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Metabolite profiles of common *Stemphylium* species

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Thirty-three isolates of *Stemphylium* spp. have been analysed for their metabolite profiles. Five metabolites, stemphylin, stemphyloxin II, stemphyperlenol, stemphol and a stemphol related compound, have been detected by high-performance liquid chromatography and thin-layer chromatography and identified by their chromatographic and spectroscopic data (R_f values, reflectance spectrum, retention index and ultraviolet spectrum). These metabolites have been used for the chemotaxonomical characterization of *Stemphylium botryosum*, *S. herbarum*, *S. alfalfae*, *S. majusculum*, *S. sarciniforme*, *S. vesicarium*, *Pleospora herbarum* and *P. tarda*.

Identification of fungi based exclusively on morphology is often a problem because of variations in conidial appearance within the same species or resemblance between different species. Thus, profiles of secondary metabolites have proven a good support to the morphology and have been used with great success in differentiating between species within *Penicillium* and *Fusarium* (Frisvad & Filtenborg, 1989; Thrane, 1989; Andersen, 1991). It is often seen that two different fungal species have one or more metabolites in common but other metabolites will discriminate between the two species (Frisvad & Filtenborg, 1989). Standards of mycotoxins and other secondary metabolites have been characterized in the same system used for establishing metabolite profiles (Frisvad & Thrane, 1987; Singh *et al.*, 1991; Frisvad & Thrane, 1993) and are used for identification of compounds in metabolite profiles.

A general method for visualizing a metabolite profile is using high-performance liquid chromatography (hplc) with a diode array detector (DAD) (Frisvad & Thrane, 1987). By means of the DAD detector the chromatographic peaks can be recognized by their uv spectra. It has been shown for both *Penicillium* and *Fusarium* that each species has a unique and specific hplc chromatogram (Frisvad & Filtenborg, 1989; Thrane, 1989). Another and more rapid method for visualizing a metabolite profile is thin-layer chromatography (tlc) using the agar plug method (Filtenborg & Frisvad, 1980; Frisvad, Filtenborg & Thrane, 1989; Singh *et al.*, 1991). The advantage of this method is that there is no chemical treatment of the metabolites and thereby less chance for formation of artefacts, which can give a distorted picture of metabolite profile. Reflectance spectra recorded from the tlc plate by a tlc Scanner makes it possible to distinguish between different metabolites with the same R_f -value and colour (Andersen, 1991). The two

methods are complementary, since some metabolites are only detectable either by tlc or hplc.

This paper deals with some of the metabolites in the metabolite profile of *Stemphylium* from pure cultures and the use of their chromatographic and spectroscopic characteristics in determining the fungal metabolite profile. For chemotaxonomic comparison of metabolite profiles from *S. botryosum* Wallr. and related species, isolates of *S. herbarum* E. G. Simmons, *S. alfalfae* E. G. Simmons, *S. majusculum* E. G. Simmons, *S. sarciniforme* (Cavara) Wiltshire, *S. vesicarium* (Wallr.) E. G. Simmons and *Stemphylium* states of *Pleospora herbarum* (Pers.: Fr.) Rabenh. and *P. tarda* Simmons were investigated as well.

MATERIALS AND METHODS

Fungi and growth conditions. The following 33 isolates of different *Stemphylium* species and their teleomorphs *Pleospora* were screened for consistent production of metabolites: 12 isolates of *S. botryosum* (represented by IBT 8226 and IBT 8213) were obtained from rapeseeds in Italy and from barley kernels in Denmark, four isolates of *S. herbarum* (represented by IBT 8224) obtained from rapeseeds in Italy, two isolates of *S. alfalfae* (represented by IBT 8227) obtained from rapeseeds in Italy, five isolates of *S. vesicarium* (represented by IBT 8220) obtained from pear in Italy (2) and barley kernels in Denmark (3), four isolates of *S. sarciniforme* (represented by CBS 335-33), four isolates of *P. herbarum* (represented by IMI 130782), one of *P. tarda* (IMI 135456) and one of *S. majusculum* (IMI 135459).

