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Isolation and determination of AAL phytotoxins from corn cultures of the fungus *Alternaria alternata* f. sp. *lycopersici*

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ABSTRACT

The fungus *Alternaria alternata* f. sp. *lycopersici* produces a group of four related host-specific phytotoxins (AAL toxins) which can be divided into two groups (TA and TB), each of which exists as an equilibrium mixture of two structural isomers. The AAL toxins were isolated from corn cultures by aqueous extraction, followed by purification on Amberlite XAD-2 resin, separation of TA from TB on silica gel and final purification on a semi-preparative high-performance liquid chromatographic (HPLC) system. A rapid, sensitive and reproducible method was developed to determine these toxins in culture material in order to monitor toxin production on corn cultures. The method consisted of aqueous extraction, C_{18} solid-phase extraction clean-up, precolumn derivatization with *o*-phthaldialdehyde and reversed-phase HPLC with fluorescence detection. An isocratic HPLC system was developed that separated the structural isomers of TA and TB within a chromatographic analysis time of 24 min.

INTRODUCTION

The fungus *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici* causes a stem canker disease in certain susceptible tomato cultivars [1]. The fungal growth occurs on the stem, where it produces dark brown to black cankers. Although the pathogen occurs only on the stem, it also causes interveinal necrosis of the leaves by the translocation of fungal toxins. The cause of the disease has been traced to the production of host-specific phytotoxins (AAL toxins) [2–4]. Cell-free culture filtrates yielded two ninhydrin-positive fractions, TA and TB, each consisting of a mixture of two structural isomers [5]. Each fraction reproduced the disease symptoms on susceptible cultivars [2,3,6]. In addition, these same toxins have been extracted from necrotic leaves of tomato plants infected with *A. alternata* f. sp. *lycopersici* [2]. A recent report, utilizing a bioassay technique, presented evidence that these toxins can also be produced in very low yield by an isolate of *A. alternata* obtained from ripe tomatoes and previously considered to be non-pathogenic [7].

The AAL toxins have been shown to be esters of propane-1,2,3-tricarboxylic acid with either 1 - amino - 2,4,5,13,14 - pentahydroxy - 11,15 - dimethylheptadecane or the C-5 deoxy analogue [5] (Fig. 1). Their structural similarity to the

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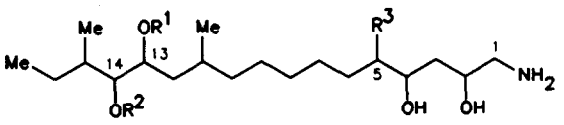

TA_1:R¹ = CO-CH_2-CH(CO_2H)-CH_2-CO_2H,R² = H,R³ = OH

TA_2:R¹ = H,R² = CO-CH_2-CH(CO_2H)-CH_2-CO_2H,R³ = OH

TB_1:R¹ = CO-CH_2-CH(CO_2H)-CH_2-CO_2H,R² = H,R³ = H

TB_2:R¹ = H,R² = CO-CH_2-CH(CO_2H)-CH_2-CO_2H,R³ = H

Fig. 1. Structures of the isomers of the AAL toxins.

recently described fumonisin mycotoxins [8] has renewed interest in these compounds. Both fumonisin B_1 and TA have similar phytotoxic properties [9,10]. In addition, fumonisins B_1 and B_2 and TA have recently been shown to be toxic to certain cultured mammalian cell lines [11].

Previously, the AAL toxins have been isolated from culture filtrates either by barium acetate precipitation, butanol extraction, gel filtration and semi-preparative thin-layer chromatography (TLC) [12] or by direct absorption and partitioning on C_{18} reversed-phase columns and clean-up by gel filtration [13]. The toxins were analysed by high-performance liquid chromatography (HPLC) of their maleyl derivatives. Although these derivatives of TA and TB could be separated by isocratic HPLC, a binary gradient (60-min analysis time) was required for partial separation of the individual structural isomers of the toxins [12].

