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Host-Specific Toxin Production by the Tomato Target Leaf Spot Pathogen *Corynespora cassiicola*

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Abstract: The culture filtrate (CF) of *Corynespora cassiicola*, the causal agent of target leaf spot of tomato, produced visible small, pinpoint, water-soaked lesions on the lower surface of wounded, detached leaves after incubation for 48 h. The extraction, isolation and purification of *C. cassiicola* tomato (CCT) toxin was achieved by anion exchange and gel filtration chromatography. Treatments of the CF with Diaion HP-20 resin and Cosmosil C_{18}-OPN gel were found to be highly effective. The toxin purified partially by cosmosil gel filtration was highly active against the tomato cultivar Momotaro used in the bioassay test, and the fraction eluted with 50% methanol showed toxicity only on the tomato plant. However, in the bioassay of the fractions after Sephadex LH-20 gel filtration no toxicity could be detected. Moreover, methanol fractions (40% and 60%) of the culture filtrate extracted by Sep-Pak cartridge were subjected to high performance liquid chromatography systems. The bioactive fraction (40% methanol) selected for analysis was eluted as double peaks. The results showed that *C. cassiicola* produced a host-specific toxin during colonization of tomato leaves and disease incidence.

Key Words: *Corynespora cassiicola*, host-specific toxin (HST), target leaf spot, tomato

Domates Yaprak Leke Hastalığı Etmeni *Corynespora cassiicola*'nın Konukçuya Özgü Toksin Üretimi

Özet: Domateste yaprak leke hastalığı etmeni *Corynespora cassiicola*'nın kültür filtratı, 48 saatlik inkubasyondan sonra yaralı, domates bitkisinden koparılmış yapraklarının alt yüzeyinde gözle görülebilir küçük, iğne ucu biçiminde, suya batırılmış şekilde lezyonlar oluşturmuştur. *C. cassiicola* domates (CCT) toksininin ekstraksiyonu, izolasyonu ve saflaştırılması, anyon değişimi ve jel filtrasyon yöntemleri ile gerçekleştirilmiştir. Kültür filtratının Diaion HP-20 ve Cosmosil C_{18}-OPN jel ile yapılan muameleleri, en etkili metodlar olmuştur. Cosmosil jel filtrasyonu ile kısmi olarak saflaştırılan toksin, bioassay denemesinde kullanılan domates çeşidine karşı son derece etkili olmuş ve % 50 metanol ile saflaştırılmış olan fraksiyon sadece domates bitkisine toksin aktivitesi göstermiştir. Ancak, Sephadex LH-20 jel filtrasyon aşamasından sonra elde edilen toksin fraksiyonlarının bioassay denemesinde, herhangi bir toksisite saptanamamıştır. Ayrıca, Sep-Pak cartridge ile ekstrakte edilmiş kültür filtratının % 40 ve % 60 fraksiyonları, HPLC (yüksek performanslı sıvı kromatografisi) sistemine maruz bırakılmıştır. HPLC analizi için seçilen biyoaktif fraksiyon (% 40 metanol), çift pik elde edilmiş ve saflaştırılmıştır. Elde edilen bu sonuçlar *C. cassiicola*'nın, domates yapraklarının kolonizasyonu ve hastalık oluşumu sırasında konukçuya özgü toksin oluşturduğunu göstermiştir.

Anahtar Sözcükler: *Corynespora cassiicola*, konukçuya özgü toksin (HST), yaprak lekesi, domates

Introduction

Target leaf spot of the leaves and fruits of the tomato, caused by *Corynespora cassiicola* (Berk. & Curt.) Wei, is a common disease in almost all tomato growing regions of the world (Ellis and Holliday, 1971). The disease occurs on a wide range of host plants in both tropical and subtropical countries. It was first reported on tomato (*Lycopersicon ecsulentum* Mill.) by F.C. Deighton in Sierra Leone (Wei, 1950), followed by India (Mohanty and Mohanty, 1955), Queensland, Australia (Simmonds, 1958), the United States (Blazquez, 1972) and southern Nigeria (Bliss et al., 1973). Tomato plants appear to be most susceptible at the seedling stage and just before and during fruiting (Bliss et al., 1973). Initially, symptoms consist of small pinpoint, water-soaked lesions on the upper surface of the leaves. They become circular and pale brown lesions surrounded by conspicuous yellow halos. The coalescence of the lesions may result in a rapid

