

Ammonia causes necrosis in tomato leaves infected with *Pseudomonas tomato* (Okabe) Alstatt

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(Accepted for publication February 1980)

Ammonia in culture filtrates of *Pseudomonas tomato* and in homogenized diseased leaves caused necrosis of healthy tomato and bean leaves. In addition, electrolyte leakage and symptoms formation in tomato leaves infected with *P. tomato* were preceded by the production of toxic quantities of ammonia. By contrast, non pathogenic pseudomonads produced ammonia only in culture. No chlorosis-inducing substance could be obtained from filtrates of any of the two isolates of *P. tomato* tested. Macromolecules in the culture filtrates, cell-free extracts and cell debris of *P. tomato* did not visibly affect tomato leaves. Necrotic spots were observed when cell-free extracts of *P. tomato* were applied to bean leaves.

INTRODUCTION

It has been reported that *Pseudomonas tomato* (Okabe) Alstatt [27], the causal agent of bacterial speck of tomato, produces an extracellular thermostable toxin(s), which causes a diffuse chlorotic halo without necrosis in tomato leaves [6, 8, 36]. So far, the structure of this toxin has not been established. Other chlorosis-inducing toxins from *P. tabaci* and *P. phaseolica* have been extensively studied [24, 32, 34]. Although many bacterial pathogens cause necrosis, no bacterial toxin has been isolated which will cause this type of symptom [16, 19].

Lovrekovich *et al.* [19, 20] suggested that ammonia produced by phytopathogenic bacteria played a rôle in tissue necrosis. Similarly, Trabulsi *et al.* [40] observed a continuous accumulation of ammonia in diseased plants infected by *P. coronafaciens*.

Recently, we have observed [2, 28] that tomatoes infected with *P. tomato* and incubated under mist developed typical speck symptoms similar to those reported by Wilkie & Dye [42], i.e. 1 to 3 mm black brown lesions surrounded by an irregular chlorotic halo. However, in commercial plots in Israel, *P. tomato*-infected plants mainly showed severely necrotic areas on leaves either with a small or without any chlorotic halo (Plate 1). In this paper, the results of some experiments, designed to study the possible presence of toxin in the development of speck symptoms in tomato are reported.

MATERIALS AND METHODS

Organisms and growth media

Cultures of the following were used: *Pseudomonas tomato* (ATCC 10852); a local isolate of *P. tomato* from infected tomato plants in Israel; *P. fluorescens*; *P. aeruginosa*

and *Pseudomonas* sp. obtained from the collection of our Department. Stock cultures were kept at room temperature on nutrient agar (Difco) slants and transferred to fresh medium every month. Cultures were grown in Erlenmeyer flasks containing one third of their volume of either yeast peptone broth [Y.P.] [34], synthetic medium for toxin production [43], tomato leaf extract medium [35] or a synthetic medium containing ($g\ l^{-1}$): glucose 10; KNO_3 10; $MgSO_4$ 1; K_2HPO_4 1. All media were maintained at pH 6.8 with 0.06 M potassium phosphate buffer. The cultures were incubated in a rotary shaker (110 strokes min^{-1}) for 4 days at 18 °C. Cells were collected by centrifugation (10 000 g for 10 min) and the supernatant was analyzed for toxic substances. Tomatoes (*Lycopersicon esculentum*), cv. VF-198, highly susceptible to bacterial speck [2] and beans (*Phaseolus vulgaris* L.) cv. Brittle Wax, were obtained from Hazera Co., Haifa, Israel. Plants were grown in an air-conditioned glasshouse (25 ± 2 °C with 12 h light/12 h dark periods), in half litre plastic pots containing 600 g sandy loam soil from Rehovot. The plants were fertilized with 10 ml of a 1% (w/v) commercial fertilizer solution (20 : 20 : 20 NPK). Tomato plants were inoculated with bacteria by rubbing the leaf surface with carborundum powder or by pre-misting [2].

