Isolation and Biological Activities of Two Host-Specific Toxins from the Tangerine Pathotype of *Alternaria alternata*

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ABSTRACT

Kohmoto, K., Itoh, Y., Shimomura, N., Kondoh, Y., Otani, H., Kodama, M., Nishimura, S., and Nakatsuka S. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. Phytopathology 83:495-502.

Two host-specific toxins (HSTs), ACT-toxin Ib and Ic, were isolated from culture filtrates of *Alternaria alternata* affecting tangerines and mandarins. The biological characteristics of ACT-toxin Ib matched the criteria for HST. ACT-toxin Ib was detected by HPLC (high-performance liquid chromatography) analysis and was one of the major components in the ethyl acetate extracts of culture filtrates and spore-germinating fluids of the pathogen. ACT-toxin Ib induced veinal necrosis and a rapid increase in electrolyte loss from susceptible leaves of Emperor mandarin, at 2×10^{-8} M, and from susceptible Japanese pear cv. Nijisseiki (a potential host), at 1×10^{-6} M, but did not affect resistant citrus and Japanese pear, at 2×10^{-4} M. Furthermore, the toxin caused plasma-membrane invaginations in susceptible Emperor mandarin and Japanese pear but not in resistant genotypes. Infection hyphae of an avirulent isolate of *A. alternata* were produced in leaf tissues when their spores were inoculated with a small amount of the toxin solution. These results suggest that ACT-toxin Ib rapidly affects plasma-membrane integrity of susceptible genotypes and plays a critical role in the infection process of the pathogen. A minor ACT-toxin, Ic, had selective toxicity, but Ic might be an artifact of ACT-toxin Ib exposed to light during purification.

Additional keywords: ACT-toxin A, ACT-toxin B.

At least two distinct pathotypes in the species *Alternaria alternata* (Fr.:Fr.) Keissl. are known to cause brown spot disease on citrus leaves and fruits (11,12,22). These pathotypes, previously included in the species *A. citri* Ellis & N. Pierce in N. Pierce, clearly have different host spectra. Isolates of one of the pathotypes were found in Queensland, Australia, in 1966 (28) and in Florida, the United States, in 1976 (29). These isolates are grouped in the tangerine pathotype, which is pathogenic to Dancy tangerine and Emperor mandarin (*Citrus reticulata* Blanco) but not to rough lemon (*C. jambhiri* Lush.) and other nonhost plants. The second pathotype is the rough lemon pathotype, which was also found in Florida in 1976 (29). In contrast to the tangerine pathotype, it is pathogenic to rough lemon but not to Dancy tangerine and Emperor mandarin. Both of these pathotypes produce host-specific toxins (HSTs) with the same host specificity as the fungal isolates (11,12). During research on pathogenicity of these pathotypes and host-specific involvement of the toxic compounds, the toxin was first called AC-toxin (derived from *A. citri*). Later, to distinguish the toxins produced by the two different pathotypes, the tangerine-pathotype toxin was named ACT- (6,8) or ACTG-toxin (2,14,15), and the rough lemon-pathotype toxin was named ACR- (9,19) or ACRL-toxin (2,3,14).

The chemical structures of the major components of ACR- and ACRL-toxins were determined independently by Gardner et al (3) and Nakatsuka et al (20). The primary action site for the major ACR-toxin I was found in the mitochondria of susceptible rough lemon (1,9). The toxin caused uncoupling of oxidative phosphorylation and changes in membrane potentials (1).

The effects of ACT-toxins appear to be more complex. Kohmoto et al (12) used several toxin preparations that differed in degree of purity and found that ACT-toxins caused veinal necrosis on leaves and induced electrolyte leakage from tissues. Electron microscopy indicated that the primary action site for ACT-toxins is on the plasma membranes (13). Interestingly, culture filtrates of the tangerine pathotype were toxic under experimental conditions to both the natural host and to Japanese pear (*Pyrus serotina* Rehd.) cv. Nijisseiki (6). In addition, Nijisseiki was sus-

ceptible to the tangerine pathotype in laboratory tests.

Kohmoto et al (11) found that the tangerine pathotype produces at least two toxins in vitro, ACT-toxin A and B, that differ in host-specificity. ACT-toxin A is selectively toxic to citrus; ACT-toxin B is not toxic to citrus but has the same selective toxicity to certain Japanese pear cultivars as has AK-toxin from the Japanese pear pathotype of *A. alternata*. Kono (14) and Kono et al (15) determined the structures of three tangerine toxins (ACTG-toxin A, B, and C) that have selective toxicity to Dancy tangerine. However, it was not clear if Kono's (14) ACTG-toxins were identical to ACT-toxins described by Kohguchi et al (6) and Kohmoto et al (8,11,12). Recent information on the structures of the ACT-toxins has led to confusion in the abbreviated toxin names. Therefore, we propose to change ACT-toxin A (11) to ACT-toxin I and to change ACT-toxin B (11) to ACT-toxin II. In addition, geometric isomers can be designated by adding a lower-case letter (e.g., a, b, or c) as a suffix to the Roman numeral. Suffixes a, b, and c indicate 2*E*,4*E*,6*Z*, 2*E*,4*Z*,6*E*, and 2*E*,4*E*,6*E* configurations in a decatrienoic acid, respectively. This system follows the precedent set for designating geometric isomers of AK- and AF-toxins (21) because ACT-toxins share the same trienoic acid moiety as these toxins (18,19). In this paper, we report the isolation of two host-specific toxins that cause rapid effects on plasma-membrane integrity. Some of these results have been presented in preliminary form (5,8), and the structural characterization of ACT-toxin I was reported by Nakatsuka et al (19).

MATERIALS AND METHODS

Plants. Emperor mandarin (*Citrus reticulata*) and Japanese pear (*Pyrus serotina*) cv. Nijisseiki were used as host and toxin-sensitive plants. Rough lemon (*C. jambhiri*) and Japanese pear cv. Chojuro were used as resistant plants. The plants were grown in a greenhouse. Young leaves of Emperor mandarin and rough lemon (5–7 days old) and cvs. Nijisseiki and Chojuro (3–5 days old) were used for the experiments.

