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Secondary Metabolite and Mycotoxin Production by the *Rhizopus microsporus* Group

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Fast-growing Zygomycetes, most notably *Rhizopus oligosporus*, are traditionally used in many food fermentations, for example, for soybean tempeh production. *R. oligosporus* is considered to belong to the *Rhizopus microsporus* group. Certain *R. microsporus* strains have been reported to produce either the pharmaceutically active rhizoxins or the highly toxic rhizonins A and B. In this study was investigated the formation of secondary metabolites by *R. microsporus*, *R. oligosporus*, and *Rhizopus chinensis* grown on a wide range of different semisynthetic and natural substrates. Liquid chromatography, combined with photodiode array detection and high-resolution mass spectrometric techniques, was used to identify secondary metabolites. Growth on maize, brown rice, and Pharma agar gave both the highest amounts and the maximum diversity of rhizoxins and rhizonins. Rhizoxins were produced by all four *R. microsporus* strains, whereas only one strain produced rhizonins. The six *R. oligosporus* and four *R. chinensis* strains investigated did not produce any of these two classes of metabolites.

KEYWORDS: Tempeh; dereplication; LC-MS; mycotoxin; chemotaxonomy; rhizonin; rhizoxin

INTRODUCTION

The fungus *Rhizopus oligosporus* Saito is traditionally used in Indonesia to ferment soybeans to produce the food tempeh (tempe). Tempeh is becoming increasingly popular as a meat substitute in North America, Japan, and Europe, where it is now produced on an industrial scale (*1*). Legumes other than soybeans, as well as cereals, can also be used to produce tempeh (*2*). *R. oligosporus* fermentations can further be used to manufacture bongkrek (tempeh bongkrek) from vegetable byproducts, tauco made from soybeans and cereals (*3*, *4*), and sufu pehtze from soy tofu (*5*). Furthermore, production of industrial enzymes, for example, phytase, by *R. oligosporus* has also been investigated recently (*6*).

The most recent major taxonomic revision of the genus *Rhizopus* was carried out by Schipper and Stalpers (*7*). Using mainly morphological criteria such as spore shape and ornamentation, supplemented with data on maximal growth temperatures, they identified three species complexes or species groups within the genus: the *R. oryzae* group (Went & Princ. Geerl.); the *R. stolonifer* ((Ehrenb.: Fr.) Vuill.) group; and the *R. microsporus* (Tiegh.) group. The taxa within the last-named group were difficult to differentiate on the basis of morphology, and Schipper and Stalpers (*7*) considered *R. chinensis* Saito and *R. rhizopodiformis* Cohn to be varieties of *R. microsporus* Tiegh. on the basis of their ability to mate and form zygospores. *R. oligosporus* did not produce zygospores with any tester strains, but was still considered to be a variety of *R. microsporus*. More recent studies have confirmed that these four taxa are related (*8*−*10*). The varietal status of *R. oligosporus* and *R. chinensis* has been a matter of discussion and also has implications for the safety of strains used in food fermentation and biotechnology (*11*). In addition, the use of zygospore morphology as a principal taxonomic criterion is debatable, and new characters must be sought.

The tempeh fungus *R. oligosporus*, suggested to be a domesticated form of *R. microsporus* (*11*), has only been isolated from tempeh and other fermented products and never from nature. It has only once been isolated from a human case of zygomycosis (*12*), but the strain involved may be a degenerated strain of *R. microsporus*. *R. chinensis* has been isolated from vegetable material and also from tempeh (*13*) and participates in fermentations of lao-chao (tape ketan) from rice (*3*). It is not associated with human or animal infections, but toxicity to rats and ducklings has been reported (*14*, *15*). *R.*

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Table 1. *Rhizopus* Strains Investigated in the Present Study

scientific name	strain[a]	no. in other collections[a]	source
R. chinensis	CBS 388.34[b]		unknown, USA
R. chinensis	CBS 394.34[b]		unknown, USA
R. chinensis	CBS 537.80[b]	MRC 1639	sorghum malt, South Africa
R. chinensis	CBS 631.82[b]	ATCC 52807, IMI 130842	bread, China
R. microsporus	CBS 699.68[b]	ATCC 52813, VKM F-733, CCRC 31140	soil, Ukraine
R. microsporus	CBS 700.68[b,c]	ATCC 52814, VKM F-774, CCRC 31141	forest soil, Georgia
R. microsporus	CBS 308.87[b]		human tissue, Australia
R. microsporus	CBS 112285[c]	MRC 303	groundnuts, Mozambique
R. oligosporus	CBS 112586[c]	ATCC 60826, NRRL 5905	tempeh, Indonesia
R. oligosporus	CBS 112587[c]	ATCC 76011, ATCC 96528	tempeh, Indonesia
R. oligosporus	CBS 112588[c]	ATCC 48012, NRRL 6495, NRRL A-9868	tempeh, Indonesia
R. oligosporus	CBS 112589[c]	ATCC 48011, NRRL 6205, NRRL A-9867	tempeh, Indonesia
R. oligosporus	ATCC 48109[c]		tempeh, The Netherlands
R. oligosporus	ATCC 64063[c]		tempeh, Indonesia

[a] ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI, CABI Bioscience Genetic Resource Collection, Egham, U.K.; MRC; South African Medical Research Council, Tygerberg, South Africa; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, IL; VKM, All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia. [b] Strains investigated in the first study. [c] Strains investigated in the second study.

