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A possible role for proteases and deaminases in the development of the symptoms of bacterial speck disease in tomato caused by *Pseudomonas syringae* pv. *tomato*

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Pseudomonas syringae pv. *tomato* produces constitutive proteases during the logarithmic phase of growth in culture, which have optimum activity at pH 7·0 and 30 °C. The addition of proteinaceous compounds to the growth medium caused only a slight increase in the specific activity of the enzyme preparation. Proteolytic activity was higher in the diseased tissue of susceptible plants than in resistant plants, reaching maximum levels during the later stages of infection. Total proteolytic activity in diseased plants appeared to be due to four proteases, two originating from the pathogen, one from the host and one which appeared to be produced during the interaction between the pathogen and its host. A correlation between disease severity and proteolytic activity in infected tissue was demonstrated, with activity being greatest around developing necrotic zones. The activity also varied with the age of the leaf at the time of infection. Total nitrogen content and soluble proteins decreased in infected susceptible tissue during disease development, with asparagine and free amino acids accumulating during the first 100 h after inoculation, but then decreasing. The pathogen utilized five amino acids as a source of nitrogen in culture, but only asparagine and glutamine appeared to be utilized in the infected plant. Five different deaminases were detected in culture, but, of the five, only asparaginase and glutaminase were detected in the infected suceptible cultivar. Liberation of gaseous ammonia in the infected susceptible cultivar tissue was first observed 72 h after inoculation. Similar changes also occurred, but on a very limited scale, in inoculated resistant plants. The addition of asparagine and glutamine to infected suceptible plants, increased disease severity and ammonia production.

INTRODUCTION

Phytopathogenic bacteria produce hydrolytic enzymes, mainly pectinases, which have been shown to be important for disease initiation and the degradation of host plant tissues [*10, 17, 33*]. While many bacteria, such as species of *Pseudomonas* and *Xanthomonas*, are capable of hydrolysing proteins in culture, few workers have implicated proteolytic activity with pathogenicity [*9, 16, 26*]. Proteolytic enzymes may be involved in the degradation of host cell walls during the early stages of infection, or cause tissue collapse at later stages of disease development.

It has been suggested that the accumulation of ammonium ions and the evolution of gaseous ammonia may contribute to the bacterial speck syndrome in tomato plants caused by *Pseudomonas syringae* pv. *tomato* [*2*]. Free amino acids in plants can be

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Abbreviations used in text: BOH, synthetic medium with galactose and asparagine as sole carbon and nitrogen sources, DI, disease index; CFU, colony forming units; GS, glucose salts medium; TCA, trichloroacetic acid; WPB, synthetic medium with sucrose as sole carbon source; YP, yeast peptone broth.

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deaminated to release ammonia by the activity of deaminases [*37*]. Ammonia can also accumulate as a result of the deamination of glutamine and asparagine, the two amino acids which serve as nitrogen reservoirs and which can be drawn on for the synthesis of new proteins [*19, 22*]. Accumulation of free amino acids in diseased plant tissues has also been reported [*29,* 38, 39]. It was suggested that amino acid accumulation during the development of angular leaf spot of cucumber, resulted from proteolytic activity [*16*], but this hypothesis was later withdrawn [*15*].

One aim of this study was to investigate the capacity of *P. syringae* pv. *tomato* to produce proteolytic enzymes, and to follow the development of proteolytic activity during the course of infection of tomato and the associated disease development. A further aim was to identify the products of proteolytic activity, and to investigate the evolution of the deaminative activity which leads to the accumulation of the ammonia, which may be an important factor in bacterial speck development. A preliminary report of this study was presented elsewhere [*25*].

MATERIALS AND METHODS

Organisms and general procedures

Pseudomonas syringae pv. *tomato* isolate (WT-1), originally isolated from infected tomato plants [*1*], was used in all experiments. Two further isolates, Bet-Dagan 134-1, and ATCC 10852 were used in some experiments. An isolate of *P. fluorescens* from healthy tomato plants, and one isolate of *P. aeruginosa* (from the Departmental collection) were also used.

Two tomato (*Lycoperiscon esculentum* Mill) cultivars were used in most experiments; the highly susceptible cv. "VF-198" and the resistant cv. "Rehovot 13". Other tomato cultivars and lines included in one experiment were: cv. Rutgers nor — acc. 364, Red Sherry, Kewalo, Rutgers rin-acc. 365, Ontario 7710, VFN–7OT–81–1 (UCD), Italian winter, Hosen VF—228, F_2 (Ont. 7710 × Rehovot 13), Step–535, Saladette, Extra Marmande, Manalucy, Tropic–VF, lines 97–3 (UCD) and line 339944–3 (UCD). A line of one other species was also used: *Lycopersicon pimpinellifolium* P.I. 126927 and a line of *L. esculentum* var. *cerasiforme.*

Growth conditions, inoculum preparation, inoculation procedures, mist chamber conditions, pathogenicity tests and disease index (DI) determinations were as described previously [*1, 42*]. Amino acids at a concentration of 10^{-3} M were applied, (1 ml per leaf), directly onto the leaf surface; the leaves were then inoculated with *P. syringae* pv. *tomato.*

Media

For the production of inocula, bacteria were grown in a yeast-peptone liquid medium; however, for the enzyme investigations they were grown in a defined synthetic medium (BOH), especially developed for *P. syringae* pv. *tomato,* which contained galactose and asparagine as sole carbon and nitrogen sources [*3*]. Other synthetic media used were a glucose–salts medium (GS) [*2*] and a sucrose–salts medium (WPB) [*40*]. In some experiments, 1% peptone, proteose peptone No. 3, casein hydrolysate, casein, bovine serum albumin, egg albumin or soluble tomato leaf proteins extracted from cv.

"VF–198" according to the method of Uritani and Stahmann [*38*] were used as C and N sources in the BOH synthetic medium.

