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Effect of phytotoxic compounds produced by Clavibacter michiganensis subsp. michiganensis on resistant and susceptible tomato plants

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Abstract

A phytotoxic fraction of high molecular weight was isolated from the culture filtrate of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, and partly purified. This high molecular weight fraction consists of sugars and a minor protein moiety and is therefore probably of similar nature to that of the toxin from *C. michiganensis* subsp. *michiganensis* reported earlier in literature.

The high molecular weight fraction was able to induce wilting, the predominant symptom of the disease, as shown in a bioassay with tomato cuttings. However, this wilting reaction turned out to be non-specific in the bioassay, since (partially) resistant and susceptible genotypes responded similarly. No correlation could be found between the degree of virulence of five *C. michiganensis* subsp. *michiganensis* strains and the amount of the phytotoxic high molecular weight fraction produced in vitro.

As the isolated high molecular weight fraction showed a phytotoxic effect on tomato plants it is worthwhile to test its potential for use as a selective agent in in vitro selection.

Additional keywords: bacterial canker, *Lycopersicon esculentum*, *Lycopersicon peruvianum*, virulence, bioassay, non-specific toxin.

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davies et al., formerly *Corynebacterium michiganense* (Smith) Jensen, causes bacterial canker of tomato. This disease occurs worldwide and affects fruit production by causing death of the plant in severe cases. Wilting of plants is usually the first and predominant symptom after infection. It is still not known how *C. michiganensis* subsp. *michiganensis* causes wilting of plants. Several modes of action have been suggested, such as plugging of the xylem vessels. Evidence that wilting of plant cuttings could be caused by large molecules excreted by the pathogen was provided by studies of Van Alfen and Allard-Turner (1979) and Van Alfen and McMillan (1982). However, in histopathological studies of infected tomato plants no indications of occlusions in the xylem vessels could be found (Wallis, 1977). At the ultrastructural level Wallis was able to show degradation of primary cell walls and secondary wall thickenings of the xylem vessels and adjacent parenchyma cells. This suggests that enzymatic breakdown of plant material might be involved in the course of wilting. Indeed, Patiño-Méndez (1964) had shown before that *C. michi-*

ganensis subsp. *michiganensis* is able to produce enzymes with pectolytic and cellulase activity both in vivo in the plant and in culture. Cell wall degradation of xylem vessels and surrounding vessels and parenchyma cells was also observed by Leyns and De Cleene (1983). They suggested that a toxin might be involved. It was supposed that this toxin, released by the pathogen, will disorder cell membranes and subsequently bacterial enzymes decompose the plant cell walls. The role of a toxin, excreted by the bacterium, in the disease process has been investigated by Rai and Strobel (1969a, b). They showed toxin activity of glycopeptides produced by *C. michiganensis* subsp. *michiganensis* in culture. Disorganization of membranes by these glycopeptides was suggested to be the primary cause of wilting. Recently an injuring effect of a wilt inducing high molecular weight toxin, produced in culture and characterized as a glycopeptide, was demonstrated on chloroplast membranes (Krämer, 1986; Krämer and Leistner, 1986). Whether the observed damage of membranes is the primary effect of the toxin is not known.

Toxins which are involved in pathogenesis have been successfully used as selective agents for in vitro selection for disease resistance. Comprehensive reviews about this subject have been published by Yoder (1983), Wenzel (1985) and Ingram and McDonald (1986). Since no absolute resistance against bacterial canker is available in cultivated tomato and the disease is difficult to control (Watterson, 1986) new approaches to the development of resistant plants are needed. It is worthwhile to evaluate the possibilities of in vitro selection of cell cultures by using a toxin of *C. michiganensis* subsp. *michiganensis*. This paper presents the results of isolation of a phytotoxic high molecular weight fraction from the culture filtrate of the bacterium and its effect on tomato.