Extraction of toxins from culture filtrates. *A. alternata* tangerine pathotype isolate AC-320 was kindly provided by J. O. Whiteside (University of Florida, Lake Alfred). Cultures were maintained on potato-dextrose agar slants in test tubes. For toxin production, pieces of mycelial mats from test tubes were placed in 500-ml bottles, each containing 200 ml of a modified Richards' solution (7) (25 g of glucose, 10 g of $KN0_3$, 5 g of KH_2P0_4, 2.5 g of $MgSO_4$, 0.02 g of $FeCl_3$, and 0.005 g of $ZnSO_4$ each per liter). After the stationary cultures were grown for 24 days at 25 C, they were harvested by filtration through four layers of gauze and a Toyo No. 2 filter paper (Toyo Roshi Co. Ltd., Tokyo) to eliminate mycelia. The culture filtrate (30 L) was adjusted to pH 5.5 with 10% NaH_2PO_4 and was stirred with 1 L of Amberlite XAD-2 resin (Aldrich Chemical Co., Inc., Milwaukee, WI) for 2 h to adsorb toxins. The Amberlite XAD-2 was packed in a column and eluted with 4 L of methanol. The eluate was evaporated under reduced pressure at 40 C until all methanol was gone. The residual concentrate (about 100 ml) was extracted five times with ethyl acetate; the extract was dehydrated with anhydrous sodium sulfate and evaporated under reduced pressure at 40 C. The final residue was dissolved in methanol and subjected to droplet countercurrent chromatography (DCC), using a Tokyo Rikakikai's model DCC-A instrument (Tokyo Rikakikai Co. Ltd., Tokyo). The sample was developed in descending mode; the solvent was carbon tetrachloride/chloroform/benzene/methanol/deionized water (3:3:6:8:2, v/v). Two-hundred fractions (each containing 10 ml) were collected. Two groups of active fractions were shown by leaf necrosis assay. The second group of active fractions (57–148), which was highly toxic to Emperor mandarin and Japanese pear cv. Nijisseiki but not to rough lemon and cv. Chojuro, was applied to thin-layer chromatography (TLC) using Kieselgel 60 F-254 (E. Merck, Darmstadt, Germany) plate (0.5 mm). After development with benzene/ethyl acetate/acetic acid (50:50:1, v/v), the active zone (Rf 0–0.25) was scraped from the plate and eluted with methanol. The eluated methanol was subjected to high-performance liquid chromatography (HPLC) on a Develosil 10/20 ODS column (20 × 250 mm, Nomura Chemical Co. Ltd., Tokyo) using methanol/deionized water/acetic acid (60:40:1, v/v) as a mobile phase at a flow rate of 5 ml/min. The absorbance of the effluent was monitored at 290 nm. Four major peaks were detected. Because the third (Rt [retention time] 11–14 min) and fourth (Rt 14–17 min) peak fractions, but neither the first (Rt 2–4 min) nor the second (Rt 4–5.5 min) peak fraction were selectively toxic to Emperor mandarin, these peaks were separately subjected to HPLC on Develosil ODS-5 column (4.6 × 250 mm), using acetonitrile/deionized water/acetic acid (40:60:1, v/v) at a flow rate of 1 ml/min.

Because the fourth peak fraction showed higher toxicity than did the third peak fraction, this fraction was isolated more directly from ethyl acetate extract of culture filtrate to obtain a higher yield. A summarized procedure is shown in Figure 1. The ethyl acetate extract of the culture filtrate was subjected directly to reverse-phase HPLC on a Develosil 10/20 ODS column (20 × 250 mm), using a gradient solvent system composed of acetonitrile/deionized water/acetic acid (from 25:75:1 to 60:40:1, v/v, in 40 min) at a flow rate of 5 ml/min. A peak fraction (Rt 27.5–30.5 min) corresponding to the fourth peak fraction was obtained. This fraction was rechromatographed by HPLC, using a Develosil 10/20 ODS column (20 × 250 mm) and a gradient solvent system composed of methanol/deionized water/acetic acid (from 60:40:1 to 100:0:1, v/v, in 40 min) at a flow rate of 5 ml/min. One major peak (Rt 21.7–23.3 min) and several minor peaks were detected by UV absorbance at 290 nm. This major peak fraction was obtained, evaporated, and dissolved in methanol. The yield was about 22 mg from 60 L of culture fluid.

Preparation of spores. Fungal isolate AC-320 was grown under still culture in potato-dextrose broth (100 ml in 500-ml bottles) for 2–3 wk at 25 C until mycelial mats covered the surface. Isolate O-94 (avirulent *A. alternata*) was grown in Richards' solution (200 ml in 500-ml bottles) for about 3 wk at 25 C. Mycelial mats were removed from the media, washed with flowing water, and dried briefly on paper towel to remove excess water. The mats were incubated for 20–24 h at 20 C in the dark. During this time, many spores formed on the surface of the mats. The spores were harvested by placing the mats in water and brushing the surface with a small painting brush, followed by filtration through four layers of gauze to eliminate mycelia. The spores were washed five times by centrifugation (400 *g*, for 3 min each). Washed spores were suspended in deionized water and adjusted to the desired concentration using a Thoma's hemacytometer. Spores were used either at once in inoculation experiments or were stored briefly for later use. For storage, the spore suspension

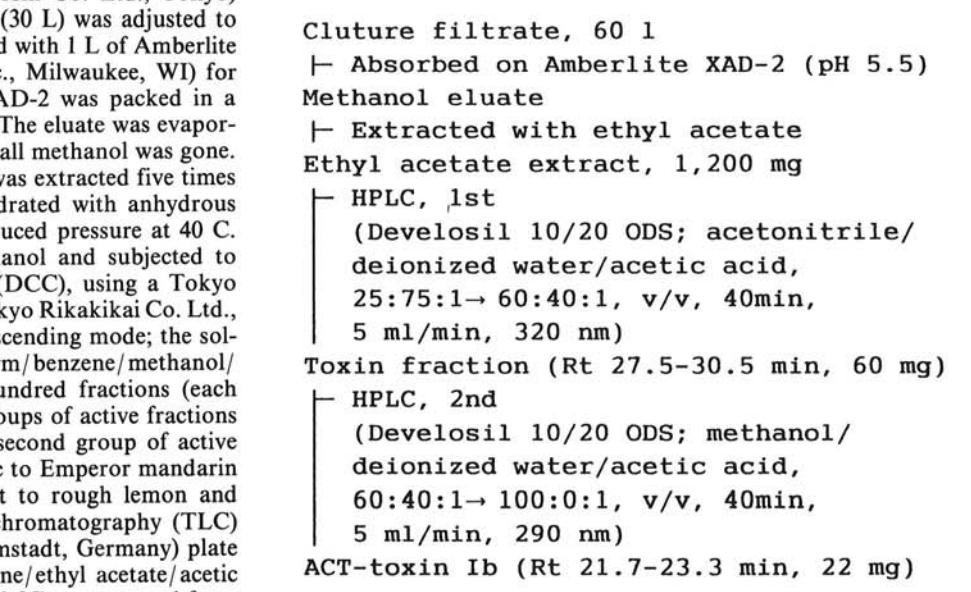

Fig. 1. Outline of an isolation procedure for ACT-toxin Ib from culture filtrates of *Alternaria alternata* tangerine pathotype (isolate AC-320).

