column). Samples were dissolved in the buffer and eluted at 30°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°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°C for 24 h (2) and dialyzed at 4°C against distilled water for 24 h. Samples from the two types of gels were lyophilized to dryness, dissolved in 100 μL or 50 μL 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 μg 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- μL 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°C for 6 h. Water conductivity of 1-mL aliquots was then measured with a Radiometer CDM2b conductivity bridge.

Cellulolytic activity

A modification of the Benefield method (5) for cellulase activity with glucose as a standard was used. The reaction mixture contained 200 mg of carboxymethyl-cellulose (CMC, BDH) and 2 mg of either commercial cellulase (Sigma) or factor preparation in 20 mL of 0.04 M citrate-phosphate buffer pH 5.0. Reaction flasks were sealed with "Parafilm" and incubated for 48 h at 50°C. After incubation the reaction mixture was centrifuged at $10\,000 \times g$ for 10 min. Aliquots (0.5 mL) of the supernatant were added to 3 mL of glucose-oxidase and peroxidase reagent (Sigma) and incubated at 25°C for 45 min, and the absorbance was recorded in a Coleman Junior spectrophotometer at 425 nm.

Pectolytic activity

The dinitrosalicylic acid method as modified by Wang and Keen (30), using galacturonic acid as a standard, was used. The reaction mixture contained 20 mg of pectin (BDH, 250 grade) and 2 mg of either commercial pectinase (Sigma) or the factor preparation in 3 mL 0.05 M acetate buffer, pH 6.8. After incubation, aliquots of 3 mL dinitrosalicylic acid were added, the mixture was boiled for 15 min, and the absorbance was recorded with a spectrophotometer at 575 nm.

Analytical methods

Protein determinations were made by the method of Lowry *et al.* (20) with chymotrypsin (Sigma) as a standard. Fatty acids were tested colorimetrically by the method of Haskins (16) or qualitatively by Sudan Black B staining (24). Polysaccharides were determined by the anthrone method (17). Strong hydrolysis (6 N HCl, 100°C, 4 h) of the necrosis-inducing factor was done. Digestion of the NIF by pronase (Sigma) was done for 1 h in a mixture of 3:1 (w/w) of these ingredients in phosphate buffer (pH 7.4) at 37°C.

Results

Properties of the crude necrosis-inducing factor of *Pseudomonas syringae* pv. *tomato*

Repeated attempts to obtain high-molecular-weight substances from a cell-free liquid culture of *P. syringae* pv. *tomato* by means of ethanol precipitation failed. It was not possible to obtain any visual lesion formation when the pathogen or a saprophytic *Pseudomonas* was sprayed on the leaves of bean, pepper, or cucumber plants. However, typical bacterial speck symptoms were obtained when *P. syringae* pv. *tomato* was inoculated on susceptible tomato leaves under high humidities.

Cell extracts of both isolates of *P. syringae* pv. *tomato* (1000 µg) but not of the saprophytic *P. fluorescens* showed necrotic activity only when applied in 1 mL buffer to leaves of nonhost, e.g. bean, pepper, cucumber, but not to either the susceptible or the resistant tomato host leaves. Lesions formed were identical with those induced in nonhost leaves after injecting 10^{10} *P. syringae* pv. *tomato* cells/mL.

Dilution of this crude extract in saline (0.85 g NaCl/L) resulted in its inactivation. Preparations of both *P. syringae* pv. *tomato* isolates produced lesions within

5 days after injection. However, the lesions caused by the ATCC 10852 isolate were less marked than those produced by the local isolate (WT-1).

Further concentration of the crude complex by lyophilization did not increase severity of necrosis. Activities of the preparations of both pathogens were nondialyzable and lost their activity after digestion by pronase or by strong hydrolysis, indicating that their activity was related to high-molecular-weight substances possibly containing proteins. It was possible to store this crude extract at -18°C without any further change in activity.