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collapse of tissue, but the leaves remain attached to the petiole. Petiole and stem lesions are brown and oblong to elongate. On fruits, the lesions appear as dark, sunken, pinpoint, brown spots with a pale brown center, which crack (Blazquez, 1993). The rapid development of the symptoms led researchers to the hypothesis that a toxin may be involved in the disease syndrome (Onesirosan et al., 1975).

Intense studies about the toxins produced by plant pathogens met with disappointment until attention was centered on host-specific toxins or host-selective toxins (HSTs) as primary determinants of pathogenicity (Otani, 2000). Most of the research begun on HSTs produced by plant pathogens was biased toward toxin isolation and purification, or the mode of biochemical action on susceptible plant tissues, because of their interesting and unique host selective phytotoxicity (Nishimura and Kohmoto, 1983a). The finding of a group of highly host selective HSTs, valuable both as tools for academic research on plant host-parasite interactions and as markers for epidemiological surveys of pathogens in fields, confirms that the search for toxins is an important part of modern plant pathology. Well-designed experiments with purified HSTs provide some clues to clarifying the role of HST in pathogenesis and the chemical basis of host-selectivity of the pathogen (Nishimura and Kohmoto, 1983b). Efforts to resolve them should contribute to a better understanding of the molecular basis of host parasite coevolution in natural agro-ecosystems. The study of fungal toxins in plant pathogenesis has made remarkable progress within the last 2 decades. Fungal cultures provided a bewildering array of low molecular weight metabolites that demonstrate toxicity to plants (Desjardins and Hohn, 1997). But although it was easy to demonstrate that fungal cultures contained toxic substances, it proved a more difficult to establish their causal role in plant disease (Yoder, 1980). However, it would appear that an understanding of the exact roles of such toxins in relation to successful pathogenesis as well as to the epidemiology of the disease is the critical need (Nishimura and Kohmoto, 1983a).

Both specific and nonspecific secondary metabolites also play an important role in symbiotic plant/microbe interactions (Walton and Panaccione, 1993). Most host-specific toxins are low molecular weight secondary compounds with diverse chemical structures and are toxic only to the host plant and at very low concentration (Quayyum et al., 2003). Known HSTs include cyclic peptides, terpenoids, oligosaccharides, polyketides and compounds of unknown biogenesis. Most HSTs from any single pathogen occur as families of closely related compounds. Genetic analysis has shown that sensitivity to particular HSTs is controlled monogenically in the host plants (Walton and Panaccione, 1993). Despite the target leaf spot of tomato being of economic importance, no information about the toxigenic pathogenesis is available with respect to *C. cassiicola* tomato (CCT) toxin extraction from the culture filtrate. The characteristic symptoms on leaves inoculated with the culture filtrate of the pathogen suggested the possible involvement of an HST in disease development. The chemical and structural elucidation of HSTs is also promoted by the widespread use of modern analytical instruments such as high performance liquid chromatography (HPLC). The objectives of this study were to (i) detect toxin produced by the pathogen in the culture filtrate, (ii) determine the sensitivity of host and nonhost plants to the toxin fraction by bioassay, and (iii) purify it from the culture filtrate by HPLC analysis.

Materials and Methods

Fungal isolates: A highly virulent isolate, LC93020, of the tomato pathotype of *C. cassiicola* was mainly used for analysis of toxin production. Isolates were obtained from naturally occurring lesions on tomato leaves, and maintained on potato-dextrose agar (PDA) medium (potato, 250 g; dextrose, 20 g; agar, 15 g; and distilled water, 1000 ml; pH 7.0).