Toxicity assays

Plants possessing at least two true leaves were used. Test solutions (2 to 50 μ l droplets) contained 0.05% agar to enable the formation of compact uniform droplets. These were placed on the upper surface of bean or tomato leaves.

Toxic effect (necrosis) was observed within 3 to 6 days. The prick assay method [26] was used for testing high molecular weight substances. Electrolyte leakage of infected leaves was done according to the method of Goodman [9].

Purification methods

Dialysis was carried out against distilled water at 4 °C with constant stirring.

Charcoal adsorption [23, 24]. Charcoal 1 : 100 (w/v) was added to filtrates under continuous stirring for 20 min. The suspension was filtered through Whatman's No. 1 filter paper in a Buchner funnel and washed with 100 ml distilled water. Substances were eluted from the charcoal with a solution of methanol : chloroform : aqueous ammonia 0.5 M (3 : 1 : 1) (MCA) 3 times, 100 ml each time.

Partition column chromatography. Sephadex (G-25 fine, Pharmacia Co.) was packed into a 61×1.2 cm column. The elution rate was $0.15\ ml^{-1}\ min$, 5 ml fractions were collected.

Ion exchange chromatography [11]. Resin (Dowex AG 50W-X8) was prepared in a column bed of 20×0.5 cm.

Paper chromatography. Amino acids were separated on Whatman No. 1 filter paper (40×60 cm) using propanol : water (2 : 1) [8] or butanol : water : glacial acetic acid (25 : 25 : 6) [10] as solvents, and 0.1% ninhydrin solution as developer. Positive fractions were cut and substances eluted from them with 50 ml water each and filtered through Whatman's No. 1 filter paper.

Thin layer chromatography (t.l.c.) was carried out on 20×20 cm silica gel G plates. Butanol : water : acetic acid (8 : 2 : 2) was used as solvents for one dimension

amino acid chromatography [30], and butanol : water : acetic acid (5 : 4 : 1) [25] for the other dimension. Also benzene : methanol : acetic acid (45 : 8 : 4) [14] was used. A 0.1% ninhydrin solution was used as developer.

For sugars, propanol : ethyl acetate : water : ammonia (5 : 1 : 3 : 1) or butanol : acetone : water (4 : 5 : 1) were used as solvents, whereas benzidine reagent was used as developer [37]. For separation of fatty acids, petrol-ether : diethyl-ether : glacial acetic acid (90 : 10 : 1) or propanol : water (9 : 1) were used as solvents, and bromothymol blue was used for developing [37].

All water solutions and fractions obtained from dialysis or chromatography were lyophilized and redissolved in distilled water (10 to 20 μ l). Organic solvent extracts were dried under reduced pressure at temperatures of 35 to 40 °C, and resuspended in water (10–20 μ l).

Cell fractionation

Cells of *P. tomato* (1 litre) grown on Y.P. at 18 °C for 5 days were pelleted at 10 000 g for 10 min, washed 3 times with sterile 0.06 M phosphate buffer pH 6.8, resuspended in 5 ml buffer and sonicated using a MSE ultrasonic disintegrator for 10 min in an ice bath. Cell debris was sedimented at 20 000 g for 30 min at 4 °C, and the cell-free extract used for toxicity tests. It contained 340 mg protein per ml.

Necrotic activity of the fractions (pellet and supernatant before and after dialysis) was tested on bean and tomato leaves.

Macromolecule extraction

Ethanol (96%) was added to a dialyzed filtrate (3 : 1, v/v) and incubated overnight under stirring at 4 °C. The precipitate was collected and washed 3 times with distilled water and sedimented at 15 000 g for 10 min. The pellet obtained was suspended in 0.5 ml H₂O.

Transfer of the necrosis inducing substance(s) from diseased to healthy plants

Bacterial speck-diseased tomato leaves (10 g) were homogenized in 10 ml water in an Omni-Mixer (Sorvall). The homogenate was centrifuged at 15 000 g for 10 min. The clear supernatant obtained was lyophilized and then dissolved in 1 ml distilled water. Ten μ l droplets were placed on healthy plants for toxicity test.