Identification of isolates. All isolates were identified on PCA (potato carrot agar) (Simmons, 1992) according to the descriptions by Simmons (1969, 1985). One isolate from each species (*S. botryosum*, *S. herbarum*, *S. alfalfae* and *S. vesicarium*) was given an IBT number and taken as a representative for the

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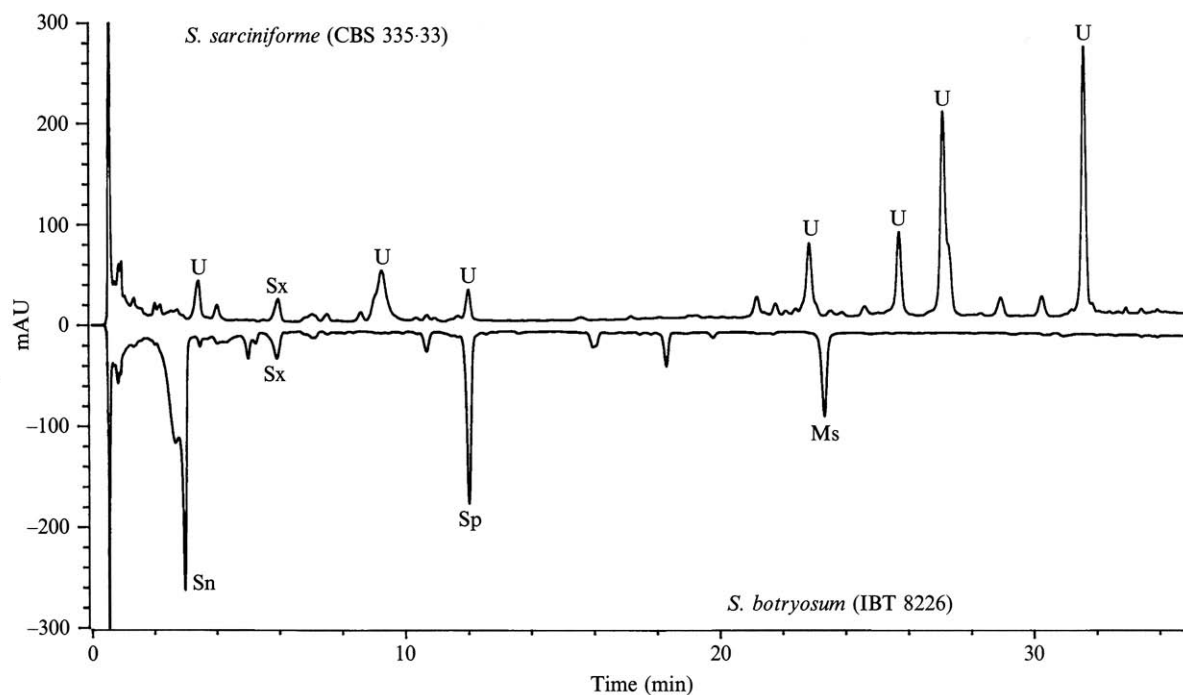


Fig. 1. Metabolite profiles of *S. sarciniforme* (IBT 8222) and *S. botryosum* (IBT 8226) from hplc. Sn, stemphylin; Sx, stemphyloxin II; Sp, stemphyperlylenol; Ms, macrosporin; U, unknown compound.

group. Isolates with IBT numbers are held at the fungal collection at Department of Biotechnology, Technical University of Denmark, CBS number is held at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands and IMI numbers are held at the International Mycological Institute, Egham, Surrey, U.K.

Metabolite profile analyses. For metabolite profile analyses, each isolate was inoculated on seven Petri dishes (90 mm diam.), each containing the following seven media: ALK (Alkaloid formation agar) (Reshetilova *et al.*, 1992), DRYES (Dichloran Rose bengal Yeast Extract Sucrose agar) (Frisvad, 1983), MEA (Malt Extract Agar) (Pitt, 1979), NS (Nitrite Sucrose agar) (Frisvad, 1981), OAT (Oat meal agar) (Gams *et al.*, 1987), PSA (Potato Sucrose Agar) (Booth, 1971) and SYES (Sigma Yeast Extract Sucrose agar) (Filttenborg, Frisvad & Thrane, 1990). All media except ALK were given 1.0 ml l^{-1} of trace metal solution ($1.0 \text{ g ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $0.5 \text{ g CuSO}_4 \cdot 5\text{H}_2\text{O}$, 100 ml water) (Smith, 1949). After inoculation the Petri dishes were incubated at 25°C in the dark for 14 d.