Plants: For bioassay tests, tomato plants (*Lycopersicon esculentum* L. cv. Momotaro) as host and cabbage (*Brassica oleracea* L. cv. Shoshu) as nonhost were used. The plants were grown in pots under greenhouse conditions. The greenhouse temperature ranged from 20 to 26 ºC, and the relative humidity from 50% to 80% during the experimental period. All experiments were repeated twice. Three leaves were used for each dilution.

Toxins extractions from the culture filtrate: For toxin production, 500-ml bottles, each containing 200 ml of a modified Richard's solution (Kohmoto et al., 1991) (25 g of glucose, 10 g of potassium nitrate, 5 g of potassium dihydrogen phosphate, 2.5 g of magnesium sulfate, 0.02

g of ferric chloride, 0.005 g of zinc sulfate, and 1000 ml of distilled water, pH 7.0) were inoculated with pieces of mycelial mats grown in PDA slants in test tubes. After 24 days of incubation under stationary conditions at 25 ºC, the culture filtrates were obtained by filtration through one layer of Whatman No. 2 filter paper (Whatman International Ltd., Maidstone, England). The culture filtrate (1 l) was stirred with Diaion HP-20 (Ion Exchange Resin, Mitsubishi Kasei Corp., Japan) for 4 h to adsorb toxins (Figure 1). The Diaion HP-20 resin was packed in a column and eluted with 4 l of methanol. The eluate was evaporated to dryness under reduced pressure at 40 ºC using a Rotary Vacuum Evaporator (Tokyo Rikakikai Co. Ltd., Japan) until all methanol was gone. In the second step, Cosmosil 75 C_{18}-OPN (a specifically prepared reagent, Nacalai Tesque Inc., Japan) was used for extraction. The HP-20 sample was washed with 25% and 75% methanol solutions and eluted with 50% methanol. The extract was evaporated to dryness under reduced pressure at 40 ºC. The partially purified toxin, after passing through the cosmosil gel, was dissolved in 1 ml of distilled water used in the bioassay as described below. In third step, gel filtration was studied with the Sephadex LH-20 (particle size 25 to 100 µm, Pharmacia Fine Chemicals, Sweden). The Sephadex bed (1.6 x 42.5 cm) was washed with 50 ml of distilled water before the use of methanol (100%) and ethanol (10%) for 3 times. For separation of the active fractions, column chromatography (1.6 x 42.5 cm) and a Fraction Collector (Advantec SF-2100, Japan) were used in this experiment.

Detached leaf necrosis assay for HST: In order to understand the role of host-specific toxin production in plant disease development, the biological activity and host specificity of toxins in sample solutions were determined by a leaf necrosis assay using susceptible and resistant leaves (Kohmoto et al., 1979). The midribs of young, detached leaves of 45-day-old tomato plants were removed, and the lower surface of the leaf lamina was scratched near the center with a sterile needle. Later, a drop (25 µl) of toxin containing solution was placed on each wounded site. Several dilutions of the solutions were tested. The leaves were incubated on moistened spongy mats in a moist chamber for 48 h at 25 ºC. After incubation, the necrosis appearing around the wounded sites was recorded as (-) = no toxicity (0), (±) = slight symptoms (1), (+)= moderate symptoms (2), and (++) = severe symptoms (3) based on the infected leaf area (Kohmoto et al., 1993).