Partial purification and estimation of the molecular weight

The combined fractions of the tested isolates, after Sephadex G-25, which showed necrotic activity, were further purified using a Sephadex G-200 column. Protein content of each fraction was monitored separately, and although nearly every fraction contained some protein, only one peak of *P. syringae* pv. *tomato* (WT-1) extract and one peak of the ATCC 10852 isolates caused visual necrosis of beans, pepper, and cucumbers (Fig. 1). These peaks were further purified by means of affinity chromatography on Con A - Sepharose column.

The yield of the active factor obtained after Con A - Sepharose was 100 µg protein equivalent per 10 g of fresh weight cells of WT-1 isolate and 5 µg per 10 g of the ATCC 10852 isolate. No detectable active fraction was obtained from *P. fluorescens* cells. No change in the necrosis-inducing-factor activity was observed when the active fractions were combined and refractionated in Sephadex G-200. Samples containing 100 µg protein were further fractionated on a linear gradient of NaCl on a column of CM-cellulose. As shown in Fig. 2, a single protein component was obtained. Electrophoretic

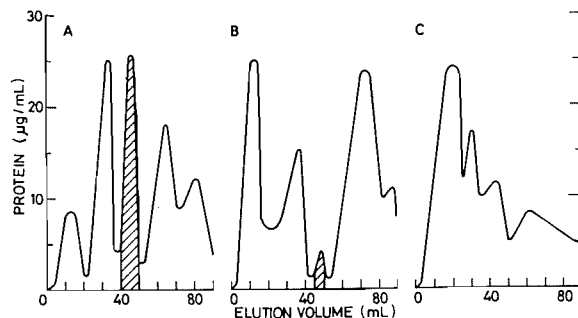


FIG. 1. Gel filtration on Sephadex G-200 of crude extract from (A) local *Pseudomonas syringae* pv. *tomato* (WT-1); (B) ATCC 10852 *P. syringae* pv. *tomato*; (C) *P. fluorescens*. Gel column was 1.5×61 cm in 0.06 M potassium phosphate buffer (pH 7.0). One millilitre sample in the same buffer was eluted at a flow rate of 0.2 mL/min. Hatched areas indicate necrosis-inductive fractions.

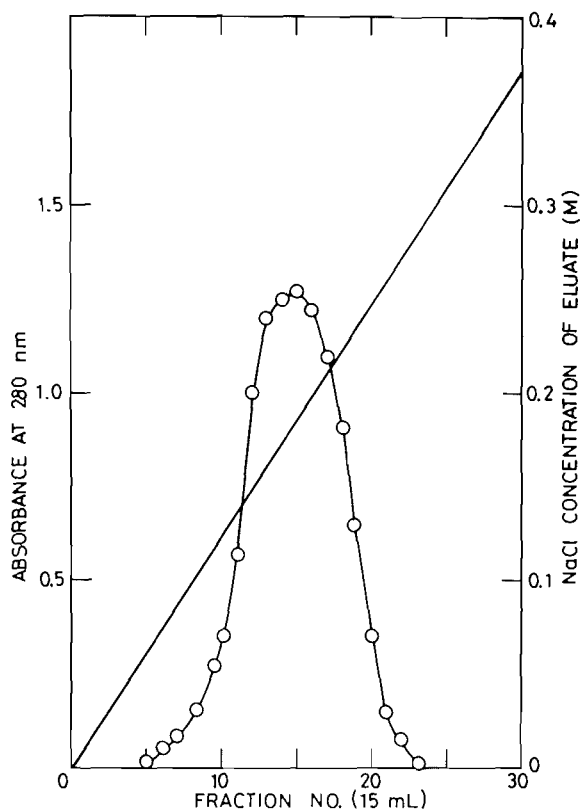


FIG. 2. Chromatography on CM-cellulose. The column was loaded with 100 μg of active fractions obtained from Sephadex G-200. Column was 2.5×40 cm in a 0.001 M Tris-maleate buffer (pH 6.4). The flow rate was 30 mL/h. \circ — \circ represent the absorbance at 280 nm of the eluate and —, the NaCl concentration of the eluate.

determination using two types of polyacrylamide gels and cellulose acetate strip yielded one band only (Fig. 3) indicating the absence of contaminating proteins. This band was extracted either by reverse electrophoresis in regular gels, by dissolving in periodic acid in DATD gels, or by shaking the strip of cellulose acetate in buffer. The material obtained after electrophoresis was referred to as the necrosis-inducing factor. The extracted band was dissolved in phosphate buffer and its protein content adjusted to 100 $\mu\text{g}/\text{mL}$. When injected into nonhost leaves, it showed a necrotic activity.