was filtered (Whatman No. 50 filter paper, Whatman International Ltd., Maidstone, England), air-dried on the filter paper at room temperature, and preserved at 20 C in the dark until use.

Effect of ACT-toxin on infection of nonpathogenic *A. alternata*. Spores (5×10^5 spores per milliliter) of an avirulent isolate (O-94) of *A. alternata* were sprayed on the lower surface of susceptible and resistant leaves of citrus and Japanese pear. The spore suspension contained small amounts of ACT-toxin Ib: The final concentration was 2×10^{-8} M for Emperor mandarin and 1×10^{-6} M for cv. Nijisseiki. Control leaves were sprayed with deionized water containing spores at the same density. Each leaf was incubated in the dark at 25 C for 48 h. After incubation, the leaves were treated with lactophenol solutions to remove color and were examined microscopically for spore germination, appressorial formation, and infection hypha.

HPLC. Analytical and preparative HPLC was carried out with a Hitachi HPLC system equipped with an L-3000 photodiode array detector, an L-5000 LC controller, an L-6000 pump, and a D-2500 chromatointegrator (Hitachi Scientific Instrument Co., Ltd., Tokyo). Sample solutions obtained from culture filtrates or spore germination fluids were filtered with a 0.2-μm Millipore filter (Millipore Corp., Bedford, MA) to remove insoluble materials and were subjected to HPLC analysis on an appropriate Develosil column at room temperature.

Quantitative analysis of ACT-toxin in spore-germination fluids. Spore suspensions (50 ml of 1×10^6 spores per milliliter) were poured onto paper towels in plastic chambers ($33 \times 23 \times 6$ cm). Spores were incubated for 6, 12, 18, and 24 h at 25 C. After germinating rate was determined by light microscopy, paper towels were squeezed to recover germination fluids; deionized water (20 ml per chamber) was added, and towels were squeezed again. The combined fluids were filtered through Toyo No. 2 filter paper to remove spores. The spore suspension was directly filtrated and designated 0-h incubation. The solutions were evaporated until dry, and the residues were washed several times with methanol to remove the water. After insoluble materials were removed by filtration with a Millipore filter (0.45 μm), the solutions were applied to a Sep-pak C_{18} cartridge (Waters Chromatography Division Millipore Corp., Bedford, MA), and the toxin was eluted with methanol. After evaporation under reduced pressure at 40 C, the residue was dissolved in methanol. ACT-toxin Ib in sample solutions was quantified by measuring the height of HPLC peaks in the elution profile and by comparing them with a standard line for authentic ACT-toxin Ib. Authentic ACT-toxin Ib at 3.2×10^{-11} mol to 2×10^{-4} mol gave a linear relationship of peak height to quantity.

Leaf necrosis assay for HST. The biological activity and host specificity of toxins in sample solutions were determined by a leaf necrosis assay using susceptible and resistant leaves (12). The midribs of young leaves were removed, and the lower surface of the leaf lamina was scratched near the center with a needle. A drop (40 μl) was placed on each wounded site. Several dilutions of the solutions were tested. The leaves were incubated on moistened mats in a moist chamber for 48 h at 25 C. After incubation, the necrosis appearing around wounded sites was recorded.

Measurement of electrolyte loss. Electrolyte loss from tissues was determined as described previously (12). Twenty leaf disks, 0.8 cm in diameter, were cut from leaves with a leaf punch. The disks were vacuum-infiltrated with toxin solution or deionized water for 30 min. Disks were rinsed with deionized water, were placed in flasks, each containing 20 ml of deionized water, and were incubated on a reciprocal shaker (120 strokes per minute) at 25 C. Conductance of ambient solutions was measured at intervals with a conductivity meter, CD-35MII (M & S Instruments Inc., Osaka, Japan; $k = 1.0$).

Electron microscopy. The upper surfaces of young leaves were slightly wounded in 3- to 5-mm-long scratches with a razor blade, and a drop (30 μl) of ACT-toxin-Ib solution was placed on the wound site. Deionized water was applied to control leaves. Treated leaves were then incubated in a moist chamber for 1, 3, 6, and 12 h at 25 C. The treated sites were trimmed with a razor blade. The tissues were prefixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 C and were postfixed with 1.5% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 C. The tissues were dehydrated with ethanol and embedded in Spurr's resin (25). Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT), using a glass knife. The sections were placed on copper grids and stained with 2% uranyl acetate for 30 min and with lead citrate for 20 min. Some sections were treated with an alkaline bismuth solution (0.05% sodium hydroxide, 0.02% potassium sodium tartarate, and 0.01% bismuth subnitrate) for 40 min at 37 C. Stained sections were observed with a JEOL JEM-100 CX electron microscope (Japan Electric Optics Laboratory, Akishima, Tokyo). Because the necrosis initially developed along the leaf veins and later extended to neighboring mesophyll cells, ultrastructural observations were focused on three types of leaf cells: vascular-bundle sheath cells, mesophyll cells adjacent to bundle sheaths, and mesophyll cells remote from bundle sheaths. About 80 cells of each type were examined.

Spectral analysis. ^{1}H-NMR spectra were obtained with a JEOL JNM-FX-200 spectrometer (Japan Electric Optics Laboratory). UV spectra were recorded on a JASCO UNIDEC-505 spectrometer (Japan Spectroscopic Co., Ltd., Hachioji, Japan).

