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Note

Analysis of phomenone in cultures of *Phoma destructiva* and *Phoma betae* by high-performance liquid and thin-layer chromatography

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Phomenone (Fig. 1) is an eremofilanic sesquiterpene with wide biological activity[1–6]. It was identified as phytotoxin of *Phoma exigua* var. *inoxidabilis* Boerema et Vegh[7] and recently as phytotoxin of *Phoma destructiva* Plowr., the causal agent of wilt disease of tomato[8]. Some attempts have been made to identify it in culture filtrates of twenty phytopathogenic *Phoma* species[9] by high-performance liquid chromatography (HPLC), on account of its possible property as taxonomic marker for these species.

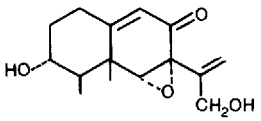

Fig. 1. Structure of phomenone.

We applied an HPLC method as described previously[10] to check the occurrence of phomenone in infected tomato plants, but this method failed to detect with reliability the occurrence of the phytotoxin in the *Phoma* species mentioned above. However, a more sophisticated investigation, utilizing both a partially modified HPLC method and a high-performance thin-layer chromatographic (HPTLC) method, as described in this paper, excluded the presence of phomenone in cultures of *Phoma betae* Frank, in contrast with previous findings[9].

The methods were applied to *P. destructiva*, to determine quantitatively the toxin, and to *P. betae* from diseased leaf-beet (*Beta vulgaris* var. *cycla* L.) and *P. betae* from diseased spinach (*Spinacia oleracea* L.) culture filtrate extracts, to check its occurrence. The procedure was based on two steps: (1) extraction of culture filtrates with ethyl acetate and (2) analysis by HPLC and HPTLC.

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EXPERIMENTAL

Reagents

All reagents were of analytical reagent grade. HPLC-grade ethanol, acetonitrile, methanol, ethyl acetate, *n*-hexane and chloroform for use as eluting solvents were purchased from Fluka. HPLC-grade water was prepared by double distillation over $KMnO_4$ and filtration on Millipore filters (GSWP, 0.22 μm).

A pure sample of phomenone was purified from culture filtrates of *P. destructiva*, as described previously[8]. Solutions of 1.9 and 0.09 mg of phomenone in 1 ml of methanol for HPLC and HPTLC analysis, respectively, were used as standards.

Culture filtrates

The culture filtrates of *P. destructiva* and *P. betae* isolates were obtained as described previously[8].

Extraction of phomenone from culture filtrates of P. destructiva and extraction of culture filtrates of P. betae from leaf-beet and spinach

Culture filtrates of *P. destructiva* (220 ml), *P. betae* from leaf-beet (1250 ml) and *P. betae* from spinach (1325 ml) were extracted with ethyl acetate (4 × 150 ml for *P. destructiva*, 4 × 900 ml for *P. betae* from leaf-beet and 4 × 900 ml for *P. betae* from spinach). The organic extracts were combined, dried over anhydrous sodium sulphate and evaporated under reduced pressure. The dry residues (15 mg for *P. destructiva*, 28.3 mg for *P. betae* from leaf-beet and 46 mg for *P. betae* from spinach) were dissolved in methanol (1 ml) and analysed by HPLC and HPTLC. The solutions used for HPTLC analysis were diluted 1:100.

HPLC

A Perkin-Elmer Series 3B liquid chromatograph equipped with a Perkin-Elmer LC-75 variable-wavelength UV–visible spectrophotometric detector was used in connection with a Perkin-Elmer Sigma 10B chromatographic data station.

Experimental conditions for the determination of phomenone in the extracts of *P. destructiva* and *P. betae* culture filtrates are reported in Table I.

HPTLC

HPTLC was performed on silica gel-coated aluminium plates (Merck 5547, 20 × 20 cm, 0.25 mm layer), previously washed with ethyl acetate–*n*-hexane (90:10, v/v) and heated at 100°C for 1 h. The plates were eluted for a distance of 10 cm with the same solvent.

A Camag TLC/HPTLC scanning densitometer set at 240 nm was used to analyse the phomenone spots *in situ*. The corresponding peaks were recorded with a Perkin-Elmer 56 recorder and the areas were calculated in square millimetres by graphic integration. Each sample was loaded twice on the plate by an automatic loader under a nitrogen flow as 4 mm spaced narrow bands 4 mm long.

For qualitative HPTLC controls plates of various lengths were used, eluted with chloroform–methanol (85:15, v/v), the phomenone spot showing $R_F = 0.6$.