Materials and methods

Bacteria. The following five strains of *C. michiganensis* subsp. *michiganensis* were used for toxin research: Cm 542 (obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK: NCPPB 1064), CM 543 (NCPPB 1379), Cm 545 (NCPPB 2034), Cm 8701 (obtained from Willie Commelin Scholten Phytopathological Laboratory, Baarn, the Netherlands) and Cm 8702 (provided by the Institute for Horticultural Plant Breeding, Wageningen, the Netherlands). All strains were originally isolated from *Lycopersicon esculentum*. The bacteria were stored at 4 °C on an agar medium, containing 1% special peptone (Oxoid L72), 0.5% NaCl and 1.5% agar. Prior to use the bacteria were cultured at 27 °C on yeast-peptone-glucose-agar (YPGA), containing 0.5% yeast extract, 1% peptone, 0.5% D(+)-glucose and 1.5% agar.

Plant material. Genotypes of the cultivated tomato *Lycopersicon esculentum* and the related *L. peruvianum* were used to test the biological activity and specificity of the isolated phytotoxic fraction. Cv. Moneymaker (supplied by Rijk Zwaan BV, De Lier, the Netherlands) was used as a susceptible tomato and Okitsu sozai 1-20 (Laterrot et al., 1978) as a partially resistant tomato. Two accessions of *L. peruvianum* were used: LA 2334 (obtained from C.M. Rick, Tomato Genetics Stock Centre, Department of Vegetable Crops, University of California, Davis, USA), with high resistance against bacterial canker (Lindhout and Purimahua, 1987), and PI 251306 (obtained from the North Central Regional Plant Introduction Station, Ames, Iowa, USA), which is susceptible.

Virulence testing of strains. Plant inoculation was performed using a modified version of a procedure described by Van Steekelenburg (1985). Bacterial suspensions in 0.85% NaCl were prepared from 2 to 3 days old cultures, grown on YPGA. A concentration of approximately 10^7 cells ml^{-1} was obtained by adjusting the optical density ($OD_{400\,nm}$) of the suspension to 0.3. Plants which had developed 5-7 leaves were double inoculated by cutting the top of the plant with a scalpel dipped in bacterial suspension and by injecting 5 μl of this suspension in the stem between the cotyledons and the first leaf. Plants were incubated in the greenhouse at a temperature of 24/18 °C (day/night) and scored for symptoms 5 weeks after inoculation on an arbitrary scale from 0 (healthy) to 5 (dead). Experiments were carried out with 9 replicates in a randomized block design.

Isolation, purification and partial characterization of a phytotoxic fraction. The phytotoxic fraction was obtained from liquid cultures. For preparation of the growth medium 10 g of yeast extract was dissolved in 25 ml distilled water and dialysed (Thomas dialysis tube, cut-off 12 000 Da, Philadelphia, USA) against 200 ml distilled water for 12 h at 4 °C. The fraction excluded by the dialysis tube was diluted 100 times and supplemented with 1.5% D(+)-glucose and 0.5% $CaCO_3$ (YGC medium). Bacteria were grown in the YGC medium for 12 days at 27 °C on a rotary shaker (150 rpm). The bacterial suspensions were centrifuged for 20 min at 15 000 *g* and the supernatant fluid passed through a 0.2 μm filter to obtain a cell free culture filtrate. The culture filtrate was subsequently filtrated through an ultrafilter with a nominal molecular weight limit of 10 000 (Minitan system, Millipore). The retained fraction of high molecular weight was used in the experiments. YGC medium, cell free culture filtrate and the high molecular weight fraction were analysed by HPLC using a 300 × 7.5 mm gel filtration column (Biogel TSK30, Biorad) with a separation range for globular proteins with a molecular weight of 1000-100 000. Samples (20 μl) with a concentration of 1 mg ml^{-1} were injected. The column was eluted with 0.05 M KH_2PO_4, pH 3.5, at a flow rate of 1 ml min^{-1} at room temperature. The column effluent was monitored at 200 nm.