RESULTS

Isolation of ACT-toxin I group. Culture filtrate of isolate AC-320 caused veinal necrosis on leaves of Emperor mandarin and on Japanese pear cv. Nijisseiki up to 32- and eight-times dilutions, respectively. Nondiluted culture filtrate did not cause any visible reaction on leaves of either rough lemon or Japanese pear cv. Chojuro. After fractionation with DCC, two active groups of fractions were obtained. The first group (12–56) was more toxic to cv. Nijisseiki than to Emperor mandarin; the second group (57–148) was highly toxic to Emperor mandarin. Neither group caused necrosis on resistant genotypes. Based on TLC analysis (K. Kohmoto et al, *unpublished data*) and biological activity, the first group was judged to contain ACT-toxin II and the second to contain ACT-toxin I. The second group was purified further with two rounds of HPLC, and two toxic compounds to Emperor mandarin were obtained. The purity of both compounds was higher than 95% after the second HPLC purification, as shown by ^{1}H-NMR analysis (data not shown). Treatment of both compounds with diazomethane in methanol produced monomethyl esters in about 60% of the yield. The UV spectra (λmax 287 nm in methanol) of both compounds and their methyl esters suggested that the decatrienoic acid moiety of AK- and AF-toxins was also present in these compounds. The detailed analysis reported by Nakatsuka et al (19) suggested that the third peak (Rt 11–14 min) and fourth peak fractions (Rt 14–17 min) contain all-*trans* (2*E*,4*E*,6*E*) and central-*cis* (2*E*,4*Z*,6*E*) decatrienoic moiety, respectively. To follow the nomencleature of AF- and AK-toxins (21), the third peak fraction was designated ACT-toxin Ic, and the fourth peak fraction was designated ACT-toxin Ib.

The ethyl acetate extract from culture filtrates was subjected to HPLC (Fig. 2B). Three fractions, A (Rt 0–15.8 min), B (Rt 15.9–17.9 min), and C (Rt 18.0–30.0 min), were obtained. Fraction B contained ACT-toxin Ib and Ic (Fig. 2A and B). The ethyl acetate extract was toxic up to 64- and eight-times dilutions on Emperor mandarin and Japanese pear cv. Nijisseiki, respectively. Fraction A did not show toxicity; B and C were toxic both on Emperor mandarin and Japanese pear cv. Nijisseiki. Fraction B showed toxicity up to 32- and eight-times dilutions on Emperor mandarin and Japanese pear cv. Nijisseiki, respectively; fraction C was toxic at two-times dilutions on both plants.

Toxicity of ACT-toxin to citrus and Japanese pear leaves. A dose-response study revealed that ACT-toxin Ib induced veinal necrosis and increased electrolyte losses from leaf tissues (Fig. 3). ACT-toxin Ib at concentrations of more than 2×10^{-8} M induced brown veinal necrosis on leaves of Emperor mandarin and at a concentration of 1×10^{-6} M induced black veinal necrosis on leaves of Japanese pear cv. Nijisseiki. ACT-toxin Ib gave no

reaction on leaves of resistant plants (rough lemon and Japanese pear cv. Chojuro) at 2×10^{-6}, 4×10^{-5}, and 2×10^{-4} M concentrations (data not provided in Fig. 3). In contrast, ACT-toxin Ic caused the same necrosis on Emperor mandarin at a concentration of 4×10^{-7} M and on pear cv. Nijisseiki at a concentration of 4×10^{-6} M; it had no effect on resistant plants at a concentration of 1.2×10^{-5} M, the highest concentration tested. ACT-toxin Ib caused a rapid increase (detected within 30 min after toxin exposure) in electrolyte loss from leaf disks of susceptible citrus and Japanese pear at concentrations of more than 2×10^{-8} M and 1×10^{-6} M, respectively. There were no differences in electrolyte losses from toxin- and water-treated resistant leaf disks when toxin was used at concentrations of 2×10^{-6} M on rough lemon and at 4×10^{-6} M on Japanese pear cv. Chojuro.

Ultrastructural changes in the cells of ACT-toxin Ib-treated susceptible citrus and Japanese pear. A small amount of ACT-toxin Ib (30 μl; 2×10^{-7} M, for citrus, and 1×10^{-5} M, for Japanese pear) caused typical veinal necrosis of susceptible leaves 24 h after toxin treatment, but no necrosis was observed on resistant leaves even after 48 h of treatment. The earliest toxin-induced ultrastructural changes appeared, 1 h after toxin exposure, in the plasma membranes of all types of susceptible citrus leaf cells. The membrane modifications became conspicuous and frequent in susceptible leaves 3, 6, and 12 h after toxin treatment (Fig. 4B and 4C). No ultrastructural changes were detected in other organelles (chloroplasts, Golgi bodies, mitochondria, vacuoles, and rough endoplasmic reticula) 12 h after treatment