The plates were analysed by UV detection at 240 nm and by visible detection, by spraying the plates first with 10% sulphuric acid in methanol and then with 3% phosphomolybdic acid in methanol, followed by heating for 10 min at 110°C.

RESULTS

HPLC

The standard deviation of the phomenone determination was 3% for eight replicate injections using two different volumes. When phomenone was analysed using an RP-18 column eluted with the mixtures described in Table I and a UV detector set at 240 nm, the detection limit was 5.5 ng. However, using a silica gel column eluted with ethyl acetate–*n*-hexane (90:10) and a UV detector set at 248 nm (Table I), the detection limit was 100 ng.

The retention time of phomenone is dependent on the experimental conditions and the values obtained are reported in Table I. The analysis of a semi-synthetic liquid medium[11] spiked with phomenone led to an average recovery of 95.1 ± 1.6% in four independent experiments.

The determination of the toxin in crude extracts of *P. destructiva* culture filtrates was accomplished by preparing two calibration graphs of average peak area versus amount of pure compound, and were linear in the range 0.1–8 μg (Fig. 2a and b). The content of phomenone in *P. destructiva* extracts, characterized by the chromatograms in Fig. 3a and b, corresponded to 5.5 μg/ml of culture.

The chromatograms of crude extracts of *P. betae* from beet and spinach culture filtrates, obtained using an RP-18 column as reported in Table I (experimental conditions 1 and 1a–e, showed peaks with the same retention time as phomenone. In contrast, the chromatograms of the same extracts obtained using a silica gel column eluted with ethyl acetate–*n*-hexane (90:10) (experimental conditions 2 and 2a in Table I) showed peaks with retention times different from that of phomenone, as shown in Fig. 4a–d.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF PHOMENONE IN *IN VITRO* CULTURES OF *P. DESTRUCTIVA* AND *P. BETAE* BY HPLC

No.	*Column*	*Chromatographic eluent (v/v)*	*Flow-rate (ml/min)*	*Retention time of phomenone (min)*	*UV detector (nm)*
1	RP-18*	Water–ethanol (70:30)	1.5	3.51	240
1a		Water–ethanol (70:30)	0.8	3.65	240
1b		Water–acetonitrile (50:50)	0.8	3.75	240
1c		Water–methanol (50:50)	0.8	4.78	240
1d		Water–acetonitrile (5:95 to 50:50 over 20 min)**	0.9	3.45	240
1e		Water–acetonitrile (15:85 to 50:50 over 10 min)***	0.8	3.32	240
2	Silica gel*	Ethyl acetate–*n*-hexane (90:10)	2.5	3.09	248
2a		Ethyl acetate–*n*-hexane (90:10)	1.2	7.60	248

* 25 × 0.46 cm I.D., 10 μm (Hibar-LiChrochart stainless steel, Merck).
** Linear gradient.
*** Concave gradient.

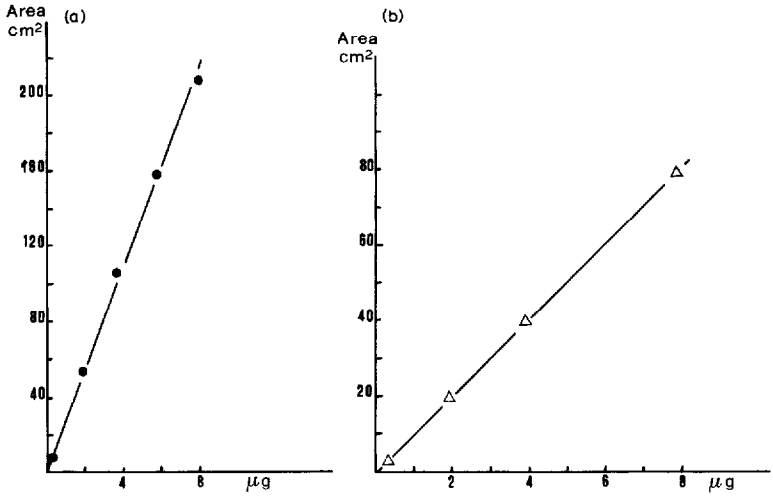

Fig. 2. (a) Calibration graph for phomenone obtained on a Merck RP-18 column. Mobile phase, water–ethanol (70:30, v/v); flow-rate, 1.5 ml/min; detector, 240 nm. (b) Calibration graph for phomenone obtained on a Merck Si-60 column. Mobile phase, ethyl acetate–*n*-hexane (90:10, v/v); flow-rate, 2.5 ml/min; detector, 248 nm.