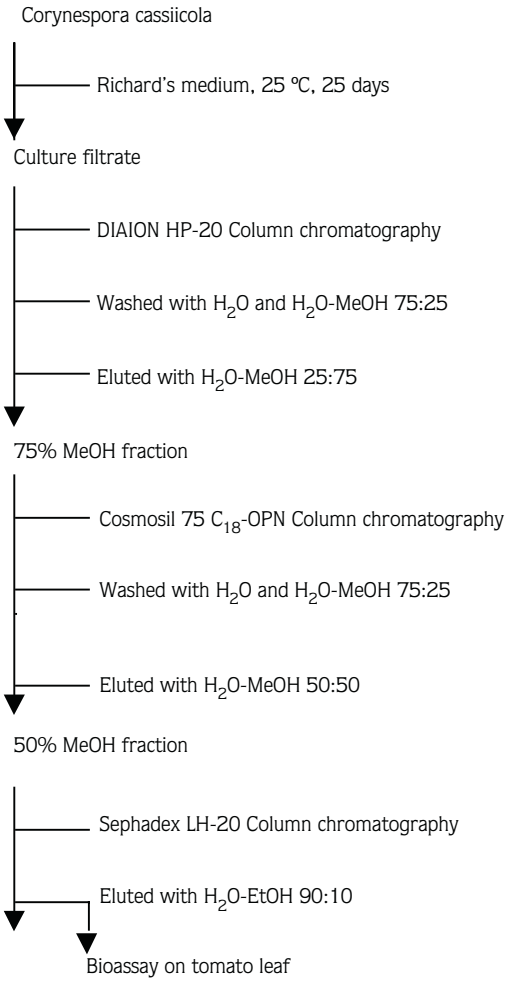

Figure 1. Purification steps for isolating CCT-toxin from the culture filtrates of *C. cassiicola* tomato pathotype (isolate LC93020).

High Performance Liquid Chromatography (HPLC): Analytical, semipreparative and preparative HPLC was performed with a Hitachi HPLC system equipped with an L-3000 Photodioide Array Detector, an L-5000 LC controller, an L-6200 Intelligent pump, and a D-2500 chromatointegrator (Hitachi Scientific Instruments Co. Ltd., Tokyo, Japan).

Sample solutions obtained from the culture filtrates were filtered with a 0.2 µm Millipore filter (Millipore Corp., Bedford, MA, USA) to remove insoluble materials. The solutions were applied to a Sep-Pak C_{18} cartridge (Waters™ Chromatography Division Millipore Corp.), and the toxin was eluted with various methanol dilutions (20%, 40%, 60%, 80% and 100%). After evaporation under reduced pressure at 40 ºC, the residue was

dissolved in 1 ml of sterile distilled water and bioassayed with host and nonhost plants as described above. Crude fractions of the active toxin were subjected to HPLC analysis on Daisopak SP-120-5-ODS-A (10 x 250 mm) and Daisopak SP-120-5-ODS-B (6.0 x 150 mm) columns using acetonitrile and potasium dihydrogen phosphate as a mobile phase at a flow rate of 0.8 ml/min, and absorbance was monitored at 200 nm. CCT-toxin in sample solutions was evaluated by measuring the height of HPLC peaks in the elution profile.

Results and Discussion

Toxin Isolation: Treatment of the CF with Diaion HP-20 (Ion Exchange resin) was an effective extraction step, because it removed many contaminating proteins without affecting the toxic activity of the extract. To be pathogenic, a pathogen must produce the specific toxin at the infection court. HSTs are a key and control event in early pathogenesis, allowing fungus access to host cells (Nishimura and Kohmoto, 1983b). A toxin also can cause either typical and atypical symptoms, depending on concentration and assay procedures. Thus, the production of typical visible symptoms is one of the least reliable criteria for evaluating a possible role for a toxin in the disease (Yoder, 1980). The resin also clarified the CF by adsorption of pigments, phenolics or other darkly colored substances present in the crude extract. The toxin fractions collected after Sephadex LH-20 gel filtration were bioassayed under stationary conditions, and evaluated after 48 h of incubation. The results of the tomato detached-leaf bioassay with different fractions extracted by column chromatography indicated that toxin activity could not be detected, indicating that the toxin was absorbed by Sephadex LH-20 gel filtration. The failure to detect the CCT toxin by Sephadex gel filtration of the culture filtrates of *C. cassiicola* in bioassay is consistent with the report by Vidhyasekaran et al. (1997), who were working on host-specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. Therefore, the activity of methanol fractions (25% and 100%) of the cosmosil 75 C_{18}-OPN gel were studied on tomato (host) and cabbage (nonhost) plants in various dilutions. Total toxin amount was 0.02594 g. The biological activity of toxin fractions of the culture filtrate is presented in Table 1. As can be clearly seen, although phytotoxicity was observed on tomato plants in dilutions of 10^{-1} and 10^{-2}, toxin activity could not be detected on the leaves of cabbage except for the 25% methanol fraction in 10^{-1} dilution. The partially purified toxin had host-specificity as crude filtrate, and was highly active on the tomato cultivar. It also produced symptoms similar to those of the pathogen under greenhouse conditions. Meaningful studies on the role of HSTs in plant diseases depend on assay systems. Assays must be considered in terms of specificity, ability to quantify, sensitivity, simplicity, and reproducibility (Yoder, 1981). Bioassays usually give qualitatively reliable data with considerable sensitivity, and quantification by bioassays often provides only relative amounts of HST in samples (Hayashi et al., 1990). The results revealed that the 25% methanol fraction contained both host- and nonhost-specific toxin activity. No toxicity was observed on the control plants. The fraction eluted with 50% methanol was toxic on the tomato plants only. This result is similar to those reported by Onesirosan et al. (1975), who indicated that the CCT toxin was partially extracted at a dilution of 25%. Thus, treatment of the culture filtrate with cosmosil gel was an effective purification step because it