Enzyme extractions

Bacterial cells were collected from liquid cultures by centrifugation at 10 000 ***g*** for 10 min at 4 °C. The pellet was washed three times in 0·06 M phosphate buffer, pH 7·0. The washed cells were then resuspended in 5 ml buffer, and sonicated in an ice bath using a 1·5 A MSE ultrasonic disintegrator (three 60 s treatments, with an interval of 60 s between each treatment). The sonicate was finally centrifuged at 30 000 ***g*** for 30 min at 4 °C. The culture filtrate was also retained and used in investigations of enzyme activity. Partial protein purification of bacterial sonicate and culture filtrate was carried out by two successive gel filtrations, first on a G–25 Sephadex column, followed by a separation on a G–200 column (61 × 2·5 cm, eluted with 0·06 M phosphate buffer, pH 7·0, elution rate 0·2 and 0·15 ml min^{-1}, respectively.) Fractions with proteolytic activity were lyophilized and redissolved in 0·06 M phosphate buffer, pH 7·0, before testing. Leaves homogenized in an ice bath, first with an Ultra Turrax (Janke and Kunkel), then in an Omnimixer (Sorvall), again the Ultra Turrax and finally with a fine homogenizer (Elda). The homogenate obtained was first centrifuged at 12 000 ***g*** for 10 min and the supernatant then subjected to two further 30 min periods of centrifugation at 30 000 ***g***. Proteins present in the final supernatant were treated as described for the bacterial extracts.

Enzyme assays

Proteolytic activity was determined colorimetrically, by measuring the increase in absorbance at 595 nm which occurs as the result of the accumulation of the soluble blue pigment which is released from insoluble crystal Hide-azure (Calbiochem) by protease activity [*27*]. The 3 ml reaction mixture consisted of 2 ml 0·06 M phosphate buffer pH 7·0, containing 5 mg ml^{-1} Hide-azure and 1 ml enzyme preparation and was incubated for 2 h at 37 °C. The reaction was stopped by the addition of 2 ml of a solution of 10% TCA. The mixtures were transferred to an ice bath for 30 min and then centrifuged at 2000 ***g*** for 10 min. Mixtures without enzyme or without substrate, or containing boiled enzyme or commercial chymotrypsin (Sigma), were used as controls. One unit of enzyme activity was defined as that amount of enzyme which produced an increase in absorbance of 0·01 h^{-1} mg^{-1} protein.

All deaminase assays were carried out using freshly prepared enzyme preparations. Glutamate dehydrogenase was assayed according to the method of King [*18*], asparaginase, glutaminase and glycine synthase were assayed according to the method of Bergmeyer *et al.* [*5*], serine dehydratase according to the method of Sagers & Carter [*30*] and threonine deaminase according to the method of Nakazawa [*23*].

The controls included reaction mixtures without enzyme, or without substrate, or with boiled enzymes. Further controls included reaction mixtures in which the enzyme was applied at the end of the incubation period. Enzyme units were defined as described elsewhere [*5, 18, 23, 30*].

Electrophoresis and protease staining

Electrophoresis was carried out on 7% polyacrylamide gels using slight modifications of the methods described by Davis [*8*] as follows After a pre-run at 1 mA, 20 µl samples

containing 10 μg protein were run at 100 v, 10 mA for 2 h. Proteases were stained according to the method of Bott [7].

Ammonia atmosphere cell

Detached tomato leaves were put in enrichment bacterial systems as described by Sharon *et al.* [32]. Before inoculation, the leaves were washed under a stream of tap water for 10 min to remove dust and dirt, soaked for 3 min in a 0·5% (w/v) solution of NaOCl and washed again for 10 min under a stream of tap water. The leaves were then placed aseptically on 0·5% (w/v) water agar plates with their adaxial side up. The leaves were inoculated by spreading 2 ml amounts of a bacterial suspension over their surface. Small plastic Petri dishes (3 cm dia.) containing crystals of NaOH were immersed in water agar contained in a large Petri dish (9 cm diameter). After the large petri dish was sealed with parafilm, aqueous solutions of various concentrations from $1–10^6$ μg ml^{-1} of NH_4Cl were injected through the plate-cover onto the NaOH crystals. The injection pore was immediately sealed and the effect of any evolution of gaseous ammonia from the mixture, on the leaves, was recorded.

Ammonia absorption from sealed cells

This was estimated using the same cells as described above, but the small plastic Petri dish was replaced with a glass dish containing 1N N_2SO_4. Ammonia determination was done directly in the Petri dish by the improved phenol-hypochlorite method[13].

Chemical analyses

Soluble proteins were extracted from plant tissues using the method of Uritani and Stahmann [38] and amino acids by the method of Keen *et al.* [16]. Total proteins were assayed using the Coomassie brilliant blue G-250 method [31], total amino acids by the method of Yemm and Cocking [41] and individual amino acids using an amino acid analyser (Technicon TSM). Asparagine and glutamine were analysed according to Bessman [6]. Ammonia was determined by the improved phenol-hypochlorite method [13] and the UV method (Kit No. 170–UV Sigma). Total nitrogen content of leaves was determined after acid digestion of dried leaves (6 N HCl for 16 h at 100 °C), using the method described above for ammonia determination.

Experimental design

All experiments were repeated 2 to 3 times with 5 to 10 replicates in each. Five to 20 plants, 3 Erlenmayer flasks or 3 sealed cells constituted a replicate. The results given are from one representative experiment in each case.