HPTLC

The detection limit when using a UV detector set at 240 nm was 10 ng, whereas the blue spot of phomenone obtained by spraying the plates first with 10% sulphuric acid in methanol and then with 3% phosphomolybdic acid in methanol followed by heating 10 min at 110°C was detectable at levels up to 19 ng. The standard deviation calculated for ten loadings [5 μl (0.013 μg/μl)] on a plate was found to be 7.8%. The linear calibration graph obtained in the range 10–200 ng is shown in Fig. 5.

The thin-layer chromatogram of a culture filtrate of *P. destructiva* is shown in Fig. 6. Phomenone appeared at R_F 0.42 with UV and visible range development

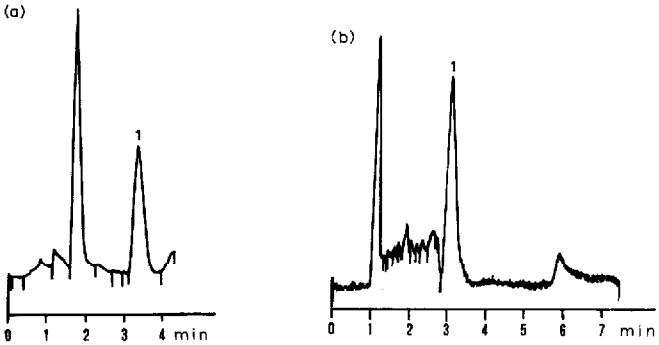

Fig. 3. (a) Chromatogram of extract from *P. destructiva* culture filtrates obtained on a Merck RP-18 column (25 × 0.46 cm I.D., 10 μm) at 20°C. Mobile phase, water–ethanol (70:30, v/v); flow-rate, 1.5 ml/min; detector, 240 nm. Peak 1, phomenone (3.51 min). (b) Chromatogram of extract from *P. destructiva* culture filtrates, obtained on a Merck silica gel column (25 × 0.46 cm I.D., 10 μm) at 20°C. Mobile phase, ethyl acetate–*n*-hexane (90:10, v/v); flow-rate, 2.5 ml/min; detector, 248 nm. Peak 1, phomenone (3.09 min).