Table 1. Biological activity of toxin fractions obtained from the culture filtrate of *C. cassiicola* on host and nonhost plants in various dilutions.

	Tomato (host)			Cabbage (nonhost)		
Methanol samples	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
25%	++1	++	-	±	-	-
50%	++	+	-	-	-	-
75%	+	+	-	-	-	-
Control[2]	-	-	-	-	-	-

[1] Toxicity was evaluated as 0 = no toxicity (-), 1 = slight symptoms (±), 2 = moderate symptoms (+), and 3 = severe symptoms (++)
[2] Sterile distilled water

removed many contaminating proteins without affecting the toxic activity of the filtrate.

HPLC: For purification of fungal toxin, the culture filtrate of *C. cassiicola* isolate LC93020 grown in test tubes containing Richard's medium for 15 days at 25 ºC was subjected to HPLC. Firstly, the diluted solutions (dilution rates 1:2, 1:4, 1:8) of the culture filtrate were assayed on tomato plants and the results are shown in Table 2. In the biological activity test of the fractions collected by gel filtration chromatography using the detached leaf bioassay, although dilutions 1:2 and 1:4 had toxin activity, no toxicity was observed on the leaves inoculated with water and Richard's medium serving as a control. The dilution end point bioassay previously employed for toxicity may give variable results, depending on the developmental and physiological conditions of the biological materials. It also is time consuming and rather qualitative (Hayashi et al., 1990). The present HPLC facilitated a more precise, rapid and easy estimation of the production of host-selective toxins during the culture filtrate of *C. cassiicola*.

Secondly, selected methanol fractions (20%, 40%, 60%, 80% and 100%) of the culture filtrate were extracted by Sep-Pak cartridge and bioassayed on host and nonhost plants (Table 3). Methanol fractions (40% and 60%) of the toxin extract showed toxicity on tomato plants only. On the other hand, sterile distilled water extraction of the toxin had toxicity on both tomato and

Table 2. Bioassay of *C. cassiicola* culture filtrate, Richard's medium and distilled water (DW) on tomato plants in various dilutions (CF, culture filtrate; RS, Richard's solution; DW, distilled water).

		Sample no.									
Treatment	Dilutions	1	2	3	4	5	6	7	8	9	10
CF	0	±	+	++	++	++	+	+	+	+	+
	1/2	+	+	+	+	+	±	+	±	±	±
	1/4	±	-	±	+	±	-	±	-	-	-
	1/8	-	-	-	-	-	-	-	-	-	-
RS	0	-	-	-	-	-	-	-	-	-	-
	1/2	-	-	-	-	-	-	-	-	-	-
	1/4	-	-	-	-	-	-	-	-	-	-
	1/8	-	-	-	-	-	-	-	-	-	-
DW	0	-	-	-	-	-	-	-	-	-	-
	1/2	-	-	-	-	-	-	-	-	-	-
	1/4	-	-	-	-	-	-	-	-	-	-
	1/8	-	-	-	-	-	-	-	-	-	-

Toxicity was evaluated as 0 = no toxicity (-), 1 = slight symptoms (±), 2 = moderate symptoms (+), and 3 = severe symptoms (++)

Table 3. Biological activity of toxin extracts of *C. cassiicola* with Sep-Pak cartridge in various methanol dilutions.