RESULTS

The production of proteases by P. syringae *pv.* tomato *in liquid cultures*

The optimal conditions for proteolytic activity in extracts, either from the cells or culture media of *P. syringae* pv. *tomato*, were determined by testing activity over a range of pH from 4–9, a range of temperatures from 4–50 °C and a range of incubation times from 0·5–10 h. The bacterial cells or culture supernatants contained maximum specific

TABLE 1

Proteolytic activity in extracts from cultures of three isolates of Pseudomonas syringae *pv.* tomato *pathogenic on tomato and two saprophytic species of* Pseudomonas

Bacteria	Proteolytic activity (Units)†				
	Peptone		Leaf proteins		
	Bacterial cells	Culture supernat-tant	Bacterial cells	Culture superna-tant	Disease index (0–3)
P. syringae pv. *tomato*					
Bet Degan 134.1	6·35‡	2·01	6·15	2·17	2·98a§
ATCC 10852	6·48	1·89	6·44	2·21	2·95a
WT-1	6·04	1·76	5·88	1·04	2·83a
Saprophytic species					
P. fluorescens	9·86	4·12	8·75	3·91	0b
P. aeruginosa	10·44	4·76	9·1	4·03	0b

†After separation of cells from the liquid culture, bacteria and culture supernatant were tested, separately. The unit of proteolytic activity was defined as an increase in absorbance at 595 nm of 0·01 h^{-1} mg^{-1} protein.

‡All values are means from 20 replicates.

§Values followed by a different letter differ significantly at $P \leqslant 0{\cdot}05$. The disease index was recorded five days after inoculation using a scale of 0 to 3.

protease activity between 16 and 18 h after inoculation, at which stage the cultures were approaching the end of the logarithmic phase of growth on the BOH medium containing 1% peptone. Activity in the culture supernatant was lower than that of the sonicated cells. Optimum activity occurred at pH 7·0 and 30 °C and activity increased linearly over the period from 1–6 h after inoculation. The effect of the addition of different proteinaceous substances to the BOH medium on enzyme production was also determined. None of the proteinaceous substances enhanced enzyme production much (6·8–7·6 activity units as compared with 5·2 units in extracts from cultures grown with asparagine as a sole N source). Thus, the proteases of *P. syringae* pv. *tomato* seem to be constitutive enzymes.

Relationship between pathogenicity on tomato and proteolytic activity in culture

The ability of three isolates of *P. syringae* pv. *tomato* and two saprophytic pseudomonads to produce proteases in culture, was compared with their ability to initiate disease symptoms in tomato plants (cv. "VF-198"). No relationship was observed between pathogenicity on tomato and the proteolytic activity of the cultures (Table 1).

Proteolytic activity in plants

Proteolytic activity was measured during disease development in response to *P. syringae* pv. *tomato* in both susceptible and resistant tomato plants. The assay conditions for protease activity in infected leaves of tomato (120 h after inoculation) were similar to

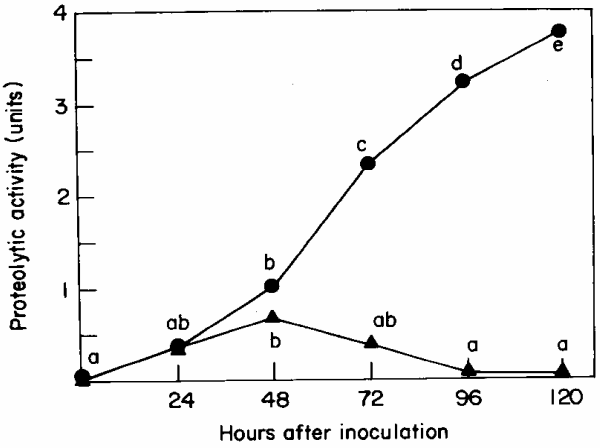

FIG. 1. Proteolytic activity in resistant and susceptible tomto plants during disease development. (●), susceptible cv. VF-198; (▲), resistant cv. "Rehovot-13". Inoculum concentration 10^5 CFU ml^{-1}. One unit of protease activity produces an increase in absorbance of 0·01 h^{-1} mg^{-1} protein. Points on the graph followed by different letters differ significantly at $P \leqslant 0{\cdot}05$.

the conditions for bacterial extractions. No activity was detected in extracts of leaves from healthy plants.

During the first 48 h after infection, low levels of specific protease activities were detectable in both susceptible and resistant plants. At later stages of infection, between 48 and 120 h after inoculation, specific activity increased in the susceptible plants but decreased in the resistant plants (Fig. 1).

Proteases in bacterial cultures and in infected tissues

In the healthy plant, a single protease was found [Fig. 2 (b) No. 6] which also appeared to be present in diseased tissue. Four intercellular proteases were detected in bacterial cultures Fig. 2(c)], two of which appeared to be present in the diseased tissue (Nos 2 and 4). A further protease was present in diseased tissues which was not detectable either in uninfected plants or in bacterial cultures [Fig. 2(a) No. 1].

Proteolytic activity in plant tissue in relation to disease severity

The highest disease index developed by the resistant cultivar was 1·0, while the disease indices of the suceptible cultivar ranged from 1·66 to 3·0. Protease activity was determined in 20 plants of each of the resistant cv. "Rehovot–13" and the susceptible cv. "VF–198" at each disease level.

A positive highly significant correlation ($r = 0{\cdot}98$) was found between disease index and the proteolytic activity of the diseased tissue (Fig. 3).

Relationships between susceptibility of different cultivars and proteolytic activity in infected tissues

Nineteen cultivars, of *L. esculentum* and one of *L. pimpinellifolium,* ranging from highly resistant to highly susceptible, were analysed for proteolytic activity in the young leaves beneath the growing tip, 5 days after inoculation. Disease severity in the leaves was also recorded. The correlation coefficient between disease severity in the analysed leaves and proteolytic activity was found to be $r = 0{\cdot}97$ (Fig. 4).

