Accumulation of Phenolic Compounds in Leaves of Tomato Plants after Infection with *Clavibacter michiganense* subsp. *michiganense* Strains Differing in Virulence

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Infection experiments using tomato (*Lycopersicon esculentum cv.* Moneymaker) plants and three strains of *Clavibacter michiganense* subsp. *michiganense* were conducted. The bacterial isolates differed in plasmid status, virulence (measured as wilting index and as reduction of plant biomass) and the ability to colonize the plants. Differences in the expression of exopolysaccharides and the exoenzymes endocellulase, polygalacturonase, and xylanase, respectively, were not found with the bacterial isolates. As infection-induced responses of the plants time-depending increases in soluble phenolic material, including chlorogenic acid and rutin, in the steroid alkaloid tomatine and in cell wall-bound cinnamic acids were found. In the latter fraction *p*-coumaric, ferulic and especially caffeic acid were the main constituents. All plant responses were preferentially expressed in the compatible interaction. Sesquiterpenoid phytoalexins were not found in the infected plant tissues. The relation between bacterial pathogenicity and plant responses is discussed.

Introduction

Clavibacter michiganense subsp. *michiganense* is a bacterial pathogen of the tomato (*Lycopersicon esculentum*) [1]. Natural infection generally occurs *via* wounds by which the bacteria invade the xylem vessels causing a systemic infection [2]. The typical disease symptoms are wilting and at later stages the appearance of canker lesions. *Clavibacter michiganense* is known to produce in culture high molecular weight exopolysaccharides which induce wilting in plant bioassays [3]. The current assumption on the mode of action of these EPS is that they may interfere with water uptake by a physical plugging of the xylem vessels [3]. Also colonization of the xylem by the bacteria may contribute to deficiencies in water transport leading to the development of wilting symptoms. Since one can assume that an effective propagation of the bacteria in the plant is necessary for establishing the disease it is of interest to test if and how the plant is reacting against this attack.

For the tomato plant various antimicrobial defence reactions have been reported. Among them the accumulation of sesquiterpenoid [4] and polyacetylenic phytoalexins [5], of chitinase and β-1,3-glucanase activities [6] and of the antifungal steroid alkaloid tomatine [7] appear to be especially pronounced. Furthermore, increased levels of soluble and cell wall-bound phenolics partly in connection with the formation of papillae have been observed [8, 9]. Such responses including the formation of proteinase inhibitors [10] have mainly been found in cases of fungal infection so that bacteria-induced defence reactions are much less known.

In this communication we have investigated EPS and exoenzyme production of three strains of *Clavibacter michiganense* subsp. *michiganense* which possess a pronounced difference in virulence and monitored their colonization of tomato plants after infection. In order to obtain information on the plant response induced by the bacterial pathogen an analysis of various possible defence reactions was conducted with special emphasis on soluble and cell wall-associated phenols.

Abbreviations: EPS, exopolysaccharide; fr. w., fresh weight; HPLC, high performance liquid chromatography; GC, gas chromatography; TLC, thin layer chromatography.

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Materials and Methods

Bacterial strains and culture conditions

Clavibacter strains NCPPB 382 and NCPPB 3123 were obtained from the National Collection of Plant Pathogenic Bacteria NCPPB, Hatching Green, Harpenten, Great Britain. Strain CMM 101 is a derivative of strain NCPPB 382 selected after a plasmid curing experiment as described by Meletzus and Eichenlaub [11].

Cultures of *C. michiganense* were grown at 24 to 26 °C in L-broth medium containing 5 g yeast extract, 10 g tryptone, and 5 g NaCl per liter (pH 7.2) or in $CaCO_3$ medium containing 15 g glucose, 10 g yeast extract and 5 g $CaCO_3$ per liter. For reisolation of *C. michiganense* from infected plants a modified SCM medium [30] was used containing 0.25 g $MgSO_4 \times 7H_2O$, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 10 g sucrose, 15 g boric acid, 30 mg nalidixic acid, 0.1 g yeast extract, and 15 g agar per liter.

Plant material

All experiments were carried out with susceptible tomato plants of *Lycopersicon esculentum cv.* Moneymaker obtained from H. and K. Hild, D-W-7142 Marbach, F.R.G. Plants were cultivated in a growth chamber at 19 °C, 8 h/25 °C, 16 h night/day regime and at 80% relative humidity.

Test for presence of plasmids

Large scale DNA purifications of plasmids from *C. michiganense* were prepared from a 1 l culture by a method of Anderson and McKay [12] with an initial lysozyme treatment (10 mg/ml) at 37 °C for 1 h.

Total *Clavibacter* DNA was prepared by the following procedure. Cells of a 5 ml overnight culture were pelleted by centrifugation at 3000 × *g* and resuspended in 500 µl protoplasting buffer (10 mg of lysozyme per ml in 6.7% (w/v) sucrose, 1 mM EDTA, 50 mM Tris/hydrochloride, pH 8.0). After incubation at 37 °C for 1 h 250 µl of lysis buffer (2% sodium dodecyl sulfate) were added and mixed for 1 min on a Vortex mixer. The cell lysate was extracted twice with an equal volume of phenol–chloroform (1:1) and the supernatant was finally dialyzed against TE buffer (1 mM EDTA, 10 mM Tris/hydrochloride, pH 8.0).

For Southern hybridization, DNA was digested with appropriate restriction enzymes, fragments were separated by agarose gel electrophoresis, and transferred to nylon membranes by blotting with an LKB 2016 Vakugen apparatus.