Protein content of the high molecular weight fraction was measured spectrophotometrically as described by Lowry et al. (1951) and with the single reagent Bio-Rad Protein Assay (Bio-Rad Laboratories, München, West-Germany) using bovine plasma gamma globulin as a standard. The amount of carbohydrates was determined spectrophotometrically by the anthrone test (Dische, 1962) using glucose as a standard.

Culture filtrate, high molecular weight fraction and the filtrate < 10 000 Da were freeze dried and stored at −20 °C.

Bioassay. Toxic activity of cell free culture filtrate, high molecular weight fraction and filtrate < 10 000 Da were assayed by testing their wilt inducing activity. Cuttings were taken from plants with 5 to 7 leaves by dissecting the tops of the plants. The cuttings, approximately 15 cm long and at least having one unfolded leaf, were immediately recut in water, 2-3 cm above the first cut, to prevent the occurrence of emboly. They were placed in 25 ml Erlenmeyer flasks with purified water (Milli-Q water system of Millipore) and 0.1 mg ml^{-1} of the antibiotic cefotaxime (Claforan, Hoechst, Frankfurt am Main, West-Germany) to avoid bacterial growth. The opening of the Erlenmeyer flasks with the cuttings was covered with parafilm to prevent direct evaporation of the water. Incubation took place in a growth chamber with continuous illumination of 6-8

W m^{-2} at 25 °C and 80% relative humidity. After 24 hours of incubation each cutting was transferred to an Erlenmeyer flask with either culture filtrate, high molecular weight fraction or filtrate < 10 000 Da, dissolved in purified water with 0.1 mg ml^{-1} cefotaxime and adjusted to pH 6.0. The weight of each individual cutting (W_1) was determined by weighing the Erlenmeyer flask before and after addition of the cutting. The cuttings were then incubated for another 24 h under the same conditions and their weight (W_2) was determined again. The relative change in weight, $(W_2 - W_1)/W_1 \times 100\%$, was used as a parameter for wilting.

Control plants were incubated in purified water with cefotaxime. All experiments were carried out in a randomized block design with 5 replicates.

Results

The biological activity of high molecular weight compounds excreted by C. michiganensis subsp. michiganensis. To demonstrate the production of phytotoxic compounds in vitro by *C. michiganensis* subsp. *michiganensis*, the wilt inducing activity of culture filtrate of Cm 542 was compared with the activity of the YGC medium in the bioassay. When cuttings of cv. Moneymaker were placed in water with increasing concentrations of YGC medium, there was a slight increase in weight of 3-5%, due to continued growth of the cuttings over the 24 h test period. In contrast, cuttings significantly ($p < 0.001$, p-value obtained from the t-distribution with 120 degrees of freedom, residual variance is 3.06) decreased in weight when incubated in culture filtrate of Cm 542 at a concentration of 5 mg ml^{-1} (Fig. 1). At this concentration wilting of the cuttings was indeed observed. This shows that a compound released by the bacterium is able to cause the most important symptom of the disease, i.e. wilting, in this bioassay.

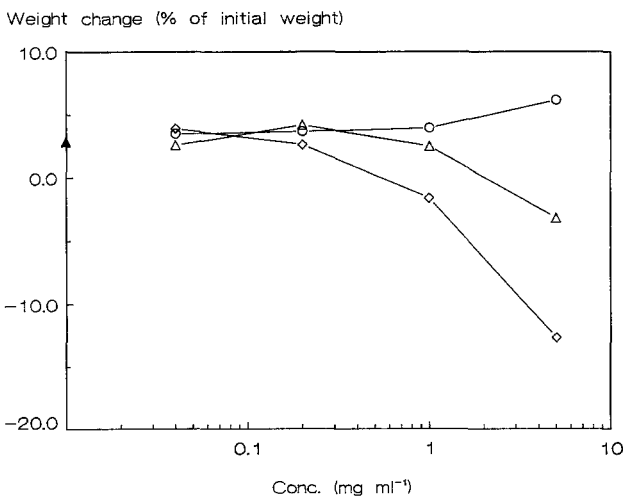

Fig. 1. Change in weight of tomato cuttings, cv. Moneymaker, after incubation for 24 h in different concentrations of culture filtrate from Cm 542 (△), YGC medium (○) or Dextran T500 (◇) at 25 °C and 80% r.h. Concentrations are based on dry weight. The weight change of the cuttings in purified water is indicated on the Y-axis (▲).