TLC. One agar plug (4 mm diam.) from each of the seven Petri dishes was applied in the same line to 0.25 mm silica gel 60 precoated plates with and without fluorescence indicator (Merck nos. 5715 and 5721, respectively). The plates were eluted in TEF (toluene/ethyl acetate/90% formic acid (5:4:1, v/v/v)). The metabolite profiles were visualized under uv light (365 and 254 nm) and sprayed with 50% H_2SO_4 in methanol or ANIS (1.8% *p*-methoxybenzaldehyde in methanol/glacial acetic acid/conc. H_2SO_4 (14:2:1, v/v/v)). Reflectance spectra were recorded on a CAMAG tlc scanner II (Muttentz, Switzerland) with CATS3 software.

HPLC. The whole agar cultures of the seven Petri dishes were extracted with 150 ml ethyl acetate and 1 ml formic acid and

then with 150 ml chloroform/methanol (2:1, v/v). The extracts were pooled and evaporated to dryness. The residue was dissolved in 3 ml acetonitrile and defatted with 6 ml hexane. The hexane was discarded. The metabolites were separated by hplc analysis on a Hewlett Packard HP 1090M high-performance liquid chromatograph equipped with two pumps, a built-in diode array detector, an autosampler injection system (10 μl) and an external computer control (HP 9000 Model 310). The column was a 100 mm \times 4.0 mm i.d. Nucleosil 5 μm C_{18} reversed phase column (Macherey-Nagel & Co., Düren, Germany). A gradient solvent system consisting of acetonitrile with 0.01% trifluoroacetic acid and water as described by Frisvad & Thrane (1987) was used with a flow rate at 2.0 ml min^{-1} . A series of alkylphenones was used as external standards for calculating a retention index (RI) for each peak in the chromatogram (Frisvad & Thrane, 1987).

Identification of metabolites. The identity of the metabolites was confirmed by comparison with the authentic material, uv spectra and/or with tlc data available from the literature. Standards of stemphylin (= altersolanol A) and macrosporin were kindly provided by Professor Akira Yagi, Faculty of Pharmacy & Pharmaceutical Sciences, Fukuyama University, Hiroshima, Japan and G. W. van Eijk, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, respectively. Stemphol was obtained from a culture of *S. botryosum* on rice at Istituto Tossine e Micotossine da Parassiti Vegetali, Italy.

RESULTS AND DISCUSSION

Metabolite profile analyses

The metabolite profile of a *Stemphylium* isolate is composed by 15–20 well-defined spots on tlc and 10–15 major peaks on hplc. Most of the spots and peaks represent unknown

