Acremonium zeae, a Protective Endophyte of Maize, Produces Dihydroresorcylide and 7-Hydroxydihydroresorcylides

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Acremonium zeae has been characterized as a protective endophyte of maize and displays antifungal activity against other fungi. Pyrrocidines A and B were discovered to be the metabolites accounting for this activity. During a population survey of *A. zeae* isolates from maize seeds produced in nine states to determine their ability to produce pyrrocidines, another metabolite of *A. zeae*, unrelated to the pyrrocidines, was found to have widespread occurrence (105 of 154 isolates) and to be produced in amounts comparable to the pyrrocidines. Further chemical studies of fermentation extracts of an *A. zeae* isolate (NRRL 45893) from maize led to the identification of a new compound, dihydroresorcylide, the saturated analogue of *cis*-resorcylide. Also identified were the two diastereomers of 7-hydroxydihydroresorcylide. Dihydroresorcylide and pyrrocidines A and B were detected by LC-APCI-MS in symptomatic maize kernels from ears that were wound-inoculated in the milk stage with *A. zeae* NRRL 34559.

KEYWORDS: Dihydroresorcylide; 7-hydroxydihydroresorcylide; pyrrocidine; *Acremonium zeae*; maize

INTRODUCTION

Acremonium zeae has been characterized as a protective endophyte of maize. On the basis of a bioassay-directed purification for antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides*, pyrrocidines A and B were discovered to be the metabolites accounting for this activity (*1*). Pyrrocidines A and B had previously been shown to be metabolites of the filamentous fungus *Cylindrocarpon* sp. LL-Cyan426. Pyrrocidine A exhibited potent antibiotic activity against Gram-positive bacteria and was also active against the yeast *Candida albicans*, whereas pyrrocidine B showed weaker activity (*2, 3*). Pyrrocidine A has also shown potent activity against major stalk and ear rot pathogens of maize, including *Fusarium graminearum*, *Nigrospora oryzae*, *Stenocarpella (Diplodia) maydis*, and *Rhizoctonia zeae* while also exhibiting potent activity against *Clavibacter michiganense* subsp. *Nebraskense*, the causal agent of Goss's bacterial wilt of maize. Again, pyrrocidine B showed less activity (*4*). An evaluation of miscellaneous *A. zeae* isolates deposited with the ARS (NRRL) and CBS Culture Collections showed that they varied greatly in their ability to make pyrrocidines. This led us to conduct a population survey of *A. zeae*, representing populations from maize seeds produced in Arizona, California,

Illinois, Indiana, Iowa, Kentucky, Michigan, Nebraska, and Texas, to determine their ability to produce pyrrocidines. During the survey, three other metabolites of *A. zeae*, unrelated to the pyrrocidines, were found to have widespread occurrence and to be produced in amounts comparable to the pyrrocidines (unpublished results). This study was designed to identify the metabolites and investigate their occurrence and biological activity in order to determine what effects they may have on commercial maize production.
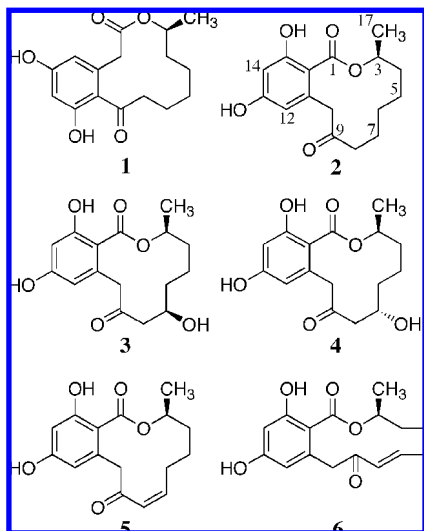
MATERIALS AND METHODS

LC-APCI-MS Analysis. A SpectraSYSTEM P4000 pump and an AS3000 autosampler were coupled to an LCQ Classic mass spectrometer via an atmospheric pressure chemical ionization (APCI) interface (Finnigan-MAT, San Jose, CA). A 150 × 2.0 mm i.d., 4 μm, Synergi Polar-RP column (Phenomenex, Torrance, CA) with a Polar-RP guard column and a 0.5 μm prefilter was used, and the entire HPLC eluent was introduced into the detector. A 10 min linear gradient from 30:50:20 (v/v/v) to 20:50:30 water/2% acetic acid in MeOH/100% MeOH, followed by a 10 min linear gradient from 20:50:30 to 0:50:50 was used, and the final composition was held for 10 min before the initial conditions were restored. The total run time was 45 min. The flow rate was 0.4 mL/min. Mass spectra were obtained by scanning from *m/z* 250 to 950 in the positive ion mode. The source voltage was 6 kV, the APCI vaporizer temperature was 350 °C, and the capillary temperature was 170 °C. The sheath gas flow was 70 arb.