DNA probes were labeled with digoxigenin-11-dUTP by nick translation as described by Maniatis *et al.* [13]. Hybridization was done at 80 °C with 1% blocking reagent, and the results were visualized by using a non-radioactive detection kit from Boehringer GmbH.

Infection procedure of plants

Inoculum was prepared from L-broth cultures of *Clavibacter* strains grown overnight at 25 °C. Cells were washed twice with 68.5 mM NaCl in 0.02 M phosphate buffer, pH 7.2 and the titer was finally adjusted to 10^9 CFU/ml. 4 week old tomato plants (4–5 leaf stage) were infected by cutting off the petiole of the first true leaf near the stem with a contaminated scalpel blade [14].

Biomass determination

The biomass of infected plants (2, 3 and 5 weeks after infection) was used as one possible parameter for virulence. Infected plants (stems and leaves) were dried at 110 °C for 3 days. The dry weight was compared to non-infected control plants (100%) ($n = 10$).

Wilting index

For the determination of the wilting index, which is another estimate for the virulence of a bacterial pathogen, 4 week old tomato plants were infected by the standard procedure. Plants were examined every day for the development of wilting symptoms, *i.e.* the occurrence of leaf curling. The wilting index was defined as the number of days after infection at which 50% of the plants displayed the first wilting symptoms ($n = 30$).

Bioassay for wilt-inducing activity (EPS test)

The wilt-inducing activity of cell-free culture filtrates of *Clavibacter* strains was determined in a bioassay with tomato epicotyle cuttings. After growth for 12 days in $CaCO_3$ medium bacterial cells were spun down by centrifugation at 15,000 × *g* for 20 min. The supernatant underwent

a steril filtration (0.45 µm filter) and was directly used for the bioassay. Four week old plants (about 10 cm long, with 4–6 leaves) were cut approximately 1 cm below the seed leaves with a scalpel. The weight of the cuttings was determined and they were then immediately placed into 1 ml of test solution containing various amounts of the sterile culture filtrate. Incubation was for 8 h at 25 °C in a growth chamber with 80% relative humidity. Control plants were incubated in $CaCO_3$ medium. Afterwards the weight of the cuttings was again determined and the weight loss in percent as compared to the initial weight was calculated.

Enzyme assays

Endocellulase was assayed by growing *Clavibacter* strains on solid M9 medium [13] lacking glucose but supplemented with 0.5% glycerol and 0.5% carboxymethylcellulose (CMC) [15]. After 3 days plates were stained by overlaying with 0.1% Congo-Red for 20 min, followed by bleaching with 1 M NaCl. Polygalacturonase was assayed as described by Collmer *et al.* [16]. Preparation of soluble xylan and screening for xylanase activity was carried out according to Ghangas *et al.* [17]. Xylan degradation was visualized by staining agar plates with Congo-Red as described for the endocellulase assay.

Determination of bacterial titers

To determine the number of viable bacteria in inoculated plants, whole plants (15–20 g fr. w.) were homogenized by grinding in a mortar with the addition of 10 ml 68.5 mM NaCl in 0.02 M phosphate buffer, pH 7.2. Serial dilutions were plated on modified SCM agar plates semiselective for *Clavibacter*. After an incubation of 5 days at 24 °C colonies were scored. To ensure that the reisolates on the SCM agar plates represented *Clavibacter michiganense* subsp. *michiganense*, sensitivity to the specific bacteriophage CMP1 [18] was assayed.

Chemicals

p-Coumaric, caffeic, ferulic and chlorogenic acid as well as tomatine, tomatidine and rutin were obtained from Roth (Karlsruhe, F.R.G.). Caffeoylglucose was kindly donated by Prof. Dr. D. Strack (Braunschweig, F.R.G.). *p*-Coumaroyltyramine and feruloyltyramine were provided by Dr. D. Scheel (Köln, F.R.G.). The sesquiterpene phytoalexins were placed at our disposal by Prof. Dr. A. Stoessl (London, Ontario, Canada) and Prof. Dr. Ch. West (Los Angeles, U.S.A.).

Extraction of plant constituents

All investigations on soluble and cell wall-bound phenolic metabolites were performed with leaf blades which had been stored at −20 °C. Leaf blades (2 g fr. w.) were homogenized with 12 ml of chloroform/MeOH (2:1, v/v) in an ultraturrax for 1 min. The plant material obtained by filtration was again extracted twice with 6 ml chloroform/MeOH (2:1). The combined organic phases were extracted with 2 × 1 vol. *Aqua dest.* After centrifugation (1500 × *g*, 5 min) the combined chloroform phases containing chlorophyll were reduced to dryness without heating and the residue dissolved in 300 ml abs. MeOH for the analysis of sesquiterpenoid phytoalexins [19]. Alternatively the combined aqueous MeOH/$CHCl_3$ phases were evaporated to dryness and dissolved in 1.5 ml MeOH. This solution was used for the determination of soluble phenolics.

Alkaline hydrolysis of cell debris obtained from the aforementioned extraction procedure for soluble phenolics was performed with 1 g debris each (not dried). The material was suspended in 15 ml 0.5 N NaOH, heated to 70 °C for 5 min and then stirred at room temperature for 24 h. The pH value of the solution was adjusted to 2 with 50% H_2SO_4, and the slurry was then extracted with 2 × 1 vol. diethylether. The combined ether phases were dried under reduced pressure and the residue taken up in 500 ml abs. MeOH for investigating cell wall-bound phenolics. As a control reference compounds of the identified phenolics were carried through the same procedure to assure their stability.

