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Identification and determination of mellein in cultures of the fungus *Septoria nodorum* (Berk.) by thin-layer and high-performance liquid chromatography

Jutta Sachse

Swiss Federal Research Station for Agronomy, Reckenholz, CH-8046 Zurich (Switzerland)

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ABSTRACT

In order to select *in vitro* wheat embryos with a high level of resistance to the fungal pathogen *Septoria nodorum* (Berk.), extracts from *Septoria* cultures grown on infected wheat grains were prepared. One of their toxins, mellein, was identified by thin-layer chromatography and its content determined by high-performance liquid chromatography using UV light and fluorescence detection. Synthetic (±)mellein was used as standard. We investigated two techniques of *Septoria* cultivation, different methods of mellein extraction and the stability of the extracted mellein. In these experiments, the content of mellein in fresh fungal cultures amounted to 46 mg/kg, but in the dry material only 21 mg per kg of dry matter were recoverable.

INTRODUCTION

Fungal infection is a cause of significant yield losses in grain crops. The fungus *Septoria nodorum*, a prominent wheat parasite [1,2], retards germination and growth of seeds and young plants and suppresses carbon dioxide assimilation [3–7]. A high level of resistance to this pathogen is, therefore, an important selection criterion for wheat breeders. Because determination of resistance using traditional techniques is a lengthy process, the development of *in vitro* methods to test embryos or plant tissues using extracts of *Septoria* cultures or of one of its identified toxins was undertaken.

Previously, we have tested extracts from infected wheat plantlets, prepared by the Kietreiber test method [8]. The extracts were separated using thin-layer chromatography (TLC) [7] and the biological activity of some of the bands visible with UV light was examined. We could confirm growth suppression of coleoptiles and roots of germinating wheat grains.

For our biological assays, a pure fungus toxin with a known content in *Septoria* cultures is necessary as a standard. Since none of the *Septoria* toxins was commercially available, mellein (3,4-dihydro-8-hydroxy-3-methylisocoumarin), which is well known and synthesizable, was chosen. For the identification and determination of mellein, we decided to use TLC and high-performance liquid chromatography (HPLC), respectively, an elegant alternative to biological tests for determining the content of numerous mycotoxins. This technique has been used particularly for the isolation and purification of mycotoxins needed for tests on plant material. As yet, no determinations of *Septoria* toxins by HPLC have been reported in the literature. However, there is one publication on the determination of mellein and other toxins in filtrate cultures of *Aspergillus ochraceus* [9]. Filtrates of cultures contain fewer interfering substances than extracts from wheat grains infected with *S. nodorum*. Therefore,

Correspondence to: Dr. J. Sachse, Swiss Federal Research Station for Agronomy, Reckenholz, CH-8046 Zurich, Switzerland.

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we first had to develop a method suited to our research; this is the subject of this paper.

EXPERIMENTAL

Cultivation of S. nodorum on wheat grains

Two procedures were used for the cultivation of *S. nodorum*:

(a) According to Fried [10], yielding a brown, finely crushed, dry powder (cultivation *a*).

(b) According to Kietreiber [8], consisting of fungus grown on grains germinating on a substrate of wet filter paper (cultivation *b*).

Extraction

(a) A 30-g aliquot of dry powder (water content 12.75%) was extracted for at least 8 h (or preferably overnight) with 450 ml of ethyl acetate in a Soxhlet apparatus. For the biological test, 100 g of the powder must be extracted and processed. It may be necessary first to remove lipids from the material by extraction with petroleum ether (b.p. 40/70°C) for 3 h.

(b) The wet grains and the filter paper from two dishes were cut into pieces of approximately 1 cm^2, crushed in a mortar and extracted in a Soxhlet apparatus.

The extracts were concentrated under vacuum at 50°C to an oily consistency. Residues of ethyl acetate, free acetic acid and oxygen were removed with a stream of nitrogen. The concentrated extract was then flushed with ethyl acetate into a 25- or 100-ml measuring flask, depending on the amount of the extracted material. If necessary, the solution was diluted prior to measurement.

Identification of mellein

Thin-layer chromatography. As a standard, (±) mellein, synthesized by Fluka (Buchs, Switzerland) (m.p. 38–40°C) was used. It had a purity of 98% (gas chromatography) and absorption maxima at 246 and 314 nm in ethanol, with molar extinction coefficients of 6740 and 4350, respectively. These data correspond to those in the literature [11,12].

Thin-layer plates (10 × 20 cm) were prepared with silica gel (Art. 7731; Merck, Darmstadt, Germany) and with silica gel with fluorescence indicator (Art. 7730, Merck) as follows: 2.75 g of silica gel suspended in ethanol–water (9:1, v/v) were poured onto the plate. The eluent was light petroleum (b.p. 40–70°C)–ethyl acetate (60:40, v/v); the R_F of 10 μg of mellein is 0.62. For polyamide plates (10 × 20 cm), 1 g of polyamide Woelm (ICN Pharmaceuticals, Eschwege, Germany) with an addition of 0.2 g of cellulose MN 300 (Macherey–Nagel, Düren, Germany) was suspended in 6 ml of 96% ethanol and dispersed on the plate. The eluent was ethanol–water (40:60, v/v), R_F 0.38. On thin-layer plates, mellein was detected with the following spray reagents: 1 *M* sodium hydroxide, ammonia, ferric chloride (1% in water) and reagents according to Krebs *et al.* [13], Nos. 3, 91, 108 and 230. For two-dimensional TLC, 20 × 20 cm plates were used with twice the amount of the sorbents mentioned above and the same eluents.