Chemical tests

Protein was determined by the Biuret method [17] or by the Folin-phenol reagent method of Lowry *et al.* [21]. Phosphate was measured according to the method of Lazarus *et al.* [18]. Hydrolysis of fractions was carried out in 6 N HCl at 100 °C for 10 h [23, 31].

The amount of ammonia in the filtrates and fractions was determined with the Nessler reagent [12, 13]. A modified Barker & Volk [1] Kjeldahl method was used to measure ammonia extracts from plants using Nessler reagent instead of titration. Ammonia was totally evaporated from solutions with 0.1 N NaOH (pH 12) at 80 °C for 1 h. The solutions were neutralized with 0.1 N HCl. The efficiency of the method

for ammonia evaporation was confirmed by measuring the solutions with Nessler reagent or by running the sample in an amino acid analyzer.

Electric conductivity of solutions was measured with a Radiometer. Spectrum of the active fraction was scanned in a Varian-Techtron spectrophotometer at 200 to 800 nm.

RESULTS

Ten to 50 μl aliquots of the centrifuged growth medium of either *P. tomato*, ATCC 10852 or the local isolate caused weak necrosis of tomato or bean leaves. A dialyzable component of this culture supernatant caused severe necrosis when concentrated to its original volume. The non-dialyzable material was not toxic to bean or tomato leaves.

Culture filtrates of *P. tomato* retained toxicity after treatment with charcoal. No toxic material was recovered from the charcoal in MCA.

Macromolecules precipitated from the cell free growth medium with ethanol and redissolved in water [7] did not cause any damage, even when applied to wounded leaves.

Filtrates of *P. tomato* cultures grown on yeast peptone and tomato leaf extract media showed necrotic activity whereas none was detected in filtrates of cultures grown on synthetic medium or NO_3 -glucose medium probably because of the poor growth in the latter media. The possibility that salts present in the medium caused the necrosis was disregarded because the growth medium contained 11.61 mM ml^{-1} salts and gave no necrotic effect, whereas the toxic dialyzed filtrate contained 9.16 mM ml^{-1} salts and produced severe necrosis.