This paper reports the isolation of the AAL phytotoxins from corn cultures of *A. alternata* f. sp. *lycopersici* using aqueous extraction and purification on Amberlite XAD-2 resin and silica gel, followed by reversed-phase semi-preparative HPLC. In order to monitor toxin production in culture, a rapid, sensitive and reproducible HPLC method, similar to that used for the structurally similar fumonisins [14], was developed using precolumn *o*-phthaldialdehyde (OPA) derivatization and fluorescence detection. Separation of all structural isomers was achieved in 24 min using isocratic conditions on a reversed-phase column.

EXPERIMENTAL

Materials

Amberlite XAD-2 and silica gel 60 (Kieselgel 60, 0.063–0.200 mm) were purchased from Merck (Darmstadt, Germany). Bond-Elut C_{18} cartridges (3-ml capacity containing 500 mg of sorbent) were obtained from Analytichem International (Harbor City, CA, USA). A reversed-phase C_{18} column packing used for desalting was prepared from silica gel 60 (0.063–0.200 mm) according to the method of Kingston and Gerhart [15]. All other reagents and solvents were of analytical-reagent grade from Merck.

Fungal cultures

Lyophilized cultures of *A. alternata* f. sp. *lycopersici* MRC 6231 (As27-3p2), supplied by Professor D.G. Gilchrist (University of California, Davis, CA, USA), were used to inoculate autoclaved, ground, moistened yellow corn. The corn cultures were incubated in the dark at 25°C for 16 days, after which the material was dried (45°C, 24 h) and ground in a laboratory mill.

Isolation of fungal toxins

A 500-g sample of culture material was extracted by blending with 1000 ml of chloroform–methanol (10:3, v/v). The mixture was filtered, washed with 300 ml of extraction solvent and dried under vacuum on the filter. Subsamples (30 g) of this dried culture material were further extracted three times in centrifuge bottles with 110 ml of water using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). After each extraction, the homogenate was centrifuged (4000 *g*, 10 min, 4°C) and supernatants from all extractions were pooled, acidified to pH 2.7 with 2 *M* hydrochloric acid and then recentrifuged. The clear supernatant was applied to a column packed with Amberlite XAD-2 resin (35 × 3.0 cm I.D.) that had previously been washed with 500 ml of methanol and 500 ml of water. After application of the sample, the resin was washed with 100 ml of water and 400 ml of methanol–water (1:3, v/v) and the toxins were eluted with 400 ml of methanol, which was removed under vacuum on a rotary evaporator at 40°C.

The residue was dissolved in 20 ml of ethyl acetate–acetic acid–water (12:6:1, v/v/v) and applied to a column packed with silica gel 60 (50 × 3.0 cm I.D.). The column was eluted with the same solvent and 20-ml fractions were collected. The fractions were tested for the presence of TA or TB by TLC using precoated silica gel 60 plates (Merck) developed with ethyl acetate–acetic acid–water (6:3:1, v/v/v). An authentic standard of TA and TB (donated by Professor D.G. Gilchrist) was used to identify the AAL toxins. The toxins were revealed as purple spots by spraying with *p*-anisaldehyde reagent [8]. Fractions containing TA or TB were separately pooled and dried under vacuum at 40°C.

Both pooled fractions were separately dissolved in 12 ml of methanol–water (1:1, v/v) and individually purified in a semi-preparative isocratic HPLC system at room temperature using a Phenomenex (Rancho Palos Verdes, CA, USA) analytical column (250 × 4.6 mm I.D.) packed with Ultracarb 7-μm ODS 30 reversed-phase (C_{18}) material incorporated in an automated system consisting of a Waters (Milford, MA, USA) WISP autoinjector, Waters Model 590 programmable pump, Waters automated switching valve and Waters Lambda-Max variable-wavelength ultraviolet detector set at 215 nm. The mobile phase for the purification of TA was methanol–0.05 *M* sodium dihydrogenphosphate (47:53, v/v) adjusted with orthophosphoric acid to pH 3.4 and pumped at a flow-rate of 1.5 ml min^{-1}. For purification of TB, the mobile phase was methanol–0.05 *M* sodium dihydrogenphosphate (53:47, v/v), also at pH 3.4. The autoinjector was programmed for repeated injections (100 μl) of aliquots of the semi-purified toxin solution and the eluate fraction containing the AAL toxin was collected using the automated switching valve. Most of the methanol was removed on a rotary evaporator and the toxins were then recovered by passing the concentrate down a short column containing C_{18} packing material (20 g). After washing the salt from the column with 150 ml of water, the toxin was eluted with 200 ml of methanol.

