A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*

Donald T. WICKLOW¹, Shoshannah ROTH², Stephen T. DEYRUP² and James B. GLOER²

¹ USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL 61604, USA.
² Department of Chemistry, University of Iowa, Iowa City, IA 52242, USA.
E-mail: wicklodzi@ncuar.usda.gov

Received 16 June 2004; accepted 25 February 2005.

The maize endophyte *Acremonium zeae* is antagonistic to kernel rotting and mycotoxin producing fungi *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests for antagonism, and interferes with *A. flavus* infection and aflatoxin contamination of preharvest maize kernels. Chemical studies of an organic extract from maize kernel fermentations of *Acremonium zeae* (NRRL 13540), which displayed significant antifungal activity against *Aspergillus flavus* and *F. verticillioides*, revealed that the metabolites accounting for this activity were two newly reported antibiotics pyrrocidines A and B. Pyrrocidines were detected in fermentation extracts for 12 NRRL cultures of *Acremonium zeae* isolated from maize kernels harvested in Illinois (4/4 cultures), North Carolina (5/5), Georgia (1/2) and unrecorded locations within the USA (2/2). Pyrrocidine B was detected by LCMSMS in whole symptomatic maize kernels removed at harvest from ears of a commercial hybrid that were wound-inoculated in the milk stage with *A. zeae* (NRRL 13540) or (NRRL 13541). The pyrrocidines were first reported from the fermentation broth of an unidentified filamentous fungus LL-Cyan426, isolated from a mixed Douglas Fir hardwood forest on Crane Island Preserve, Washington, in 1993. Pyrrocidine A exhibited potent activity against most Gram-positive bacteria, including drug-resistant strains, and was also active against the yeast *Candida albicans*. In an evaluation of cultural antagonism between 13 isolates of *A. zeae* in pairings with *A. flavus* (NRRL 6541) and *F. verticillioides* (NRRL 25457), *A. zeae* (NRRL 6415) and (NRRL 34556) produced the strongest reaction, inhibiting both organisms at a distance while continuing to grow through the resulting clear zone at an unchanged rate. Maximum colony diameters for *A. zeae* (NRRL 6415) and (NRRL 13540), on potato dextrose agar after 14 d, were attained within the range of 25–30 °C, with less growth recorded at 15 °C and 37.5 °C and no growth at 5 °C. Potential interactions between *A. zeae* and other maize endophytes are considered and the significance of these interactions relative to the aflatoxin and fumonisins contamination of preharvest maize is presented. This is the first report of natural products from *Acremonium zeae*.