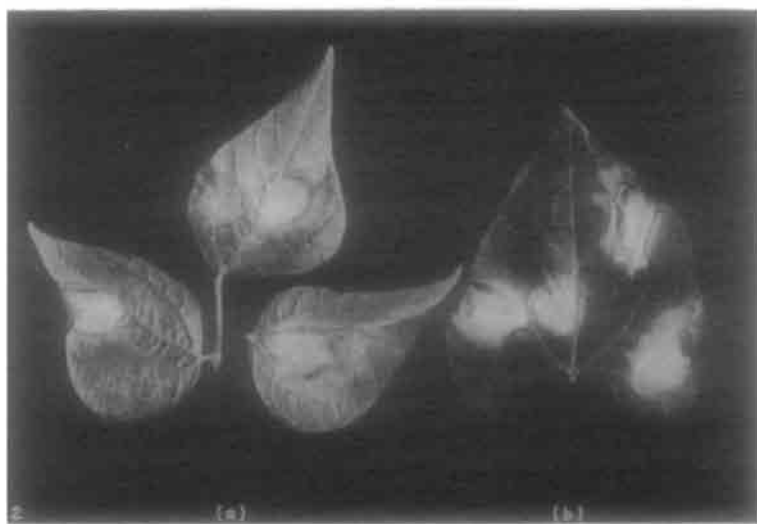
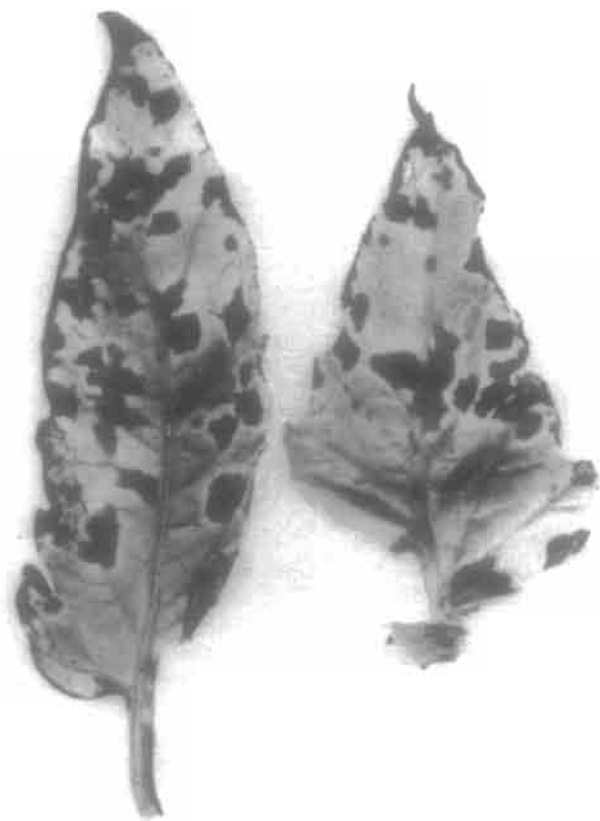
The necrosis-causing substances which passed through the dialysis membrane and those present in the fraction that was not adsorbed by charcoal, were applied to a Sephadex G-25 column. In both cases only one fraction eluted (55 to 60 ml from the beginning of column washing) showed necrotic activity. No fraction caused chlorosis, although the necrotic spots were sometimes surrounded by narrow chlorotic edges. The active fraction eluted from the Sephadex G-25 column was subjected to paper and thin layer chromatography. Five amino acids were identified as proline, glutamic acid, tyrosine, phenylalanine and arginine, but none of these spots or any of the identified amino acids caused necrosis in tomato or bean leaves at $100 \mu\text{g ml}^{-1}$. Hydrolysis of the active fraction did not yield additional amino acids. No protein and peptide was revealed in this fraction by the Lowry or Biuret methods or by spectrum analysis at 200 to 800 nm. Phosphorus was not detected. When the active fraction was analyzed in an amino acid analyzer, ammonia at a concentration of $100 \mu\text{g ml}^{-1}$ was detected, as well as the above mentioned five amino acids at concentrations 1 to $4 \mu\text{g ml}^{-1}$.

The ammonia content of the fraction eluted from the Sephadex column was measured with Nessler's reagent. Only the active fraction contained ammonia at a toxic level ($>120 \mu\text{g ml}^{-1}$). Fractions 1 to 10 contained $0.5 \mu\text{g ml}^{-1}$, fraction 12 $15 \mu\text{g ml}^{-1}$ and 13 to 16 $0.5 \mu\text{g ml}^{-1}$.

The active fraction was unable to cause necrosis after evaporation of ammonia at pH 12. Furthermore, when the active fraction was passed through a Dowex AG 50W-X8 resin column which adsorbed ammonia, it lost its activity.

PLATE 1. Bacterial speck of tomato, caused by *Pseudomonas tomato* in a leaf from a diseased field in Israel, showing extensive necrosis.

PLATE 2. Necrosis in bean leaves produced by (a) ammonia $100 \mu\text{g ml}^{-1}$ and (b) by an active fraction of a Sephadex filtered culture filtrate of *Pseudomonas tomato* containing $120 \mu\text{g ml}^{-1}$ ammonia.



PLATES 1 and 2

Ammonia (2.5 to 250 000 $\mu\text{g ml}^{-1}$), ammonium salt solutions [NH_4Cl , NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4COOH] adjusted to pH 5, 7, 8 (100 $\mu\text{g ml}^{-1}$) and amino acids (ornithine, tryptophane, valine, alanine, phenylalanine, glutamate, threonine, glycine, tyrosine, aspartate, proline, serine, methionine, cystine, cysteine, asparagine, histidine and arginine) at 1 M concentration were tested for toxicity on tomato and bean leaves. Only ammonia (pH 8.5) produced necrosis $\geq 25 \mu\text{g ml}^{-1}$; the necrosis being morphologically similar to the active fraction obtained from *P. tomato* active fraction (Plate 2).

