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Note

Rapid high-performance liquid chromatographic analysis of phytotoxins from *Phoma lingam*[a]

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Phoma lingam (Tode ex Fr.) Desm., [perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.] is the causative agent of blackleg disease of the rapeseed/canola (*Brassica napus* and *B. campestris*) oilseeds. The phytotoxins produced by virulent isolates of *P. lingam* were recently reported [1]. The metabolite profiles of several fungal isolates were analyzed and correlated with the virulence of the pathogen [1]. There is a good indication that only virulent isolates of *P. lingam* produce phytotoxins; however, to establish such a correlation, a large number of fungal isolates should be screened. If the production of phytotoxic compounds proves to be a characteristic of virulent isolates, it may be used as a chemotaxonomical marker of particular strains of *P. lingam*.

In order to carry on a wide screening programme, it is essential to have a rapid and reliable analytical method for the detection of phytotoxins. In this note we report a high-performance liquid chromatography (HPLC) method for the rapid analysis of phytotoxins produced by *P. lingam*.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Spectra-Physics solvent delivery system, Model SP8700, equipped with pump and injector, and a Kratos Analytical Instruments absorbance detector (variable wavelength), Model Spectroflow 773. Chromatograms and retention times were recorded on a Hewlett-Packard integrator, Model 3392A. The column used was a Partisil PXS 5/25 (5-μm particle size silica, 25 cm × 4.6 mm

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I.D.; Whatman, Clifton, NJ, U.S.A.), equipped with a guard column filled with the same stationary phase.

Reagents

The organic solvents (Table I) were HPLC grade and were degassed during operation through continuous bubbling of a stream of helium.

TABLE I

RETENTION TIME OF SIRODESMIN PL (MINIMUM DETECTABLE AMOUNT 0.1 μg) UNDER DIFFERENT EXPERIMENTAL CONDITIONS [COLUMN PARTISIL PXS 5/25 (WHATMAN); ISOCRATIC ELUTION; 235 nm]

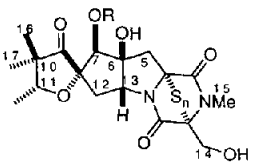
Solvent system	Flow-rate (ml/min)	Retention time (min)
(A) Hexane–2-propanol (90:10)	2.0	6.8
(B) Hexane–2-propanol (92:8)	2.0	8.4
(C) Dichloromethane–2-propanol (94:6)	2.0	2.4
(D) Dichloromethane–2-propanol (97:3)	2.0	3.0
(E) Dichloromethane–2-propanol (98:2)	2.0	4.7
(F) Dichloromethane–ethyl acetate (50:50)	2.0	3.5
(G) Ethyl acetate–hexane (60:40)	2.0	5.0

Preparation of extracts

The isolates of *P. lingam* (obtained from G. A. Petrie, Agriculture Canada Research Station, Saskatoon, Canada) were grown in liquid minimal medium supplemented with thiamine [1]. Initially the fungus was grown in still culture for three weeks, because the phytotoxicity of the culture filtrate was highest at that stage. Subsequently, one to four weeks old still cultures and five to seven days old shake cultures were investigated. The liquid cultures were filtered through cheesecloth, and the broth was freeze-dried. The freeze-dried broth was diluted with distilled water to one tenth of the initial volume and extracted four times (separatory funnel) with an equivalent volume of ethyl acetate (yields from 20–200 mg of crude extract per liter of broth) [1]. The crude broth extracts were subjected to a preliminary "clean-up" by filtering ethyl acetate solutions (100 mg/ml) through a mini-silica gel column (Pasteur pipette containing silica gel about 4 cm high) eluted with ethyl acetate (10 ml). After evaporation of the ethyl acetate, the extracts were dissolved in dichloromethane and/or in the mobile solvent system and filtered through a cotton plug (2.5 mg/ml; injection volume 1–5 μl).

Preparation of natural standards

The naturally occurring phytotoxins sirodesmin PL, deacetylsirodesmin PL, sirodesmins H, J and K, and phomalirazine (structures in Fig. 1) were isolated from the crude broth extracts of *P. lingam* liquid cultures and purified as previously described [1]. Solutions of these compounds prepared in dichloromethane and filtered through a cotton plug were used as natural standards.