INTRODUCTION

*Acremonium zeae* (Gams 1971), also reported as *Acremonium* sp., *A. strictum*, *Cephalosporium* sp., or *Cephalosporium acremonium*, which was mistaken by Manns & Adams (1923) for *C. sacchari* (syn. *Fusarium sacchari*), along with *F. verticillioides* (syn. *F. moniliforme*), are the most prevalent colonists of preharvest maize (*Zea mays*), typically producing symptomless kernel infections (Reddy & Holbert 1924, Harris 1936, King 1981). In the following text, the modern names *A. zeae* and *F. verticillioides* have been substituted for earlier names assigned to these fungi. The significance of high levels of infection by *A. zeae* and *F. verticillioides* in asymptomatic maize kernels is not well understood (King 1981, Munkvold et al. 1997). These fungi are frequently isolated as colonists of the same kernel. Because *F. verticillioides* grows more rapidly from kernels, it can mask the presence of *A. zeae* and the infection frequency may be underestimated (Manns & Adams 1923, Sumner 1968a, King 1981). Histopathological studies and fungal isolations from dissections of ‘sound-appearing’ kernels revealed that *F. verticillioides* was primarily confined to the pedicel and abscission layers of maize while *A. zeae* was more frequently isolated from excised embryos and endosperm (Sumner 1968a). Might *A. zeae* and *F. verticillioides* therefore function as protective endophytes of maize? Endophyte establishment within the germ and endosperm would present an immediate defense against pathogen attack at the seed and seedling stage, where natural enemies of the plant host would have the greatest impact on fitness (Faeth 2002).
Mycotoxin contamination of maize affects the quality and safety of human food and animal feeds thereby lowering the value of the grain and resulting in substantial economic losses to maize growers, livestock and poultry producers, grain handlers, and food and feed processors (Council for Agricultural Science and Technology 2003). Prominent among the mycotoxins associated with such losses in maize are aflatoxins produced by Aspergillus flavus and fumonisins produced by F. verticillioides. Individual kernels infected with F. verticillioides were less likely to be infected with A. flavus or other fungal pathogens of maize (Wicklow 1988, Rheeder et al. 1990). Suppression of A. flavus infection and aflatoxin in preharvest maize by A. zeae and F. verticillioides was first demonstrated by Wicklow et al. (1988) who inoculated maize ears produced on plants grown in an environmental chamber. Similar results were obtained by Zummo & Scott (1992) when they inoculated F. verticillioides and A. flavus onto maize ears produced by plants growing in field plots. Fumonisin and aflatoxin levels in grain samples may also bear a negative relationship to each other, suggesting the interaction of fumonisin producers (e.g. F. verticillioides) and aflatoxin producers (e.g. A. flavus) impacts kernel infection and mycotoxin production (Yoshizawa et al. 1996, Ono et al. 2001). The presence of F. verticillioides in the ear was also negatively correlated with Diplodia maydis (Rheeder et al. 1990) and Gibberella zeae (Blaney et al. 1986), leading Rheeder et al. (1990) to suggest that F. verticillioides may have a “protective effect” by suppressing the growth of more destructive pathogens of maize with the potential for use as a ‘biocontrol agent.’ However, because F. verticillioides may produce symptoms of Fusarium kernel rot or ear rot of maize (White 1999) and contaminate the grain with mycotoxins, this fungus is considered less attractive for use in biocontrol (Rheeder et al. 1990).

A. zeae is not recognized as causing ear, kernel or storage rots of maize (White 1999) and there have been no reports that A. zeae isolates from maize produce any metabolites toxic to animals or plants. A. zeae (NRRL 6415) strongly interfered with Aspergillus flavus (NRRL 6412) colony growth when paired on agar in Petri dishes (Wicklow et al. 1980). Furthermore, experimental inoculations of maize ears with a mixed conidial inoculum consisting of three cultures of Acremonium zeae (NRRL 6415, NRRL 13540, NRRL 13541), limited Aspergillus flavus colonization and aflatoxin contamination of the intact grains removed from wound-inoculated ears (Wicklow et al. 1988). At the same time, we could find no reports of research to investigate competitive or cooperative interactions between Acremonium zeae and F. verticillioides. Our objective was to describe the isolation and characterization of antifungal metabolites produced by A. zeae which inhibit growth of Aspergillus flavus and/or F. verticillioides, molds capable of infecting and rotting maize kernels while contaminating the grain with mycotoxins harmful to livestock as well as humans.

**MATERIALS AND METHODS**

**Fungal strains**

13 maize kernel isolates of Acremonium zeae were obtained from the USDA Agricultural Research Service Culture Collection, Peoria (NRRL). Culture collection records listed these strains as Acremonium strictum or Cephalosporium; NRRL 6515, NRRL 13540, NRRL 13541, NRRL 34554 (=A-24200), and NRRL 34555 (=A-24202), identified by D.T.W. as Acremonium strictum, were isolated by R. Rogers from maize sampled at harvest in North Carolina in 1977, each isolate coming from a different crop field; NRRL 34556 (=A-25103), identified as A. strictum by B. W. Horn, was isolated by B. W. Horn from maize left standing in a field near Tifton, Georgia until ears were hand-harvested in October 1981; NRRL 34557 (=A-25141), identified as A. strictum by B. W. Horn, was isolated by B. W. Horn from a maize ear that was hand-harvested in 1981 from a field near Tifton, Georgia, placed on the ground, and retrieved in January 1982; NRRL 34558 (=A-25722), identified as Cephalosporium sp. by D.G. White, was isolated by D.G. White from maize sampled at harvest in 1980 from a field at Champaign, Illinois; NRRL 34559 (=A-21613) and NRRL 34560 (=A-21614), identified as Cephalosporium sp. by D.I. Fennell, were isolated by R.J. Bothast from separate ears of maize that were hand-harvested in 1974 from a field in Peoria County, Illinois; NRRL 34561 (=A-21769), identified as Cephalosporium sp. by J.J. Ellis, was isolated by R. Rogers from a sample of whole maize kernels that was fed to pigs near Springfield, Illinois; NRRL 34562 (=A-18709), identified by T.A. Toussoun as Cephalosporium sp., was isolated by R. Rogers from a kernel of blight-infected hybrid maize, USA; NRRL 34563 (=A-18737), identified by T.A. Toussoun as Cephalosporium sp., was isolated by R. Rogers from a sample of whole maize kernels received from a corn mill, locality not specified, USA.