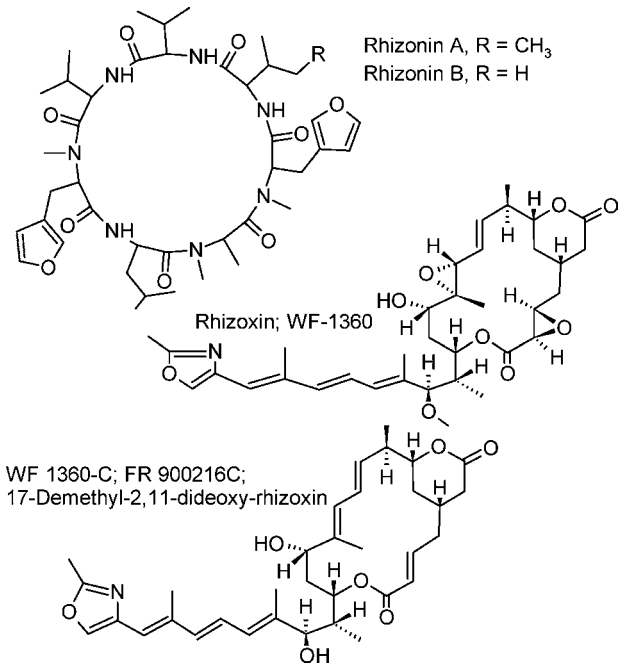

Figure 1. Structures of rhizonins A and B and some of the rhizoxins.

microsporus is found in soil (*7*) and in food from tropical climates (*16*), and it is able to cause zygomycosis (*17*).

Compared with other microfungi, members of the *R. microsporus* group have so far not been shown to produce many secondary metabolites, especially when false, methodologically unsound reports of aflatoxin production (*18*−*20*) are discounted. The rhizoxins (**Figure 1**) are potent anticancer drugs, and nine analogues have been isolated from *R. microsporus* strain Rh-2 (deposited as *R. chinensis* at American Type Culture Collection, ATCC, Manassas, VA) and *Rhizopus* sp. strain F-1360 (deposited at ATCC) (*21*−*24*). Interestingly, rhizoxins have also been isolated from *Pseudomonas* spp. (*25*, *26*). An artifact of rhizoxin formed by hydrolysis of the C_2-C_3 epoxy group has also been described (*27*). Two cyclic peptides rhizonins A and B (**Figure 1**), which are mycotoxins with strong hepatotoxic activity, have been isolated from *R. microsporus* MRC 303 (CBS 112285; see **Table 1**) (*28*, *29*). Finally, an antibacterial protein has been described from *R. oligosporus* (*30*).

None of the metabolites described above should be permitted to occur in foods, making it important to investigate if members of the *R. microsporus* group can produce these metabolites. In the present study, we investigated secondary metabolite formation by four strains of *R. microsporus*, six strains of *R. oligosporus*, and four strains of *R. chinensis*. For this analysis, strains were grown on a wide range of semisynthetic and natural substrates. The results have obvious implications for the safety of *Rhizopus*-fermented food and could also contribute toward a taxonomic revision of this genus.

MATERIALS AND METHODS

Solvents and Reagents. Solvents were of HPLC grade, and other chemicals were of analytical grade. Rhizoxin was obtained from Sigma-Aldrich (St. Louis, MO). Water was purified from a Milli-Q system (Millipore, Bedford, MA).

Fungal Strains. Fourteen *Rhizopus* strains from the *R. microsporus* group were selected (**Table 1**). The strains have previously been identified according to their morphology, temperature requirements, and mating abilities (*7*). *R. oligosporus* strains were maintained on silica beads (*16*, *31*) at 4 °C, and *R. chinensis* and *R. microsporus* were stored lyophilized. Cultures were transferred, grown and maintained on slants of 2% malt extract agar (MEA), and stored at 4 °C until used for preparing inocula.

Culture Media. To identify media promoting secondary metabolite production, eight different standard cultivation media were screened: Czapek yeast extract agar (CYA), MEA, oatmeal agar (OMA), potato−carrot agar (PCA), potato−sucrose agar (PSA), rice meal agar (RA), V-8 agar (V8), and yeast extract sucrose agar (YES) (*31*). Trace metal solutions were added to all agar media (*31*). Bacto 212750 yeast extract [Becton Dickinson (BD), Sparks, MD] and Bacto 218630 malt extract (BD) were used as ingredients. In addition to the standard cultivation media, Pharma agar, brown rice, white rice, and maize were prepared as described below. Pharma agar (*30*) contained 40 g of cottonseed flour (Sigma-Aldrich), 30 g of maltose monohydrate, 10 g of Bacto 211677 peptone (BD), 2.5 g of KH_2PO_4, 7.5 g of K_2HPO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 2 g of $(NH_4)_2SO_4$, 15 g of agar, and water to 1 L. Both brown rice and white rice (purchased in local supermarkets) were prepared by mixing 200 g of rice, 25 g of finely ground rice, and 92 g of tap water. Maize (purchased in a feed store) was prepared by mixing 150 g of maize kernels and 150 g of tap water. Brown rice, white rice, and maize were soaked for at least 30 min and autoclaved two times on two consecutive days. Rice (23 g) and maize (30 g) were aseptically transferred to Petri dishes.

Inoculation and Incubation. All Petri dishes were incubated in perforated plastic bags in the dark. All substrates were inoculated at a single point with a small piece of mycelium from cultures grown on MEA at 30 °C for 3 days.

