Antimicrobial Activity of Pyrrocidines from *Acremonium zeae* Against Endophytes and Pathogens of Maize

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ABSTRACT

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Acremonium zeae produces pyrrocidines A and B, which are polyketide-amino acid-derived antibiotics, and is recognized as a seedborne protective endophyte of maize which augments host defenses against microbial pathogens causing seedling blights and stalk rots. Pyrrocidine A displayed significant in vitro activity against *Aspergillus flavus* and *Fusarium verticillioides* in assays performed using conidia as inoculum, with pyrrocidine A being more active than B. In equivalent assays performed with conidia or hyphal cells as inoculum, pyrrocidine A revealed potent activity against major stalk and ear rot pathogens of

maize, including *F. graminearum*, *Nigrospora oryzae*, *Stenocarpella (Diplodia) maydis*, and *Rhizoctonia zeae*. Pyrrocidine A displayed significant activity against seed-rotting saprophytes *A. flavus* and *Eupenicillium ochrosalmoneum*, as well as seed-infecting colonists of the phylloplane *Alternaria alternata*, *Cladosporium cladosporioides*, and *Curvularia lunata*, which produces a damaging leaf spot disease. Protective endophytes, including mycoparasites which grow asymptotically within healthy maize tissues, show little sensitivity to pyrrocidines. Pyrrocidine A also exhibited potent activity against *Clavibacter michiganense* subsp. *nebraskense*, causal agent of Goss's bacterial wilt of maize, and *Bacillus mojaviense* and *Pseudomonas fluorescens*, maize endophytes applied as biocontrol agents, but were ineffective against the wilt-producing bacterium *Pantoea stewartii*.

The fungal endophyte *Acremonium zeae* W. Gams & D. R. Sumner 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 (*Zea mays*) seed (56, 57, 59). 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 (Fig. 1) (59). The pyrrocidines were previously reported from the fermentation broth of an unidentified species of *Cylindrocarpum*, isolated from a mixed Douglas fir forest on Crane Island Preserve, WA, in 1993 (6, 18). Pyrrocidines were detected in fermentation extracts for 12 NRRL cultures of *Acremonium zeae* isolated from maize seed harvested in the United States. Furthermore, pyrrocidine B was detected by liquid chromatography–tandem mass spectrometry in whole symptomatic maize seed removed at harvest from ears of a commercial hybrid that were wound inoculated in the milk stage with *A. zeae*. This was the first report of natural products from *A. zeae* (59).

Dihydroresorcyllide, a previously unknown macrocyclic lactone isolated from *A. zeae* and not related to the pyrrocidines (Fig. 1), was shown to be phytotoxic to maize using a leaf-puncture wound assay; however, no fungistatic activity was detected (35). Our objective was to compare the in vitro inhibitory activity of pyrrocidines A and B against common fungal endophytes and pathogens of maize which have been functionally classified as belong-

ing to different ecological groups (54), including molds capable of infecting and rotting maize kernels while contaminating the grain with mycotoxins harmful to livestock as well as humans. Because pyrrocidine A exhibits potent activity against medically important gram-positive bacteria (18), we also wanted to evaluate the activity of pyrrocidines against selected bacterial pathogens and endophytes of maize, the latter having been identified for their biocontrol potential.