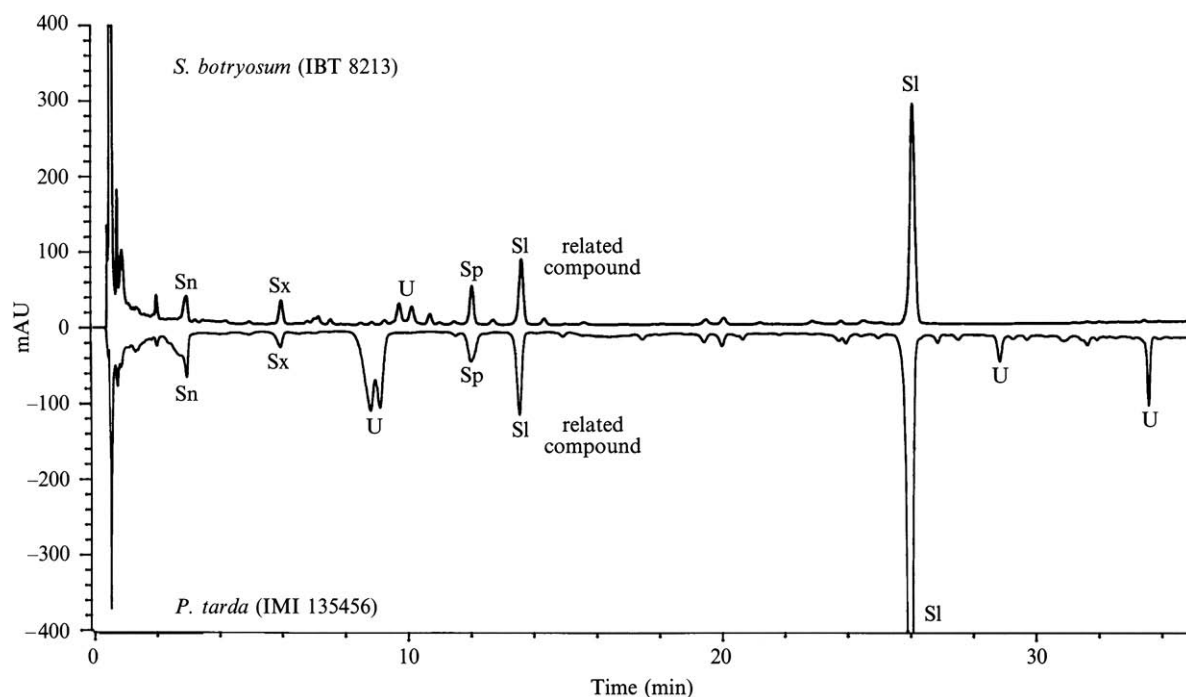


Fig. 2. Metabolite profiles of *S. botryosum* (IBT 8213) and *P. tarda* (IMI 135456) from hplc. Sn, stemphylin; Sx, stemphyloxin II; Sp, stemphperylenol; Sl, stemphol; U, unknown compound.

metabolites that are consistently produced and recognized in every isolate of the same species and can be used in construction of the metabolite profile. For comparison of metabolite profiles from hplc two profiles (*S. botryosum* and *S. sarciniforme*, respectively) are shown in Fig. 1. The two isolates have only few metabolites in common, meaning that both the retention indices and the uv spectra have to be identical. Stemphyloxin II is the only known metabolite the two isolates have in common. A chemotaxonomic segregation can be possible because each species produced metabolites that are not found in the other one. Figure 2 shows metabolite profiles from hplc of *S. botryosum* and its teleomorph *Pleospora tarda*. The two isolates have at least six metabolites in common. It is often seen that the teleomorph state produces a number of additional metabolites (Wicklow, 1988) but the metabolite profile is recognizable. Unknown metabolites are marked with a 'U' in Figs 1 and 2.

Identification of metabolites

Five major compounds, namely stemphylin, stemphyloxin II, stemphperylenol, stemphol and a stemphol related compound have been identified and placed in the metabolite profile of *Stemphylium* species on the basis of their uv spectral characteristics, tlc and hplc properties and by comparison with the authentic material. The chromatographic and spectroscopic data for all compounds are given in Tables 1 and 2. The structures of the first four metabolites are given in Fig. 3.

Stemphylin. The phytotoxin, stemphylin, is visible on tlc plate as a yellow spot in daylight without spraying. Stemphylin is identical to altersolanol A (Assante & Nasini, 1987) and has been isolated from *S. botryosum* var. *lactucum* (Barash *et al.*, 1978), *Alternaria porri* and *A. solani* (Montemurro & Visconti, 1992).

Table 1. Chromatographic and spectroscopic data of metabolites in the metabolite profile on tlc^a

	R_f	R_{fg} ^b	Detection			Reflectance ^c spectra
			At 366 nm	H ₂ SO ₄ ^d	ANIS and heat ^e	
Stemphylin	16	47	Orange brown	Brown	Yellow brown	217* (64), 235 (38), 270* (100), 328 (10), 442* (55)
Stemphyloxin II	23	72	— ^f	— ^f	— ^f	194* (40), 229 (23), 276* (100)
Stemphperylenol	34	105	Yellow	Orange	Orange red	200 (30), 218* (42), 234 (23), 262* (100), 291 (13), 341* (59)
Stemphol	70	219	Yellow green	Orange red	Red	197* (84), 219sh (57), 237 (50), 272* (100), 300 (44), 352* (88)
Stemphol related compound	44	136	Yellow green	Orange red	Red	198* (92), 266sh (54), 239 (40), 271* (100), 300 (32), 352* (68)

^a All data are recorded on tlc plates with fluorescence indicator eluted in TEF.