The NMR spectra were recorded for solutions in [$^2H_6$]dimethyl sulphoxide on a Bruker AC-300 spectrometer operating at 7.0 T.

Determination of TA and TB in corn culture

A 5-g sample of culture material was blended with 50 ml of water using a Polytron homogenizer and then centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was filtered, acidified to pH 2.9 with 2 *M* hydrochloric acid and then recentrifuged at 3000 *g*. A 2-ml aliquot of this supernatant was applied to a Bond-Elut C_{18} solid-phase extraction cartridge that had been preconditioned by washing with 5 ml of methanol and 5 ml of water. The cartridge was washed with 8 ml of water, drained under vacuum and then eluted with 10 ml of methanol. The eluate was evaporated to dryness at 60°C in a stream of nitrogen and the residue dissolved in 2 ml of methanol, prior to derivatization and HPLC analysis.

Chromatographic analysis

The AAL toxins in the sample residue were determined by reversed-phase HPLC of preformed OPA derivatives. The OPA reagent was prepared by dissolving 40 mg of OPA in 1 ml of methanol followed by addition of 5 ml of 0.1 *M* sodium borate and 50 μl of 2-mercaptoethanol. The reagent was stored in the dark for up to 1 week without deterioration. Derivatives were prepared by mixing a 50-μl aliquot of purified sample extract with 200 μl of OPA reagent.

The analytical HPLC system consisted of a Waters M-45 pump and U6K injector. The fluorimetric detector was a Perkin-Elmer (Norwalk, CT, USA) Model 650S with an 18-μl flow cell. The excitation and emission wavelengths were 335 and 440 nm, respectively. Quantification was achieved by peak-area measurement using a Waters Model 745 data module. Chromatographic separation was achieved using either of two systems. One system, which separated the derivatives of TA and TB but not their structural isomers, consisted of a Phenomenex Ultracarb 7-μm ODS 30 analytical column and a mobile phase of methanol–0.1 *M* sodium dihydrogenphosphate (75:25, v/v) with the pH adjusted to 3.4 with orthophosphoric acid and

pumped at a flow-rate of 1 ml min^{-1}. The other system, which separated the structural isomers, consisted of a Phenomenex Ultracarb 5-μm ODS 30 column (250 × 4.6 mm I.D.) eluted with methanol–0.1 *M* sodium dihydrogenphosphate (70:30, v/v) at a flow-rate of 1 ml min^{-1}. The pH was measured at 6.1 and was not adjusted.

RESULTS AND DISCUSSION

The method for the determination of the AAL toxins in corn culture material was developed to monitor toxin production by various *A. alternata* f. sp. *lycopersici* strains and to select suitable batches for isolation purposes. The HPLC systems developed were also used to monitor the elution of the toxins from preparative columns during isolation. The first HPLC system developed was based on an analytical column packed with Ultracarb 7-μm ODS 30 packing and separated TA and TB. Elution under conditions that suppress the ionization of the carboxylic acid groups (pH 3.4) failed to separate the individual isomers of each AAL toxin. The use of a mobile phase of similar composition but at pH 6.1 achieved a partial separation of these isomers. In the second HPLC system, an improved separation of the structural isomers was achieved using a column of higher efficiency packed with Ultracarb 5-μm ODS 30 packing. Fig. 2a shows such a separation of the isolated AAL toxins and Fig. 2b shows the separation of the AAL toxins in a cleaned-up culture sample. Confirmation of the identity of the TA and TB peaks is shown in Fig. 2c, where the same culture sample was spiked with authentic toxin. These separations were achieved by isocratic HPLC within 24 min as opposed to the previously published method, which required a binary gradient over a 60-min period [12].