Fungal Culture and Fermentation Conditions. *A. zeae* (NRRL 45893), isolated as "Az-115" from a sample of whole maize seeds

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Figure 1. Structures of curvularin (**1**), dihydroresorcylide (**2**), (*R*)-7-hydro-xydihydroresorcylide (**3**), (*S*)-7-hydroxydihydroresorcylide (**4**), *cis*-resorcylide (**5**), and *trans*-resorcylide (**6**).

received from Hopkinsville, KY, was grown on several slants of potato dextrose agar (PDA) for 14 days (25 °C). A hyphal fragment-spore suspension (propagule density = 10^6/mL of sterile distilled water) prepared from the PDA slants served as the inoculum. Fermentations were carried out in 10 Fernbach flasks, each containing 200 g of rice. Distilled water (200 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min. The flasks were cooled to room temperature, inoculated with 3.0 mL of spore inoculum, and incubated for 21 days at 25 °C.

Isolation and Identification of the Fungal Metabolites. The fermented substrate in each flask was first fragmented with a spatula and then extracted three times with EtOAc (200 mL each time). The extract from each flask was separately filtered and evaporated. Two groups of five extracts were combined to give two samples weighing 1115 and 877 mg, and each combination was separated as follows. A 2 g silica Sep-Pak cartridge (Waters Corp., Milford, MA) was conditioned with 20 mL of hexane and the extract in 50 mL of EtOAc/hexane (10:90, v/v) loaded onto the column. The column was eluted with 20 mL portions of EtOAc/hexane (10:90, 15:85, 25:75, 30:70, 60:40, v/v) and 20 mL of EtOAc. Each fraction was analyzed by LC-MS. The 60:40 and EtOAc fractions were combined and separated as described below. The 25:75 EtOAc/hexane fraction was dried under N_2, dissolved in 20 mL of EtOAc/hexane (10:90, v/v), and loaded onto a 2 g silica cartridge that had been conditioned with 20 mL of hexane. The column was eluted with 20 mL portions of EtOAc/hexane (10:90, 2 × 20:80, 30:70, 60:40, v/v) and 20 mL of EtOAc. After the second sample was separated the same way, the four 20:80 fractions were combined, dried under N_2, and further purified using a 10 g tC18 Sep-Pak cartridge (Waters Corp.). The cartridge was conditioned with 100 mL of MeOH followed by 100 mL of acetonitrile/water (30:70, v/v). The combined 20:80 fraction was dissolved in 3 mL of EtOAc plus 27 mL of acetonitrile. Just before loading onto the conditioned cartridge, the fraction was diluted with 70 mL of water. The cartridge was eluted with 100 mL portions of acetonitrile-/water (30:70, 40:60, 50:50, 60:40, 80:20, v/v) and acetonitrile. The 40:60 and 50:50 fractions were combined, diluted with 250 mL of water, and loaded onto a new 10 g tC18 cartridge that had been conditioned with 100 mL of MeOH followed by 100 mL of acetonitrile/water (20:80, v/v). The cartridge was washed with 100 mL of acetonitrile/water (20:80, v/v). Air was pulled briefly through the cartridge to remove the solvent, and it was then eluted with 50 mL of acetonitrile. The acetonitrile solution was dried under N_2 to give 34.7 mg of dihydroresorcylide (**2**, **Figure 1**).

The 60:40 EtOAc/hexane and EtOAc fractions from the first silica cartridges were combined and dried under N_2. A 2 g silica cartridge was conditioned with 20 mL of hexane and the extract in 20 mL of EtOAc/hexane (35:65, v/v) loaded onto the column. The column was eluted with 20 mL portions of EtOAc/hexane (2 × 30:70, 40:60, 50:

50, 60:40, 70:30, v/v) and 20 mL of EtOAc. The 50:50 and 60:40 fractions were combined and reduced to 10 mL, at which point precipitate formed. The solution was transferred to a syringe equipped with a 25 mm, 0.45 μm, nylon filter, and the vial was rinsed twice with 3 mL portions of EtOAc. After filtration, an additional 3 mL of EtOAc was used to rinse the vial and filter. The sample was dried and dissolved in 3 mL of acetonitrile. A 2 g tC18 Sep-Pak cartridge was conditioned with 20 mL of MeOH, followed by 20 mL of acetonitrile/water (15:85, v/v). The sample was diluted with 17 mL of water just before it was loaded on the cartridge. The cartridge was eluted with 20 mL portions of acetonitrile/water (15:85, 20:80, 30:70, 40:60, 50:50, v/v) and acetonitrile. A new 2 g tC18 cartridge was conditioned as above. The 30:70 fraction was diluted with 20 mL of water and loaded onto the cartridge. The cartridge was washed with 20 mL of acetonitrile/water (15:85, v/v). Air was pulled briefly through the cartridge to remove the solvent, and it was then eluted with 10 mL of acetonitrile. The acetonitrile solution was dried under N_2 to give 23.3 mg of a mixture consisting of equal amounts of the 7-hydroxydihydroresorcylides (**3** and **4**).

The structure of dihydroresorcylide was determined by analysis of ^{1}H NMR, ^{13}C NMR, two-dimensional NMR (HMBC), and mass spectrometric data. The optical rotation was measured with a Rudolph Research Autopol III automatic polarimeter. ^{1}H, ^{13}C, and HMBC NMR data were collected using a Bruker AMX-600 spectrometer. Chemical shift values were referenced to the solvent signals for CD$_3$OD (δ_H 3.31/ δ_C 49.15). HRESIMS data were recorded on a Micromass Autospec instrument. Dihydroresorcylide has the following characteristics: it is a colorless oil; [α]$_D$ = +15 (*c* 0.33; MeOH; 25 °C); positive ion EIMS, M^+ observed at *m/z* 292.1316 (calculated for C$_{16}$H$_{20}$O$_5$, 292.1310); ^{1}H NMR (600 MHz, CD$_3$OD) δ 12.01 (s, OH-15), 6.47 (br s, position variable, OH-13), 6.27 (d, *J* = 2.5 Hz, H-14), 6.02 (d, 2.6, H-12), 5.13 (ddq, *J* = 2.7, 6.3, 6.3 Hz, H-3), 4.64 (d, *J* = 18.3 Hz, H-10a), 3.66 (d, *J* = 18.4 Hz, H-10b), 2.59 (ddd, *J* = 1.9, 9.9, 15 Hz, H-8a), 2.37 (ddd, *J* = 1.9, 9.5, 15 Hz, H-8b), 2.00 (m, H-7a), 1.78 (m, H-7b), 1.67 (m, H-4a), 1.57 (m, H-4b), 1.47 (m, H$_2$-5 and H$_2$-6), 1.29 (d, *J* = 6.3, H$_3$-17). ^{13}C NMR (150 MHz, CD$_3$OD) δ 209.6 (C-9), 170.9 (C-1), 165.6 (C-15), 160.7 (C-13), 138.3 (C-11), 112.7 (C-12), 106.3 (C-16), 103.0 (C-14), 73.6 (C-3), 50.8 (C-10), 41.8 (C-8), 31.7 (C-4), 26.9 (C-6), 21.3 (C-7), 21.1 (C-5), 19.1 (C-17); HMBC H-3 → C-1, 17, 4, 5; H$_3$-17 → C-3, 4; H-4a → C-3, 5, 6, 17; H-4b → C-3, 5, 6, 17; H-7a → C-5, 6, 8, 9; H-7b → C-5, 6, 8, 9; H-8a → C-6, 7, 9, 10; H-8b → C-6, 7, 9, 10; H-10a → C-9, 11, 12, 16; H-10b → C-9, 11, 12, 16; H-12 → C-10, 13, 14, 16; H-14 → C-12, 13, 15, 16.

Antifungal Bioassay. Dihydroresorcylide was evaluated for antifungal activity by placing 0.25 mg onto individual paper discs. Dihydroresorcylide, dissolved in MeOH, was pipetted onto analytical grade filter paper discs (13 mm diameter) in individual Petri dish lids and dried for 30 min in a laminar flow hood. Disks were placed on the surface of PDA Petri plates seeded with *Aspergillus flavus* (NRRL 6541) conidia or *Fusarium verticillioides* (NRRL 25457), each giving a final conidial/hyphal cell suspension of approximately 100 propagules/mL. The plates were incubated for 4 days at 25 °C and examined for the presence of a zone of inhibition surrounding the disk, which is a measure of fungistatic activity.