End-point titer of the partially purified factor was measured by recording the capacity of dilutions of the active fraction to cause cell collapse. By this method it was found that the complex was still active at a protein-equivalent concentration of 0.5 $\mu\text{g}/\text{mL}$ (Table 1).

Molecular weight of the active principle was found to be approximately 108 000 using six marker enzymes as references. The active band had neither pectinase nor

cellulase activity, whereas commercial pectinase and cellulase decomposed the substrate.

The chemical properties of the factor

Chemical analysis (Table 2) indicates that the main component in the active fractions of both *P. syringae* pv. *tomato* isolates is a protein, which comprises some 90% of the factor. Sugars consist of some 10%; other yet unidentified components are present in a small proportion (less than 2%). No lipids could be detected in preparations of any of the tested isolates.

Production of the necrosis-inducing factor on various growth media

Pseudomonas syringae pv. *tomato* isolate (WT-1) was grown on three complex media: nutrient broth, yeast-peptone, sucrose-nutrient (very rich medium), and glucose-salts medium. The bacteria grew abundantly on yeast-peptone and sucrose-nutrient media, moderately on nutrient broth medium, and poorly on glucose-salts medium (Table 3).

One gram (fresh weight) of cells from each culture was sonicated and the supernatant of the sonicate was fractionated by passing through a Sephadex G-25 and G-200 and Con A - Sepharose columns. Factor production depended on medium composition; the richer the medium, the larger the amount of necrosis-inducing factor that could be obtained (Table 3).

Effect of culture age on the necrosis-inducing-factor production

The WT-1 isolate was grown on sucrose-nutrient medium for 10 days. Every day a sample of 1 g (fresh weight) of bacterial cells was examined as previously described for the factor content. The factor was at a maximum 24 h after inoculation and slowly decreased as the culture became older (Fig. 4).

Leakage of electrolytes from treated nonhost tissues

Pepper leaves were either inoculated with 10^9 colony-forming units (CFU)/mL of *P. syringae* pv. *tomato* or injected with 1000 μg of the partially purified factor. Leakage of electrolytes was observed in the plant tissue within 5 h after bacterial inoculation and markedly increased after 11 h (Fig. 5). Tissue cells collapsed about 14 h after inoculation. On the other hand, the leaves treated with the factor leaked only 24 h after injection, and conductivity was increased only slightly. Forty-eight hours and 72 h after injection stronger electrolyte leakage was observed, but in any case it did not reach the level observed in bacterial-treated leaves 11 h after inoculation (Fig. 5). Approximately 75 h after inoculation, tissue began to collapse. Similar phenomena were obtained by using bean or cucumber leaves but not in tomato leaves. Also, the rate of electrolyte leakage was not quantitatively related to the dose of the