For the determination of caffeoylglucose leaf blades (2 g fr. w.) were successively extracted with 12 ml and 6 ml −15 °C cold acetone, followed by 6 ml 50% MeOH at room temperature. For the removal of chlorophyll the combined organic phases were extracted with 2 × 1 vol. petrolether (40–60 °C). The acetone/MeOH phases were then brought to dryness and the residues dissolved in 50% MeOH.

Tomatine was estimated after extraction of leaf material in an ultraturrax (2 × 1 min, room temperature) with 10 ml and 5 ml MeOH/acetic acid/*Aqua dest.* (94:2:4, v/v) per g fr. w. [20]. Chlorophyll was removed from this solution by extraction with petrolether (40–60 °C, 2 × 1 vol.) and the combined organic phases were brought to dryness and taken up in 1 ml hot MeOH.

Identification of compounds

The cell wall-bound phenolics *i.e.* caffeic, *p*-coumaric and ferulic acid as well as *p*-coumaroyl- and feruloyltyramine were identified by chromatographic comparison and cochromatography with reference material using HPLC and GC. Soluble phenolics, *i.e.* chlorogenic acid and rutin were identified by HPLC and TLC. In case of rutin UV spectra were recorded in the presence of diagnostic reagents [21]. Tomatine was determined by TLC and caffeoylglucose by HPLC. For the detection of sesquiterpenoid phytoalexins GC and TLC were used.

Folin test for soluble phenolics

According to the method of Swain and Hillis [22] 20 ml samples were diluted with 8.5 ml *Aqua dest.*, 0.5 ml Folin Ciocalteu reagent (Merck) was added and the solution vigorously mixed. Three minutes later 1 ml saturated Na_2CO_3 solution was added and again well mixed. After standing for 1 h at room temperature the extinction at 760 nm was measured. For quantitative determinations a calibration curve set up with chlorogenic acid (20 to 200 nmol) was used. Rutin was determined spectrophotometrically with shift reagents as described by Mabry *et al.* [21]. The data obtained were: λ_{max} (CH_3OH) nm: 257, 358, shoulder: 267, 296; λ_{max} (+CH_3ONa) nm: 271, 411, shoulder: 315; λ_{max} (+$AlCl_3$) nm: 274, 428, shoulder 302; λ_{max} (+$AlCl_3$ + HCl) nm: 270, 400, shoulder: 300, 360; λ_{max} (+$CH_3COONBa$) nm: 269, 379, shoulder: 323; λ_{max} (CH_3COONa + H_3BO_3) nm: 262, 381, shoulder: 300.

HPLC

High performance liquid chromatography (Waters, Milford, Massachusetts, U.S.A.) on prepacked RP_{18} columns (Lichrosolv, 18 cm × 5 mm, i.d., and Lichrospher, 13 cm × 5 mm, Merck, Darmstadt, F.R.G.) was used. Elution gradient: from 5% solvent B (CH_3CN) in solvent A (1%, v/v, H_3PO_4 in *Aqua dest.* or pure *Aqua dest.*) to 20% B in 30 min and then from 20% B to 50% B in 30 min; flow rate 0.8 ml × min^{-1}; detection with a photodiode array detector at suitable wavelengths. Quantification of compounds was achieved by using the phenolic references as external standards.

Gas chromatography

For the detection of sesquiterpenoid phytoalexins gas chromatography was performed using a Varian GD 3600 machine (Darmstadt, F.R.G.). Column: PB 1, 50 m × 250 mm, i.d. injector 220 °C, detector (FID) 260 °C, temperature gradient: 150–250 °C with 3 °C/min, 250 °C 5 min fixed. Sensitivity 2×10^{-11}, flow (N_2) 30 ml/min, split 10 ml/70 s, injection volume 1 ml. The R_t values of the sesquiterpene reference compounds were as follows: solavetivone 14.30 min, rishitin 14.64 min, phytuberin 14.80 min, lubimin 16.45 min, capsidiol 17.06 min, 15-hydroxy-lubimin 18.95 min and phytuberol 19.49 min. Methylstearate (R_t 22.99 min) was used as internal standard.

Qualitative confirmation of the identified cell wall-bound phenolics also was performed by GC on an OV 225 column (3%); length 1.8 m. Injector 150 °C; detector (FID) 250 °C; temperature gradient 100–230 °C with 6 °C per min; sensitivity 1×10^{-9}; flow (N_2) 30 ml/min; injection vol. 1 ml.

Prior to GC aliquots of the methanolic stock solution (150–300 µl) were dried and silylated with 30 ml N-trimethylsilyl-N-methyltrifluoroacetamide (85 °C, 15 min). When kept at 4 °C the silylated derivatives were stable for some hours. R_t values of the references (*cis* and *trans* isomers) were as follows: caffeic acid 14.34 and 17.02 min, ferulic acid 14.76 and 17.83 min, *p*-coumaric acid 12.44 and 15.03 min.

Thin layer chromatography

Tomatine/tomatidine. TLC was performed with silica gel plates without fluorescence indicator (Merck, Darmstadt, F.R.G.) which had been activated by heating at 80 °C for 10 min. Solvents were: S I: isopropanol/formiate (conc.)/*Aqua dest.* (73:3:24); S II: *n*-butanol/NH_3 (25%)/*Aqua dest.*

(4:1:5); S III: ethyl acetate/MeOH/acetic acid/*Aqua dest.* (30:20:10:1); R_f values of tomatine: S I 0.81; S II 0.09; S III 0.52 and of tomatidine S I 0.90; S II 0.95; S III 0.96. Compounds were detected as coloured spots after treatment with J_2 vapour (yellowish-brown), Dragendorff's reagent (orange on yellow background) and 0.2% anthrone in conc. H_2SO_4 after heating (dark violet-blue/green).