MIC Determination. Fungal cultures representing different species isolated from maize, including *Alternaria alternata* NRRL 6410, *Aspergillus flavus* NRRL 6541, *A. niger* NRRL 6411, *Cladosporium cladosporioides* NRRL 6421, *Curvularia lunata* NRRL 6409, *Eupenicillium ochrosalmoneum* NRRL 6568, *Fusarium graminearum* NRRL 13188, *F. proliferatum* NRRL 6413, *F. verticillioides* NRRL 25457, *Nigrospora oryzae* NRRL 6414, *Penicillium funiculosum* NRRL 6417, *P. oxalicum* NRRL 6416, *P. pinophilum* NRRL 6420, *P. variable* NRRL 6419, *Rhizoctonia zeae* NRRL 40186, *Stenocarpella (Diplodia) maydis* NRRL 13615, *Trichoderma viride* NRRL 6418, and *Verticillium lecanii* NRRL 26576, were grown as PDA slant cultures (6 days, 25 °C). A suspension of conidia and/or hyphal cells was prepared from these cultures and used as inoculum to seed potato dextrose broth (PDB), giving a final propagule density of approximately 4 × 10^4/mL. Dihydroresorcylide and 7-hydroxydihydroresorcylides were evaluated in 96-well plates at concentrations of 0.25, 1, 2, 3, 5, 10, 25, and 50 μg/mL as previously reported (5). A minimal inhibitory concentration

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(MIC) was assigned to those treatment concentrations for which no fungal growth was recorded, whereas an IC_{50} was designated when fungal growth inhibition exceeds 50% of the growth recorded for MeOH control wells.

Leaf-Puncture Wound Assay. Dihydroresorcylide (**2**) and 7-hydroxydihydroresorcylides (**3** and **4**) were evaluated using a leaf-puncture assay (*6*). A droplet (5 μL) of a MeOH/water solution (1:1, v/v) containing 5 μg of test compound was placed over each of six needle puncture wounds (~0.25 mm) on the upper surface of a maize leaf blade cut from 4-week-old maize seedlings of Mandan Bride, Gaspe, and FS 6873RR, grown in the greenhouse. Oxalic acid (5 μg) served as a positive control (*7*). The leaf blades were incubated (72 h; 21–23 °C) on moistened filter paper in a sealed Petri dish. The lengths of the necrotic lesions spreading from needle puncture wounds were measured under a stereomicroscope. No symptoms were observed with 1:1 MeOH/water control solutions.

Inoculation of Maize Ears. Maize ears were wounded by a single inoculation with *A. zeae* (NRRL 34559) in the late milk to early dough stage of kernel maturity (21 days after mid silk; July 22, 2006) for a commercial maize hybrid (FS 6873RR) grown at Kilbourne, IL. Following natural dry-down in the field, ears were hand-harvested (September 21, 2006), and the seeds nearest each wound-site were sampled. Seeds with visible discoloration of 50% or more of the kernel surface, but none of the wounded seeds, were selected for analyses of dihydroresorcylide content.

Analysis of Inoculated Maize. The 50–100% discolored kernels were ground in a Stein mill (Steinlite, Atchison, KS). A 10 g sample was extracted with 50 mL of EtOAc for 1 h on an orbital shaker, then gravity filtered through Whatman 2V filter paper, and 25 mL of the filtrate was transferred to a glass vial and dried under a filtered air stream at room temperature. The residue in the vial was dissolved in 10 mL of hexane and transferred to a 30 mL separatory funnel. It was then partitioned with three 5 mL portions of acetonitrile, each portion used to rinse the vial before being added to the separatory funnel. The acetonitrile was collected in a vial and dried under an air stream. The residue was dissolved in 2 mL of EtOAc and 1 mL of the solution filtered through a 0.45 μm, 13 mm, nylon syringe filter (Gelman) and analyzed by LC-MS and LC-MS/MS.