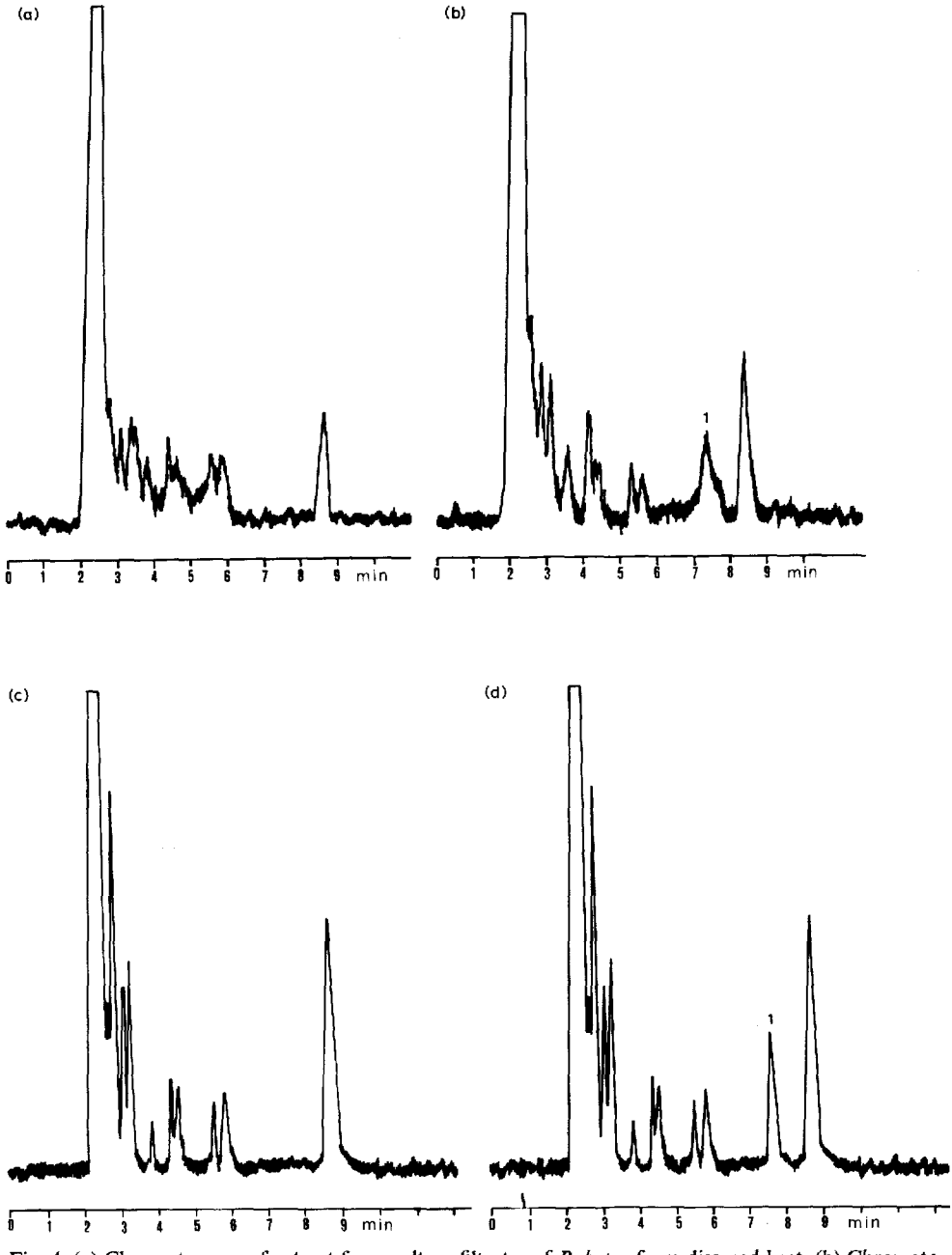

Fig. 4. (a) Chromatogram of extract from culture filtrates of *P. betae* from diseased beet. (b) Chromatogram of extract from culture filtrates of *P. betae* from diseased beet, after co-injection of phomenone. Peak 1, phomenone (7.60 min). (c) Chromatogram of extract from culture filtrates of *P. Betae* from diseased spinach beet. (d) Chromatogram of extract from culture filtrates of *P. betae* from diseased spinach beet, after co-injection of phomenone. Peak 1 phomenone (7.60 min). Merck silica gel column (25 × 0.46 cm I.D., 10 μm) at 20°C. Mobile phase, ethyl acetate–*n*-hexane (90:10, v/v). flow-rate 1.2 ml/min; detector, 248 nm.

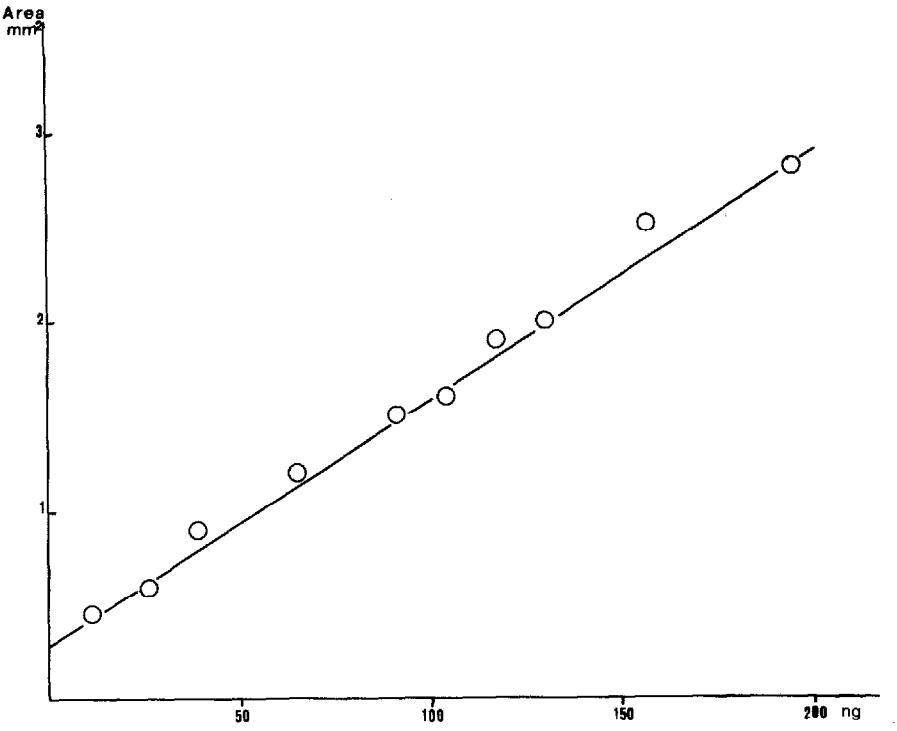

Fig. 5. Calibration graph for phomenone. Merck HPTLC plate (20 × 20 cm, 0.25 mm). Mobile phase, ethyl acetate–*n*-hexane (90:10, v/v). UV detector, 240 nm.