Rutin and chlorogenic acid. 2-dim. TLC with (1st dim.) butanol/acetic acid/*Aqua dest.* (4:1:5) (upper phase) and (2nd dim.) ethyl acetate 2-butanon/formiat/*Aqua dest.* (5:3:1:1) on cellulose (Merck) and silica gel plates. Detection of chlorogenic acid (blue) and rutin (purple) under UV light (366 nm). R_f values were as follows: rutin (1st dim.) 0.54 on silica gel and 0.46 on cellulose plates; (2nd dim.) 0.40 on silica gel and 0.13 on cellulose plates; chlorogenic acid (1st dim.) 0.47 on silica gel and 0.56 on cellulose plates; (2nd dim.) 0.54 on silica gel and 0.55 on cellulose plates.

Sesquiterpenoids. The chloroform phases used for GC investigations on sesquiterpenoids were also tested by TLC on silica gel plates with ethyl acetate/cyclohexane (1:1). After drying, the plates were sprayed with anisaldehyde (100 ml dissolved in 10 ml acetic acid + 200 ml H_2SO_4) and heated at 105 °C for 3 min. The tested references gave the following R_f values and colours: 15-OH-lubimin (0.07; pink/violet); capsidiol (0.10; blue); rishitin (0.17; pink/violet) and lubimin (0.23; blue/violet).

Bioassay

The chloroform solution from the GC investigations on sesquiterpenoids was subjected to TLC as described. After drying the plate was covered with a thin layer of potato dextrose agar which was then sprayed with a conidial suspension of *Cladosporium cucumerinum* (5×10^6 conidia/ml). The fungus was cultivated at 25 °C and high humidity for several days until clear inhibition zones became visible.

UV/VIS spectroscopy

UV/VIS spectra were recorded with an Uvicon spectrophotometer (Kontron, München, F.R.G.).

Results

Physiological and genetic characterization of three strains of Clavibacter michiganense subsp. michiganense

In the present study we have investigated three strains of *Clavibacter michiganense* subsp. *michiganense,* two wild type isolates NCPPB382 and NCPPB3123, and strain CMM101 which is a derivative of NCPPB382 [11]. All three strains are identical in terms of orange pigmentation, morphology of colonies and growth. The generation time on L-broth medium at 25 °C was in the range of 150–180 min for all three strains (Fig. 1).

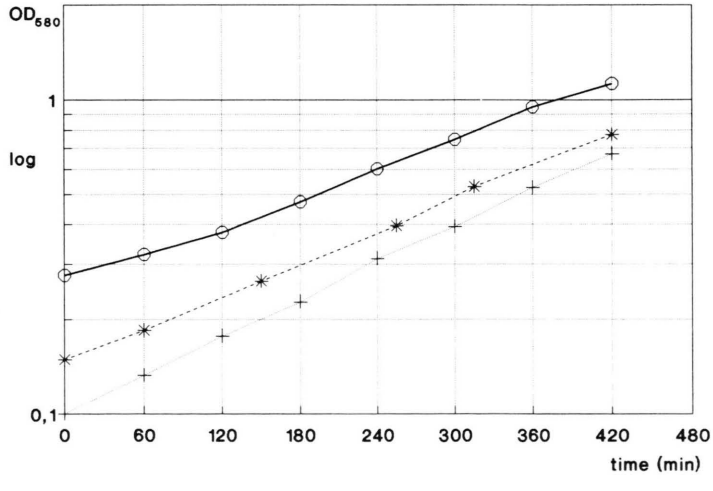

Fig. 1. Growth rates of *Clavibacter michiganense* subsp. *michiganense* on L-broth medium. (○) NCPPB382; (+) CMM101; (∗) NCPPB3123.

However, there are differences in the plasmid status of the strains. Strain NCPPB382 contains two plasmids of 27.5 kb (pCM1) and 72 kb (pCM2) [11]. Strain CMM101 which was selected after a plasmid curing experiment with NCPPB382 carries only the plasmid pCM1 [11]. This is demonstrated by Southern hybridization carried out with total DNA and a mixture of the purified endogenous plasmids pCM1 and pCM2 as probes (Fig. 2, lanes 3–5). Strain NCPPB3123 which was included in this experiment lacks any detectable plasmid (data not shown) but significant homologies were found between its chromosomal DNA and plasmids pCM1/pCM2 of strain NCPPB382 as indicated by the two hybridization signals in Fig. 2, lane 5. Also the chromosomal DNA of strain NCPPB382 and its derivative CMM101 share some homology with plasmids pCM1/pCM2 (see Fig. 2, arrows). Three hybridization signals are observed which are different to those obtained for strain NCPPB3123 allowing a differentiation of the two NCPPB strains.

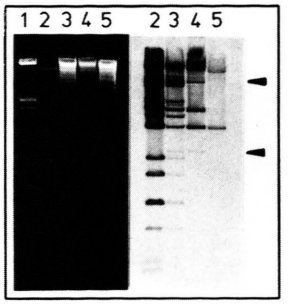

Fig. 2. Southern hybridization of total *Clavibacter* DNA and plasmids pCM1 and pCM2 as probes. DNA's were digested with *Bgl*II. Lane 1: 1 *Eco*RI/*Hind*III; Lane 2: pCM1/2; Lane 3: NCPPB382; Lane 4: CMM101; Lane 5: NCPPB3123. Arrow indicate chromosomal DNA fragments homologous to the plasmid probe.