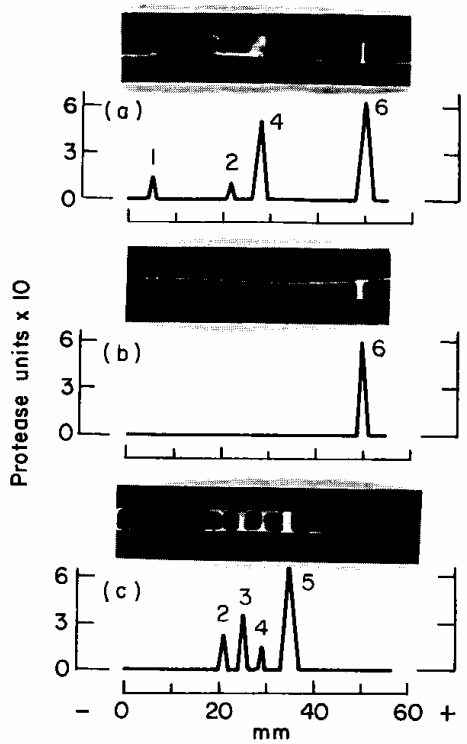

FIG. 2. Proteases isolated from (*a*) diseased plants 120 h after inoculation; (*b*) control healthy plants; (*c*) bacteria (18 h cultures). Peaks on diagrams of a gel scans followed by the same number are identical.

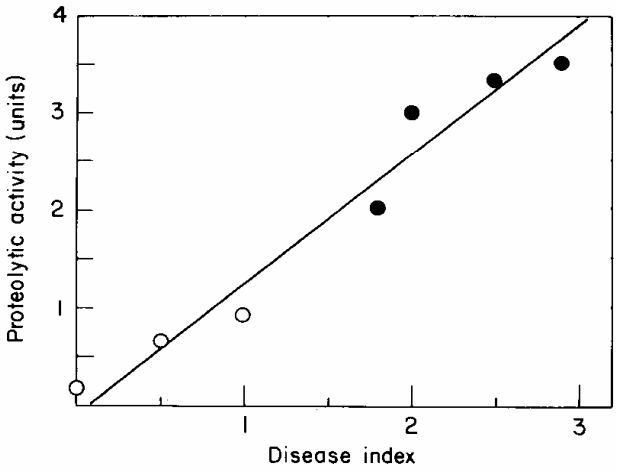

FIG. 3. Relationship between proteolytic activity in tomato tissue and disease index. (○), resistant cv. "Rehovot-13"; (●), susceptible cv. "VF-198". Proteolytic activity units were defined as in Fig. 1. $y = 2{\cdot}59x - 0{\cdot}04$; $r = 0{\cdot}98$.

Variations in proteolytic activity in relation to leaf age

It is known that mature leaves of tomato are less susceptible than younger leaves to infection by *P. syringae* pv. *tomato* [*42*]. Twenty plants of the susceptible cultivar cv. "VF–198" were inoculated 6 weeks after the production of the first true leaf. Five days later, proteolytic activity was measured in each leaf. Activity in the lower, older leaves

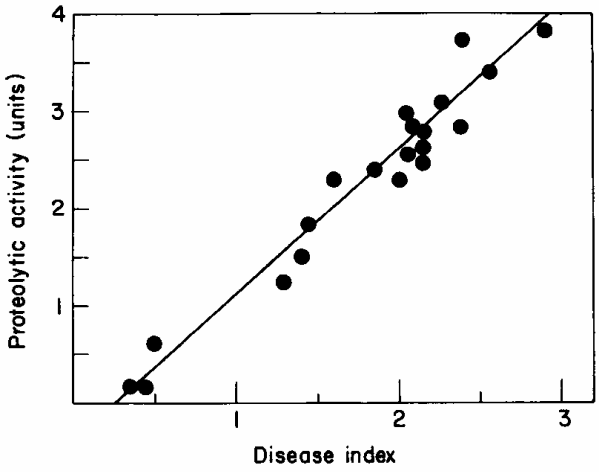

FIG. 4. Correlation between proteolytic activity and resistance to bacterial speck in 19 cultivars and lines of *Lycopersicon esculentum* and one line of *Lycopersicon pimpinellifolium*. Each cultivar was analysed for proteolytic activity and disease severity in the young leaves beneath the growing tip, 5 days after inoculation. Proteolytic activity units were defined as in Fig. 1 and disease index as in Table 1. $y=3{\cdot}01x-0{\cdot}77$; $r=0{\cdot}97$.

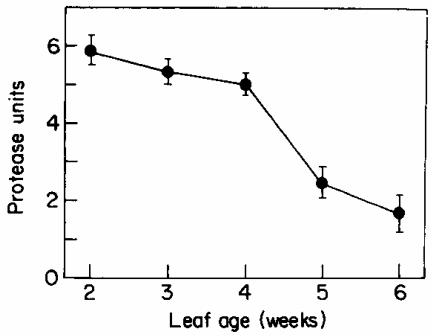

FIG. 5. Proteolytic activity in diseased tissue of leaves of tomato inoculated with *Pseudomonas syringae* pv. *tomato* at different ages. The tomato plants cv "VF-198" were inoculated six weeks after the production of the first true leaf. Proteolytic activity was defined as in Fig. 1. Bars represent standard error.

was found to be lower than that in the upper, more susceptible young leaves (Fig. 5). Similar relationships were observed in plants of different ages, which were inoculated at the same time.

Location of proteolytic activity in diseased tissues

After symptoms first appeared in susceptible plants, 5 replicates each of 20 leaf discs were cut with a 6 mm cork borer from different parts of diseased leaves. Proteolytic activity was very low within necrotic tissue but high activity was found around this tissue, with the greatest activity being found around the smaller, still expanding necrotic areas. Activity was low in tissue distant from the necrosis, even when most of the plant was visibly infected (Table 2).

Changes in total nitrogen, soluble protein, asparagine, glutamine and free amino acids in infected tissues

The total nitrogen content of infected plants showed a marginal but steady decrease, which accelerated slightly towards the later stages of disease development [Fig. 6(a)].