**Fermentation conditions**

_Acremonium zeae_ (NRRL 13540) was grown on several slants of potato dextrose agar (PDA) for 14 d (25°C). A hyphal fragment-spore suspension (propagule density 10⁶ ml⁻¹ of sterile distilled water) prepared from the PDA slants served as the inoculum. Fermentations were carried out in forty 500 ml Erlenmeyer flasks, each containing 50 g of whole maize kernels (Kelly’s Seed, Peoria). Distilled water (50 ml) was added to each flask, and the contents were soaked overnight before being autoclaved at 1.055 kgf cm⁻² for 30 min. After the flasks had cooled to room temperature, they were inoculated with 1 ml of the hyphal fragment-spore suspension and incubated for 30 d at 25°C. The remaining 12 isolates of _A. zeae_ were grown as above, but maize kernel fermentations were limited to two 500 ml flasks (100 g whole maize kernels) per isolate. Two flasks...
containing autoclaved maize kernels provided an uninoculated control.

**Bioassay of extractable residue**

Following incubation, the fermented whole maize kernel substrate in each flask was first fragmented with a large spatula and then extracted three times with ethyl acetate (50 ml each time). The combined ethyl acetate extracts were filtered and evaporated. Following evaporation of the ethyl acetate, approximately 5 mg of the residue was redissolved in methanol for antifungal activity assays. The remaining dried extract was stored at −20 °C. One mg equivalents of extractable residue, dissolved in methanol, were pipetted onto analytical-grade filter paper discs (13 mm diam) in individual Petri dish lids and dried for 30 min in a laminar flow hood. Discs were placed on the surface of PDA seeded with *Aspergillus flavus* (NRRL 6541) conidia or *Fusarium verticillioides* (NRRL 25457), each giving a final conidial/hyphal cell suspension of approximately 100 spores per ml. The bioassay plates were incubated for 4 d at 25 °C and examined for the presence of a zone of inhibition surrounding a disc, which is a measure of fungistatic activity. This bioassay procedure was used to guide the isolation of those *A. zeae* metabolites which accounted for the antifungal activity. Pure compounds were evaluated for antifungal activity by placing 0.25 mg onto individual paper discs.

**Isolation of pyrrocidines A and B from solid-substrate fermentation cultures**

A 100 g sample of freeze-dried fermented corn substrate was pulverized using a mortar and pestle and extracted overnight with 1.3 l of ethyl acetate. The ethyl acetate solution was then filtered, dried with magnesium sulfate, and the ethyl acetate was evaporated leaving an orange oil. This procedure was repeated two times with hexanes (10 ml each), and the acetonitrile layer was washed twice with hexanes (20 ml each). The acetonitrile layer was also washed twice with hexanes (20 ml each), and the acetonitrile layer was washed twice with hexanes (20 ml each). The acetonitrile layer was then evaporated to afford 0.91 g of crude material. The acetonitrile-soluble material was subjected to fractionation by flash chromatography on a silica gel column. The column was eluted successively with 2% methanol in CHCl3 (500 ml), 1% methanol in CH3CN (500 ml), 2% methanol in CH3CN (500 ml), 2% methanol in CH3CN (500 ml), 3% methanol in CH3CN (400 ml), 4% methanol in CH3CN (400 ml), 4% methanol in CH3CN (400 ml), 5% methanol in CH3CN (400 ml), 10% methanol in CH3CN (400 ml), 20% methanol in CH3CN (400 ml), 50% methanol in CH3CN (400 ml), and finally 90% methanol in CH3CN (400 ml). Bioassay results, together with 1H nuclear magnetic resonance (NMR) analysis suggested that compounds of interest eluted with 2% methanol in CH3CN. A 12 mg portion of this 43 mg fraction was further purified by semipreparative reversed-phased high-performance liquid chromatography (HPLC) (Alltech 8u C-18 column; 250 × 10 mm; 20 to 100% CH2CN in H2O in 45 min). Pyrrocidines A and B eluted as two broad, partially overlapped peaks with retention times of 47.0 and 49.5 min under these conditions, but collection of the first part of peak 1 and the latter part of peak 2, followed by evaporation of the solvent, afforded samples of pyrrocidines A (1.4 mg) and B (2.4 mg), respectively. Collection of the valley between the two peaks afforded another 6.2 mg of a mixture of the two compounds.