Virulence test: Determination of wilting index and biomass of infected tomato plants

Virulence of the *Clavibacter* strains was compared by the determination of the wilting index and the biomass of infected tomato plants. The results are summarized in Table I. Strain NCPPB382 is highly virulent. Usually 8 days after infection some plants already display the typical wilting symptoms, *i.e.* leaf curling. After 12 days 50% of 30 plants infected in a standard experiment show these disease symptoms.

On the other hand, infection with strain NCPPB3123 did not result in wilting of tomato plants. Generally the experiments were terminated after 4 weeks but even after 6–7 weeks no wilting occurred.

The NCPPB382 derivative CMM101 exhibited an intermediate virulence phenotype, wilting of infected plants was retarded by about a week as compared to the parent strain.

Differences between bacterial strains were also observed when the biomass of infected tomato plants was determined (Table I). Infection by NCPPB382 resulted in a rapid and drastic reduction of the biomass during the course of the experi-

Table I. Wilting index and biomass of tomato plants after infection.

Strain	Biomass at different times[a] after infection			Wilting index[b]
	2 weeks [%]	3 weeks [%]	5 weeks [%]	[days]
NCPPB382	76.0	69.7	35.2	12
CMM101	103.9	81.1	62.2	18
NCPPB3123	112.5	76.5	69.0	>28[c]

[a] Biomass was expressed as dry weight of tomato plants as compared to control plants (100%). Mean values were calculated from experiments with 10 plants.

[b] Days after infection at which 50% of the plants show wilting symptoms (n = 30).

[c] After 28 days the experiment was terminated, even later wilting symptoms never occurred.

ment, due to growth retardation and massive wilting of the plants. Five weeks after infection the biomass was only 35% of the uninfected control plants (100%).

Slower development of wilting symptoms after infection by strain CMM 101 seems to result in a less pronounced reduction in the biomass of 62% of the control.

Although after infection by NCPPB 3123 the typical bacterial wilt is not observed and the plants appear to be comparatively healthy, a significant reduction of the biomass results (69% of the control) which is only slightly higher as compared to an infection by CMM 101. Thus NCPPB 3123 can still be considered as a pathogen, however, the degree of virulence is reduced as compared to NCPPB 382.

Production of exopolysaccharides and exoenzymes

Plant pathogenic bacteria may require cell wall-degrading exoenzymes for a successful infection and exploration of the host plant as energy source. On the other hand, these enzymatic activities may cause elicitation of a specific plant response by production of endogenous elicitors. Since a difference in pathogenicity of the three *Clavibacter* strains was observed after infection of tomato plants we have compared their production of the exoenzymes endocellulase, polygalacturonase, and xylanase. In plate assays on solid medium all strains produced these exoenzymes with no difference in their amounts (Table II).

The production of exopolysaccharides is a characteristic trait of *Clavibacter michiganense* subsp. *michiganense*. These EPS have been shown to act as phytoaggressins which induce wilting in plant bioassays with epicotyle cuttings [14]. Since virulence may depend on the production of EPS the three strains were tested accordingly. However, it was found that they appear to produce about the same quantity of wilt-inducing exopolysaccharides in culture (Table II).

Colonization of infected tomato plants

Since no correlation between pathogenicity and an otherwise detectable phenotype could be found so far we tested whether the strains differ in their ability to colonize the host plant. Various times after infection the tomato plants were homogenized and the bacterial titer per gram plant tissue was determined on a semiselective medium for *Clavibacter michiganense* (Table III). The propagation of strain NCPPB 382 and its derivative CMM 101 in the infected plants is quite rapid, because after two weeks the titer is as high as 10^9 cells per gram plant tissue. Only a slight increase of this titer is observed during the later stages of infection.

On the other hand, strain NCPPB 3123 is not able to colonize the host plant to the same extent. The titer is much lower and reaches only 3×10^4 cells per gram plant tissue. It is conceivable that the inability to effectively colonize the plant is the reason for the reduced pathogenicity of NCPPB 3123. There are at present no obvious explanations for the deficiency of strain

Table III. Colonization of tomato plants after infection with *Clavibacter michiganense* strains.

Strain	Bacterial titer at different times after infection [cfu/g plant tissue] 2 weeks	3 weeks	5 weeks
NCPPB 382	1.4×10^9	3.6×10^9	2.9×10^9
CMM 101	1.1×10^9	2.1×10^9	3.6×10^9
NCPPB 3123	$<1 \times 10^4$	$<1 \times 10^4$	3.3×10^4

Table II. Production of wilt-inducing exopolysaccharides and exoenzymes.

Strain	Wilt induction by EPS [% weight loss[a]]	Endo-cellulase	Polygalact-uronase	Xylanase
NCPPB 382	15.0/4.6	+	+	+
CMM 101	17.1/7.8	+	+	+
NCPPB 3123	17.7/7.1	+	+	+

[a] Weight loss of tomato cuttings in percent after 8 h of incubation. First value undiluted, second value 1 : 10 diluted culture filtrate. Data represent mean values of 10 assays.

NCPPB3123 to propagate in the plant since growth in culture and the production of EPS and exoenzymes under standard conditions are not impaired. However, it is entirely possible that strain NCPPB3123 may respond more sensitively to defence reactions of the plant which are induced by the bacterial infection. Therefore, tomato plants were analyzed for the production of sesquiterpenoid, alkaloid and phenolic compounds possibly elicited by the infection with either of the three *Clavibacter* strains.