TABLE 2.
Proteolytic activity at various sites on the leaf in relation to lesions in infected susceptible tomato plants

Site in leaf†	Proteolytic activity (units)‡
Healthy leaves	0d§
Necrotic area of lesions	0·5d
Tissue surrounding large necrotic lesions (3–5 mm diameter)	4·15b
Tissue surrounding small necrotic lesions (1 mm dia.)	5·45a
Tissue of infected leaflet some distance from necrosis)	1·12c
Apparently healthy leaves from an infected plant	0·88cd

†After symptoms had appeared, leaf discs were cut from each site with a cork borer and analysed for proteolytic activity.
‡Unit as in table 1.
§Values followed by a different letter differ significantly at $P \leqslant 0{\cdot}05$.

During the first 100 hours after inoculation, soluble protein content decreased. There was a concomitant increase in asparagine levels during the first 72 hours, after which they also sharply decreased [Fig. 5(b)], with a corresponding increase in ammonia, first detected just before the appearance of necrotic spots (Fig. 7 and Ref. 2). Accumulation of free amino acids in the diseased tissue paralleled the increase in proteolytic activity until 96 hours after inoculation, when their levels decreased sharply, although proteolytic activity continued to increase [Fig. 6(c)]. Only a slight increase in free amino acid levels was observed in inoculated resistant plants. No measurable changes were detected in uninoculated plants during the 5 day experiment. Changes in asparagine, glutamine and free amino acids were measured 8 days after inoculating leaves of different ages. Figure 6(d) shows that total free amino acid content was lowest in the leaves which were oldest at the time of inoculation. Similar trends were found in asparagine and glutamine levels in relations to leaf age at the time of inoculation. In addition, the number of necrotic lesions produced from a given level of inoculum decreased with leaf age at the time of inoculation.

Utilization of amino acids as nitrogen sources in culture and in infected plants

Twenty-one different amino acids were tested as sole nitrogen sources at concentrations of 1 g l^{-1}, in BOH medium containing 0·06 M potassium phosphate buffer, pH 6·8. The experiment was repeated three times with 10 replicates in each treatment. Bacterial growth was estimated after 24 h by measuring the absorbance of the culture. The amino acids fell into three groups: (a) asparagine, glutamine, serine, threonine and glycine supported good growth (1·01, 0·98, 0·28, 0·93 and 0·35 absorbance units, respectively); (b) glutamate and lysine supported little growth (0·08 and 0·12 absorbance units, respectively); (c) the remaining 13 amino acids did not support any growth.

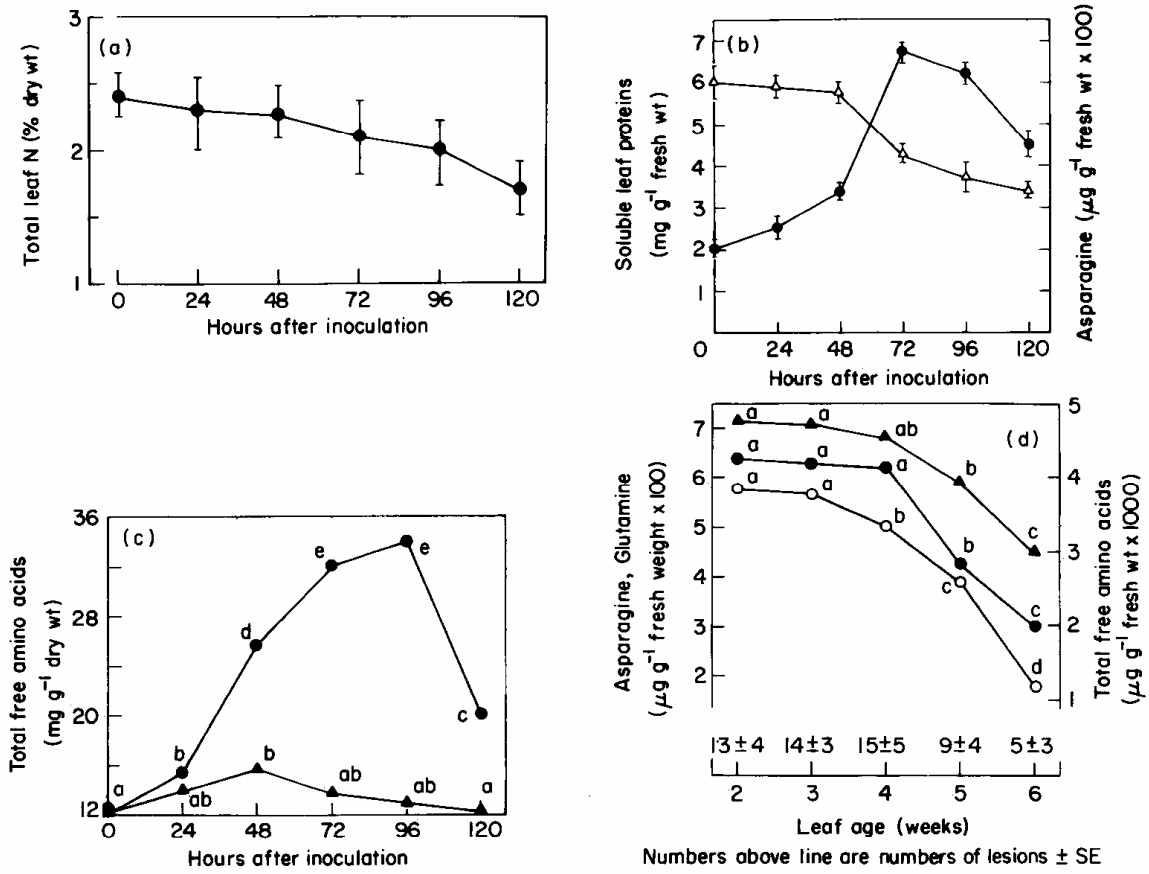

FIG. 6. Changes in levels of the different forms of nitrogen in tomato leaves during bacterial speck development. The plants were inoculated with 10^6 CFU ml^{-1}. (a) Changes in total nitrogen. (b) Changes in soluble proteins and asparagine (●), asparagine; (△), soluble proteins. Bars represent standard error. (c) Changes in free amino acids. (●), susceptible cv. "VF-198"; (▲), resistant cv. "Rehovot-13". Points on the graph followed by a different letter differ significantly at $P \leqslant 0{\cdot}05$. (d) Changes in asparagine (●), glutamine (○) and free amino acids (▲) in leaves inoculated with *P. syringae* pv. *tomato* at different ages. The plants were inoculated 6 weeks after the production of the first true leaf. Points on each line followed by different letters differ significantly from each other at $P \leqslant 0{\cdot}05$.