Because of the difficulty in separating the two components in quantity, a slightly altered procedure was employed using fractions obtained from a larger-scale fermentation extract. In this case, a 400 g sample of freeze-dried fermented corn substrate was extracted with ethyl acetate (2.0 l) and partitioned between acetonitrile and hexane (50 ml each) as described above. After washing the acetonitrile layer three times with hexane (50 ml each time), the acetonitrile fraction was concentrated to afford 2.0 g of extract that was subjected to column chromatography on silica gel using the same solvent step gradient described above. A 61 mg fraction eluting with 3% methanol in CH3CN clearly consisted mostly of pyrrocidine A accompanied by some pyrrocidine B and other impurities. Fortuitously, at this stage, pyrrocidine B was found to be more soluble in CHCl3 than pyrrocidine A. Thus, simple trituration of this fraction with CHCl3 (0.5 ml) and subsequent filtration was found to remove pyrrocidine B, some of the pyrrocidine A, and other impurities leaving a much larger pure sample of pyrrocidine A (30 mg) as a residual white solid.

The molecular formula of the pyrrocidine B sample obtained from *A. zeae* was established by high resolution fast atom bombardment mass spectrometry (HRFABMS). The structure was independently established by detailed, two-dimensional (2D) NMR analysis using 1H NMR, 13C NMR, 1H-1H correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and 2D nuclear Overhauser enhancement spectroscopy (NOESY) data. The structure of pyrrocidine A was assigned by spectral comparison to pyrrocidine B. During the course of this work, pyrrocidines A and B were independently reported from a different fungal source by researchers at Wyeth-Ayerst who assigned the names pyrrocidines to these metabolites (He et al. 2002). Comparison of data obtained for the compounds isolated from *Acremonium zeae* with those reported for pyrrocidines A and B confirmed the identities of these metabolites.

**Inoculations of maize ears**

Pioneer 3394 maize kernels were harvested in 2001 from ears that were wound-inoculated with *Acremonium zeae* NRRL 13540 (10 plants) or NRRL 13541 (10 plants) in the milk stage to early dough stage of kernel
RESULTS AND DISCUSSION

Chemical studies of the organic extract from maize kernel fermentations of *Acremonium zeae* (NRRL 6415) were performed 48 h before *Aspergillus flavus* (NRRL 6541) and *Fusarium verticillioides* (NRRL 25457). Conidial suspensions, representing a given test pair, were transferred with an inoculating loop onto the agar surface at points separated by 3 cm according to predesignated markings on the underside of a Petri dish. *Acremonium* interference with *A. flavus* and *F. verticillioides* was recorded after 4, 6, and 8 d. Colony reaction types were assigned to each pairing after Wicklow et al. (1980).

**Evaluation of cultural antagonism**

Cell and conidial suspensions of each fungal isolate, made from 14-d old slants of PDA, were used as inoculum. Cultures were paired on triplicate plates of PDA in Petri dishes incubated at 25 °C (8 d); *Acremonium zeae* inoculations were performed 48 h before *Aspergillus flavus* (NRRL 6541) and *Fusarium verticillioides* (NRRL 25457). Conidial suspensions, representing a given test pair, were transferred with an inoculating loop onto the agar surface at points separated by 3 cm according to predesignated markings on the underside of a Petri dish. *Acremonium* interference with *A. flavus* and *F. verticillioides* was recorded after 4, 6, and 8 d. Colony reaction types were assigned to each pairing after Wicklow et al. (1980).