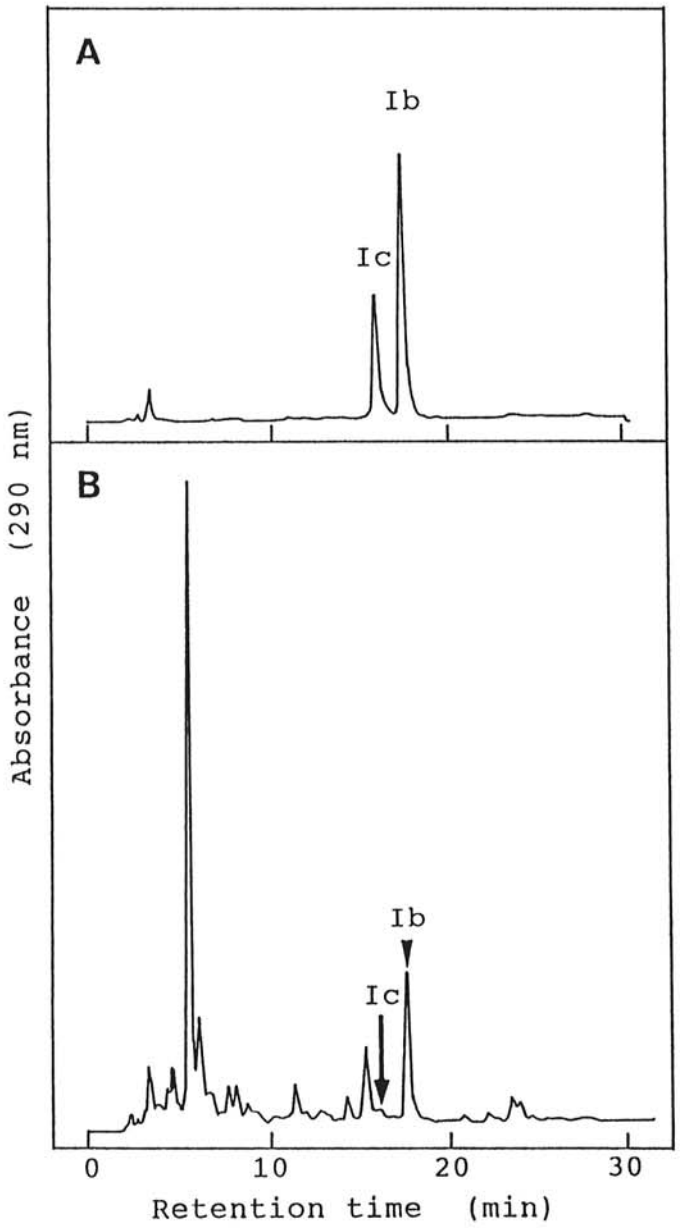

Fig. 2. A, HPLC (high-performance liquid chromatography) of authentic ACT-toxin Ib and Ic. **B,** HPLC of ethyl acetate extract from culture filtrate. HPLC was carried out using a Develosil ODS-5 4.6- × 250-mm column, an acetonitrile/deionized water/acetic acid (30:70:1 to 60:40:1, v/v, in 30 min) solvent system, a flow rate of 1 ml per minute, and 290-nm detection.

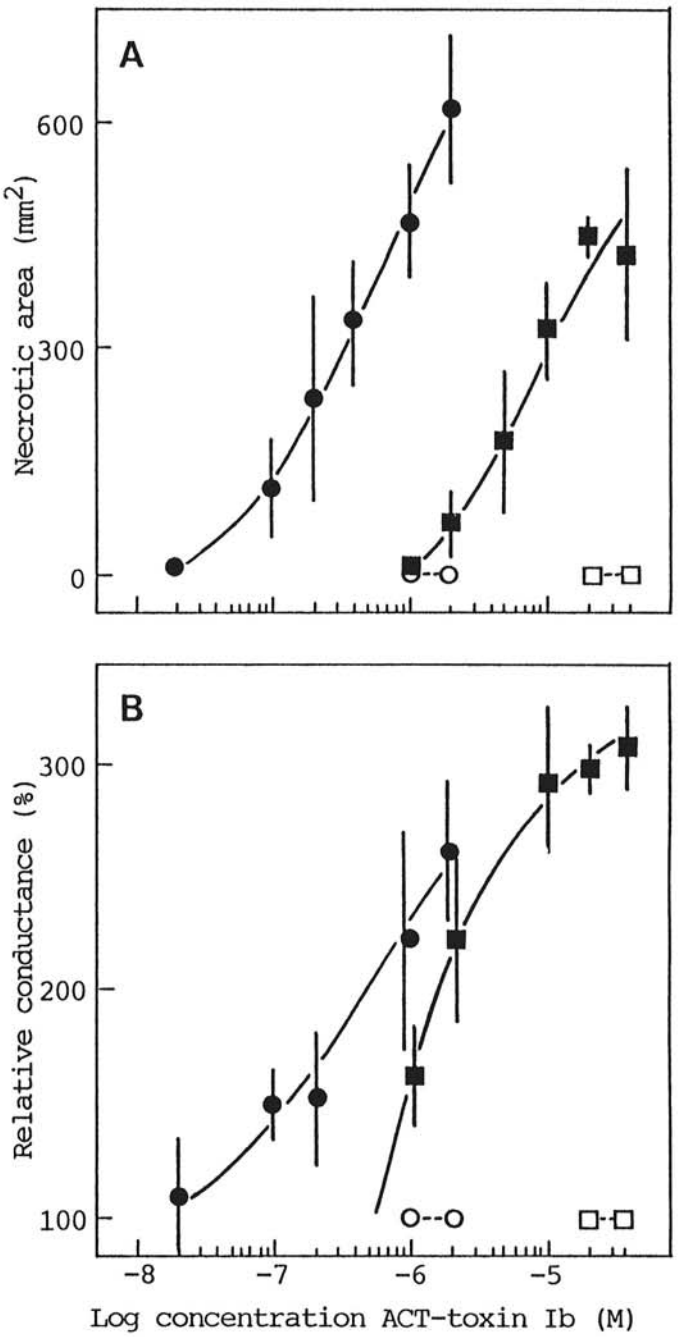

Fig. 3. A, Relationship between log concentration of ACT-toxin Ib and formation of veinal necrosis on leaf tissues, and **B,** electrolyte loss from leaf disks. Leaves of susceptible citrus Emperor mandarin (-●-), resistant citrus rough lemon (-○-), susceptible Japanese pear cv. Nijisseiki (-■-), and resistant Japanese pear cv. Chojuro (-□-) were treated with 40 μl of ACT-toxin Ib solution. Area of veinal necrosis was measured 48 h after toxin treatment. Electrolyte loss was measured every hour after toxin treatment and was represented as a value of relative conductance for 6 h between toxin-treated leaves and deionized water-treated leaves (control). The vertical bars represent the standard diviations of three experiments.

(Fig. 4B, 4C, and 4E). The plasma membranes of the toxin-treated susceptible leaves were invaginated prominently near the plasmodesmata (Fig. 4B and 4C). The invaginated regions contained desmotubles that extended from plasmodesmata (Fig. 4C). Membrane fragments and vesicles were stained positive with alkaline bismuth solution. No ultrastructural changes were evident in the vascular-bundle sheath and mesophyll cells of toxin-treated resistant plants and water-treated susceptible plants 12 (Fig. 4A and 4F) and 48 h after toxin treatment.