Accumulation of phenolic and secondary compounds by infected tomato plants

Analyses of soluble phenolics in leaf blades showed an enhanced accumulation depending on time (Fig. 3A). Thus, the tomato plants treated with the three *Clavibacter* strains only showed very small differences 2 weeks after infection. But in the older stages a pronounced increase in phenolic material was observed. The infection with the highly virulent strain NCPPB382 resulted in the highest values followed by the two other interactions.

The results obtained with the photometric Folin test in Fig. 3A were confirmed by HPLC using a photodiode array detector. Again the 2 week old samples and plants infected with NCPPB3123 measured after 3 weeks contained relatively small amounts of phenolic metabolites. All samples of the avirulent interactions, of control plants as well as the preparations of the 2 week old plants infected with CMM101 and NCPPB382 contained two dominant phenolic compounds which were identified as chlorogenic acid (caffeoylquinic acid) and rutin (quercetin-3-rhamnoglucoside). Fig. 3B and 3C depict the general increase in chlorogenic acid and rutin accumulation in the three infection experiments. In case of chlorogenate plants infected with the avirulent strain NCPPB3123 contained the highest amounts whereas infection with the moderately virulent strain CMM101 favoured rutin accumulation.

The pronounced stimulation of secondary product accumulation in the plants especially in case of strains NCPPB382 and CMM101 (Fig. 3A) comprises a considerable number of new metabolites as shown in Fig. 4. Up to 10 compounds with UV absorption maxima between 280 and 320 nm were detected 3 to 5 weeks after onset of infection and several of these exceeded chlorogenic acid and

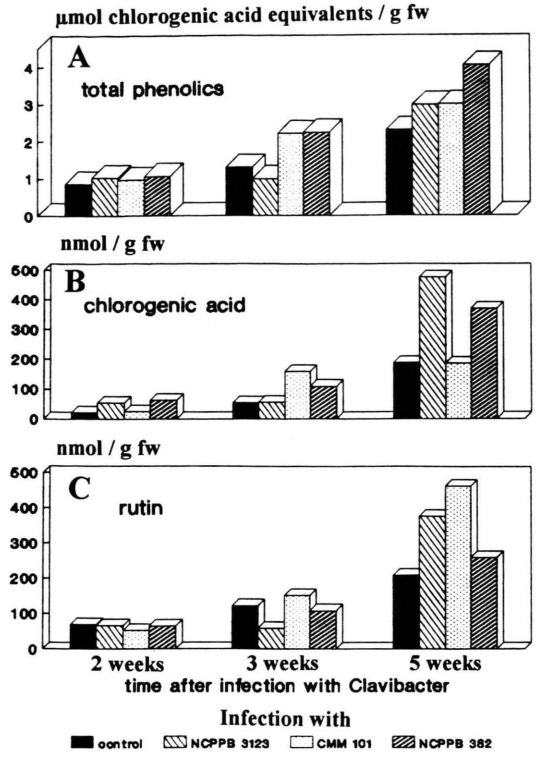

Fig. 3. Accumulation of A) soluble phenolics (µmol equivalents of chlorogenic acid/g fr. w.); B) chlorogenic acid (nmol/g fr. w.) and C) rutin (nmol/g fr. w.) in leaf blades of *Lycopersicon esculentum cv.* Moneymaker 2, 3, and 5 weeks after infection with differently virulent strains of *Clavibacter michiganense* subsp. *michiganense*.

rutin in quantity. These newly formed compounds remained unidentified so far and some of them may possibly result from bacterial metabolism.

From plant material harvested 5 weeks after infection with strain NCPPB382 considerable amounts of 1-O-caffeoylglucose (518 nmol/g fr. w.; controls 233 nmol/g fr. w.) and traces of 1-O-feruloylglucose could be isolated. In tissue extracts from the other infection stages and interactions no such increased levels of these cinnamoylesters could be demonstrated and 1-O-coumaroylglucose failed to accumulate at all. The caffeoyl and feruloyl conjugates are of interest with regard to phenolic incorporation in wall structures (see below).

Careful investigations on the plant material from all infection stages with the three bacterial strains failed to demonstrate the occurrence and accumulation of sesquiterpenoid phytoalexins.