**Determination of temperature optima for fungal growth**

Multiple hyphal tip transfers, taken from colonies of *Acremonium zeae* (NRRL 6415, NRRL 13540) grown on plates of PDA for one week at 25 °C, were used to inoculate test plates containing 15 ml of the same medium. Three plates of each fungus selected for study were incubated in the dark at each of the following temperatures: 5, 15, 25, 30, and 37.5 °C. The diameters of developing colonies were measured at intervals of 3–4 d over a 2 wk period.
13540), which displayed significant antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* in conventional paper disc assays, revealed that the metabolites accounting for this activity were two newly reported antibiotics pyrrocidines A and B (Fig. 1). Pyrrocidine A at 250 \( \mu \text{g disc}^{-1} \) and pyrrocidine B at 200 \( \mu \text{g disc}^{-1} \) were more strongly inhibitory to the growth of *F. verticillioides*, as measured by the zone of inhibition surrounding each disc, than to *A. flavus*. The pyrrocidines were first reported from the fermentation broth of an unidentified species of *Cylindrocarpon LL-Cyan426*, isolated from a mixed douglas dir hardwood forest on Crane Island Preserve, Washington, in 1993 (He et al. 2002, Bigelis et al. 2003). The pyrrolidinone function has been reported in another antifungal compound, talaroconvolutin A produced by *Talaromyces convolutus* (Suzuki et al. 2000) along with four tetramic acid derivatives including ZG-1494, a compound first isolated from *Penicillium rubrum* (West et al. 1996). *P. rubrum* also produces rubratoxin B which interferes with cell wall synthesis and leads to hyphal deformation in many fungi (Reiss 1972).

The presence of pyrrocidines A and B in ethyl acetate extracts of maize kernel fermentations was determined for 13 cultures of *Acremonium zeae* isolated from maize kernels maintained by the ARS Culture Collection (Table 1). This is the first report of natural products from *A. zeae*. Pyrrocidines were detected in fermentation extracts for twelve cultures of *A. zeae* isolated from maize kernels harvested in Illinois (4 cultures), North Carolina (5), Georgia (1) and unlisted locations within the USA (2). Pyrrocidine A and B were detected in nine of the cultures while pyrrocidine B alone was detected in three cultures. The production of pyrrocidines by the majority of *A. zeae* isolates from maize suggests that the ability to produce these antibiotics could be important to fungal endophyte interactions.

Table 1. Detection of pyrrocidines A and B in organic extracts of corn kernels fermented with cultures of *Acremonium zeae* and evaluation of cultural antagonism in pairings with *Aspergillus flavus* and *Fusarium verticillioides*.

<table>
<thead>
<tr>
<th>Strainb</th>
<th>Received as</th>
<th>Source</th>
<th>Pyrrocidines</th>
<th>A. flavus</th>
<th>F. verticillioides</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL 6415</td>
<td><em>Acremonium strictum</em></td>
<td>North Carolina</td>
<td>A,B</td>
<td>‘E’</td>
<td>‘E’</td>
</tr>
<tr>
<td>NRRL 13540</td>
<td><em>Acremonium strictum</em></td>
<td>North Carolina</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 13531</td>
<td><em>Acremonium strictum</em></td>
<td>North Carolina</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34554</td>
<td><em>Acremonium strictum</em></td>
<td>North Carolina</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34555</td>
<td><em>Acremonium strictum</em></td>
<td>North Carolina</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34556</td>
<td><em>Acremonium strictum</em></td>
<td>Tifton, Georgia</td>
<td>A,B</td>
<td>‘E’</td>
<td>‘E’</td>
</tr>
<tr>
<td>NRRL 34557</td>
<td><em>Acremonium strictum</em></td>
<td>Tifton, Georgia</td>
<td>ND^{a,b,g}</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34558</td>
<td><em>Cephalosporium sp.</em></td>
<td>Champaign, Illinois</td>
<td>B</td>
<td>‘E’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34559</td>
<td><em>Cephalosporium sp.</em></td>
<td>Peoria Co., Illinois</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34560</td>
<td><em>Cephalosporium sp.</em></td>
<td>Peoria Co., Illinois</td>
<td>A,B</td>
<td>‘E’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34561</td>
<td><em>Cephalosporium sp.</em></td>
<td>Springfield, Illinois</td>
<td>B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34562</td>
<td><em>Cephalosporium sp.</em></td>
<td>USA</td>
<td>A,B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
<tr>
<td>NRRL 34563</td>
<td><em>Cephalosporium sp.</em></td>
<td>USA</td>
<td>B</td>
<td>‘B’</td>
<td>‘B’</td>
</tr>
</tbody>
</table>