An analysis of the concentrations of different amino acids in inoculated susceptible tomato cv. "VF–198" 72 and 120 h after inoculation indicated that after 72 h most of the 20 amino acids had accumulated in the infected tissue, even those which the bacterium did not utilize in culture. However, after 120 h asparagine and glutamine decreased markedly and threonine, serine and glycine showed slight decreases. The levels of the other amino acids either increased or remained stable (Fig. 7).

Deaminase activity in cultures of P. syringae *pv.* tomato *and in the infected host*

The ability of the specific substrate amino acid to induce the production of glutamate dehydrogenase, asparaginase, glutaminase, glycine synthase, serine dehydratase and threonine dehydratase was tested. Bacteria were grown in BOH medium, in the presence of 0·05% of each inducer amino acid. The location of the enzyme in the bacterial culture was also tested. Asparaginase, glutaminase and threonine

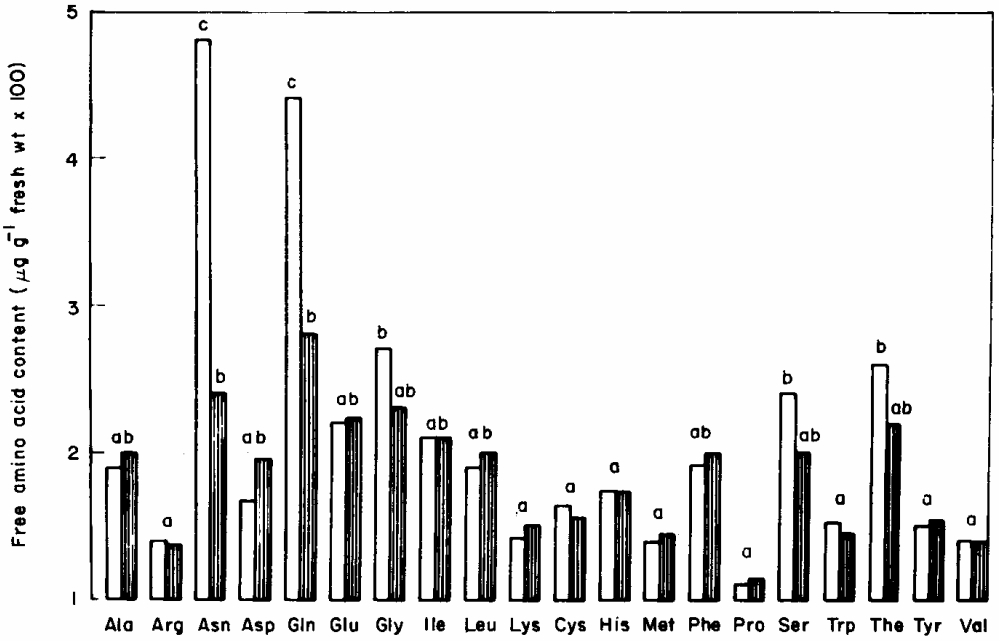

FIG. 7. Accumulation of free amino acids in diseased tomato tissue of the susceptible cv. "VF-198" 72 h (□) and 120 h (▥) after inoculation with 10^6 CFU ml^{-1}. Alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cystine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); isoleucine (Ile); leucine (Leu); lysine (Lys); histidine (His); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); tryptophan (Trp); threonine (The); tyrosine (Tyr); valine (Val). Columns followed by different letters differ significantly at $P \leqslant 0{\cdot}05$.

dehydratase were found to be inducible to high specific activity, but remained totally within the bacterial cell ($4{\cdot}02 \pm 0{\cdot}03$, $3{\cdot}85 \pm 0{\cdot}05$ and $3{\cdot}77 \pm 0{\cdot}12$ units, at 200 mM substrate concentration, respectively). Glycine synthase and serine dehydratase showed the same characteristics, although their specific activities were lower ($1{\cdot}56 \pm 0{\cdot}06$ and $1{\cdot}96 \pm 0{\cdot}08$ units, respectively). In contrast, the specific activity of glutamate dehydrogenase remained low (0·02 units), even in the presence of its substrate amino acid.

The activities of these enzymes were also investigated in infected plants of the susceptible cv. "VF–198" and the resistant cv. "Rehovot–13", 100 h after inoculation. Leaves showing symptoms and leaves without symptoms from inoculated plants were separately homogenized. Leaves from healthy plants were included as controls. Asparaginase and glutaminase were only detected in infected plants, their activities being higher in leaves of the suceptible cultivar than in the resistant cultivar and higher in leaves showing symptoms, than in leaves without symptoms (Table 3).

