Determination of Host-Selective Toxin Production During Spore Germination of *Alternaria alternata* by High-Performance Liquid Chromatography

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ABSTRACT

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The Japanese pear pathotype of *Alternaria alternata*, which causes black spot disease, produces multiple host-selective toxins (AK-toxins I and II) in culture; the strawberry pathotype, which causes black spot disease, also produces a series of analogous host-selective toxins (AF-toxins I, II, and III). Analysis by high-performance liquid chromatography of toxin production during spore germination showed that virulent isolate A85-10 of the Japanese pear pathotype produced about 0.02 pg of AK-toxin I per spore in the first 6 hr after germination and a trace amount of AK-toxin II after 24 hr. Most of the virulent isolates produced only AK-toxin I during the spore germination. Isolate YM-19 of the strawberry pathotype released about 0.08 pg of AF-toxin I per spore in the initial 6 hr of germination and a small but detectable amount of AF-toxin II, but no detectable AF-toxin III, within 18 hr of incubation. From the point of view of pathological importance of early host-parasite interactions, we propose that AK-toxin I and AF-toxin I, respectively, are the major toxins in yield and biological activity which play a critical role as host recognition factors at primary infection sites in each pathosystem.

Additional keywords: bioassay, *Helminthosporium victoriae*, victorin.

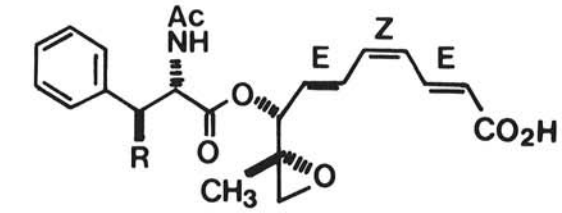
Knowledge of the release of host-selective toxins from the germinating spores of fungal plant pathogens aids in understanding the early participation of these toxic metabolites in host-parasite interactions (5,8,10). As early as 1965, Nishimura and Scheffer (6) reported that virulent spores of *Helminthosporium victoriae* Meehan & Murphy, the causal agent of Victoria blight of oats, released a host-selective toxin, victorin, on glass slides or on oat leaves during germination. Similar results have since been

obtained by a series of investigations working with *Alternaria alternata* (Fr.) Keissler (5).

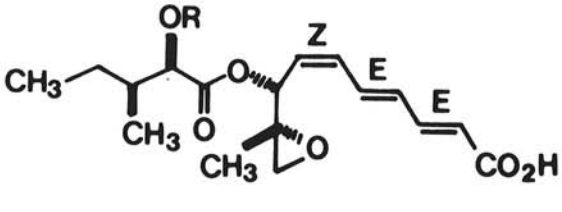
Meaningful studies on the role of host-selective toxins in plant diseases depend on assay systems (11). Conventional methods for qualitative and quantitative determination of toxin production by germinating spores of *A. alternata* have been based on bioassays—for example, inhibition of seedling root growth and induction of necrosis on detached leaves. Assays must be considered in terms of specificity, ability to quantify, sensitivity, simplicity, and reproducibility (11). Bioassays usually give qualitatively reliable data with considerable sensitivity. The quantification by bioassays, however, often provided only relative amounts of host-selective toxins in samples but not absolute ones, especially in the case of unknown compounds. In addition, the sensitivity of bioassays varies with growth conditions of test plants.

AK-toxins are produced during culture and spore germination of the Japanese pear pathotype of *A. alternata*, the causal agent of black spot, and AF-toxins are produced during culture and spore germination of the strawberry pathotype, which causes black spot of strawberry (1,5,7,9). AK-toxins were isolated from the culture filtrate and characterized as two related molecular species, AK-toxins I and II (2). AF-toxins also were isolated from the culture filtrate as three active components, AF-toxins I, II, and III, along with inactive isomers (1,3,4). These host-selective toxins share a common ester of epoxy-decatrienoic acid (Fig. 1). Although the existence of the toxins in spore-germination fluids of the pathogens has been demonstrated by the bioassays, the type and amount of each toxin molecule in the fluid remains to be determined.

We describe here an improved method for determining production of these compounds by germinating spores of *A. alternata*. Regardless of the structural similarity and complexity, this protocol (high-performance liquid chromatography [HPLC]) is useful for rapid and quantitative analyses of extremely small amounts of pathologically important materials. In this study, the form and amount of AK- and AF-toxins released during spore germination were measured by HPLC to provide an increased understanding of the participation of toxins in the pathogenesis.