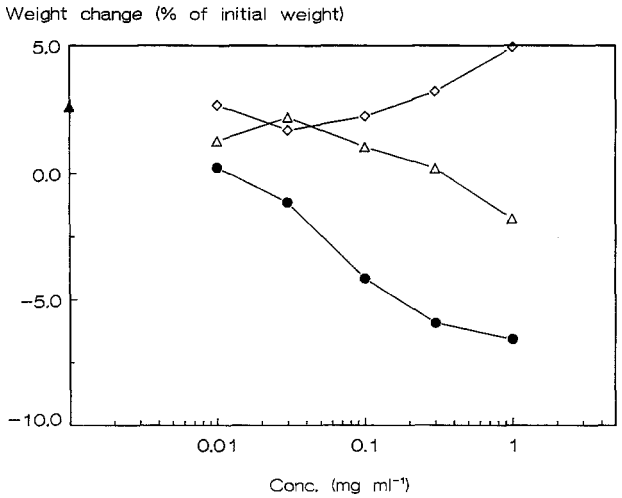

Fig. 2. Change in weight of tomato cuttings incubated for 24 h at 25 °C in different concentrations of culture filtrate (▵), high molecular weight fraction (•) or filtrate < 10 000 D (◇) produced by isolate Cm 542. Change in weight in purified water is indicated on the Y-axis (▲). Concentrations are based on dry weight.

The culture filtrate of Cm 542 was fractionated by ultrafiltration and, based on dry weight, 3-4% of culture filtrate was retained. The cell free culture filtrate, the (retained) high molecular weight fraction and the filtrate with molecular weight < 10 000 were tested in the bioassay (Fig. 2). The filtrate < 10 000 Da did not show wilt inducing activity. The high molecular weight fraction showed a much higher activity compared with culture filtrate ($p < 0.001$ for 0.03 mg ml^{-1} and higher, t-test with 70 degrees of freedom, residual variance is 0.26). At the highest concentration used in this experiment (1 mg ml^{-1}), the culture filtrate induced a decrease in weight of approximately 2%. A comparable decrease in weight was already reached with the high molecular weight fraction at a concentration of approximately 0.04 mg ml^{-1}. Hence the purification factor is about 25. This is in reasonable agreement with the percentage of material retained after ultrafiltration, 3-4% of the culture filtrate, from which a purification factor of 25-33 could be expected.

A decrease in weight of the cuttings could also be induced by adding the polymer Dextran T500 (Fig. 1), but similar concentrations of high molecular weight fraction induced a much stronger decrease in weight (Fig. 2).

The enrichment of the phytotoxic high molecular weight fraction was verified by HPLC chromatography. Comparison of HPLC elution profiles of YGC medium (Fig. 3A) with culture filtrate (Fig. 3B) showed the appearance of substance(s) in the latter (retention time approximately 6 min.). This substance(s) runs in the void volume of the column, indicating material of high molecular weight. After filtration and concentration the phytotoxic fraction showed mainly this peak (Fig. 3C).

The protein and carbohydrate measurements showed that the phytotoxic high molecular weight fraction contains about 93% glucose equivalents sugar and 0.3-0.4% gamma globulin equivalents protein.