\(^{a}\) Culture reference nos. in the ARS Culture Collection, Peoria, IL (NRRL).

\(^{b}\) Ethyl acetate extracts of whole corn kernel fermentations (25°; 30 d).

\(^{c}\) Detected by NMR analysis of the solvent-partitioned crude extracts.

\(^{d}\) Detected by LCMSMS analysis of the solvent-partitioned extracts.

\(^{e}\) Cultures paired on potato dextrose agar in Petri dishes incubated at 25° (8 d). *A. zeae* inoculations were performed 48 h before *Aspergillus flavus* (NRRL 6541) and *Fusarium verticillioides* (NRRL 25457).

\(^{f}\) Reaction types (Wicklow et al. 1980): ‘B’ mutual inhibition on contact, the space between the two colonies is small, but clearly marked; ‘E’, inhibition of one organism at a distance, the antagonist continues to grow through the resulting clear zone at an unchanged rate.

\(^{g}\) ND, not detected.

![Fig. 1. Structures of pyrrocidine A (1) and B (2).](image-url)
with competing microbes and contributes to fungal survival in maize cultivation.

Pyrocidine B was detected by LCMSMS in pick-out kernel samples removed at harvest from ears of a commercial hybrid that were wound-inoculated with A. zeae strains NRRL 13540 or NRRL 13541. The samples consisted of whole symptomatic maize kernels showing some discoloration of the endosperm or the presence of white chalky stripes on the surface of the pericarp (Koehler 1942). Our LCMSMS method enabled us to determine the presence or absence of pyrocidines A and B in an organic extract but not the concentrations of these compounds.

Regional aflatoxin outbreaks are commonly accompanied by outbreaks in fumonisin (Mubatanhema et al. 2002) and therefore, aflatoxin and fumonisin can be present at unacceptable levels in the same grain samples at harvest (Chamberlain et al. 1993, Chu & Li 1994). Aflatoxins are primarily associated with hepatic disease and are powerful hepatotoxins, teratogens, mutagens, and carcinogens, while also causing decreased milk and egg production and suppression of immunity in animals consuming low dietary concentrations (Council for Agricultural Science and Technology 2003). Fumonisins have been reported to induce several diseases in animals, including leukoencephalomalacia in horses (Marasas et al. 1988), pulmonary edema in swine (Harrison et al. 1990), act as tumour promoters in rats (Gelderbloom et al. 1996) and may also be associated with oesophageal cancer in humans (Rheeder et al. 1992, Chu & Li 1994). The occurrence of these mycotoxins may increase the cancer risk of aflatoxin exposure. Fumonisin B1 was recently shown to promote aflatoxin B1-initiated liver tumours when fed to rainbow trout (Carlson et al. 2001). Therefore, in considering among ‘protective’ maize endophytes as relevant biocontrol agents, consideration should be given to microbes that can simultaneously prevent both aflatoxin- and fumonisin-contamination of the grain at harvest.