Deaminative activity of saprophytic pseudomonads

Deaminative activity, expressed as ammonia production from glutamine or asparagine, by combined glutaminase and asparaginase activities of *P. syringae* pv. *tomato*, was compared with that of the saprophytes *P. fluorescens* and *P. aeruginosa*. The bacteria were grown in YP medium maintained in 0·1 M sodium phosphate buffer pH 6·8 [*11*] for 72 h. All the pseudomonads tested showed high deaminating activity in culture (5·64, 7·03, 6·81 and 4·88 units, respectively) and produced ammonia in large quantities

TABLE 3.
Asparaginase and glutaminase activity in inoculated resistance and susceptible tomato plants

Plant†Symptons	Asparaginase	Specific activity (units)‡ Glutaminase	
Resistant cv. "Rehovot-13"	+§	0·98±0·11	0·52±0·12
Inoculated	—	0·13±0·1	0·1±0·1
Non-inoculated control	—	0	0
Susceptible cv. "VF-198"	+	3·82±0·13	3·02±0·06
Inoculated	—	1·46±0·08	1·11±0·1
Non-inoculated control	—	0	0

†One hundred hours after inoculation; leaves showing symptoms and symptomless leaves were analysed separately for deaminase activity.

‡200 mM substrate concentration. One enzyme unit catalyses the production of 1 μg ammonia h^{-1} mg^{-1} protein.

§(+), Symptoms present on leaflets; (—), symptomless leaflets.

(120, 95, 180 and 115 μg ammonia, respectively). However, when inoculated into tomato leaves maintained in sealed cells, only *P. syringae* pv. *tomato* showed deaminative activity in infected plants (5·88±0·17 enzyme units and 960±80 μg ammonia g^{-1}, leaf disease index 2·89±0·11).

The effect of medium composition on ammonia production by P. syringae *pv.* tomato

The effect of the initial pH of the BOH medium, supplemented with either 0·06 M acetate or phosphate buffer [*11*] on bacterial growth and ammonia production after 24 hours growth was tested. No correlation between growth and ammonia production was detected. Best growth occurred between pH 6·0 and 7·0, but the highest levels of ammonia production occurred at pHs over 7·0. On the other hand, bacterial growth decreased at those pH levels.

Growth and ammonia production were examined in various other media, including two complex media YP and tomato leaf extract media and three synthetic media, WPB, GS and BOH. It was found that, 24 h after inoculation, most growth occurred and most ammonia was produced on the two complex media (1·1±0·01 and 1·05±0·02 absorbance units and 130±10 and 135±15 μg ammonia produced per ml, in YP and tomato leaf extract media, respectively). Of the synthetic media, the best growth and ammonia production was obtained in BOH medium (0·82±0·03 absorbance units and 100±8 μg ammonia per ml, respectively). The other synthetic media supported poor growth and ammonia production (0·15±0·01 and 0·13±0·02 absorbance units and 5±2 and 4±2 μg ammonia per ml, in WPB and GS media, respectively).

Production of ammonia by diseased tissues

Leaves of resistant and susceptible tomato cultivars were placed in enrichment sealed cells and inoculated with *P. syringae* pv. *tomato*. Liberation of ammonia by suceptible

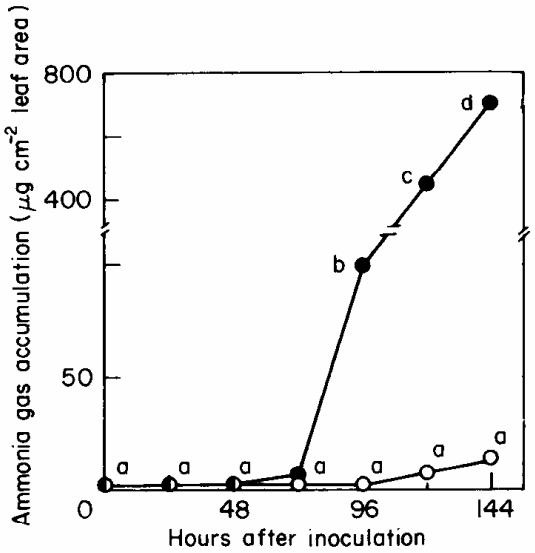

FIG. 8. Leakage of gaseous ammonia from leaves of infected tomato plants during disease development in enrichment sealed cells. (●), susceptible cv. "VF-198"; (○), resistant cv. "Rehovot–13". Values followed by different letters differ significantly from each other at $P \leqslant 0{\cdot}05$.

tissues began 72 h after inoculation and then increased dramatically (Fig. 8). Only small amounts of ammonia were produced by resistant plants (15 μg cm^{-2} leaf area as compared to 700 μg cm^{-2} in leaf area in susceptible plants).

Effect of the addition of amino acids to leaves before inoculation on disease severity

Low concentrations (10^{-3} M) of asparagine, glutamine, serine, threonine, glycine, arginine or methionine were applied to leaves before inoculation with *P. syringae* pv. *tomato*. Inoculated and uninoculated untreated leaves and uninoculated leaves treated with either 10^{-3} M or with 1 M amino acids were used as controls. All amino acids which induced deaminative activity in culture also induced severe necrosis, and ammonia accumulation (Fig. 9). Asparagine, glutamine and serine caused the most severe effects. Arginine and methionine, which did not induce deamination, had no effect on disease severity.

DISCUSSION

Recently, we have demonstrated that *P. syringae* pv. *tomato* produces ammonia in infected tomato plants and that this ammonia may contribute to the general syndrome of necrosis [*2*]. Production of ammonia during pathogenic infections has been reported for other phytopathogenic bacterial systems as well [*12, 14, 20, 21, 24, 34, 35, 36*]. In healthy plants, ammonia accumulation in tissues is negligible because of its fast incorporation into compounds such as asparagine and glutamine. The evolution of ammonia in diseased tissue probably results from deamination of the amino acids produced by the proteolytic breakdown of cell proteins.