RESULTS AND DISCUSSION

Three new metabolites of *A. zeae* were identified during a population survey of *A. zeae* isolates to determine their ability to produce pyrocidines. The major metabolite was produced by more of the isolates (105 of 154 isolates) than were the pyrrocidines (52 of 154 isolates) (unpublished results). Inspection of MS, ^{1}H NMR, and ^{13}C NMR data for the major metabolite initially suggested that this compound was the known fungal metabolite curvularin (**1**, **Figure 1**). However, comparison of the NMR data with those reported for curvularin (*8*) showed minor differences in some of the chemical shifts. Thus, a full set of 2D NMR data was obtained to unambiguously establish the structure. Analysis of these data led to identification of the compound as a regioisomer of curvularin. A search of the literature revealed that the compound has not been previously reported, although a related compound called resorcylide (*9*) that incorporates a double bond between carbons 7 and 8 is known. The new analogue from *A. zeae* was assigned the name dihydroresorcylide (**2**).

Both dihydroresorcylide and curvularin contain the same set of proton spin systems, but they differ in that the ketone carbonyl in curvularin is directly linked to the aromatic ring rather than to the isolated CH_2 corresponding to C-10 in dihydroresorcylide. Conversely, the isolated benzylic CH_2 in curvularin is linked to the ester carbonyl, rather than to the ketone carbonyl.

The absolute stereochemistry at C-3 was not directly determined, but is presumed to be the same as that of resorcylide. Resorcylide was originally assigned the *S*-configuration on the

basis of the chemical degradation of *cis*-resorcylide (*9*), and this was later confirmed by total synthesis (*10*). A minor peak occurring in some of the chromatograms was also analyzed and found to consist of a mixture of stereoisomers that differ from dihydroresorcylide by the addition of a hydroxyl group at carbon 7 (in both possible relative stereochemical orientations). These compounds, 7-hydroxydihydroresorcylides (**3** and **4**), have been reported previously, and their structures were confirmed by comparison of MS and NMR data with literature values (*11*). As expected, these values were different from those of the corresponding curvularin analogues (*12*).

Although dihydroresorcylide has not been previously reported, the closely related *cis*- and *trans*-resorcylides (**5** and **6**) have been isolated from *Penicillium* spp. (*9, 11*), *Pyrenophora teres*, the cause of net-type net blotch of barley (*13*), and *Drechslera phlei*, a pathogenic fungus on timothy grass (*14*). *cis*-Resorcylide has also been isolated from *Penicillium roseopurpureum* (*15*). The 7-hydroxydihydroresorcylides have been isolated from *D. phlei* (*14*) and a *Penicillium* species (*11*).

The resorcylides have been shown to have several different biological activities. *trans*-Resorcylide is a plant growth inhibitor and is >10 times more effective than *cis*-resorcylide at inhibiting seedling root elongation (*16*). *trans*-Resorcylide is cytotoxic and is an inhibitor of 15-hydroxyprostaglandin dehydrogenase, whereas *cis*-resorcylide does not inhibit that enzyme (*16*). *cis*-Resorcylide is an inhibitor of activated factor XIII (FXIIIa), which is an enzyme that catalyzes a number of covalent cross-linking reactions of fibrin in blood clots (*15*).

trans-Resorcylide also showed antimicrobial activity against *Pyricularia oryzae* using a paper disk assay (*16*). When dihydroresorcylide was tested using a paper disk assay against *A. flavus* or *F. verticillioides*, no fungistatic activity was detected. It has been previously shown that virulent fungal pathogens of maize were most sensitive to pyrrocidine A, whereas other fungal endophytes and mycoparasites displayed little or no sensitivity at the concentrations tested (*4*). MIC tests were performed using dihydroresorcylide and the mixed 7-hydroxydihydroresorcylides to see if they showed a similar pattern of activity against these fungi. No growth inhibition (MIC > 50 μg/mL) was recorded for any of the test fungi.

trans-Resorcylide and (*R*)-7-hydroxydihydroresorcylide have been shown to be phytotoxic to several plant species including maize using the leaf-wound puncture assay. *trans*-Resorcylide was the more active compound, whereas *cis*-resorcylide and (*S*)-7-hydroxydihydroresorcylide showed little or no effect on these hosts (*14*). A leaf-puncture wound assay showed that dihydroresorcylide and the 7-hydroxydihydroresorcylides were phytotoxic, producing elongated lesions extending in both directions along the length of the leaf. Dihydroresorcylide, 7-hydroxydihydroresorcylides, and oxalic acid produced lesions averaging 1.37, 1.10, and 1.94 mm long, respectively. The controls exhibited only the 0.25 mm needle puncture wounds. Thus, the dihydroresorcylides are phytotoxic, but appear to be less active than *trans*-resorcylide.