In an evaluation of cultural antagonism between 13 isolates of A. zeae in pairings with Aspergillus flavus (NRRL 6541) and Fusarium verticillioides (NRRL 25457), Acremonium zeae (NRRL 6415 and NRRL 34556) inhibited both organisms at a distance while continuing to grow through the resulting clear zone at an unchanged rate, designated reaction type ‘E’ (Table 1). A. zeae (NRRL 34558) and (NRRL 34560) also produced reaction type ‘E’ in cultural pairings with Aspergillus flavus, but in cultural pairings with Fusarium verticillioides showed mutual inhibition on contact, the space between the two colonies being small but clearly marked as designated for reaction type ‘B’. Cultural pairings with the remaining Acremonium zeae isolates and Aspergillus flavus or F. verticillioides produced reaction type ‘B’. This is the first demonstration of Acremonium zeae interference with the faster growing F. verticillioides. A. zeae and F. verticillioides compete with one another over the same range of temperatures supporting growth, and share the same optimum for fungal growth. F. verticillioides isolates show temperature optima for growth on agar media of 22.5–30 °C with a minimum of 2.5–5 °C and a maximum of 35–37.5 °C with no growth at 40–45 °C (Marin et al. 1995, Samson et al. 1995). Maximum colony diameters for A. zeae isolates NRRL 6415 (Fig. 2) and NRRL 13540, on PDA after 14 d, were attained within the range of 25–30 °C, with less growth recorded at 15 °C and 37.5 °C, and no growth at 5 °C. We could not determine the temperature optimum for pyrocidine production because there is presently no method for measuring the concentration of pyrocidines.

Aflatoxin outbreaks in preharvest maize are associated with high temperatures during grain development (Anderson et al. 1975), especially high evening temperatures associated with stress to maturing maize kernels (Smart et al. 1990, Wicklow 1994). Experimental maize inoculations in an environmental growth room showed that Acremonium zeae and F. verticillioides could prevent the spread of Aspergillus flavus infection in preharvest maize at 30 °C day and 20 °C night temperatures (Wicklow et al. 1988). Under these mild or moderate temperatures Acremonium zeae and F. verticillioides can compete successfully with Aspergillus flavus and limit the frequency of kernel infection and aflatoxin contamination. F. verticillioides and F. proliferatum were generally very competitive and dominant against A. flavus over a range of temperatures (10, 15, 25, 30 °C) and 0.994 to 0.96 a w (water availability) (Marin et al. 1998). At the same time, A. flavus isolates show a higher temperature optima for growth on agar media of 35–37 °C with a minimum of 6 °C and a maximum of 45 °C (Schindler et al. 1967, Magan & Lacey 1984). Aspergillus flavus may overcome the competitive effects of A. zeae and F. verticillioides when temperatures equal or exceed the 35–37.5 °C maximum growth temperatures recorded for these endophytes.

![Fig. 2. Growth-increment curves for Acremonium zeae (NRRL 6415).](image-url)
Antifungal antibiotics in *Acremonium zeae* 616

Pyrocidine A exhibits potent activity against most Gram-positive bacteria (He et al. 2002) and could have an important role in interactions between *Acremonium zeae* and bacterial pathogens or endophytes of maize. Gram-positive bacterial pathogens of maize include *Clavibacter michiganense* subsp. *nebraskense* (syn. *Corynebacterium nebraskense*), causal agent of Goss’s bacterial wilt and leaf freckles in maize (Ngong-Nasah et al. 2002). It will be interesting to learn whether pyrocidine antibiotics are produced by *A. zeae* in healthy maize tissues and contribute to the suppression of bacterial or fungal pathogens. Fisher et al. (1992) were the first to establish that symptomless populations of bacteria exist in healthy maize stem tissues in conjunction with the endophytic fungal biota. *A. zeae* and *Ustilago* sp. were the most frequent fungal isolates from stem core tissues. The authors recorded a low incidence of fungal infections in the lower parts of the stems which coincided with the tissues yielding the highest bacterial counts. The bacterial endophytes isolated by Fisher et al. (1992) were all Gram-negative (e.g. *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Vibrio*). However, Zinniel et al. (2002) characterized 300 bacterial isolates from 90 maize plants as Gram-positive (164 isolates; 55%) or Gram-negative (136 isolates; 45%). Furthermore, among 15 genera isolated as endophytes of maize and sorghum three Gram-positive genera, *Bacillus*, *Corynebacterium*, and *Microbacterium* were most prevalent, having more assigned isolates. Might pyrocidines negatively impact non-pathogenic bacterial endophytes of maize (Fisher et al. 1992, Zinniel et al. 2002), including Gram-positive actinomycetes (Araujo et al. 2000) and bacteria which have been identified for their biocontrol potential in maize (e.g. *Bacillus subtilis*, Chang & Kommedahl 1968; *B. mojavensis*, Bacon & Hinton 2002, *Clavibacter xyli* subsp. *cynodontis*, Tomasinio et al. 1995)?