Leaves showing necrotic specks were homogenized in sterile water and fractionated as described for filtrates. When tested by the same procedure as for culture filtrates, a fraction of the homogenized leaves caused necrosis of leaves of healthy tomato or bean plants and also contained ammonia. The pH of a diseased tomato leaf homogenate was 8 ± 0.5 , whereas the pH of healthy leaves was 6.3 ± 0.1 . No chlorosis-inducing fraction could be obtained from diseased leaves.

P. fluorescens, *P. aeruginosa* and *Pseudomonas* sp. grown on yeast peptone also produced ammonia in amounts sufficient to cause necrosis (Table 1). However, inoculation of tomato plants with 10^8 cells ml^{-1} of these cultures did not cause any disease, whereas with *P. tomato* (ATCC 10852 or the local isolate) speck symptoms always developed. A slight increase in the electrolyte leakage level could be observed with both organisms after 20 h. However, a strong leakage of electrolytes could be observed only after 120 h in leaves inoculated with *P. tomato* coinciding with the highest point of ammonia accumulation. (Fig. 1).

TABLE 1

Ammonia, pH, and toxicity to tomato leaves of culture filtrates of some pseudomonads, growing on yeast peptone medium

Bacterial species	Final pH	Ammonia ($\mu\text{g ml}^{-1}$)	Toxicity
<i>Pseudomonas fluorescens</i>	7.4	95	+
<i>P. aeruginosa</i>	8.6	180	+
<i>Pseudomonas</i> sp.	7.6	115	+
<i>P. tomato</i>	8.0	120	+

Bacteria were grown at 25 °C for 4 days.

Increase in ammonia in plants inoculated with the pathogen could be observed after 20 h. This increase continued until the appearance of symptoms (necrosis). At this stage the pH of the leaves was above 8. *Pseudomonas fluorescens* did not affect ammonia concentration or pH of leaves. The possibility of a direct effect of cell constituents of *P. tomato* on tomato leaves was examined. Cell-free extracts (340 mg protein per ml) and cell residues (280 mg dry wt ml^{-1}) from washed cells of *P. tomato* grown on yeast peptone medium were tested for necrotic activity on tomato and bean leaves by the prick method. Necrosis was caused only by the cell-free extracts on bean leaves.