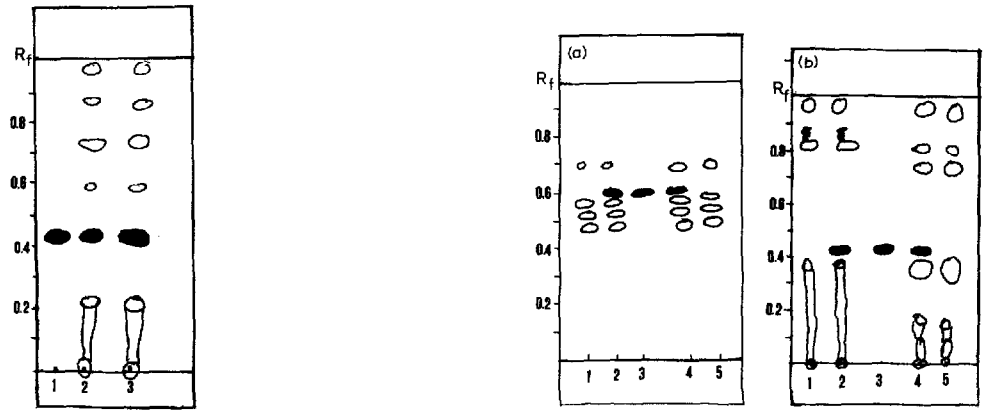

Fig. 6. Chromatogram of extract from *P. destructiva* culture filtrates. 1, Phomenone as reference (R_F 0.42); 2, *P. destructiva* extract by co-loading of phomenone; 3, *P. destructiva* extract. Mobile phase, ethyl acetate–*n*-hexane (90:10, v/v); detector, 240 nm; visible detection by development with 10% sulphuric acid and 3% phosphomolybdic acid in methanol by heating for 10 min at 100°C.

Fig. 7. Chromatograms of extracts from culture filtrates of the two isolates of *P. betae*. Detector, 240 nm; visible detection by development with 10% sulphuric acid and 3% phosphomolybdic acid in methanol by heating for 10 min at 100°C. 1, *P. betae* extract from beet; 2, *P. betae* extract from beet by co-loading of phomenone; 3, phomenone as reference; 4, *P. betae* extract from spinach beet by co-loading of phomenone; 5, *P. betae* extract from spinach beet. Mobile phase: (a) chloroform–methanol (85:15, v/v); (b) ethyl acetate–*n*-hexane (90:10, v/v).

[when chloroform–methanol (85:15, v/v) was used as the eluent, phomenone appeared at R_F 0.60]. Quantitative analysis, performed as described under Experimental, gave an amount of phomenone up to 5.5 μg/ml of (*P. destructiva*) culture.The thin-layer chromatograms of culture filtrate extracts of *P. betae* are shown in Fig. 7a and b; the phomenone spot could not be detected with either a UV or a visible-range detector.

DISCUSSION

Reversed-phase and adsorption TLC methods were not suitable, because compounds with the same R_F as phomenone were not separated. On the other hand, the HPTLC method allowed the separation of phomenone from compounds having an R_F value similar to that of phomenone (Figs. 6, 7a and 7b). This method showed a resolution higher than that of reversed-phase HPLC, although its sensivity was similar (10 and 5 ng, respectively). Finally, the HPLC method with a UV detector and a silica gel column gave good resolution, but it exhibited a low sensitivity (100 ng) on account of the interference of the UV absorbance of the solvent (ethyl acetate–*n*-hexane) with phomenone. Therefore, by combining the use of HPTLC plates with a scanning densitometer and an automatic loader, it was possible to perform rapid and sensitive analyses with a precision comparable to that of HPLC.

The two methods were used independently for the analysis of *P. destructiva* cultures, whereas HPTLC was used in combination with HPLC to determine phomenone in *P. betae* cultures. Using HPLC on silica gel the detection limit of the toxin was 100 ng, whereas using silica gel HPTLC it was 10 ng, because in the latter instance ethyl acetate was removed by evaporation on the plate and the analysis was performed *in situ*. Such conditions should be considered when an eluent interferes in UV detection. Moreover, if a scanning densitometer is not available, HPTLC can be used in a preliminary step to establish a suitable mobile phase for the HPLC column to be used.

In conclusion, for the analysis of *Phoma* phytopathogenic species, the application of these two methods, separately or together, should provide reliable results.

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