*A. zeae* has been associated with stalk rot in mature maize plants, but the damage is not always apparent as stalk rot (Christensen & Wilcoxson 1966), and the fungus is not regarded as an aggressive parasite, especially when compared with fungi recognized as the primary cause of stalk rot *Diplodia maydis* or *Gibberella zeae* (Harris 1936, Summer 1968a, White 1999). Reddy & Holbert (1924) proposed that *A. zeae* is seed-borne in maize, beginning its development with the germinating seed, and resulting in a systemic infection of the plant through the vascular system, eventually invading the ears and seeds of plants that appear outwardly healthy. They isolated *A. zeae* from blackened vascular bundles and identified it as the causal agent of ‘black-bundle disease’ of maize. Even so, the symptoms were inconsistent and subsequent research has shown the black-bundles to be associated with the variety of maize planted and influenced by environmental conditions such as severe drought (Harris 1936). *A. zeae* grew systematically on maize grown in growth rooms and the greenhouse without significantly impacting the mean weight of grain produced per plant (Summer 1968a). In field grown maize, seeds infected with *A. zeae* did not produce plants having a greater incidence of stalk rot or any other obvious symptoms of disease than uninfected seeds (Harris 1936, Summer 1968a, King 1981). Stalk rot is a ‘disease complex’ and it is often difficult to determine the cause of stalk rot in the field as many kinds of fungi and bacteria, both primary pathogens and secondary invaders, can often be isolated from a small piece of necrotic tissue (Christensen & Wilcoxson 1966). Therefore, it should not be surprising that two of the most common systemic fungal endophytes of maize, *A. zeae* and *F. verticillioides*, are also commonly recorded as colonists of senescent or dead maize stalk tissues (Christensen & Wilcoxson 1966, Summer 1968a).

*A. zeae* and *F. verticillioides* endophytes of commercial corn hybrids should escape the negative effects of pathologist/breeder selection when their infections are symptomless and have no noticeable impact on yield. *A. zeae* could represent a confounding variable in evaluating varietal resistance to bacterial or fungal diseases. Grain harvested from irrigated fields was more heavily contaminated with *A. zeae*, whereas the incidence of *F. verticillioides* was much higher in corn grown under dryland conditions (Summer 1968b, Arino & Bullerman 1994). For some locations and years, the selection of disease resistant lines may be the indirect result of the presence of pyrocidine-producing *A. zeae* endophytes. For example, in a study of maize genotype effect on internal fungal infection, the incidence of *A. zeae* and *F. verticillioides* differed significantly (*P<0.01*) among maize hybrids grown under irrigation in test plots at six locations in Nebraska (Arino & Bullerman 1994). *Acremonium* was more often isolated from grain harvested in 1991 from the ‘T1 genetic group’, while *Fusarium* was isolated significantly less often. The authors suggest that the ‘T1 genetic group’ was more resistant to *Fusarium* colonization without considering interference by *Acremonium* as a potential confounding variable. One might also ask, are maize varieties selected for their resistance to *F. verticillioides* in field inoculation trials (e.g. King 1981, King & Scott, 1981, Ochor *et al.* 1987), generally more susceptible to *Aspergillus flavus* infection and aflatoxin?

In the present study we have shown that *Acremonium zeae* interferes with *F. verticillioides* and *Aspergillus flavus* colony growth in cultural pairings and produced a pyrocidine antibiotic in preharvest maize kernels which was active against *F. verticillioides* and *A. flavus* in disc assays. We have characterized *A. zeae* as a ‘protective endophyte’ of maize, thereby bringing attention to the biocontrol potential of *A. zeae*, which has never been linked to any mycotoxicosis in cultivated cereals.

**ACKNOWLEDGEMENT**

Support for this work from the National Science Foundation (CHE-0079141 and CHE-0315591) is gratefully acknowledged.
REFERENCES


Koehler, B. (1942) Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. *Journal of Agricultural Research* **64**: 421–442.


*Corresponding Editor: N. Hywel-Jones*