	Methanol fractions					
Plant	DW	20%	40%	60%	80%	100%
Tomato	+[1]	NT[2]	++	++	NT	NT
Cabbage	±	NT	NT	NT	NT	NT
Control	NT	NT	NT	NT	NT	NT

[1] Toxicity was evaluated as 0 = no toxicity (-), 1 = slight symptoms (±), 2 = moderate symptoms (+) and 3 = severe symptoms (++)
[2] NT, No toxicity

cabbage plants. No symptoms were observed on the wounded leaves treated with sterile water.

The methanol fractions (40% and 60%) selected for HPLC analysis were diluted in Acetonitrile (CH3CN) and potassium dihydrogen phosphate (KH_2PO_4) mixtures and subjected to HPLC (Figure 2). Only one methanol sample (40%) had 2 toxin fractions, A (Rt 14.4-15.5) and B (Rt 16.03-17.5). Recent progress on the chemistry and biology of HSTs indicates that HSTs are ordinary natural products. The chemical structures, biosynthetic pathways, and molecular genetics of HSTs are similar to those of other microbial secondary metabolites, whether they be called antibiotics, antimetabolites, nonspecific toxins, xenobiotics, growth regulators, teratogens, mutagens, pigments, etc. (Walton and Panaccione, 1993).

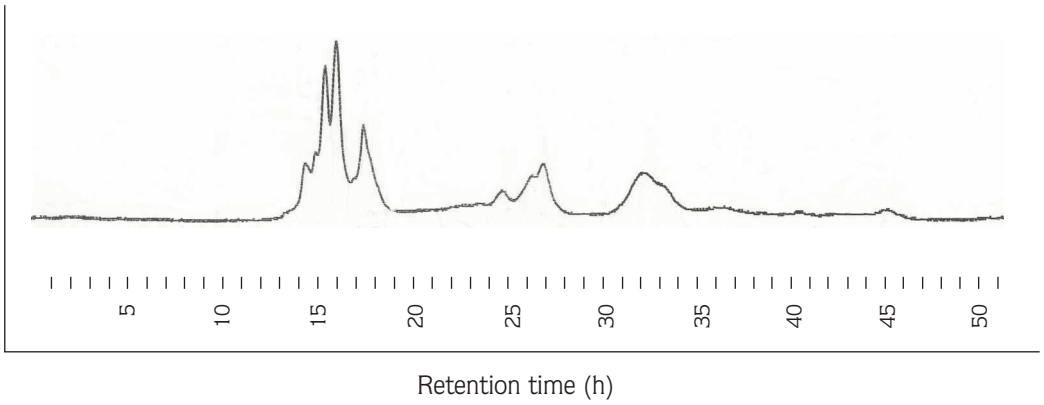

Figure 2. High performance liquid chromatography of the culture filtrate (CF) of the tomato pathotype of *C. cassiicola*. The CF was extracted after 2 weeks of incubation, carried out using Daisopak SP-120-5-ODS-A (10 x 250 mm) and Daisopak SP-120-5-ODS-B (6.0 x 150 mm) columns, an acetonitrile-potassium dihydrogen phosphate solvent system, a flow rate of 0.8 ml per minute, and 220 nm detection.

Conclusions

Much research concerning *C. cassiicola* HSTs seems warranted for explaining their significant role in the pathogenesis and specificity of co-evolutional parasitism in host-parasite combinations. The tomato-specific toxin may be a useful tool in plant breeding. It could be used as a quick method for screening tomato lines for resistance to *C. cassiicola*. Such HST research may also be useful for understanding many other types of host-recognition factors produced by plant pathogens in other pathosystems, for identifying detoxification genes from plants and microorganisms and for studying the etiology and disease resistance mechanisms against the pathogen, involved in disease development. According to these results, further characterization and developed purification methods for each toxin should be assessed for their similarities to each other.

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