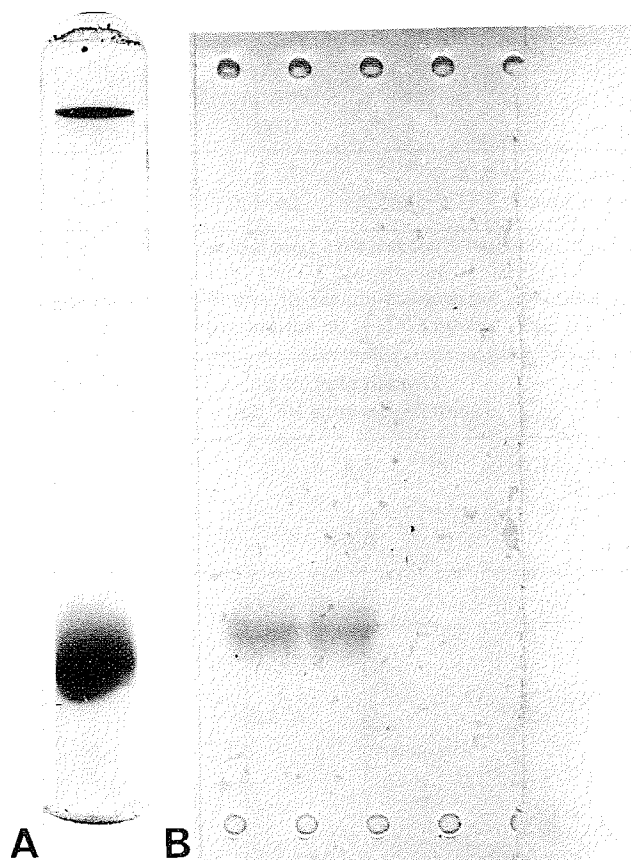


FIG. 3. (A) Polyacrylamide gel electrophoresis and (B) electrophoresis on cellulose-acetate strip of the NIF that causes necrosis in nonhost leaves.

TABLE 1. Production of the necrosis-inducing factor by various bacterial isolates

Bacteria	Fraction	Volume (mL)	Protein weight (μg)	End-point titer of activity ^a
<i>P. syringae</i> pv. <i>tomato</i> WT-1	Sonicated	1	1000	1:1 ^b
<i>P. syringae</i> pv. <i>tomato</i> ATCC 10852				1:1
<i>P. fluorescens</i>				No activity
<i>P. syringae</i> pv. <i>tomato</i> WT-1	After Sephadex G-25	1	500	1:50
<i>P. syringae</i> pv. <i>tomato</i> ATCC 10852				1:2
<i>P. syringae</i> pv. <i>tomato</i> WT-1	After second Sephadex G-200	1	100	1:100
<i>P. syringae</i> pv. <i>tomato</i> ATCC 10852				1:5
<i>P. syringae</i> pv. <i>tomato</i> WT-1	After CM-cellulose	4	10 ^c	1:20
<i>P. syringae</i> pv. <i>tomato</i> ATCC 10852				1:1

^aActivity measured as a 1-mm-diameter necrosis after 5 days on each species of plant.

^bThe protein weight was diluted to an end-point titer of activity.

^cFinal end-point titer was measured at this fraction.

TABLE 2. Composition of the active fraction obtained from CM-cellulose column^a

Bacteria	Protein (μg)	Sugar (μg)	Lipid	Unidentified components (μg)
<i>P. syringae</i> pv. <i>tomato</i> WT-1	27.6	2.34	—	0.06
<i>P. syringae</i> pv. <i>tomato</i> ATCC 10852	26.4	3.0	—	0.6

^aThirty micrograms (dry weight) of materials was assayed from each isolate.

TABLE 3. Production of the necrosis-inducing factor (NIF) by *Pseudomonas syringae* pv. *tomato* (WT-1) on various growth media

Media	Growth after 24 h (absorbance units 540 nm)	NIF content after 24 h ($\mu\text{g/g}$ fresh weight cells)
Yeast-peptone	1.1	9.8
Sucrose-nutrient	1.2	10.2
Nutrient broth	0.6	2.1
Glucose-salts	0.08	0.1

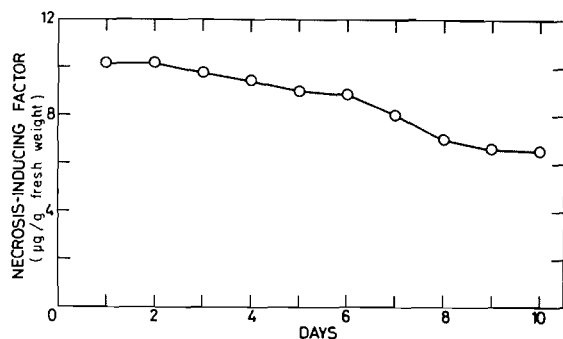


FIG. 4. Relation between necrosis-inducing-factor production by *Pseudomonas syringae* pv. *tomato* (WT-1) as related to culture age. The factor was assayed using the end-point titer of activity method.

factor because increase of the factor dose to 2000 μg did not result in parallel increase in electrolyte leakage.