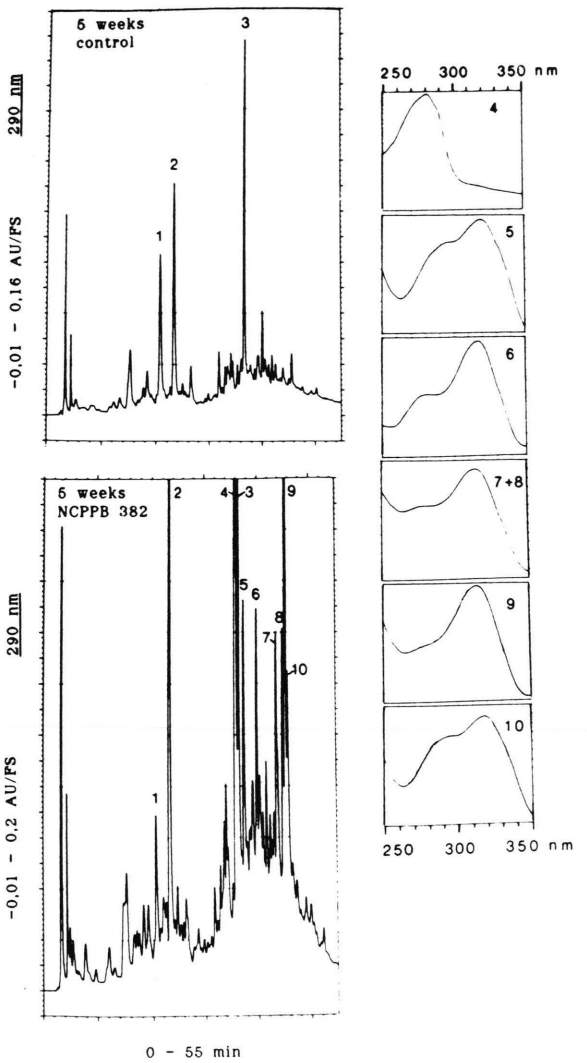

Fig. 4. Left: HPLC chromatogram of soluble phenolics extracted from leaf blades of *Lycopersicon esculentum cv.* Moneymaker 5 weeks after infection with the highly virulent strain NCPPB382 (below) and control (above). Compounds are: 1 = L-Trp, 2 = chlorogenic acid, 3 = rutin, 4–10 = unknown. Right: UV spectra (in CH_3CN/H_3PO_4) of compounds 4–10.

These studies were performed by gas chromatography as well as by bioassays using *Cladosporium cucumerinum* mycelial growth on TLC-separated tissue extracts.

Accumulation of the steroid alkaloid tomatine was analyzed in the leaf samples from the three infection experiments after 2 weeks. Inoculation with the highly virulent strain NCPPB382 resulted in approx. 1600 nmol tomatine/g fr. w. compared to 775 nmol/g fr. w. for the controls. Infection with strains NCPPB3123 and CMM101 also stimulated tomatine accumulation but much less effectively. Although quantitative tomatine determinations by TLC are moderately precise the enhanced accumulation of this claimed antifungal alkaloid in case of strain NCPPB382 was very evident. The aglycone tomatidine could not be detected.

Cell wall-bound phenolics

Leaf material from control and tomato plants infected with the three *Clavibacter* strains were analyzed for cell wall-bound cinnamic acids and

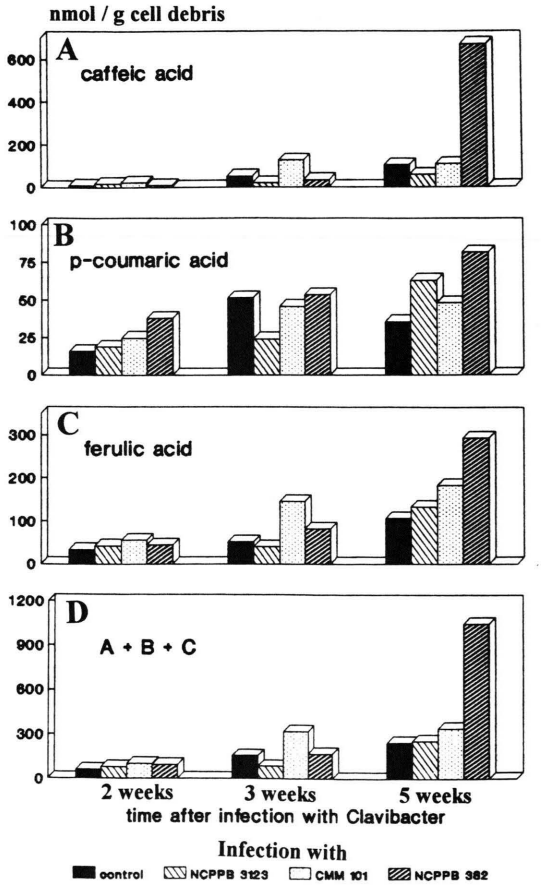

Fig. 5. Accumulation of cell wall-bound A) caffeic acid; B) *p*-coumaric acid; C) ferulic acid and D) total amount of these metabolites, in leaf blades of *L. esculentum cv.* Moneymaker after infection with differently virulent strains of *Clavibacter michiganense* subsp. *michiganense* in comparison to controls 2, 3 and 5 weeks after infection.

other phenolics. Upon alkaline hydrolysis of cell debris and subsequent HPLC and GC analyses *p*-coumaric, caffeic and ferulic acid, respectively, were found as the dominant wall phenolics. Although a steady increase in the amount of these acids was found in the controls, the levels in the infected plants were clearly much higher (Fig. 5). This became especially evident in the plant material from 5 week infection experiments where the highest amounts of all acids were induced by the most virulent strain NCPPB382 (Fig. 5D); in this latter case caffeic acid was found in exceptionally large amounts. The recovery rate of the cinnamic acids in these hydrolysis experiments was determined in appropriate control assays to be approx. 95 ± 3%.

In addition to the cinnamic acids two other phenolics were hydrolytically liberated from the plant material infected for 5 weeks with *Clavibacter* strain NCPPB382 (Fig. 6). Compounds 1 (λ_{max} 240–242, 275 nm) and 5 (λ_{max} 280–282, 290 shoulder nm) are presently unidentified. Furthermore, cell wall-bound *p*-coumaroyltyramine and feruloyltyramine well known as inducibly formed wall components from other infected solanaceous plants [23, 24] were only detected in small traces (about 2 nmol/g cell debris) in some samples from the 3 and 5 week infection experiments.