The frequency of cells with invaginated plasma membranes was determined. Sufficient cells containing a total of about 100 plasmodesmata in leaf tissues were observed for each treatment. As shown in Table 1, the percentage of invaginated plasma membranes per total plasma membranes at the plasmodesmal region differed in the three types of cells. One hour after toxin treatment of Emperor mandarin, the percentages of invaginated (or affected) plasma membranes were 76 in bundle sheaths, 11 in mesophyll cells adjacent to bundle sheaths, and 0 in mesophyll cells remote from bundle sheaths. The percentages increased somewhat with time: 81, 28 and 5, respectively, 6 h after treatment. Invaginations of plasma membranes were not observed in toxin-treated leaves of rough lemon and the untreated leaves of susceptible and

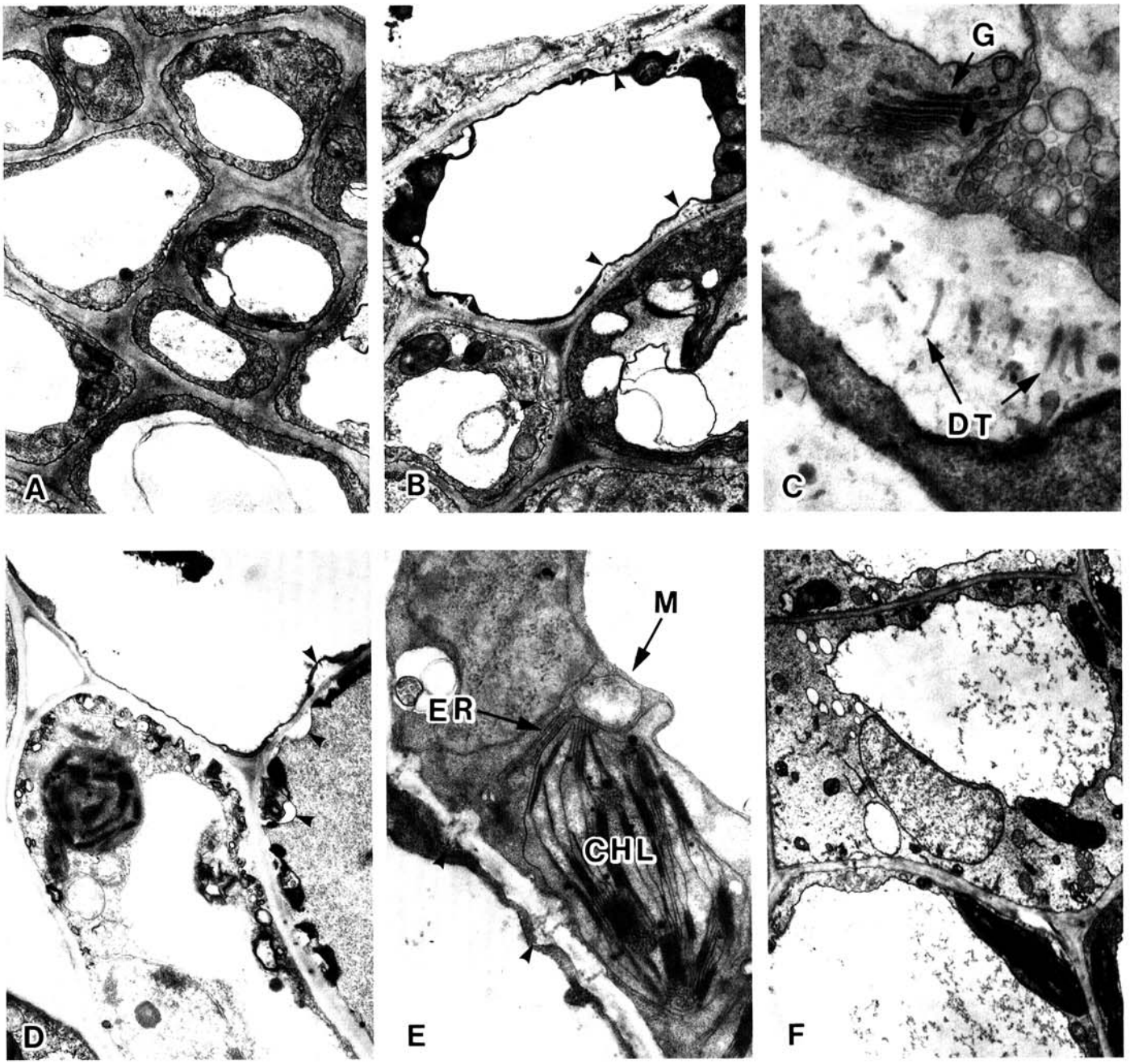

Fig. 4. Electron micrographs of citrus and pear leaf tissues treated with ACT-toxin Ib and with water. **A,** Ultrastructure of vascular-bundle sheath cells of susceptible Emperor mandarin leaf treated with water for 12 h (10,000×). **B,** Ultrastructure of vascular-bundle sheath cells of susceptible Emperor mandarin leaf treated with 2×10^{-7} M ACT-toxin Ib for 12 h. Plasma-membrane invaginations (arrows) were found but no structural changes of mitochondria and vacuoles were observed (10,000×). **C,** Typical invagination of plasma membrane at plasmodesmata in susceptible Emperor mandarin leaf treated with 2×10^{-7} M ACT-toxin Ib for 12 h. Plasma-membrane invaginations resulted in extensions of desmotubles (DT). The Golgi body (G) maintained original configuration (20,000×). **D,** Plasma-membrane invaginations (arrows) in mesophyll cells of susceptible Japanese pear leaf (cv. Nijisseiki) treated with 1×10^{-5} M ACT-toxin Ib for 6 h (8,200×). **E,** Mesophyll cells of susceptible Emperor mandarin leaf treated with 2×10^{-7} M ACT-toxin Ib for 12 h, showing plasma-membrane invaginations (arrows). Chloroplasts (CHL), mitochondrion (M), and rough endoplasmic reticulum (ER) had no apparent changes (14,000×). **F,** Resistant rough lemon leaf cells treated with 1×10^{-5} M ACT-toxin Ib for 12 h. Ultrastructural changes were not observed in the cells (5,200×).