No correlation was found between the ability of different *Pseudomonas* species to produce proteases in culture and their ability to infect and cause disease in tomato plants. On the other hand, a high correlation was found between proteolytic activity in

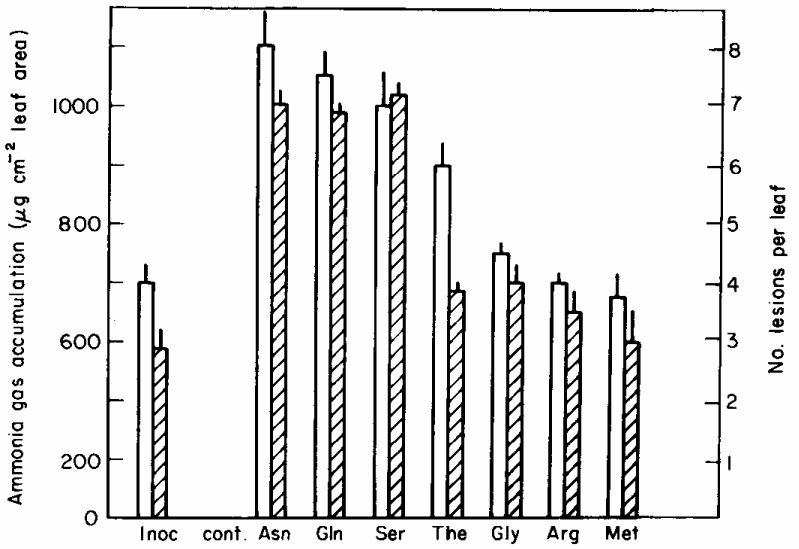

FIG. 9. The effect of the addition of 10^{-3} M amino acids to the leaf surface prior to inoculation on disease development (▨) and ammonia accumulation in enrichment sealed cells (□) in tomato leaves, 7 days after inoculation. Inoculated plants (Inoc); uninoculated controls (Cont); plants treated with asparagine (Asn); glutamine (Gln); serine (Ser); threonine (The); glycine (Gly); arginine (Arg); methionine (Met). Bars represent standard error.

diseased tissue and disease severity in plants infected with *P. syringae* pv. *tomato*. Our studies showed that both *P. syringae* pv. *tomato* and *P. fluorescens* can grow on tomato leaves. However, surface sterilization of the leaves, showed that only the pathogen was present within the leaf [*32*]. Proteolytic activity in the infected leaf decreased as the leaf aged, and other studies have shown that the older tomato leaves have greater resistance to bacterial speck [*42, 43*]. A high correlation was also observed between the proteolytic activity of infected tissue of 20 tomato cultivars infected by *P. syringae* pv. *tomato* and disease severity, the proteolytic activity of the most resistant cultivars being much lower than that of the more susceptible ones. Proteolytic activity was located mainly around the necrotic tissue and, as shown by electrophoretic pattern, was due to contributions from both the pathogen and the host. In addition, a new protease was detected in the infected plant which could have been produced by the bacterium or by the plant (Fig. 2).

Pseudomonas syringae pv. *tomato* produces both extracellular and intracellular proteases in culture, and so both types of enzyme may be produced in the infected plant. However, such a distinction is probably of little importance in the infected plant, since enzymatic activity was found mainly at the later stages of disease development (48–72 h after inoculation), when the tissue was fully colonized by the bacteria [*4*] and by which time some of the bacteria may have autolysed, releasing intracellular proteases into the tissue.

The data presented so far suggest that proteolytic activity does not play a key role in the initiation of an infection, but it is probably significant in terms of symptom development. In a recent study it was reported that a mutant of *Cladosporium cucumerinum*, which did not secrete protease, was pathogenetic to cucumber, indicating that extracellular proteases of this pathogen do not play a role in the establishment of infection [*28*].

Proteolytic activity which takes place during the later stages of disease development caused a reduction in soluble proteins in the tomato leaf and an accumulation of free amino acids, mainly asparagine and glutamine. These changes took place just before ammonia accumulation began. Although *P. syringae* pv. *tomato* was found to produce several deaminases in culture, only asparaginase and glutaminase were detected in the diseased plant. Moreover, the application of asparagine or glutamine to tomato leaves prior to inoculation with *P. syringae* pv. *tomato* induced earlier production of symptoms and increased disease severity, either by inducing pathogen deaminating enzymes or by supporting a faster multiplication of the pathogen in the tissue. Similar effects of amino acids on the virulence of *Erwinia carotovora* have been reported [*44*].

The main factor determining tissue degradation may be the level of the bacterial population in the tissue. It has been shown that, at the times symptoms first became visible, the bacterial population in the tissues is much higher in susceptible plants (10^5–10^6 CFU g^{-1} tissue, wet wt), than in resistant plants (10^2 or less CFU g^{-1} tissue) [*4*]. The massive bacterial population deaminates and then utilizes the liberated carbon for energy and some of the ammonia as a nitrogen source for multiplication. Some of the excess ammonia, which evaporates, may be released as vapour and diffuse through the intercellular spaces, damaging the cells. This damage may lead to liberation of more amino acids and so the deamination cycle continues.

We have demonstrated that deamination of amino acids to produce ammonia can occur through the activity of bacterial enzymes, but it is not clear yet whether the ammonia produced in the diseased plant is the result of host or bacterial enzyme activity. O'Brien and Wood [*24*] showed that ammonia plays no part in the hypersensitive response of bean leaves to incompatible species of *Pseudomonads* and claimed that the accumulation of ammonia is a secondary consequence of the killing of the plants cells. However, the ammonia concentration was high enough to explain symptoms in susceptible bean leaves and may give further support to our findings in bacterial speck of tomato.

The data presented here suggest that after the establishment of a massive *P. syringae* pv. *tomato* population in plant tissue (after recognition, penetration, multiplication, primary cell collapse and possible liberation of proteins and amino acids), the pathological interaction produces proteolytic enzymes which degrade plant proteins to amino acids. These amino acids are then deaminated, particularly asparagine and glutamine by asparginase and glutaminase, resulting in the production of large quantities of ammonia. This leads to an increase in plant tissue pH at later stages of disease development [*2*]. At pH 8·0 and above, the ammonia is liberated as gaseous ammonia, which diffuses through the intercellular spaces and causes leakage of electrolytes. This chain of events results in cell death and in the initiation of the necrotic lesion.

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