High-performance liquid chromatography. The experimental conditions for mellein determination were as follows: pump series 3B; UV detector LC 75 with autocontrol, 246 nm, 16 a.u.f.s.; fluorescence detector LS-3B, excitation 326 nm, emission 458 nm (Perkin-Elmer, Norwalk, CT, USA); sample injector 7125 with 20-μl loop (Rheodyne, Cotati, CA, USA); recorder W + W 320 (Scientific Instruments, Münchenstein, Switzerland); integrator 4290 (Spectra-Physics, San Jose, CA, USA); column ODS Hypersil 5 μm, 250 × 4 mm I.D. (Hewlett-Packard, Corvallis, OR, USA); eluent acetonitrile (Merck Art. 14291), double-distilled water (proportions below), Fontavapor 285 (Büchi Laboratoriums-Technik, Flawil, Switzerland), flow-rate 1 ml/min, chart speed 10 cm/h.

A 0.5-μg aliquot of pure mellein was eluted by acetonitrile–water (80:20, v/v) in 3 min. However, for its detection in the above-mentioned extracts, an acetonitrile–water ratio of 30:70 (v/v) was necessary. Its retention time in this case amounts to 21 min (see Fig. 1). Prior to injection, the extracts were filtered through a 0.5-μm filter FHLP 01300 (Millipore, Bedford, MA, USA). The mellein peak is identified in four ways: by comparison with an extract from non-infected wheat grains, by addition of pure mellein to an extract, by recording the absorption spectrum with the UV detector and the autocontrol and fluorescence detection.

Determination of mellein content

A plot of the extinction at 246 nm of 10–50 μg of mellein per ml of ethanol results in a straight line.

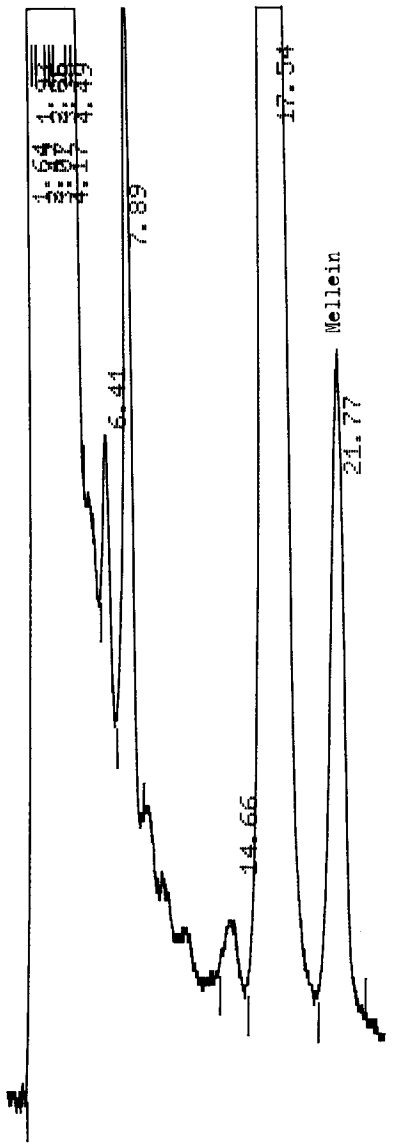

Fig. 1. HPLC of a *Septoria* extract from cultivation *a*. A 100-g aliquot of powder extracted 15 h according to Soxhlet. The concentrate was dissolved in 50 ml of ethyl acetate and diluted 1:10. Detection by fluorescence. For chromatographic conditions, see the Experimental section. Numbers at peaks indicate retention times in min.

But in this investigation, the more intensive and specific fluorescence was preferred. Here, a linear response is obtained between 0.01 and 1 μg of mellein per ml of ethanol. The detection limit was 5 ng at a signal-to-noise ratio of 2. As the external standard 0.2 μg of mellein was chosen for peak integration.

RESULTS AND DISCUSSION

For mellein extraction, ethyl acetate is superior to more polar and non-polar solvents. An extractant with a boiling point above 75°C and a medium polarity is indicated. Under such conditions, the sample must be extracted in a Soxhlet apparatus for at least 8 h, or preferably overnight. Prior extraction with petroleum ether does not cause a loss of mellein. Extraction by stirring at room temperature or at 50°C gives significantly smaller mellein yields from cultivation *a*. The isolation of mellein from fungal cultures on wheat grains depends on their consistency. Extraction is more difficult from the powder obtained by cultivation method *a* than from the wet material of method *b*. Stirring the latter at room temperature for 4 h yields more mellein than a Soxhlet procedure of equal duration. The result is different when the material is carefully dried in a desiccator before extraction. Then, despite grinding, mellein from the fungus culture *b* is less easily extracted. This is apparently because of shrinking of the grains, resulting in a stronger bond or destruction of the toxin (Table I).