TABLE 1. Plasmodesmatal invaginations induced by ACT-toxin Ib in leaf tissues of Emperor mandarin, rough lemon, and Japanese pear cvs. Nijisseiki and Chojuro

Plant and cultivar	Treatment	Time (h)	Plasmodesmata with plasma-membrane invagination					
			Vascular-bundle sheath cells		Mesophyll cells adjacent to bundle sheath		Mesophyll cells remote from bundle sheath	
			No. of plasmodesmata	No. of plasmodesmata invaginated	No. of plasmodesmata	No. of plasmodesmata invaginated	No. of plasmodesmata	No. of plasmodesmata invaginated
Citrus								
Emperor mandarin	Toxin[a]	1	112	85 (76)[b]	102	11 (11)	119	0 (0)
	Toxin[a]	3	99	51 (52)	114	32 (28)	124	0 (0)
	Toxin[a]	6	114	92 (81)	71	20 (28)	103	5 (5)
	Toxin[a]	12	44	37 (84)	73	19 (26)	133	26 (20)
	Water	12	111	0 (0)	85	0 (0)	117	0 (0)
Rough lemon	Toxin[a]	12	105	0 (0)	102	0 (0)	82	0 (0)
	Water	12	113	0 (0)	95	0 (0)	119	0 (0)
Japanese pear								
Nijisseiki	Toxin[c]	1	104	28 (27)	100	0 (0)	99	0 (0)
	Toxin[c]	3	79	34 (43)	69	0 (0)	46	0 (0)
	Toxin[c]	6	127	102 (80)	47	11 (23)	148	0 (0)
	Toxin[c]	12	71	59 (83)	55	11 (20)	52	7 (13)
	Water	12	102	0 (0)	94	0 (0)	68	0 (0)
Chojuro	Toxin[c]	12	66	0 (0)	29	0 (0)	100	0 (0)
	Water	12	96	0 (0)	91	0 (0)	73	0 (0)

[a] Concentration of toxin was 1×10^{-5} M.
[b] Numbers in parentheses indicate the percentage of plasmodata invaginated.
[c] Concentration of toxin was 1×10^{-4} M.

TABLE 2. Time course of spore germination and ACT-toxin Ib production from spores of isolate AC-320 of *Alternaria alternata* tangerine pathotype

Incubation period (h)	Spore germination (%)	Detected toxin (pg/germinating spore)
0	...	ND[a]
6	82.3 ± 1.2[b]	ND
12	84.1 ± 3.2	0.035 ± 0.015
18	83.7 ± 3.9	0.257 ± 0.010
24	86.7 ± 2.0	0.447 ± 0.045

[a] Not detected.
[b] Values are the means ± SD of three experiments.

resistant plants. The effects of ACT-toxin Ib (1×10^{-5} M) on plasma-membrane ultrastructure of susceptible Japanese pear were almost the same as the effects on susceptible citrus. The toxin caused no modifications of organelles in susceptible cells, except the invagination of plasma membranes (Fig. 4D). The invagination rate was highest for vascular-bundle sheath cells. The invagination rate significantly increased with time after toxin treatment (Table 1). No effect was detected in cells of resistant Japanese pear cv. Chojuro.

Release of ACT-toxin Ib during spore germination. Toxin at the infection site is required if toxin is to have a role in initial pathogenesis (11,24). A time-course study on the release of ACT-toxin Ib and Ic was carried out by germinating spores of the virulent isolate AC-320. The germination rate was about 80% within 6 h after incubation (Table 2). ACT-toxin Ib was detectable after 12 h; the amount was estimated at about 0.035 pg per germinating spore. The amount increased with time: 0.257 pg per spore at 18 h and 0.447 pg per spore at 24 h. However, only traces of ACT-toxin Ic were detected in the germinating fluids, and those were found only after 24 h. ACT-toxin Ib and Ic were not detected in the methanol extracts of air-dried dormant spores previously washed five times with deionized water.

Effects of ACT-toxin Ib on infection of susceptible citrus and Japanese pear leaves by a nonpathogenic *A. alternata*. Toxin treatment increased the production of infection hypha by spores of a nonpathogenic isolate of *A. alternata* on Emperor mandarin and Japanese pear cv. Nijisseiki but did not affect the number of infection hypha on resistant plants (Table 3). There was no effect of toxin on germination and appressorial formation. The toxin seldom caused necrosis on both susceptible leaves when it was sprayed on leaves at the concentrations described in these experiments.

DISCUSSION

Two host-specific toxins, ACT-toxin Ib and Ic, were isolated from culture filtrates of a virulent isolate of *A. alternata* tangerine pathotype. Most of the toxicity of the ethyl acetate extract from culture filtrate was attributed to the existence of ACT-toxin Ib and Ic (Fig. 2; in text). However, the presence of ACTG-toxins in culture filtrates of the pathogen remains an open question. The toxins differ in structure (Fig. 5) (19) from the ACTG-toxins reported by Kono (14) and Kono et al (15) but are similar to AK-toxins produced by *A. alternata* Japanese pear pathotype (17,18) and AF-toxins produced by *A. alternata* strawberry pathotype (21). ACT-toxin Ib and Ic are geometric isomers of 9,10-epoxy-9-methyl-decatrienoic acid (19): ACT-toxin Ib has the same central-*cis* decatrienoic acid as that of AK-toxin I and II (17,18), and ACT-toxin Ic has an all-*trans* decatrienoic acid that corresponds to type C isomers of AF-toxins (21).

ACT-toxin Ib is highly toxic to young leaves of susceptible Emperor mandarin and Dancy tangerine. Its dilution end point for leaf necrosis is estimated to be 2×10^{-8} M, which is comparable to the dilution end point of AK-toxin I (23). Resistant citrus tolerates 2×10^{-4} M ACT-toxin Ib. More than 60 species, cultivars, and lines of citrus plants were examined for ACT-toxin sensitivity. Toxin sensitivity correlates in all cases with susceptibility to the pathogen (7).