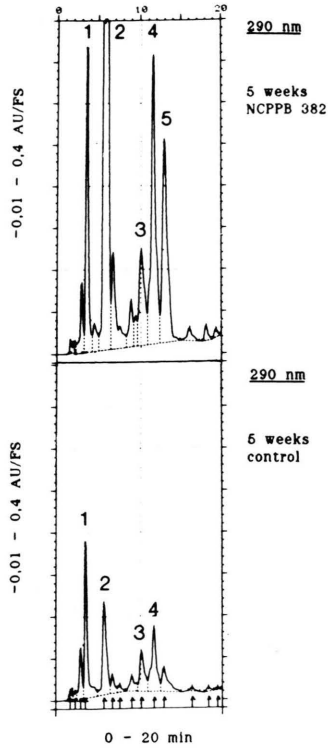

Fig. 6. HPLC chromatogram of cell wall-bound phenolics obtained from leaf blades of *L. esculentum* 5 weeks after infection with the highly virulent strain *Clavibacter michiganense* subsp. *michiganense* NCPPB382 (above) and control (below). Compounds are: 1 = unknown, λ_{max} 240/2 and 275 nm; 2 = caffeic acid; 3 = *p*-coumaric acid; 4 = ferulic acid, and 5 = unknown, λ_{max} 280/2, shoulder 290 nm.

Discussion

The majority of literature data on infection-induced responses in *Lycopersicon esculentum* plants and cell cultures deal with fungal and viral infection systems [25]. The data in this investigation now clearly show that tomato plants also readily respond to bacterial pathogens and that among the intensified reactions phenolic metabolism appears to be of major importance. In this respect various fungi-induced responses are very similar because soluble and insoluble phenolic structures were also affected [7, 9, 26]. However, the induced responses outlined in Fig. 3 and 5 only show partial correlation with the degree of virulence of the *Clavibacter* strains (Table I). Thus, not all responses should be interpreted as clear defence reactions but rather as pathogenesis-related phenomena.

Among the pool of the total phenolics shown to increase during the infection experiments (Fig. 3A) chlorogenic acid and the flavonol glycoside rutin could be identified. Both compounds are claimed to be antimicrobial metabolites [27]. However, it appears doubtful whether their increase could explain the reduced potential of strain NCPPB3123 to colonize the tomato plants (Table III). Similarly, the fungi toxic steroid alkaloid tomatine was only found in increased amounts upon infection with virulent *Clavibacter* strains. The contribution to defence of this compound previously found to be increased in plant-fungus interactions [7] appears to be limited because the quantitative increase is rather small.

Since the tissue and cellular distribution of the quantitatively enhanced phenolic and other secondary compounds remain to be elucidated a direct relation between increased phenolic pools and bacterial growth inhibition might not exist. The

complex pattern of aromatic constituents found in the tissues infected with the most virulent and best growing strain NCPPB382 (Fig. 4) may partly result from bacterial production either *de novo* or by transformation of plant constituents. Although such phenolics appear to be absent form pure bacterial cultures (unpublished data) bacterial metabolism *in planta* remains to be elucidated. Increased concentrations of phenolic compounds possibly contribute to the phenolic occluding material known to accumulate in the tomato vascular system [28] in response to fungal challenge. Such occlusion may add to the EPS-caused wilting observed in the present work (Tables I and II). The general increase in chlorogenic acid in some of the infection stages (Fig. 3 B) contrasts with the disappearance of this compound observed in other studies with challenged tomato [29] or *L. peruvianum* cells [24].

Although enzymatic investigations remain to be conducted our data (Fig. 3–6) indicate a pronounced stimulation of phenylpropanoid metabolism. This is also corroborated by the detection of 1-O-caffeoyl- and 1-O-feruloylglucose in infected tissues. These compounds are most likely precursors for the cell wall-bound cinnamic acids (Fig. 5) [23–25] where caffeic acid was shown to be the most prominent unit.

The pronounced increase of the bound cinnamic acids, especially caffeate, occurs positively correlated with the duration of the experiments and the degree of bacterial virulence (Fig. 5). It is suggested that bacteria-derived signals such as exopolysaccharide, cellulase or polygalacturonase which are expressed according to the degree of virulence elicite this process of cell wall modification. The resulting rigidity of wall structures [25] will possibly also contribute to reduced growth and biomass production (Table I). Although the cell wall incorporation of ester-bound cinnamates is known from other solanaceous fungi-infected plants such as *Solanum tuberosum* [23] and *Lycopersicon peruvianum* [24] an important difference exists. This refers to the absence of cinnamoyltyramines, *p*-hydroxy-benzaldehyde and vanillin from the cell wall hydrolysates of the bacteria-infected tomato plants. The acyltyramines are thought to contribute to massive cross-linking of wall structures and the aldehydes possibly represent suberin-like, new structures separating infection sites [9, 23]. Since *Clavibacter* obviously fails to induce this additional wall impregnation reaction the structural difference in response between fungal and bacterial infections should further be investigated.

The absence of sesquiterpenoid phytoalexins from the infected tomato leaf tissues is difficult to explain because these compounds are considered to be a characteristic response of infected tomato tissues [4]. Phytoalexins are either not synthesized during pathogenesis or the *Clavibacter* strains are able to metabolize them. In future work it will especially be necessary to search for phytoalexins during the very early stages of infection.

Questions arising from our work should deal with the mechanism by which the growth of the avirulent strain NCPPB3123 is prevented *in planta* and also with the strategy by which the virulent strains may overcome the plant defence reactions. Such work should further include histological studies to reveal the exact localization of the pathogen and the observed reactions. It may also be hypothesized that the plant biochemical reactions described here are not defence reactions but rather represent responses typical for susceptible genotypes and found as early symptoms of plant decay. Therefore, comparative investigations with resistant genotypes will be helpful.

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