^b R_f values relative to griseofulvin, which has been given the value 100 calculated on plates with fluorescence indicator.

^c Reflectance maxima (*), shoulders (sh) and minima recorded expressed in nm and relative intensities in per cent (in parentheses).

^d Colours on plates irradiated at 366 nm.

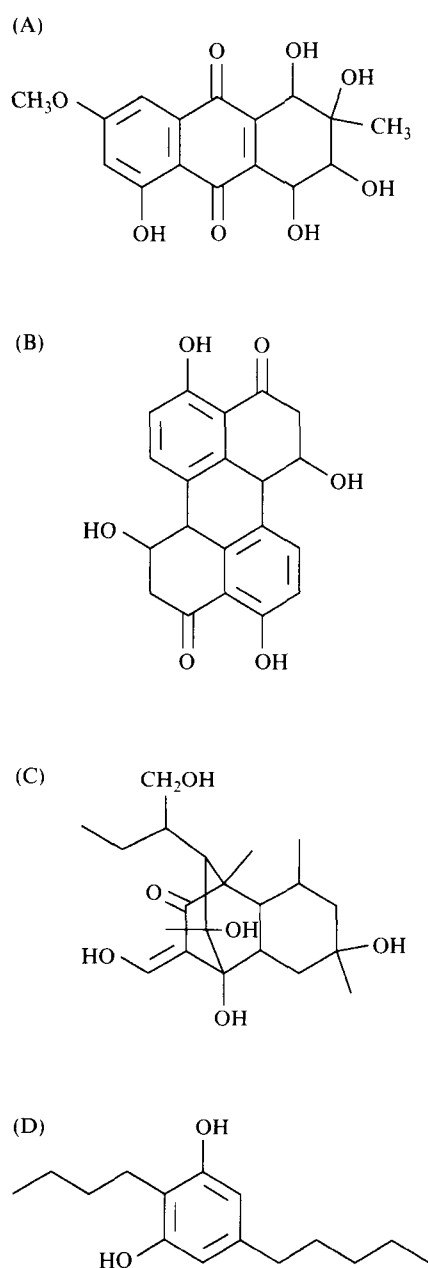
^e Colours on plates after heating and exposed to daylight.

^f Only detectable on F₂₅₄ plates at 254 nm and detected only from extracts as a dark spot.

Table 2. Chromatographic and spectroscopic data of metabolites in the metabolite profile on hplc

	uv-VIS spectra ^a	Retention index value
Stemphylin	203 (54), 221* (100), 241 (28) 268* (43), 331 (3), 429 (13)	726
Stemphyloxin II	215 sh (100), 239 (32), 277* (88)	787
Stemphyperlenol	216* (100), 239 (23), 261* (77), 291 (6), 340* (23)	909
Stemphol	205* (100), 231 sh (19), 251 (1), 273* (2)	1200
Stemphol related compound	205* (100), 231 sh (18), 251 (1), 271* (2)	921

^a Absorption maxima (*), shoulders (sh) and minima recorded in acetonitrile/water gradient. Wavelengths are expressed in nm. Percentages are given in parentheses.

**Fig. 3.** The structures of the four known metabolites. (A) Stemphylin, (B) stemphyperlenol, (C) stemphyloxin II and (D) stemphol.

Stemphyloxin II. Stemphyloxin II is not considered to be a natural metabolite, but an artefact of stemphyloxin I, which is easily converted to its tautomer, stemphyloxin II, by acid or base (Barash *et al.*, 1982). Stemphyloxin I shows an uv maximum at 282 nm in acidic solution and an uv maximum at 303 nm in basic solution. This shift in absorbance that was not exhibited by the partially purified compound. Since the agar cultures are extracted with a strong acid it is assumed that all the produced stemphyloxin I is transformed to stemphyloxin II. Stemphyloxin I and II have previously been isolated from *S. botryosum* f. sp. *lycopersici* (Barash *et al.*, 1982; Manulis *et al.*, 1984).