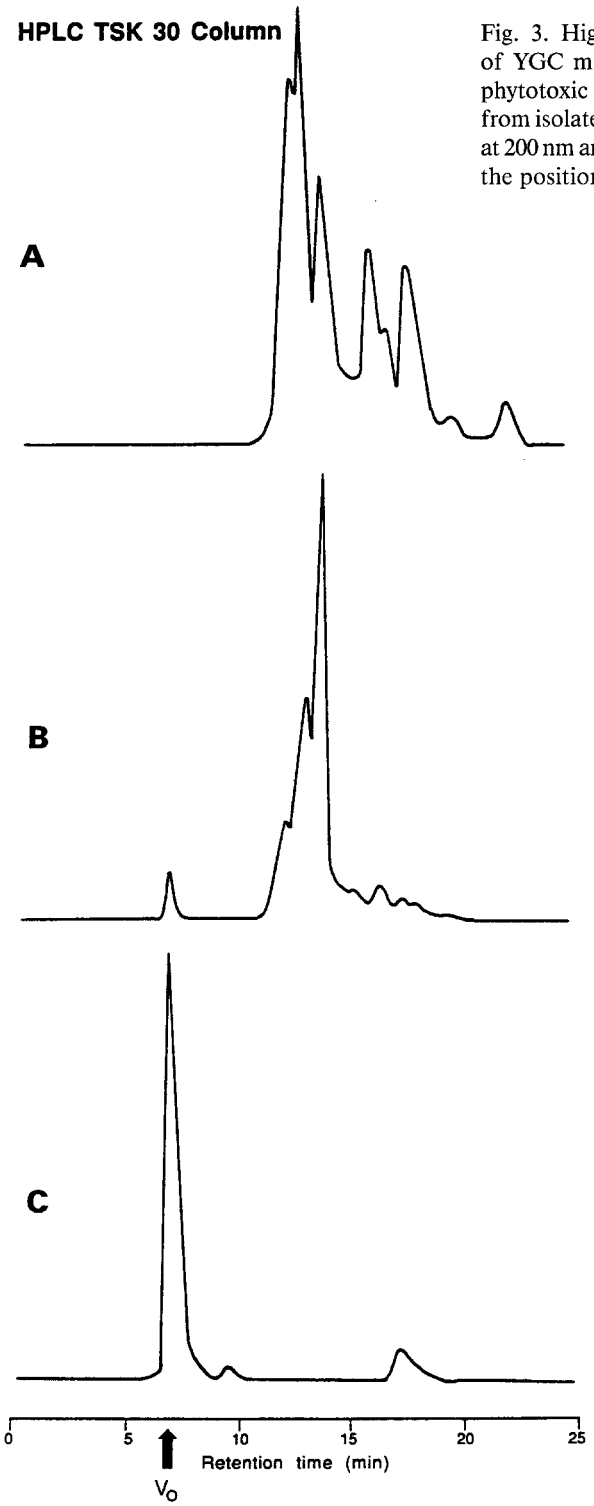

Fig. 3. High-performance gel filtration profiles of YGC medium (A), culture filtrate (B) and phytotoxic high molecular weight fraction (C) from isolate Cm 542. Absorbance was measured at 200 nm and 0.2 AUFS. The arrow (↑) indicates the position of the void volume (Vo).

Table 1. Number of plants per disease index class 5 weeks after inoculation of tomato plants, cv. Moneymaker, with different isolates of *C. michiganensis* subsp. *michiganensis* and amount of phytotoxic high molecular weight fraction produced by these isolates in vitro.

Isolate	Numbers of plants per disease index class[1]						Production phytotoxic fraction[2]	
	0	1	2	3	4	5	absolute	corrected
Cm 8701	0	0	0	0	0	9	30.3	32.3
Cm 542	0	0	0	0	2	7	35.8	35.8
Cm 8702	1	0	1	5	2	0	16.2	12.7
Cm 545	2	0	1	5	0	0	50.8	55.7
Cm 543[3]	1	2	2	0	0	0	26.0	nd[4]

[1] Disease index classes: 0 - healthy; 1 - one leaf partially wilted; 2 - two leaves wilted; 3 - more leaves wilted; 4 - most leaves + top wilted; 5 - dead.
[2] Expressed as mg dry weight per 100 ml culture filtrate (absolute) and corrected for the bacterial concentration (corrected).
[3] Data for Cm 543 were obtained from another experiment.
[4] nd = not determined.