The assay was validated with respect to precision and accuracy. Six replicate determinations of TA and TB in a corn culture sample of *A. alternata* f. sp. *lycopersici* MRC 6231 containing 0.34 mg g^{-1} of TA and 0.05 mg g^{-1} of TB gave a precision of 1.4% [relative standard deviation (R.S.D.)] for TA and 2.2% for TB. The accuracy of the method was assessed by spiking an extract of a corn culture of *A. alternata* strain

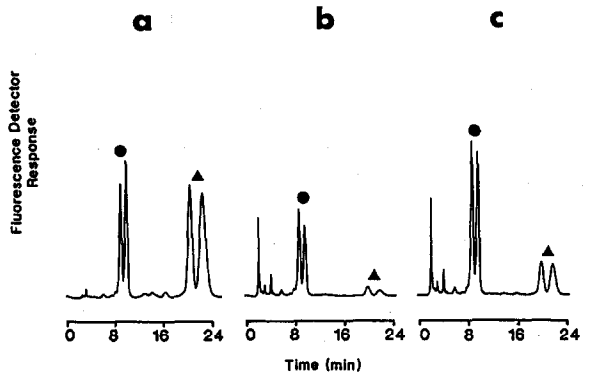

Fig. 2. Chromatograms of OPA-derivatized (●) TA and (▲) TB. In this chromatographic system, TA_2 elutes before TA_1 and TB_2 before TB_1. (a) Sample of isolated toxins (200 ng of TA, 280 ng of TB); (b) purified corn culture extract (130 ng of TA, 20 ng of TB); (c) the same purified corn extract spiked with authentic TA and TB.

MRC 5494, which did not contain AAL toxins, with the equivalent of 0.18 mg g^{-1} of TA and 0.28 mg g^{-1} of TB. The recoveries for six replicates were found to be 94.2% ± 5.4% R.S.D. and 99.8% ± 6.1% R.S.D for TA and TB, respectively.

The detection limit of the method was found to be of the order of 2 ng of injected toxin with a signal-to-noise ratio of 3:1. The absence of interfering peaks was shown by the analysis of a corn culture of *A. alternata* MRC 5494 which did not produce the AAL toxins. The OPA derivatization procedure is rapid, but the resulting derivatives are unstable and must be injected at a reproducible time within 2 min of preparation. The detector response was found to be linear over the range tested (5–130 ng of injected toxin). The method was applied to assess culture conditions for optimum toxin production and hence to select suitable cultures for toxin isolation.

Initial studies indicated that water or methanol–water (1:3, v/v) achieved similar extraction efficiencies for these toxins from corn cultures. The use of methanol–water extraction solvents with methanol proportions of 50%, 75% and 100% resulted in extracted toxin levels of 90%, 65% and 30%, respectively, of the levels achieved with aqueous extraction. The use of water as extraction solvent facilitated the adsorp-