Highly purified ACT-toxin Ib is not only selectively toxic to the known natural hosts (Emperor mandarin and Dancy tangerine) but also to Japanese pear cv. Nijisseiki, which is not known to be affected by the producing fungus in nature. Inoculation experiments proved that the *A. alternata* tangerine pathotype isolate AC-320 has the same host selectivity as has *A. alternata* Japanese pear pathotype (6). The unexpected pathogenicity and host range of the tangerine pathotype appears to correspond to production of ACT-toxin II. Also, selective toxicity of ACT-toxin Ib to pear cv. Nijisseiki apparently is involved in the unusual

TABLE 3. Effect of ACT-toxin Ib on infection behavior of a nonpathogenic isolate (O-94) of *Alternaria alternata*

Plants and inoculation[a]	Spore germination (%)	No. of germ tubes per spore	Appressoria per germ tube (%)	Infection hyphae per appressorium (%)
Citrus				
Emperor mandarin				
O-94+Tox[1]	91.0 ± 3.5[b]	1.5 ± 0.1	35.7 ± 4.3	36.9 ± 3.6
O-94	89.9 ± 3.3	1.4 ± 0.1	32.8 ± 3.4	8.9 ± 2.3
Rough lemon				
O-94+Tox[1]	86.1 ± 1.5	1.4 ± 0.0	35.9 ± 4.8	8.6 ± 2.1
O-94	87.4 ± 1.2	1.5 ± 0.0	38.7 ± 1.0	9.3 ± 1.3
Japanese pear				
Nijisseiki				
O-94+Tox[2]	88.6 ± 3.6	1.5 ± 0.0	27.5 ± 2.5	32.9 ± 1.1
O-94	88.8 ± 5.3	1.4 ± 0.0	28.9 ± 2.0	9.3 ± 0.1
Chojuro				
O-94+Tox[2]	88.7 ± 1.0	1.4 ± 0.0	33.3 ± 2.4	1.6 ± 1.2
O-94	88.2 ± 0.2	1.4 ± 0.1	33.6 ± 3.8	5.7 ± 1.7

[a] Spore suspension (5×10^5 spores/ml) was sprayed on the lower surface of leaves. Concentration of Tox[1] was 2×10^{-8} M, and Tox[2] was 1×10^{-6} M.
[b] Values are the means ± SD of 300 spores.

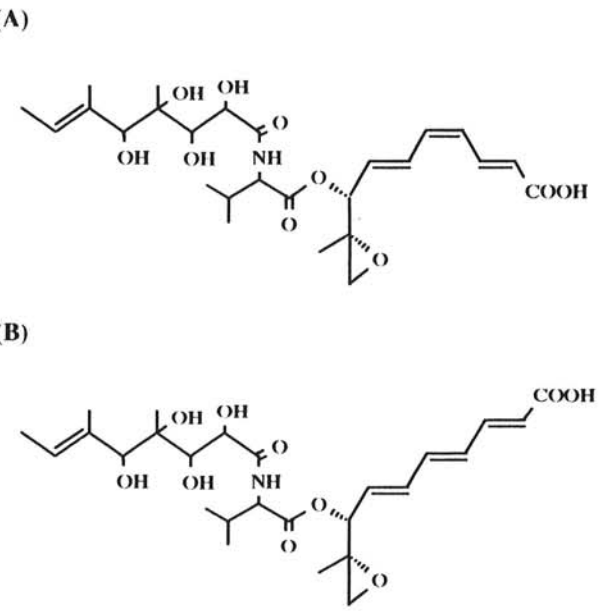

Fig. 5. Molecular structures of **A,** ACT-toxin Ib and **B,** ACT-toxin Ic.

pathogenicity of the tangerine pathotype. Thus, pear cv. Nijisseiki is a potential host in nature for the tangerine pathotype of *A. alternata*.

A similar relationship is known for AF-toxins (16). AF-toxin I is toxic to strawberry cv. Morioka-16 and to Japanese pear cv. Nijisseiki. AF-toxin II is toxic only to Japanese pear cv. Nijisseiki, and AF-toxin III is toxic only to strawberry cv. Morioka-16. The highly selective activity of the three host-specific toxins that contain decatrienoic acid is confirmed; however, decatrienoic acid itself is not toxic (K. Kohmoto et al, *unpublished data*). A further comparative study of the structure-activity relationships of host-specific toxins containing decatrienoic acid should provide important clues for understanding the precise molecular basis of host specificity and recognition in the pathogenic *Alternaria* species.

To be pathogenic, a pathogen must produce the specific toxin at the infection court (10,22). Toxin release by spores of the tangerine pathotype was tested. HPLC showed that the extracts of the germination fluids contain ACT-toxin Ib (one of the major components of detected peaks); ACT-toxin Ic was barely detected (data not shown). This is similar to the situation found in culture filtrates (Fig. 5). ACT-toxin Ic increased during the isolation procedure; also, ACT-toxin Ib isomerized to ACT-toxin Ic in light (data not shown). Therefore, it is likely that ACT-toxin Ib is the original metabolite, and ACT-toxin Ic may be, in part, an artificial isomerization product from ACT-toxin Ib.

A germinating spore released about 0.035 pg of ACT-toxin Ib within 12 h of incubation. This amount may be enough to affect several host cells adjacent to the spore, as in the case of *A. alternata* Japanese pear pathotype (4,24). This conclusion is supported by experiments with nonpathogenic spores of *A. alternata*. A dilute solution of ACT-toxin Ib (Table 3) in the infection court leads to penetration and colonization of Tox$^-$, avirulent spores of *A. alternata*. The same observations were reported previously for toxin-producing forms of *Alternaria* and *Bipolaris* (10,23,30). We concluded that ACT-toxin Ib is essential for successful establishment of the pathogen in susceptible citrus.

The effect of ACT-toxin Ib on loss of electrolyte from leaf tissues paralleled development of necrosis. This indicates a relationship between necrosis and dysfunction of plasma membranes in susceptible leaf tissues. ACT-toxin Ib induced rapid increases in electrolyte loss from susceptible tissues and an invagination of the plasma membranes. The membrane fragments found in the vicinity of plasmodesmata were stained with an alkaline bismuth solution that is specific for cell walls, plasma membranes, Golgi vesicles, starch grains, and lamellae of chloroplasts (25–27). These results indicate that the membrane fragments are not artifacts of fixation but are derived from the plasma membranes after exposure to toxin. Toxin-induced changes in the ultrastructure of susceptible cells of mandarin are almost identical to AK-toxin-induced changes in susceptible cells of Japanese pear (23,26). Changes in toxin-treated cells over time (Table 1) are compatible with physiological data. Therefore, the data suggest that the initial action site of ACT-toxin Ib is on plasma membranes of susceptible cells. This mode of action by ACT-toxin seems to be compatible with the hypothesis that plasma-membrane disorders caused, directly or indirectly, by HSTs are a key and central event in early pathogenesis, allowing the producing fungus access to host cells (10).

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