Virulence and the production of high molecular weight compounds. Five strains of *C. michiganensis* subsp. *michiganensis* were assayed for their virulence by inoculating the bacterial canker susceptible cv. Moneymaker and scoring for wilting (Table 1). Strain Cm 542, used throughout this study for production of the phytotoxic high molecular weight fraction, indeed is a highly virulent isolate. Differences in virulence were found, ranging from highly virulent (Cm 542 and Cm 8701) to moderately virulent (Cm 543). These 5 strains were grown in YGC medium and the amount of the phytotoxic high molecular weight fraction produced was determined (Table 1). Comparison of the virulence of strains with the production of phytotoxic fraction did not show any correlation between these two parameters. This suggests that the amount of the phytotoxic high molecular weight fraction produced in vitro does not determine virulence.

Specificity of the phytotoxic high molecular weight fraction. The host specificity of the phytotoxic high molecular weight fraction isolated from Cm 542 was studied in a bioassay, using bacterial canker susceptible and partially resistant genotypes of *L. esculentum* and *L. peruvianum*. No differences in response between partially resistant and susceptible genotypes could be detected (Fig. 4). Analysis of variance of the data only showed a difference ($p < 0.001$ at 0.1 mg ml^{-1} and $p < 0.2$ at 1.0 mg ml^{-1}, t-test with 90 degrees of freedom, residual variance is 1.20) in response between the *L. esculentum* genotypes and the *L. peruvianum* genotypes, suggesting the sensitivity to the phytotoxic fraction is not the same for different species. As there are no differences between partially resistant and susceptible genotypes within the same species the activity of the phytotoxic fraction is non-specific.

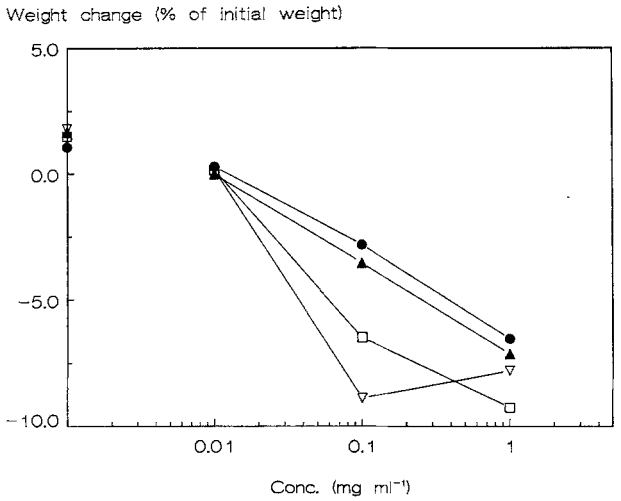

Fig. 4. Response of different genotypes, expressed as change in weight, after 24 h incubation with different concentrations of phytotoxic high molecular weight fraction. The phytotoxic fraction was isolated from isolate Cm 542. Moneymaker (▲) and Okitsu sozai (•) are genotypes of *L. esculentum*, whereas PI 251306 (□) and LA 2334 (▽) are *L. peruvianum* genotypes. Change in weight for each genotype in purified water is indicated on the Y-axis.

Discussion

The use of plant cuttings to assay for wilt inducing activity of isolated compounds is quite common in plant pathology. Since quantitative estimation of wilting by visual assessment is not very reliable, Van Alfen and McMillan (1982) used transpiration as a parameter for wilting. However transpiration is mostly measured only as the uptake of test solution. Change in weight of the cuttings as measured in this study is a better parameter for wilting, because it is composed of uptake of water by the stem and evaporation by the leaves. These two factors together determine the degree of wilting.