In a first study, *R. chinensis* strains CBS 388.34, CBS 394.34, CBS 537.80, and CBS 631.82 were inoculated onto CYA, MEA, OMA, PCA,

PSA, RA, V8, YES, brown rice, white rice, and maize. *R. microsporus* CBS 699.68, CBS 700.68, and CBS 308.87 were also inoculated onto Pharma agar. Brown rice, white rice, and maize were incubated for 5 days at 30 °C, whereas all agar media were incubated for 5 days at both 25 and 30 °C.

In a second study, *R. microsporus* CBS 700.68 and CBS 112285 and *R. oligosporus* CBS 112586, CBS 112587, CBS 112588, CBS 112589, ATCC 48109, and ATCC 64063 were cultivated on CYA, YES, Pharma agar, brown rice, and maize. Agar media were incubated at 30 °C for 5 days and the natural substrates at 25 °C for 7 and 21 days.

R. microsporus CBS 112285 was also cultured in an Erlenmeyer flask with maize for 21 days in the dark according to a procedure previously used to isolate rhizonin A (*29*).

Extraction. The microscale extraction method of Smedsgaard (*32*) was modified to suit the substrates and cultures used in this study. Extracts from cultures on agar media were prepared by cutting three (first study) or seven (second study) plugs of 6 mm diameter from the rim to the center of a colony, so that both new and old mycelia were extracted. In the first study, 4−5 rice grains and 2 maize kernels were extracted, and in the second study, 9−11 grains and 4−6 kernels were extracted (also selected from rim, middle, and center of the colonies). Plugs or kernels were transferred to screw-cap vials, then 0.7 mL (first study) of solvent mixture methanol/dichloromethane/ethyl acetate (1:2:3) containing 1% (v/v) formic acid or 1.4 mL (second study) was added, and vials were ultrasonicated for 60 min. The extracts were transferred to clean vials and the organic phases evaporated to dryness with nitrogen at room temperature overnight. Unincubated media were extracted and used as negative controls.

Analysis. Samples were redissolved in 400 μL of methanol, filtered through a 13 mm 0.45 μm PTFE syringe filter (SRI, Eatontown, NJ), and analyzed on an Agilent (Torrance, CA) 1100 liquid chromatographic system equipped with a photodiode array detector (DAD).

Samples, 5 μL, from the first study were separated on a 100 × 4 mm i.d., Hypersil BDS-C_{18} 3 μm, column (Agilent), using a flow rate of 1 mL/min and a binary gradient starting with 85% water and 15% acetonitrile (CH_3CN) and going to 100% CH_3CN in 40 min, maintaining 100% CH_3CN for 5 min (*32*). Trifluoroacetic acid, 50 ppm, was added to both solvents (*32*).

Samples from the second study were analyzed on the same Agilent system, now equipped with a 100 × 2 mm i.d., 3 μm, Luna C_{18} II column (Phenomenex, Torrance, CA) fitted with a Phenomenex SecurityGuard C_{18} precolumn. The flow rate was 0.4 mL/min, and CH_3CN was increased from 15 to 100% in 20 min, keeping this for 5 min; 3 μL of sample was injected.

Samples from the second study were also analyzed by LC−high-resolution mass spectrometry (LC-HR-MS) on an Agilent 1100 LC system equipped with DAD and a 50 × 2 mm i.d., 3 μm, Luna C_{18} II column. The flow rate was 0.3 mL/min, and the gradient was the same as used on the other Luna column, but the water was instead buffered with 20 mM formic acid and 10 mM ammonium formate and the CH_3CN with 20 mM formic acid. The system was coupled to an LCT orthogonal time-of-flight mass spectrometer (Micromass, Manchester, U.K.), equipped with a Z-spray electrospray source and a LockSpray probe. The MS was operated in the positive electrospray ionization (ESI^+) mode, tuned to a resolution >6500 (at half-peak height), and data were collected as centroid data from *m*/*z* 100 to 900 as described by Nielsen and Smedsgaard (*33*). The desolvation temperature was 450 °C, the source block temperature 120 °C, the capillary potential 3000 V, and the desolvation flow of nitrogen (99.9%) 0.430 m^3/h.

Data Analysis. Peaks, excluding those found in negative controls, were matched against an internal reference standard database (~550 compounds) (*33*), which does not include rhizonins A and B or rhizoxins other than rhizoxin itself. Metabolites not available as reference standards were dereplicated (tentatively identified) by UV spectra combined with high-resolution ESI^+ spectra as illustrated in Nielsen and Smedsgaard (*33*) and matched against literature data for *Rhizopus* metabolites (*21*−*24*) as well as Antibase 2003 (~30000 microbial secondary metabolites, Wiley, Hoboken, NJ).

Extracted ion chromatograms (±0.01 *m*/*z*) were reconstructed from $[M + H]^+$ and $[M + Na]^+$ ions of rhizonins A and B (*28*, *29*). For the rhizoxins, $[M + H]^+$, $[M + Na]^+$, and $[M + H - CH_4O]^+$ ion traces were plotted and compared to the characteristic UV absorptions in the 294−316 nm range from the UV trace.

RESULTS AND DISCUSSION

LC-UV Screening of Media. *R. microsporus* was grown on eight different standard cultivation media (first study), as well as Pharma agar, brown rice, white rice, and maize substrates, to identify media enhancing secondary metabolite production (**Table 2**). The three *R. microsporus* strains CBS 699.68, CBS 700.68, and CBS 308.87 produced rhizoxins, and this production did not seem to be influenced by the incubation temperature. After identification of rhizonins by LC-HR-MS in the second study, reanalysis of the LC-UV data files from the first study did not reveal peaks corresponding to rhizonin A or B in any of the strains. The four *R. chinensis* strains studied did not produce rhizoxins or rhizonins under any of the tested conditions.