Discussion

Nonhost plants may react to a bacterial plant pathogen either by a hypersensitive reaction (HR) or by a reaction of chlorosis and (or) necrosis caused by nonspecific toxins. The hypersensitive reaction was demonstrated by electron micrographs showing granular substances binding the bacteria to the plant cell (13, 18). Although the mechanisms involved in the induction of this phenomenon *in vivo* are not yet known, three high-molecular-weight substances produced *in vitro* have been suggested so far as the causative agents of this event in nonhost plant tissue (4, 9, 26). The modes of action of the nonspecific toxins of different types in an artificial inoculation of plants causing chlorosis or necrosis *in vivo* are largely speculative (29).

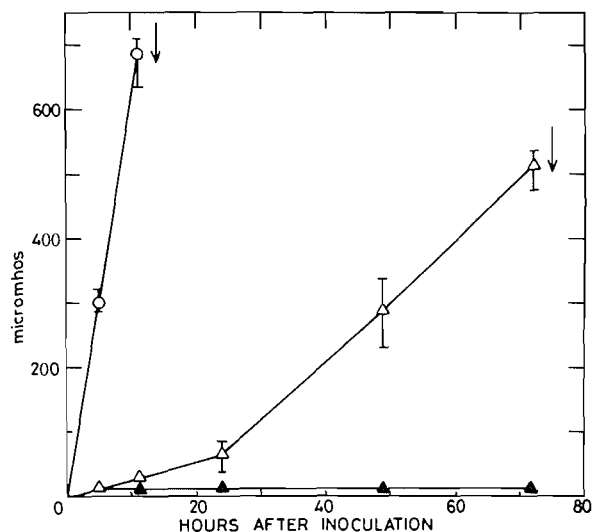


FIG. 5. Electric conductivity of pepper leaf discs (0.5 g fresh weight in 20 mL twice-distilled water) after inoculation with 10^9 CFU/mL *Pseudomonas syringae* pv. *tomato* ○—○, injection of the NIF △—△, and incubation for 6 h at 27°C. Distilled water controls ▲—▲, standard error I. Experiment was carried out twice in four replicates each. Graph was obtained from one experiment. Arrows indicate cell collapse.

Cook and Stall (8) and Wheeler and Black (31) suggested the permeability test of tissue for measuring effects of pathogenic bacteria or toxins in plant materials. Although *P. syringae* pv. *tomato* produced electrolyte leakage of pepper leaves very similar to that of *Xanthomonas campestris* pv. *vesicatoria* on resistant pepper (8), the factor extracted from this tomato

pathogen acted differently on the plant. It produced loss of electrolytes within 48 h or more after injection. According to the Müller criteria for HR (22) this effect could not be recognized as a typical HR. Obviously it is also not a disease because of the incompatible combination of the system.

The necrosis-inducing factor obtained from both isolates of *P. syringae* pv. *tomato* completely lost its activity after digestion by pronase or by acid hydrolysis indicating that the factor is a proteinaceous compound. In spite of both isolates of *P. syringae* pv. *tomato* being aggressive to tomato plants, there was a significant difference in their necrosis-inducing-factor content, and its production varied with culture age and with medium composition. The richer the medium, the higher is the factor content per constant cell weight. Therefore, it can be proposed that the extraction of a high-molecular-weight component from *P. syringae* pv. *tomato* (4) capable of producing necrosis in bean, pepper, and cucumber leaves at low concentrations causes a different interaction between materials from that of pathogenic bacteria and plants. Thus, the necrosis-inducing factor might play an unknown role in starting events which finally lead to necrosis. The fractions obtained from a saprophytic *P. fluorescens* which had been isolated from tomato leaves had no necrotic activity even when applied at high concentrations, indicating that this compound is not generally produced by all species of pseudomonads.

The effect of nutrition on the necrosis-factor production, its role in bacterial cell metabolism, its location, and its role in the induction of necrosis by *P. syringae* pv. *tomato* *in vivo* require further study. The *P. syringae* pv. *tomato* factor had neither pectolytic nor cellulolytic activity; thus the reason for its capacity to induce necrosis is still not known.

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