Dihydroresorcylide and pyrrocidines A and B were detected by LC-APCI-MS in intact, discolored maize kernels that were removed at harvest from ears that were wound-inoculated in the milk stage with *A. zeae* NRRL 34559. Pyrrocidines A and B were easily detected in extracts of *A. zeae* infected maize kernels on the basis of ion chromatograms of the $(M + H)^+$ ions, *m/z* 488 and 490, respectively. There were no interfering compounds in the maize extracts. Although the $(M + H)^+$ ion at *m/z* 293 was the most intense ion in the spectrum of dihydroresorcylide when analyzed by LC-APCI-MS, the pres-

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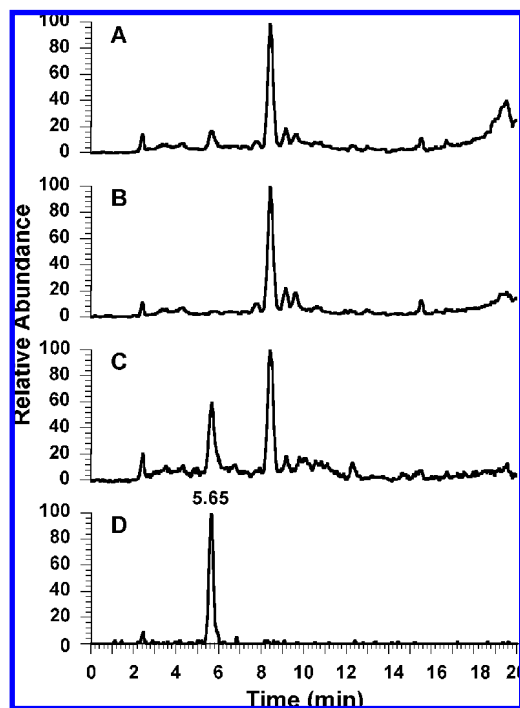
Figure 2. LC-APCI-MS/MS chromatograms of the cleaned-up EtOAc extract of *A. zeae* infected maize kernels: (**A**) TIC; (**B**) *m/z* 293; (**C**) *m/z* 275; (**D**) *m/z* 231.

ence of dihydroresorcylide was more difficult to establish because there were other compounds in the maize extracts that showed an ion at *m/z* 293. Thus, LC-APCI-MS/MS was found to be a better method for detecting dihydroresorcylide. Under the LC-APCI-MS/MS conditions used, the *m/z* 293 ion of dihydroresorcylide is completely fragmented to give two major ions at *m/z* 275 and 231 (**Figure 2**). Some of the other compounds in the corn extract also give fragments at *m/z* 275, so this mass is not useful for distinguishing the other compounds from dihydroresorcylide. However, the fragment ion at *m/z* 231 is characteristic of dihydroresorcylide. This is particularly useful, because in some extracts there is a compound with $(M + H)^+$ of *m/z* 293 that has almost the same retention time as dihydroresorcylide and gives a fragment at *m/z* 275, but not at *m/z* 231. Therefore, dihydroresorcylide was readily identified in these extracts by LC-APCI-MS/MS analysis and could be confirmed by comparing the mass spectra. This demonstrates the potential for dihydroresorcylide to occur in field corn. When cultured on potato dextrose agar, rice, or corn, some *A. zeae* strains obtained from the ARS and CBS Culture Collections produced greater quantities of pyrrocidines and dihydroresorcylide than *A. zeae* NRRL 34559, whereas other strains produced neither (unpublished results). *A. zeae* strains showing a greater ability to produce such compounds in laboratory culture presumably have the potential to produce higher levels in preharvest grain.

A. zeae and *F. verticillioides* are the most prevalent colonists of preharvest maize (*1*). During a population survey of *A. zeae* isolates from maize seeds produced in nine states to determine their ability to produce pyrrocidines, dihydroresorcylide was identified as a major metabolite of *A. zeae*. Dihydroresorcylide was produced by 68% of the isolates, whereas only 34% of the isolates produced pyrrocidines. Among the pyrrocidine-producing isolates, 85% also produced dihydroresorcylide (unpublished results). Dihydroresorcylide, in addition to pyrrocidines A and B, was detected in the EtOAc extract of maize kernels infected

by *A. zeae*. Although it is not known if dihydroresorcylide contributes to the symptoms observed for kernels infected with *A. zeae,* such as endosperm discoloration, its detection in maize kernels warrants further study into the frequency and levels at which it may occur.

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