In the first study, rhizoxins could be identified to only group level on the basis of their specific UV spectra, but it was obvious, however, that among the culture media tested, Pharma agar and brown rice yielded the greatest diversity and the highest quantities of rhizoxins, although they were also produced on OMA and RA (**Table 2**). Consequently, Pharma agar and brown rice were chosen for study 2, in which they were supplemented with CYA and YES, as well as maize, which had previously been used to isolate rhizonin A (*29*).

The complexity of the nutrient composition seemed to influence the patterns of secondary metabolite production. *R. microsporus* growth on brown rice gave significantly higher amounts of rhizoxins than growth on white rice. This is presumably due to the bran, which contains more nitrogen and trace elements than are found in the starch endosperm that constitutes most of the white rice kernel. However, the presence of a complex carbohydrate source might also be important, as YES (15% sucrose and a complex nitrogen source) gave poor yields compared to Pharma agar, which contained both a complex nitrogen source (peptone) and cottonseed flour as complex carbohydrate source.

Dereplication of Rhizoxins and Rhizonins. Culture extracts (study 2) from the original rhizonin producer, *R. microsporus* CBS 112285 (*28*), were inspected for compounds with UV spectra similar to those of the rhizoxins and rhizonins. These could then be tentatively identified by UV and high-resolution ESI^+ mass spectra, as illustrated in **Figure 2** and described in detail for other metabolites by Nielsen and Smedsgaard (*33*). In this process it is not possible to differentiate isomers unless they have different UV spectra or specific fragmentation patterns, for example, as is the case with peptides where fragmentations of the peptide bonds can show the amino acid sequence. **Table 3** shows the UV and MS data of the major peaks identified from *R. microsporus* CBS 112285 and CBS 700.68 cultivated on Pharma agar, maize, and brown rice. From the ESI^+ traces it appeared that rhizonin A ($[M + H]^+$ *m*/*z* 812.4822−812.5022) and four rhizoxins (290−320 nm UV trace and MS traces of all the rhizonin ions described in **Table 3**) were the dominant secondary metabolites of *R. microsporus* CBS 112285 (**Figure 2**).

For rhizonins, as is common with many cyclic peptides, $[M + H]^+$and $[M + Na]^+$ were the predominant ions. To produce fragment ions (in source) more abundant than $[M + H]^+$, a very high potential difference between the two skimmers needed to be applied (>50 V, corresponding to 75−100 V on other Micromass instruments). In **Table 3**, 10 major ions (including 8 fragment ions), produced by ion-source fragmentation, for the

Table 2. Production of Rhizoxins and Rhizonins from *R. microsporus* CBS 699.68, CBS 700.68, and CBS 308.87 on Various Media after 5 Days of Incubation at 25 and 30 °C (First Study)

		CBS 700.68			CBS 699.68			CBS 308.87		
			rhizoxin-like peaks[a]			rhizoxin-like peaks[a]			rhizoxin-like peaks[a]	
medium	temp (°C)	rhizonins A and B[b]	no.	∑ peak area (mAu·S)	rhizonins A and B[b]	no.	∑ peak area (mAu·S)	rhizonins A and B[b]	no.	∑ peak area (mAu·S)
brown rice	30	ND[c]	4	1280	ND	3	880	ND	1	640
CYA	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
CYA	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
maize	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
MEA	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
MEA	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
OMA	25	ND	1	160	ND	1	120	ND	ND	ND
OMA	30	ND	1	160	ND	ND	ND	ND	ND	ND
PCA	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
PCA	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pharma	25	ND	5	3518	ND	1	560	ND	1	96
Pharma	30	ND	7	3520	ND	2	880	ND	1	104
PSA	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
PSA	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
RA	25	ND	1	160	ND	1	128	ND	ND	ND
RA	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
V8	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
V8	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
white rice	30	ND	ND	ND	ND	ND	ND	ND	ND	ND
YES	25	ND	ND	ND	ND	ND	ND	ND	ND	ND
YES	30	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Peak area at 290–320 nm, from LC-UV (Luna C_{18} II) screening with the TFA-buffered system. [b] Based on reanalysis of LC-UV data from first study after knowing where the rhizonins eluted in the chromatograms of the second study. [c] Not detected.

two rhizonins are shown. All except the *m*/*z* 180 fragment could be explained by breaking two amide bonds, validating the primary structure of the two rhizonins known to be produced by this particular strain CBS 112285(*29*). The UV spectra of the rhizonins were, as previously described, nonspecific end-absorption spectra, and consequently specific analyses for these can be performed only by LC-MS. Besides rhizonins A and B, two presumed peptides (PEP 1 and 2) (**Table 3**) were also detected. As the peak areas of these correlated (**Table 4**) with the peak areas of rhizonins A and B, and they were not detected in the six *R. oligosporus* strains or the four *R. chinensis* strains, we hypothesize that they could be related to the rhizonins.

CBS 700.68 did not produce detectable quantities of rhizonin A or B or PEP 1 (**Table 4**). However, PEP 2 was produced along with unique PEP 3 (**Table 3**). In-source induced fragmentation of these presumed peptides showed few fragment ions, from which the primary structure could not be unambiguously determined. A search using the Scifinder software and Antibase did not reveal any natural peptides with the same mass as PEP 1−3, and thus are we now in the process of isolating sufficient quantities for structure elucidation by NMR techniques.