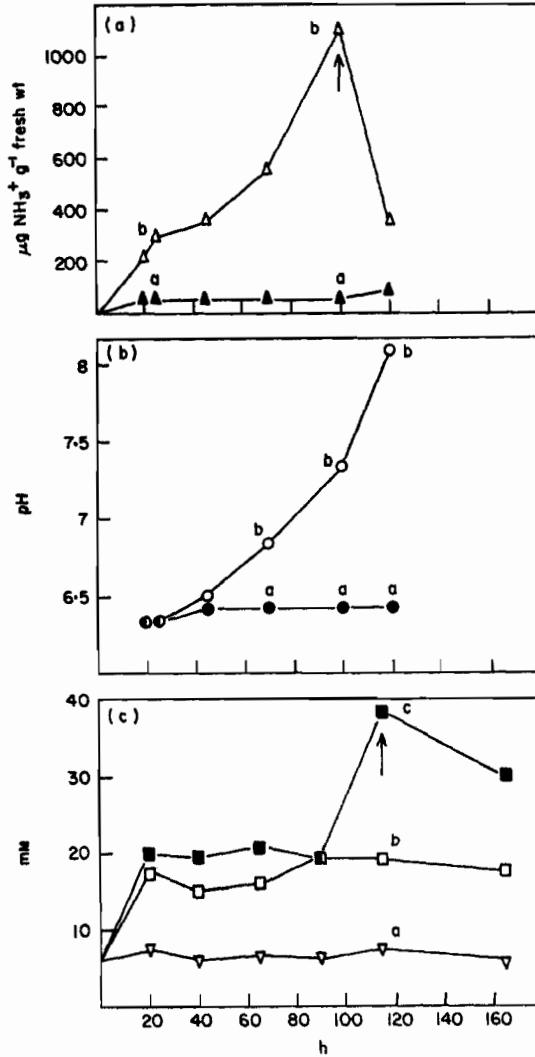


FIG. 1. Ammonia accumulation (a), pH (b) and electrolyte leakage (c) symptoms appearance in tomato plants infected with *Pseudomonas tomato* (arrow) and *P. fluorescens*. Δ , ammonia accumulation with *P. tomato*; \circ , pH of leaves infected with *P. tomato*; \blacktriangle , ammonia accumulated with *P. fluorescens*; \bullet , pH of leaves infected with *P. fluorescens*; \blacksquare , electrolyte leakage of leaves infected with *P. tomato*; \square , electrolyte leakage of leaves infected with *P. fluorescens*; ∇ , electrolyte leakage of untreated leaves.

Points in the graph are mean of 2 experiments each carried out in six replicates. Different letters in the graph show significant differences at $P = 0.05$.

DISCUSSION

The main symptoms observed in tomato leaves attacked by *Pseudomonas tomato* are black necrotic specks with small diffuse chlorotic edges [28]. The necrotic factor is a thermostable, low molecular weight substance. The possibility of necrosis caused by the salts present in the growth medium was disregarded, because their concentration was smaller in the active culture filtrate than in the non-active fresh medium.

Purification of the toxic substance(s) from liquid cultures was carried out by several procedures. Sinden & Durbin [36] reported on the production of a chlorosis-inducing toxin by *P. tomato*. No chlorosis-inducing toxin, however, could be obtained from our pathogenic isolates of *P. tomato* using methods similar to those reported by Mitchell [24] for purification of phaseolotoxin produced by *P. phaseolicola*. It is possible that other *P. tomato* strains produced a chlorosis-inducing toxin. No toxic factor other than ammonia was found in the buffered filtrate of *P. tomato*. The active fraction contained mainly ammonia, and when the ammonia was evaporated the fraction lost its activity. Furthermore, necrotic activity of *P. tomato in vivo* was accompanied with the production of large quantities of ammonia in the diseased plants.

Induction of necrosis by toxic bacterial volatiles was reported by Cook & Stall [4] using several bacterial species.

There are also many reports on ammonia toxicity and on ammonia in diseased plants [3, 29, 33, 39, 41]. Ammonia accumulation in the plant can reach a toxic level as the pH increases [9, 19, 20]. Lovrekovich *et al.* [20] found a correlation between toxicity, pH and the amount of ammonia in diseased plants. We show here that ammonia and pH increase to high levels in the leaves of tomato following inoculation with the pathogen *P. tomato* but not with the saprophyte *P. fluorescens*. Furthermore, the period of electrolyte leakage appearance coincided with ammonia accumulation. Thus, giving a further indication that the onset of these irreversible events which result in symptom formation are caused by ammonia.

On the other hand, our results support the observations [20] that the ammonium ion or amino acids are not toxic in concentration of about $100 \mu\text{g ml}^{-1}$ whereas ammonia has a necrotic effect at this concentration.

P. tomato produced ammonia at relatively high levels either in the host or when grown on media containing short-chain peptides, or tomato leaf extracts.

The possibility of *P. tomato* cells possessing a necrosis- or chlorosis-inducing toxin was also investigated. A cell-free extract of *P. tomato* cells did not affect tomato leaves, it caused, however, necrosis-like symptoms when applied to bean leaves, probably because of a hypersensitive reaction occurring in the non host plant [5, 15, 22].

This research was partly supported by grant no. 823/026 from the Agricultural Research Organization, Ministry of Agriculture, Israel. We thank Miss Batia Goc and Miss Rumia Guvrin for technical assistance, and Dr Z. Harduf and Dr R. Bielorai (Div. of Animal Nutrition, ARO, Volcani Center, Bet Dagan, Israel) for amino acid analysis.

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