tion, after acidification, of these toxins on Amberlite XAD-2 resin, as no change in solvent composition was required. Chromatography on silica achieved a separation of TA and TB. The separately pooled toxin fractions were further purified without derivatization on an automated semi-preparative isocratic HPLC system. Fractions collected from the semi-preparative system were analysed for the individual structural isomers, TA_1, TA_2, TB_1, and TB_2, on the second analytical HPLC system described above. It was shown that the semi-preparative system separated the non-derivatized TA and TB toxins into their respective individual isomers TA_1 and TA_2, and TB_1 and TB_2, which differ in that either the C-13 (TA_1 and TB_1) or the C-14 (TA_2 and TB_2) hydroxy group is involved in the ester linkage with the terminal carboxy group of the tricarballylic acid moiety (Fig. 1). The location of the ester group in the toxins was deduced from the ^1H chemical shift values in the respective NMR spectra and from the proton–proton connectivity pattern established in a COSY-45 NMR experiment [16]. Thus the signals at δ 3.227dd (J 6.9 and 4.5 Hz) and 4.904m ppm in the ^1H NMR spectrum of TA_1 were assigned to the C-14 and C-13 protons, respectively, and establish that in TA_1 it is the C-13 hydoxy group that is involved in the ester linkage. In contrast in TA_2, it is the C-14 hydroxy group that is involved in the ester linkage: the signals of the C-14 and C-13 protons in the ^1H NMR spectrum appear at δ 4.583dd (J 6.4 and 5.0 Hz) and δ 3.558m ppm, respectively. The ester linkage in the TB_1 and TB_2 toxins was established in a similar manner.

The structural isomers undergo a slow isomerization via an intramolecular transesterification mechanism to form an equilibrium mixture of the C-13 and C-14 esters. This process is analogous to the well characterized isomerization of the mono-esters of glycerol in which the acid moiety is transferred by an intramolecular mechanism to a vicinal hydroxy group [17]. The formation of an equilibrium mixture of the isomers of TA and TB was also evident in each instance from the signals in the ^1H and ^{13}C NMR spectra of these toxins. Two sets of signals were discernible in each instance and for both TA and TB an equilibrium ratio of 55:45 in favour of ester

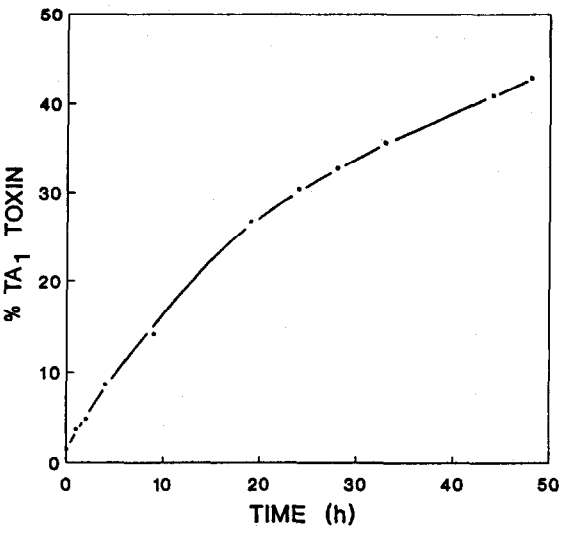

Fig. 3. Formation of TA_1 by isomerization during incubation of TA_2 in the HPLC mobile phase (methanol–0.05 *M* sodium dihydrogenphosphate, 47:53, v/v, pH 3.4) at 45°C.

formation with the C-13 hydroxy group is established. Fig. 3 illustrates the rate of the isomerization reaction in which TA_2, collected from the semi-preparative HPLC system, was incubated at 45°C in the HPLC mobile phase. The formation of TA_1 was monitored by HPLC over a period of 2 days, during which time its concentration in the sample increased from 1% in the starting material to over 40% after 48 h.

After elution from the semi-preparative HPLC system, the samples were desalted on a reversed-phase column. Based on HPLC analysis, the isolated TA and TB were *ca.* 95% pure. The identity of the isolated compounds was confirmed both by HPLC after spiking a sample with authentic toxin donated by Professor D.G. Gilchrist and observing its co-elution with the isolated compound and also by comparison of the ^1H and ^{13}C NMR spectra with published data [5].

CONCLUSIONS

A convenient procedure has been developed for the isolation of the AAL phytotoxins from corn cultures of *A. alternata* f. sp. *lycopersici*. The determination of these toxins in culture material by the proposed HPLC procedure is

sensitive, reproducible and accurate. The individual structural isomers of TA and TB can be separated as OPA derivatives on a reversed-phase isocratic HPLC system. The isolation of sufficient amounts of these toxins will enable their biological activities to be compared with those of the structurally similar fumonisins.

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