Most ESI^+ spectra of the rhizoxins showed predominant loss of 32.026 Da (**Table 3**), which corresponds to the loss of CH_4O, presumably due to the methoxy moiety. The molecular composition could therefore be calculated from $[M + H]^+$, $[M + Na]^+$, and $[M + H - CH_4O]^+$ ions. This provided a basis for assignment of the various cluster ions and subsequently the molecular mass of the rhizoxins present in small quantities. Interestingly, the extracts did not contain rhizoxin itself but an isomer with identical UV and MS spectra. Leaving the rhizoxin reference standard in pure or acidic methanol, evaporating it to dryness, or exposing it to sunlight for 5 days did not result in any artifacts that could be detected in the fungal extracts, supporting the conclusion that the rhizoxins detected in this study are not artifacts.

Influence of Media on Rhizonin and Rhizoxin Production by *R. microsporus*. In the second study, maize and brown rice were found to be the most productive substrates for formation of rhizoxins and rhizonins. No unique metabolites were produced on CYA, YES, and Pharma agar; and these media gave much lower quantities of the fungal metabolites than maize and brown rice. The high production of rhizoxins on maize, compared with Pharma agar, in the second study is different from the findings of the first study. Possible explanations for this include the different incubation times, different maize types, or, more likely, different moisture levels in the maize. It is difficult to standardize the evaporation of moisture during autoclaving of complex biological materials such as maize. However, complex natural substrates did still give the highest amounts of rhizoxins in both studies.

The rhizoxin profile did not change when *R. microsporus* CBS 112285 and CBS 700.68 were grown on the different media. Instead, there was an overall increase in the quantities of the individual components produced. Although some of the 23 different rhizoxin peaks may have originated from artifacts resulting from hydrolysis of epoxy groups (*27*), numerous undescribed analogues appears to be present, as previously only 9 naturally produced rhizoxins are described in the literature. The high number of novel rhizoxins is presumably a result of different culture conditions as solid media tend to promote formation of a diverse chemical profile (*34*) compared to the submerged conditions used in most other studies (*22*, *27*). Precise identification of all these rhizoxins will, however, require isolation and subsequent structure elucidation and is beyond the scope of this study, as all of these compounds are undesirable in food products.

The rhizonin quantities based on the peak areas of CBS 112285 grown on brown rice increased 2-fold from day 7 to 21, similar to ergosterol (result not shown), indicating that the rhizonin production followed the biomass production.