Stemphyperlenol. Stemphyperlenol gives the most conspicuous spot in the metabolite profile on tlc when the plate is sprayed with H₂SO₄. The spot is bright orange under 365 nm uv light. Stemphyperlenol has been previously been isolated from *S. botryosum* var. *lactucum* (Arnone *et al.*, 1986).

Stemphol. Stemphol from *S. botryosum* (IBT 8213) proved to be identical to a standard of stemphol derived from rice cultures of *S. botryosum* (Solfrizzo *et al.*, 1994). It exhibits a dark red spot on tlc in daylight, when sprayed with ANIS and heated with a hair dryer. The red spot fades to a reddish brown spot after cooling at room temperature. Stemphol is also produced by *S. majusculum* (Stodola *et al.*, 1973).

Stemphol related compound. An unknown stemphol derivative was isolated from *S. botryosum* (IBT 8213). The structure of this compound could not be fully elucidated because of the small amount of compound available. However, its uv spectrum was identical to that of authentic stemphol. Treatment with H₂SO₄ and ANIS gave the same reaction for stemphol and the stemphol related compound (see Table 1 and 2). These findings indicate that the stemphol related compound must have the same chromophore and the same functional groups as stemphol. A mass spectrum of the stemphol related compound showed a molecular ion at 252 mass units compared with that of stemphol at 236 mass units. This could suggest a stemphol structure with an addition -OH, which is in agreement with the increased polarity of the compound.

Table 3 shows the result of the screening of 33 *Stemphylium* isolates for production of the five metabolites considered herein. Within the species of *S. botryosum* two different chemotypes were identified based on their metabolite profiles detected by tlc and hplc analyses. *S. botryosum* Type I which is able to produce all five metabolites and *S. botryosum* Type II only producing stemphylin, stemphyloxin II and stemphyperlenol. *S. botryosum* Type II is also able to produce macrosporin, a metabolite known from *Alternaria* spp. (Montemurro & Visconti, 1992). *S. herbarum* produces only stemphyloxin II and stemphyperlenol. Table 3 also shows that the screened isolates of *S. vesicarium* and *S. alfalfae* are able to produce stemphol, which has not been reported previously.

CONCLUSION

Chemotaxonomy and morphology is a powerful combination for identification of *Stemphylium*. Both hplc and tlc

Table 3. Metabolite production in different species of *Stemphylium*

Species ^a (numbers of isolates)	Stem- phylin	Stem- phyloxin II	Stemphy- peryleneol	Stemphol	Stemphol related compound
<i>P. tarda</i> (1)	+	+	+	+	+
<i>S. botryosum</i>					
Type I ^b (5)	+	+	+	+	+
Type II ^c (7)	+	+	+	ND	ND
<i>P. herbarum</i> (4)	+	+	+	ND	ND
<i>S. herbarum</i> (4)	ND	+	+	ND	ND
<i>S. majusculum</i> (1)	ND	+	+	+	+
<i>S. alfalfae</i> (2)	ND	+	ND	ND	ND
<i>S. sarciniforme</i> (4)	ND	+	ND	ND	ND
<i>S. vesicarium</i> (5)	+	+	+	+	ND

^a *P.*, *Pleospora*; *S.*, *Stemphylium*.
^b Type I group is represented by isolate IBT 8213.
^c Type II group is represented by isolate IBT 8226.
 ND, not detected on tlc or hplc.

analyses of the metabolite profile provide useful information in the chemotaxonomic characterization of *Stemphylium* species. However, chromatographic and spectroscopic data (R_f values, reflectance spectra, retention indices and ultraviolet spectra) of the metabolites investigated and the other unknown metabolites in the profile should always be used together and in combination with morphological characteristics in order to avoid misidentification of metabolites or isolates. The current investigation represents an example of the combined application of hplc and tlc to analyse the metabolite profiles of a limited number of *Stemphylium* isolates, which enables the mycologist to identify or confirm different species of *Stemphylium* on the basis of five known metabolites. Moreover, the two distinct metabolite profiles within the species *S. botryosum* suggest further studies to test the two chemotypes for other differences, e.g. pathogenicity towards different host plants.

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