AK-toxin I : R = CH_3
II : R = H



AF-toxin I : R = $COCH(OH)C(CH_3)_2OH$
II : R = H
III : R = $COCH(OH)CH(CH_3)_2$

Fig. 1. Structures of AK- and AF-toxins.

MATERIALS AND METHODS

Fungal isolates. Virulent isolate A85-10 of the Japanese pear pathotype of *A. alternata* was used mainly for analysis of toxin production. Isolates, including nonpathogenic mutants as shown in Table 1, also were employed for the comparison of AK-toxin productivity. Isolate 15B is a nonpathogenic mutant isolated from a stock culture of highly virulent isolate 15A. Isolate 0-94, which is a stock culture of our laboratory, was obtained from the air. Other isolates were obtained from naturally occurring lesions on leaves of Japanese pear cultivar Nijisseiki collected in Aichi prefecture (isolates A8II-82, A85-1a, A85-1b, A85-5, A85-6, A85-7a, and A85-10) and Tottori prefecture (isolates TAK-1, TAK-41, TAK-42, TAK-43, TAK-44, TAK-96, TAK-151, TAK-152, and TAK-154) in 1985. Virulent isolate YM-19 of the strawberry pathotype was obtained from a naturally occurring lesion on a leaf of strawberry cultivar Morioka-16 collected in Yamagata prefecture in 1984. Fungi were cultured and maintained in potato-dextrose agar.

Preparation of spores and spore-germination fluids. Fungi were cultured in 1-L Roux bottles containing 400 ml of potato-dextrose broth for 2 wk at 26 C without shaking. Mycelial mats were taken from the bottles, washed well with tap water to remove culture medium, and placed on filter paper in a moist chamber at 26 C. After incubation for 1 day, the mycelial surfaces, laden with spores, were brushed with distilled water, and the resulting spore suspension was filtered through two layers of gauze to eliminate mycelial debris. The spores were washed three times with distilled water by centrifugation at 600 *g* for 5 min each and finally suspended in distilled water to give a concentration of 5×10^5 spores/ml. The 100-ml spore suspension was sprinkled uniformly onto paper towels and incubated in a moist chamber for appropriate periods at 26 C. The percentage of germinated spores was measured microscopically at the time when the spore-germination fluids were taken. The spore-germination fluid was harvested by squeezing the towels and filtering the liquid through a filter paper to remove the spores. The filtrate was adjusted to pH 5.5 with 1 M KH_2PO_4 and extracted twice with diethyl ether. This extraction permitted transfer of more than 95% of toxins from the filtrates to the ether phase. After removing ether

TABLE 1. AK-toxin production during spore germination of 18 isolates of *Alternaria alternata* and their pathogenicity

Isolate	Toxin production[a]		Pathogenicity[b]
	AK-toxin I	AK-toxin II	
15A	0.949	0.025	42.9
15B	ND[c]	ND	0.0
A85-1a	ND	ND	0.0
A85-1b	0.023	ND	32.0
A85-5	ND	ND	0.0
A85-6	0.006	ND	8.6
A85-7a	ND	ND	0.0
TAK-1	ND	ND	0.0
TAK-41	0.325	0.001	29.8
TAK-42	ND	ND	0.0
TAK-43	0.064	ND	44.3
TAK-44	0.095	ND	9.3
TAK-96	0.095	ND	16.2
TAK-151	0.020	ND	10.5
TAK-152	0.019	ND	11.4
TAK-154	0.076	ND	30.7
A8II-82	0.008	ND	2.0
0-94[d]	ND	ND	0.0

[a] Toxin production was represented as the amount (pg) of AK-toxins produced by one germinated spore after 24 hr of incubation at 26 C. The values are the averages of two replicated experiments.

[b] Pathogenicity was represented as the number of necrotic spots per square centimeter of leaves of pear cultivar Nijisseiki inoculated with spores of isolates of *A. alternata*. The values are the averages of six determinations.

[c] Not detected.

[d] Saprophytic isolate of *A. alternata*.

by vacuum evaporation, the residue was dissolved in 1 ml of methanol, and 1- to 20-μl portions of the sample (0.1–2% volume of the sample) were subjected to HPLC.