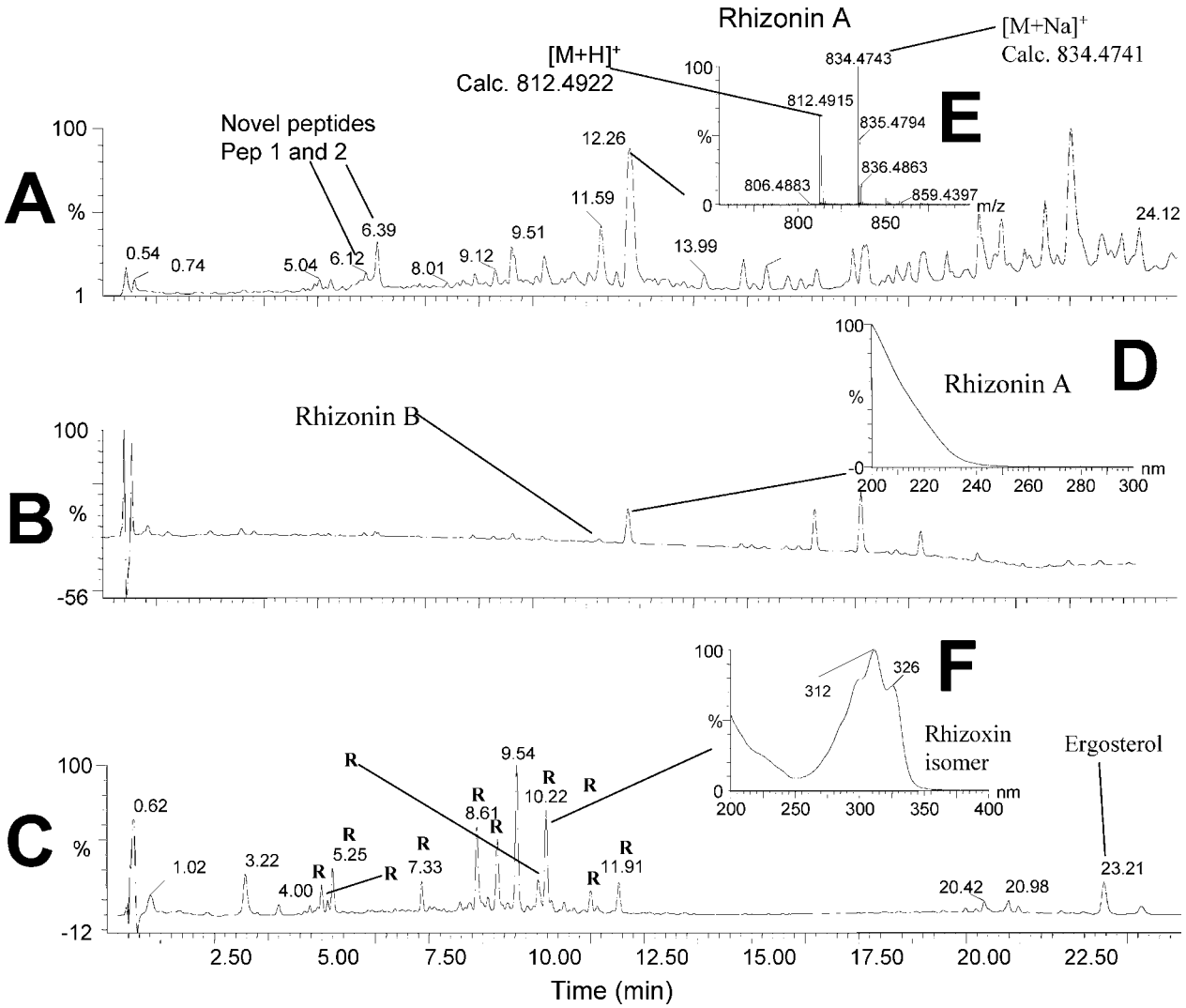

Figure 2. Liquid chromatography–diode array-positive electrospray analysis of *R. microsporus* CBS 112285 grown on maize for 21 days at 25 °C. Trace **A** is the total ion chromatogram and trace **B** the extracted wavelength chromatogram (200–220 nm) showing especially rhizonins. Trace **C** is an extracted wavelength chromatogram (290–320 nm) showing especially rhizoxins (marked **R**). Trace **D** is the UV spectrum of rhizonin A and trace **E** its positive electrospray spectrum; trace **F** is the UV spectrum of one of the rhizoxins. There is a time delay of ~0.05 min between the MS and UV signals.

No Production of Rhizonins, Rhizoxins, or Other Mycotoxins by *R. oligosporus* and *R. chinensis*. Both LC-UV and LC-HR-MS showed that none of the six *R. oligosporus* strains studied produced detectable quantities of rhizoxins or rhizonins under any of the conditions investigated (5 days on CYA, YES, and Pharma agar at 30 °C, and 7 or 21 days on brown rice and maize at 25 °C). A comparison of chromatograms of strains of *R. oligosporus* and *R. microsporus* cultivated on brown rice for 21 days at 25 °C is seen in **Figure 3**. Fungal biomass formation (estimated from the ergosterol peaks) was similar for both species.

As the rhizoxin reference standard was first available when most of the study had been finished, real detection limit experiments from spiked matrix were not performed; however, on the basis of LC-UV and LC-MS, it can be concluded that any undetected production of rhizonins and rhizoxins must have been at least 1000 times lower for the *R. oligosporus* strains than the four *R. microsporus* strains.

For the pure rhizoxin, detection limit (on column) by LC-UV was determined to be ~1 ng (s/n 10) when using the 290−320 nm chromatogram. Using LC-MS the detection limit was ~10−20 times lower. On the basis of data for compounds such as fumonisins, cyclosporin, and valinomycin (*33*), we estimate the detection limits by LC-MS for the rhizonins to be 1−10 pg (on column).

As expected, there was no sign of production of other mycotoxins (e.g., ochratoxins, aflatoxins, trichothecenes) by the six *R. oligosporus* strains analyzed with LC-UV and LC-HR-MS (*33*) in the second study or by the four *R. chinensis* strains analyzed with LC-UV in the first study. It should be noted that two of the *R. chinensis* strains have been stored as lyophilized cultures for 70 years (CBS 388.34 and CBS 394.34) and two for 25 years. Therefore, it cannot be excluded that they have lost their ability to produce some metabolites, although the four *R. microsporus* strains that were stored in the same way for 20−35 years have not lost their rhizoxin-producing ability. Also, the strain known to produce rhizonins still did this after 20 years.

Toxicity of *Rhizopus* Metabolites. Only *R. microsporus* strain Rh-2 (deposited as *R. chinensis*) and *Rhizopus* sp. strain F-1360 have been reported to produce rhizoxins (*21*−*24*). During the past two decades, *R. chinensis* has been frequently referred to as a rhizoxin producer (*35*, *36*). However, none of the *R. chinensis* strains we investigated produced rhizoxins, even when grown under a wide range of cultural conditions. Taxa belonging to the *R. microsporus* group are morphologically similar, and considerable expertise is needed for proper iden-

Table 3. Chromatographic Properties and UV and Positive-Electrospray[a] Mass Spectral Data for the Investigated Metabolites