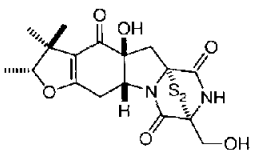
1 Sirodesmin PL R=Ac; n=2

2 Deacetylsirodesmin PL R=H; n=2

3 Sirodesmin H R=Ac; n=1

4 Sirodesmin J R= Ac; n=3

5 Sirodesmin K R= Ac; n=4



6 Phomalirazine

Fig. 1. Chemical structures of phytotoxins from *Phoma lingam*. Ac = Acetyl; Me = methyl.

RESULTS AND DISCUSSION

The chemical structures of the putative phytotoxins isolated from *P. lingam* extracts [1] are shown in Fig. 1. Sirodesmins H, J and K are unstable in methanol and other polar solvents [1,2], therefore a normal-phase column was used in the HPLC analysis. Table I lists several of the solvent systems tried with a Partisil PXS column. Compounds **1** and **4** showed identical retention times in systems A and B (Table I); similarly compounds **2** and **3** were indistinguishable in systems F and G. In dichloromethane–2-propanol (systems C–E) each phytotoxin (**1–6**) had a different retention time and could be readily differentiated. The minimum detectable amount for each phytotoxin was *ca.* 0.1 μg (experimental conditions on Table II).

The chromatograms of Fig. 2 represent extracts of the virulent isolates "Leroy" and "FRA 88" (three-week-old cultures) and were obtained under identical conditions. Sirodesmin PL (**1**) is the major component of the "Leroy" extract. The components of the "FRA 88" extract are deacetylsirodesmin PL (**2**), and sirodesmins PL, J and K (**1, 4, 5**). Two other virulent isolates examined showed chromatographic pro-

TABLE II

RETENTION TIMES OF PHYTOTOXINS OF *PHOMA LINGAM*; UV DETECTION SET AT 235 nm; COLUMN PARTISIL PXS 5/25 (WHATMAN); SYSTEMS A AND D

Phytotoxin	Retention time (min)	
	A	D
Sirodesmin PL (**1**)	6.8	2.4
Deacetylsirodesmin PL (**2**)	6.4	3.8
Sirodesmin H (**3**)	13.5	4.0
Sirodesmin J (**4**)	6.8	3.4
Sirodesmin K (**5**)	8.5	6.4
Phomalirazine (**6**)	10.9	16.6

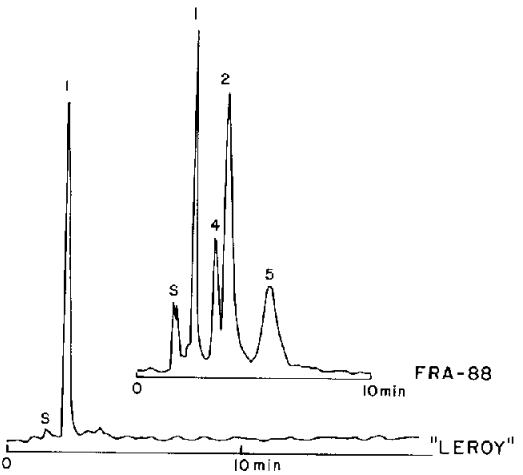


Fig. 2. HPLC chromatograms of extracts of two virulent isolates ("Leroy" and "FRA 88") of *Phoma lingam*. Peak numbers refer to structures in Fig. 1 and S to solvent peak. For experimental conditions see Table II, system C.

files identical to the isolate "Leroy". No phytotoxins (or other UV-absorbing compounds) were detected in five avirulent isolates. The limit of detection of phytotoxins per g of extract (dry material) was *ca.* 0.1%.

Three-week-old cultures are impractical (slow growth) to be used in a wide screening programme. To find a faster and yet characteristic fungal growth stage, the chromatographic profiles of still and shake cultures of different ages were compared. The metabolite profiles of seven-day-old shake cultures and three-week-old still cultures were similar. Thus, seven-day-old shake cultures are more convenient to carry out a rapid screening of a large number of fungal isolates.

Considering the worldwide occurrence and importance of the disease caused by *P. lingam* it is of interest to differentiate virulent isolates from avirulent ones with reliable methods, which at present are insufficient [3]. The simplicity of the HPLC method reported here, coupled with the use of seven-day-old shake cultures, will allow a rapid and reliable screening of isolates of *P. lingam*.

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