HPLC analysis. The HPLC system used was a Model BIP-1 instrument of Japan Spectroscopic Co. Ltd. (Tokyo, Japan). The column was stainless steel (4.6 × 250 mm) and packed with reverse-phase Finpak SIL-C_{18} (Japan Spectroscopic Co.). HPLC grade solvents were used for analysis. Elution was with a mixture of methanol, acetic acid, and water (60:1:39, v/v/v) at a rate of 1 ml/min, and detection was by monitoring absorbance at 290 nm. The amount of each AK- and AF-toxin in samples was calculated by comparing the peak heights with those of authentic samples. The smallest amounts of toxins detectable by the HPLC were approximately 10 ng. The regression line for the relationship between the amount of toxins and peak height was linear in the range of 10–200 ng. The following formula was used to calculate the amount of each toxin (in picograms) released from one germinated spore:

$$\frac{\text{Total amount of toxin in sample (pg)} \times 100}{(5 \times 10^7 \text{ spores}) \times \text{Percentage of germinated spores}}$$

Measurement of ultraviolet (UV) absorption spectra of samples. Fractions separated by HPLC and corresponding to each authentic toxin were collected and evaporated to dryness. The UV absorption spectra of the residual substances were recorded in methanol with a Ubest-30 spectrophotometer (Japan Spectroscopic Co.) and compared with that of each authentic toxin. The UV absorption maxima of authentic toxins are follows: UVmax (MeOH) = 284 nm (AK-toxin I), 286 nm (AK-toxin II), and 287 nm (AF-toxins I, II, and III) (2,4).

Bioassays. Toxicity of samples was tested by a leaf puncture assay with the susceptible Japanese pear cultivar Nijisseiki and the susceptible strawberry cultivar Morioka-16. Leaves of the resistant Japanese pear cultivar Chojuro and the resistant strawberry cultivar Hokowase were used as the controls. Young, detached leaves were wounded slightly by pricking the center of the underside with a needle. The wounded portion was treated immediately with 20 μl of the prepared sample solution to be tested and incubated for 24 hr at 26 C in a moist chamber. Host-selective activity of the sample solution was judged by characteristic veinal necrosis, which developed around the treated portion of the susceptible leaves but not on the resistant ones.

Pathogenicity of the Japanese pear pathotype was tested by spraying spore suspensions (5×10^5 spores/ml) on freshly harvested young leaves of a susceptible Nijisseiki pear with a glass atomizer. After the leaves were incubated in a moist chamber for 24 hr at 26 C, the number of necrotic spots that appeared on the leaves was counted and calculated as the number per square centimeter.

RESULTS

AK-toxin production by the germinating spores of the Japanese pear pathotype. Although AK-toxins I and II are closely related to each other, they were separable by the HPLC system (Fig. 2). More than 90% of the spores of isolate A85-10 germinated within the initial 6 hr of incubation. After 6 hr or more of incubation, the spore-germination fluid contained a distinct peak at a retention time of 17.5 min, which corresponds to that of authentic AK-toxin I. A compound in the peak was confirmed to be AK-toxin I by bioassay for host-selective toxicity to Nijisseiki pear and by comparison of UV absorption spectra with the authentic toxin. During the first 6 hr of incubation, one germinated spore released approximately 0.02 pg of AK-toxin I. The amount of toxin in the fluids increased rapidly with incubation time after 6 hr. The isolate also produced a trace amount of AK-toxin II during the spore germination, in a similar manner as above. We also employed the HPLC system for detecting AK-toxins in dormant spores. About 5×10^8 spores collected from mycelial mats were suspended in methanol and extracted for 24 hr at 26 C. When aliquots of the methanol extract were subjected to HPLC analysis, no AK-toxins were detected in the extracts.

Other isolates of the Japanese pear pathotype, including nonpathogenic and saprophytic *A. alternata*, were surveyed for AK-toxin production by spores allowed to germinate for 24 hr (Table 1). All of 11 pathogenic isolates released AK-toxin I in their germination fluids, whereas the nonpathogenic isolates produced no AK-toxins. A small amount of AK-toxin II was detected in the fluids of two pathogenic isolates: No. 15A and TAK-41. The AK-toxin production during spore germination varied greatly among pathogenic isolates and did not positively correlate with the degree of pathogenicity to Nijisseiki pear leaves.