tentative identity	retention time (min)	molecular composition	mass[b] (Da)	predominant monoisotopic ions[c] (Da) in % of base peak	UV absorption (nm) in % of UV max
PEP1[d]	6.12	$C_{43}H_{64}N_8O_9$	836	837, 859 (35)	end[e] (to 235)
PEP2[4]	6.39	$C_{43}H_{64}N_8O_9$	836	837, 859 (60)	end (to 235)
PEP3[4]	7.17	$C_{44}H_{66}N_8O_9$	850	851, 873 (25)	end (to 235)
rhizonin B	11.59	$C_{41}H_{63}N_7O_9$	797	798, 820 (95)	end (to 235)
rhizonin B[6]	11.59	$C_{41}H_{63}N_7O_9$	797	820, 798 (30), 350 (80), 534 (40), 180 (20), 265 (10), 449 (10), 501 (8), 600 (7), 685 (7)	end (to 235)
rhizonin A	12.26	$C_{42}H_{65}N_7O_9$	811	834, 812 (95)	end (to 235)
rhizonin A[f]	12.26	$C_{42}H_{65}N_7O_9$	811	350, 834 (90), 812 (80), 548 (65), 180 (50), 501 (40), 463 (45), 237 (30), 265 (30), 699 (25)	end (to 235)
17-demethyldehydroxyrhizoxin	7.37	$C_{34}H_{47}NO_9$	613	596, 578 (40), 636 (20), 614 (10)	296sh[g] (80), 312 (100), 328 (75)
dihydroxyrhizoxin	8.65	$C_{35}H_{49}NO_{10}$	643	612, 644 (37), 594 (30), 666 (28)	296sh (80), 312 (100), 326 (75)
dihydroxyrhizoxin	9.12	$C_{35}H_{49}NO_{10}$	643	612, 644 (56), 666 (40), 594 (38)	296sh (80), 312 (100), 326 (75)
undescribed rhizoxin	9.00	$C_{38}H_{55}NO_{11}$	701	724, 701 (80), 397 (80), 670 (45)	296sh (80), 312 (100), 326 (75)
dihydroxydeoxyrhizoxin	9.60	$C_{35}H_{49}NO_9$	627	596, 578 (40), 650 (25), 628 (20)	296sh (80), 312 (100), 326 (75)
dihydroxydeoxyrhizoxin	10.07	$C_{35}H_{49}NO_9$	627	596, 578 (90), 650 (80), 628 (60)	296sh (80), 312 (100), 328sh (75)
rhizoxin isomer	10.27	$C_{35}H_{47}NO_9$	625	594, 626 (90), 648 (42)	298sh (80), 310 (100), 326 (75)
rhizoxin, reference standard	11.10	$C_{35}H_{47}NO_9$	625	594, 626 (90), 648 (42)	298sh (80), 310 (100), 326 (75)
WF-1360A	11.31	$C_{36}H_{51}NO_9$	641	610, 664 (80), 592 (60), 642 (20)	296sh (80), 312 (100), 324 (75)
2-deoxyrhizoxin	11.91	$C_{35}H_{47}NO_8$	609	578, 643 (70), 610 (78)	302sh (80), 310 (100), 326 (75)

[a] At 25 V between skimmers, only inducing fragmentation of labile molecules. [b] Monoisotopic mass. [c] Isotope ions not included. [d] Presumed peptide. [e] UV max < 200 nm, which is the minimal wavelength the DAD detector can detect. [f] At 50 V between skimmers, to induce fragmentation. [g] Shoulder.

Table 4. Production of Rhizonins and Rhizoxins (Second Study)

			rhizonines			peptides[a]			rhizoxin-like peaks	
medium	temp (°C)	days	A peak area[b] (mAu·S)	A peak area[c] (ions)	B peak area (ions)[c]	PEP 1[c] (ions)	PEP 2[c] (ions)	PEP 3[c] (ions)	no.	∑ peak area[d] (mAu·S)
CBS 112285										
Pharma	30	5	457	1813	500	76	800	ND[e]	4	3360
YES	30	5	288	790	325	50	220	ND	ND	ND
CYA	30	5	132	1612	484	39	210	ND	ND	ND
maize	25	7	871	3850	900	127	530	ND	4	4240
maize	25	21	1230	3850	950	200	1900	ND	12	16960
maize, EF[f]	25	21	209	2200	548	96	560	ND	ND	ND
brown rice	25	7	692	3500	700	127	1200	ND	9	3840
brown rice	25	21	1230	3900	1000	200	2000	ND	8	8800
CBS 700.68										
Pharma	30	5	ND	ND	ND	ND	300	500	4	7360
YES	30	5	ND	ND	ND	ND	200	650	1	2000
CYA	30	5	ND	ND	ND	ND	100	342	1	1040
maize	25	7	ND	ND	ND	ND	430	724	9	14400
maize	25	21	ND	ND	ND	ND	900	60	16	98400
brown rice	25	7	ND	ND	ND	ND	800	1401	23	33360
brown rice	25	21	ND	ND	ND	ND	1500	2310	19	58960

[a] Presumed peptides. [b] Peak area at 200–210 nm from LC-UV analysis on the Luna C_{18} II with the TFA-buffered solvents. [c] Peak areas from LC-MS chromatograms of $[M + H]^+$ ion ±0.01 amu. [d] Peak area at 290–320 nm from LC-UV analysis on the Luna C_{18} II with the TFA-buffered solvents. [e] Not detected. [f] Erlenmeyer flask.

tification. Rabie et al. (*14*) reported that some *R. chinensis* strains found to be toxic to rats and ducklings did not produce rhizonin A. These strains could very well have been misidentified *R. microsporus* strains producing rhizoxins and/or the unidentified peptides found in this study. Wilson et al. (*29*) found *R. microsporus* MRC 303 (= CBS 112285) to be toxic to rats and ducklings, but concluded that rhizonin A did not account for all of the toxicity seen. The toxicity may have been due to the combination of the rhizonins, the possible related peptides, and/or the various rhizoxins also detected in this study.

All four *R. microsporus* strains produced rhizoxins (CBS 700.68, CBS 699.68, CBS 308.87, and CBS 112285), and one strain also produced rhizonins (CBS 112285), whereas *R. oligosporus* and *R. chinensis* did not. *R. oligosporus* might have lost the toxin-producing ability during centuries of cultivation (*11*), just as *Aspergillus oryzae* has lost the aflatoxin-producing capability of its wild-type progenitor, *Aspergillus flavus* (*37*). Our results also indicate that *R. oligosporus* is more closely related to *R. chinensis* than it is to *R. microsporus,* a relationship also reflected in patterns of isoenzymes and proteins seen within the group (*10*, *38*). Seviour et al. (*38*) have suggested that *R. oligosporus* and *R. chinensis* should be considered conspecific and *R. microsporus* recognized as a separate species. This suggestion is practical, as the toxin-producing organism would then be separated from the non-toxin-producing taxa.