The detection, identification and determination of mellein by TLC and HPLC is more handicapped by interference by other substances —possibly resulting from oxidation during the drying process— when cultivation *a* is used. Thus, for this material, it is necessary to carry out a two-dimensional TLC in the same eluent or to use silica gel plates 35 cm long. With cultivation *b* there are fewer problems with both techniques.

The quick technique of TLC was used for preliminary investigations of the possible occurrence of mellein. To date, only the unspecific reagent ferric chloride has been applied to detect mellein on thin-layer plates. We were interested in identifying mellein with a higher degree of certainty using different colour reactions. On thin-layer plates mellein is visible as a white–blue spot under long-wave UV light and it quenches the fluorescence on plates with a fluorescence indicator. With ammonia, it appears cornflower blue; with ferric chloride violet–red; with Echtblausalz B No. 91 [13] brown-orange; with

TABLE I

CONTENT OF MELLEIN IN FUNGUS CULTURES OF *SEPTORIA NODORUM* (BERK.), CULTIVATED ON WHEAT GRAINS (mg PER kg OF DRY MATTER)

The values are the results of 2–4 total analyses.

Extraction procedure and duration	Cultivation *a*		Cultivation *b* with filter paper[a]			
	Sample amount (g)	Dried and ground	Sample amount (g)	Wet and crushed	Dried	Dried and ground
Soxhlet						
4 h	100	10.5	13.5[b]			68.9
			20.25	29.2		Extraneous infection
8 h	100	15.4				
	30	20.0	13.5	45.9		37.2
	15	20.4				
15 h	100	20.9	6.75			
Stirring for 4 h						
25°C	100	1.6	13.5	32.3	19.5	19.9
50°C	100	3.0	13.5	34.0		

[a] The whole filter paper with wheat grains was cut into 1-cm^2 pieces before each subsequent treatment.
[b] Pro plastic sheet 200 grains = 6.75 g.

alumium chloride No. 3 [13] intensive blue. With Folin–Ciocalteus reagent No. 108 [13] a weak greyish-blue spot develops slowly. The most suitable indicator is Pauly's reagent No. 230 [13], which stains mellein yellow, like sunflowers. Such a spot changes to brown with 1 *M* sodium hydroxide solution. Thus, mellein is detectable successively by four colour tests on the same plate: UV light, ammonia, Pauly's reagent (after drying the plate) and, finally, by sodium hydroxide solution.

The experimental conditions described for HPLC were optimal for the determination of mellein. Using methanol instead of acetonitrile as eluent, altering the acetonitrile–water proportion or the flow-rate, or substituting the ODS Hypersil column by a LiChrospher RP-18 did not improve the separation of mellein from the other substances in the crude extract.

Mellein is most stable in concentrated oily extracts treated with nitrogen and stored at −20°C in the dark. Mellein is not volatile under vacuum (water-jet pump) and is thermostable. The nitrogen treatment removes ethyl acetate, free acetic acid and oxygen residues; without this treatment, only 60% of the mellein content can be determined. Half the mellein was lost within a week when the extracts were diluted with ethyl acetate and stored in the refrigerator or at room temperature in day light or in the dark.

Using the described methods, the mellein content of the analysed material amounted to 21 mg per kg of dry matter from cultivation *a*, but 46 mg/kg from cultivation *b*. The latter material is less homogeneous; its mellein content depends on the degree of *Septoria* infection of the single grain, the fungus growth and the mellein production. The mellein content of cultivation *b* is considerably increased when the grains are infected with other fungi in addition to *Septoria* (Table I). The relative standard deviations of the determination of pure mellein, within and between extracts, are shown in Table II. Recovery tests were performed with uninfected wheat grains spiked with different amounts of mellein: 96% recovery was obtained from 20 mg of mellein per kg of wheat, 94% from 5 mg/kg and 89% from 1 mg/kg (n = 3; relative standard deviation 1.85, 2.46, 3.74%, respectively). The detection limit was 500 μg of mellein per kg of wheat at a signal-to-noise ratio of 2. Mellein is not detectable on heavily infected wheat grains without *Septoria*

TABLE II

PRECISION AND ACCURACY OF MELLEIN DETERMINATION

Amounts of mellein	Relative standard deviation (%) (n = 3)	
	Within extracts	Between extracts
Pure mellein		
50 ng per 20 μl of ethanol	1.40	
100 ng per 20 μl of ethanol	1.35	
200 ng per 20 μl of ethanol	1.32	
Extracts from		
Cultivation *a*		
10 mg per kg of dry matter	2.90	5.85
15 mg per kg of dry matter	2.75	5.79
20 mg per kg of dry matter	2.67	5.70
Cultivation *b*		
20 mg per kg of dry matter	2.59	8.35
30 mg per kg of dry matter	2.55	8.10
45 mg per kg of dry matter	2.50	7.65

cultivation. This result and those in Table I show that mellein is produced in the mycelium and is destroyed to different degrees when the mycelium perishes.

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