AF-toxin production by germinating spores of the strawberry pathotype. AF-toxins I and II were detected easily as separate peaks by HPLC with a reverse-phase C_{18} column (Fig. 3). Most spores of the virulent isolate YM-19 germinated within 6 hr of incubation. AF-toxin I from such spores was detected as a distinct peak at a retention time of 15.5 min, corresponding to that of authentic AF-toxin I. The compound in the peak was identified as AF-toxin I by bioassay for host-selective toxicity and by comparison of the UV absorption spectra with that of the authentic toxin. The amount of AF-toxin I increased with incubation time up to 18 hr. Production of AF-toxin II was less than 1% of that of AF-toxin I. AF-toxin II was not produced during early

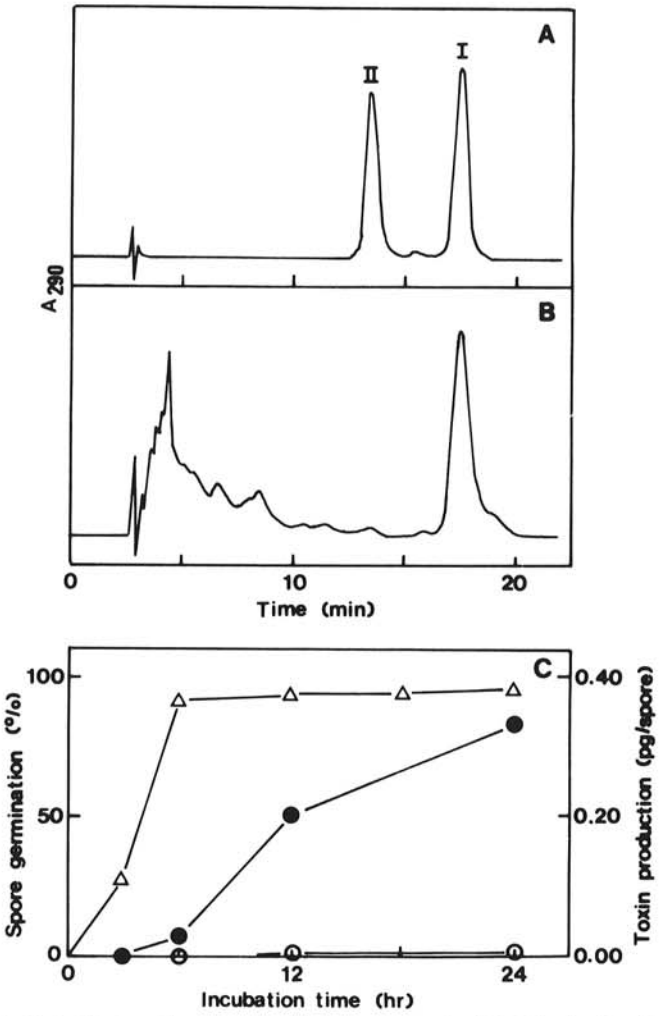


Fig. 2. A, High-performance liquid chromatography (HPLC) of authentic AK-toxins I and II. **B,** HPLC of spore-germination fluid of the Japanese pear pathotype, isolate A85-10. Fluid was harvested after 24 hr of incubation and subjected to HPLC analysis as described in the Materials and Methods section. **C,** Time course of germination (△) and production of AK-toxins (I = ●, II = ○) by spores of the Japanese pear pathotype, isolate A85-10.

stages of spore germination and was first detected after 18 hr of incubation. No AF-toxin III (retention time = 20.5 min) was detected in the spore-germination fluids. Avirulent isolates never produced any of the AF-toxins in fluids of germinated spores (data not shown).

DISCUSSION

The dilution end point bioassay previously employed for toxicity may give variable results, depending on developmental and physiological conditions of biological materials (detached leaves and seedlings). It also is time consuming and rather qualitative. The present HPLC method facilitated a more precise, rapid, and easy estimation of production of host-selective toxins during spore germination of two *Alternaria* pathogens. The HPLC system could separate each analog of AK- and AF-toxins in test materials and provide quantitative data without necessitating complex and laborious preparation steps for analysis.