It is essential that the *Rhizopus* strains used during tempeh fermentation do not produce toxins. Because *Rhizopus* spores can be isolated from the air (R. A. Samson, personal observation), it is necessary to find methods that ensure pure starter cultures of *R. oligosporus*. Our results indicate that toxins are not produced during fermentations with *R. oligosporus.* None of the substrates normally used by *R. oligosporus* in fermentation

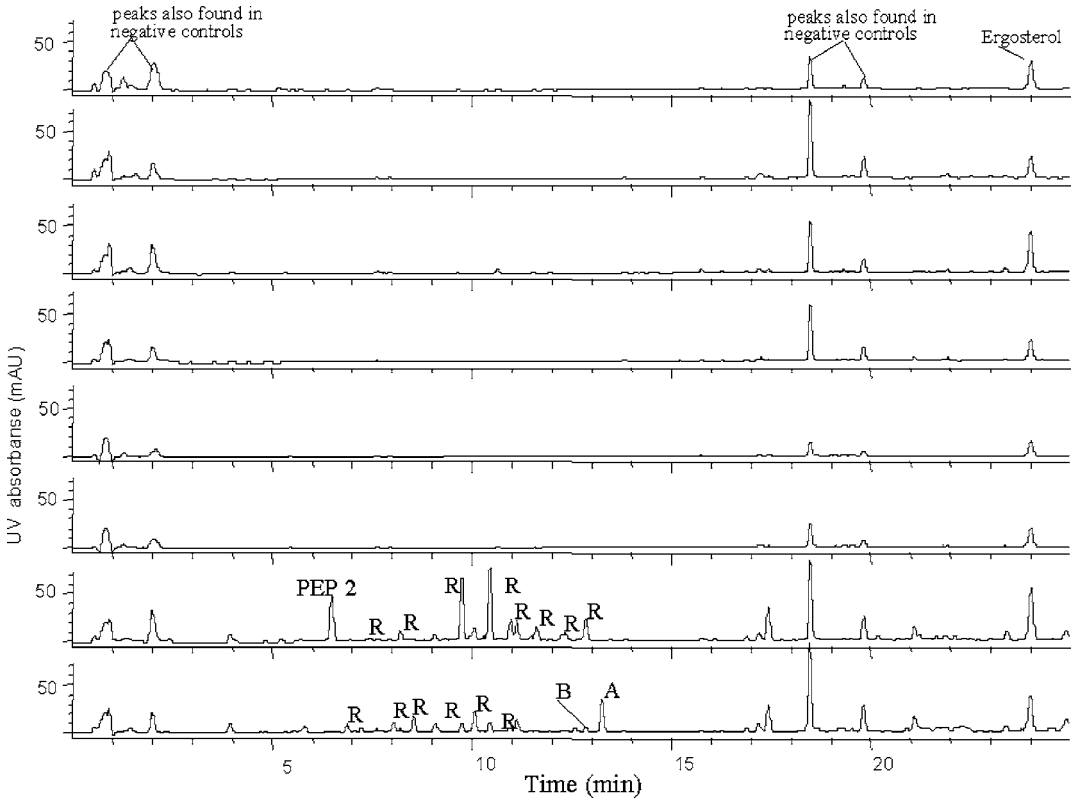

Figure 3. Liquid chromatography–diode array detection (Luna C_{18} II column) analysis chromatograms (200–350 nm) of strains grown for 21 day at 25 °C on brown rice: from the top down, traces of *R. oligosporus*: ATCC 48109, ATCC 64063, CBS 112586, CBS 112587, CBS 112588, and CBS 112589; *R. microsporus* CBS 700.68 and CBS 112285. Major rhizoxins are marked with an **R**, and the rhizonins by **A** and **B**. No rhizonins or rhizoxins were detected from any of the *R. oligosporus* strains, and the very small peaks originate from the brown rice matrix. PEP2 is an unidentified peptide.

were used in this study. However, current research at our departments shows that rhizoxins and rhizonins are not produced in barley tempeh (X. Feng, K. F. Nielsen, and J. Schnürer, J., personal communication). During the present study, incubations were run for a much longer time than that usually run in tempeh fermentation. Because toxins were not produced within 5−21 days of incubation, they are not likely to be produced during a 24 h tempeh fermentation process.

Several new species and varieties of the *R. microsporus* group have been described since the last revision of the genus in 1984 (*39*−*43*). It is not known if any of these taxa produce rhizoxins or rhizonins. *Rhizopus azygosporus* is a recently described species, isolated both from tempeh and as an agent of human infection (*42*, *44*), and suggested to be closely related to *R. oligosporus* (*10*). There is thus a need to continue to characterize the secondary metabolite production of more strains from the *R. microsporus* group. Additionally, a new taxonomic revision of the *R. microsporus* group is needed, as well as methods, other than the use of morphology and maximum growth temperature, to differentiate the members of the group. Metabolite profiling will be valuable here as indicated in this study as well as in studies on genera such as *Penicillium*, *Aspergillus*, *Fusarium*, and *Alternaria* (*45*), especially when combined with other characters such as DNA sequences, carbon source utilization data, and spore morphology.

Overall, we conclude that *R. microsporus* strains produce several of the pharmaceutically active rhizoxins, whereas only some strains produce the highly toxic rhizonins. These metabolites are not likely to be produced by the biotechnologically important *R. oligosporus* or *R. chinensis*. Care should thus be taken to avoid contamination with *R. microsporus* during production of fermented food.

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