With this bioassay, production of phytotoxic compounds by *C. michiganensis* subsp. *michiganensis* was shown. This confirms the results described by Rai and Strobel (1969b) and Krämer (1986). A concentration of 0.01-0.1 mg ml^{-1} of the phytotoxic fraction gave a significant decrease in weight of the cuttings in the bioassay without visible wilting. At higher concentrations wilting of the cuttings was also observed visually. HPLC analysis showed that this phytotoxic fraction contains compound(s) of high molecular weight, presumably larger than 100 000 as this is the upper separation limit of the gel filtration column. This is in agreement with the results of Rai and Strobel (1969a) who reported estimated molecular weights for their toxic glycopeptide fractions mainly over 100 000. Moreover, a preliminary characterization indicated that the phytotoxic fraction used in this study also consists of sugars and a minor protein moiety. A further characterization will establish the chemical composition of this fraction.

The high molecular weight of the phytotoxic fraction could implicate that the wilt inducing activity of this material is based on plugging of xylem vessels. This is also

suggested by the effect of Dextran T500 in the bioassay, but the phytotoxic fraction exerts its effect at a much lower concentration.

No correlation was found between the production of the phytotoxic compounds of high molecular weight by 5 strains grown in vitro and degree of virulence of the strains. Such a correlation has been reported for the extracellular polysaccharides producing, wilt causing bacteria *Pseudomonas solanacearum* (Husain and Kelman, 1958) and *Erwinia stewartii* (Bradshaw-Rouse et al., 1981), indicating an important role for these polysaccharides in pathogenesis. For *C. michiganensis* subsp. *michiganensis* the importance of the produced high molecular weight compounds in pathogenesis could not be shown. It is possible that the amount of these high molecular weight compounds produced in vivo differs from the amount produced in vitro as a result of plant-bacterium interactions.

Host specificity is defined as the ability of a toxin to affect susceptible genotypes of the hosts and the inability to affect resistant genotypes or non-hosts (Scheffer, 1976). Host specificity of a toxic compound indicates a primary role in pathogenesis. Our experiments showed that the isolated phytotoxic high molecular weight compounds react non-specifically in the bioassay and therefore resistance present in some of the plants is not directed against these phytotoxic compounds. Non-specificity was also reported by Rai and Strobel (1969b) for their toxin.

Of certain non-specific toxins it is known that they are not required for pathogenicity, but contribute to virulence (Scheffer, 1983). Thus these toxins do play a role in pathogenesis, but not a primary one. This might be the case with the phytotoxic compounds produced by *C. michiganensis* subsp. *michiganensis*.

Further studies will be carried out to purify and characterize the phytotoxic high molecular weight fraction and to investigate its effect at the cellular level, in order to evaluate its potential as a selective agent for in vitro selection.

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Samenvatting

Effect van toxische componenten geproduceerd door Clavibacter michiganensis subsp. michiganensis op resistente en vatbare tomateplanten

Een fytotoxische fractie werd geïsoleerd uit cultuurfiltraat van *Clavibacter michiganensis* subsp. *michiganensis*, de veroorzaker van de bacterieverwelkingsziekte bij tomaat. Een eerste karakterisering toonde aan dat deze toxische fractie hoog-moleculaire component(en) bevat, bestaande uit polysacchariden en een gering percentage eiwit. Dit is in overeenstemming met toxines van *C. michiganensis* subsp. *michiganensis* die al eerder beschreven zijn.

Deze hoogmoleculaire toxische fractie was in staat verwelking te induceren van stengeltoppen van verschillende *Lycopersicon esculentum* en *L. peruvianum* genotypen in

een bioassay. Gewichtsverandering van de stengeltoppen, uitgedrukt als percentage ten opzichte van het begingewicht, werd gebruikt als parameter voor verwelking. De toxische fractie reageerde niet-specifiek in de bioassay, want er werd geen verschil gevonden in respons van (partieel) resistente en gevoelige genotypen. Er bleek geen correlatie te zijn tussen de mate van virulentie van verschillende isolaten van *C. michiganensis* subsp. *michiganensis* en de hoeveelheid van de toxische fractie geproduceerd in vitro.

Het mogelijke gebruik van deze hoogmoleculaire toxische fractie als selectief agens bij in vitro selectie zal nader onderzocht worden.

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