Our previous data by bioassay could detect AK-toxin released in spore-germination fluid of the Japanese pear pathotype but could not distinguish between the toxin forms (7). The HPLC data indicated that the dormant spores contained no AK-toxins and produced the toxin, mainly AK-toxin I, during the germination; very little of AK-toxin II was produced within 24 hr of the germination. Most pathogenic isolates of the Japanese pear pathotype released only AK-toxin I in their spore-germination fluids. In the strawberry pathotype, the germinated spores produced AF-toxin I as the predominant form; AF-toxins II and III were minor metabolites during spore germination. These results reveal the interesting and potentially significant fact that spores of the Japanese pear pathotype and the strawberry pathotype produce predominantly AK-toxin I and AF-toxin I among the toxins, respectively, during germination, suggesting a potential importance of AK-toxin I and AF-toxin I in the early steps of infection by the pathogens. However, they do not rule out the involvement of other forms of the toxins in the steps because the condition for spore germination on paper towels employed in this study may not equal that on plant tissues.

We previously proposed that the role of host-selective toxins in the early stages of fungal infection is to lead to suppression of induction of disease resistance and ultimately to induced susceptibility in affected plant cells; toxins from germinated spores are required, not for killing the host cells, but for causing their dysfunction (5). AK-toxin production during spore germination greatly varied among the pathogenic isolates and did not positively correlate with the degree of their pathogenicity to Nijisseiki pear leaves. To precisely interpret this phenomenon, it will be necessary to determine exactly the amount of toxins required for induction of susceptibility in host cells, measure the amount of toxin produced by spores on plant tissues, and evaluate the potential of other factors for pathogenicity (for example, aggressiveness).

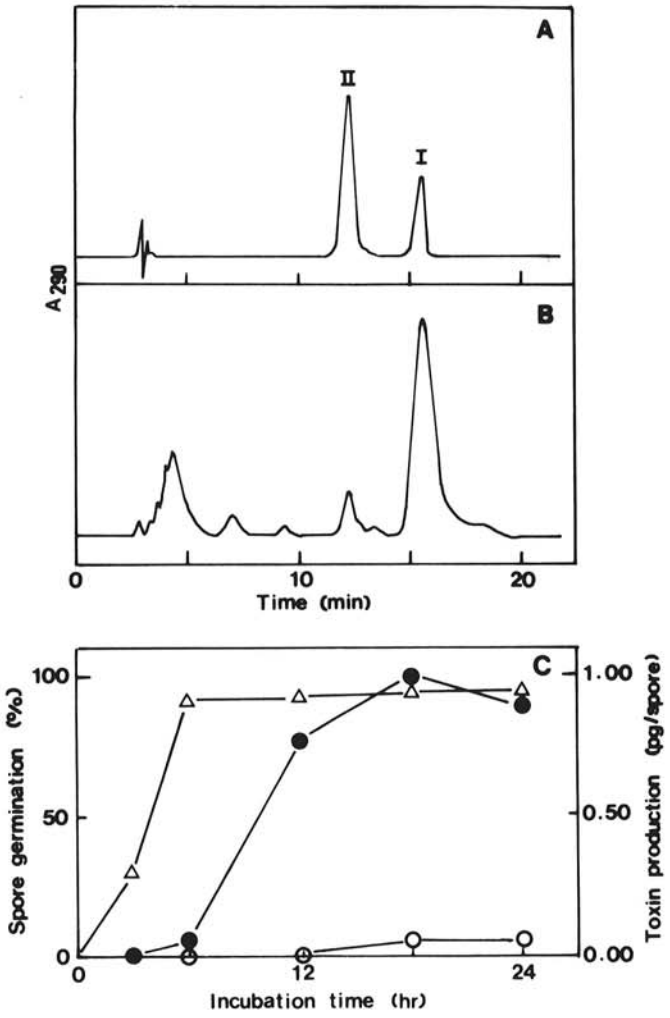


Fig. 3. A, High-performance liquid chromatography (HPLC) of authentic AF-toxins I and II. **B,** HPLC of spore-germination fluid of the strawberry pathotype, isolate YM-19. Fluid was harvested after 24 hr of incubation and subjected to HPLC analysis as described in the Materials and Methods section. **C,** Time course of germination (△) and production of AF-toxins (I = ●, II = ○) by spores of the strawberry pathotype, isolate YM-19.

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