MATERIALS AND METHODS

Fungal inoculum and culture conditions. The cultures of fungi and bacteria used in this study (Tables 1 to 3) were obtained from the Agricultural Research Service Culture Collection, Peoria, IL. All of the cultures were isolated from maize with the exception of *Verticillium lecanii* NRRL 26576, isolated by D. T. Wicklow from the mushroom *Amanita bisporigera* (43), and reported to be a seedborne endophyte of maize (12). In our initial evaluation of *Aspergillus flavus* (NRRL 6541) and *F. verticillioides* (NRRL 25457) sensitivity to pyrrocidines A and B with traditional paper disc assays, plates were seeded with a suspension of filtered conidia (59). An equivalent filtered conidial inoculum was produced from the same fungal strains to evaluate the in vitro fungistatic activity of pyrrocidines A and B in experiments described below. *A. flavus* and *F. verticillioides* were grown in Roux bottles containing potato dextrose agar (PDA) for 14 days at 25°C. Hyphal fragments and conidium-bearing structures (e.g., phialides and conidiophores) suspended in sterile distilled water were removed by filtering through a double layer of sterile cheese cloth. Potato dextrose broth (PDB) was seeded with *A. flavus* or *F. verticillioides* conidia, giving a final conidial suspension of $\approx 4 \times 10^4$ ml⁻¹ of PDB. Twenty fungal species representing different ecological groups isolated from corn (Table 2) were grown as PDA slant cultures for 6 days at 25°C. A suspension of conidia or hyphal cells was prepared from these

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cultures and used as inoculum to seed PDB, giving a final propagule density of $\approx 4 \times 10^4 \text{ ml}^{-1}$. Seed inoculum for bacterial cultures (Table 3) was produced in 100 ml of tryptone glucose yeast extract (TGY) broth (5 g of tryptone, 5 g of yeast extract, 1 g of dextrose, 1 g of K_2HPO_4 , and 1 liter of water) in a 500-ml Erlenmeyer flask shaken at 130 rpm for 18 to 36 h at 25°C.

Isolation and purification of pyrrocidines A and B. The ethyl acetate extract from six freeze-dried 15-day-old cultures of *Acremonium zeae* NRRL 13540 grown on 200 g of corn at 25°C with a total weight of 55 g was separated by flash chromatography on silica. Two extracts were combined and each pair separated as follows. The extract was dissolved in 200 ml of hexane and centrifuged at 1,700 rpm for 5 min, and the hexane was decanted. A 10-g silica Sep-Pak cartridge (Waters Corp., Milford, MA) conditioned with 100 ml of hexane and the extract in hexane was loaded onto the column. The column was eluted with 100 ml of hexane, 100 ml of ethyl acetate/hexane at 20/80, 4 $\times$ 50 ml at 30/70, 2 $\times$ 50 ml at 40/60, 2 $\times$ 50 ml at 50/50, 100 ml at 70/30, 100 ml of ethyl acetate, and 100 ml of methanol. Each fraction was analyzed by liquid chromatography–mass spectrometry (LC-

MS). Pyrrocidine A was eluted with ethyl acetate/hexane at 30/70 and pyrrocidine B with ethyl acetate/hexane at 40/60 and 50/50. Each fraction was dried down with a rotary evaporator, transferred to vials with ethyl acetate, evaporated to dryness under a stream of filtered air, and stored in a freezer. The three pyrrocidine A fractions had a total weight of 589 mg and the pyrrocidine B fractions weighed 611 mg. Each of the six fractions was further purified using a 10-g tC18 Sep-Pak cartridge (Waters Corp.). The cartridge was conditioned with 100 ml of methanol followed by 100 ml of acetonitrile/water (50/50). Pyrrocidine A fractions were dissolved in 2 ml of ethyl acetate plus 48 ml of acetonitrile. Pyrrocidine B fractions were dissolved in 50 ml of acetonitrile. Before loading onto the conditioned cartridge, each fraction was diluted with 50 ml of water. The cartridge was eluted with 100 ml of 50/50 acetonitrile/water, 2 $\times$ 50 ml at 60/40, 2 $\times$ 50 ml at 70/30, 2 $\times$ 50 ml at 80/20, 100 ml of acetonitrile, and 100 ml of ethyl acetate. Each fraction was analyzed by LC-MS. Pyrrocidine A was eluted in both of the 2 $\times$ 40/60 fractions and the first only of the 2 $\times$ 50/50 fractions. Pyrrocidine B was eluted from both of the 2 $\times$ 40/60 fractions and the 70/30 fraction. The total weights of the three pyrrocidine A and B fractions were 263 and 402 mg, respectively. The pyrrocidine A fractions were stored in a freezer for 6 months before they were combined using ethyl acetate. LC-MS analysis showed that, during this time, some of the pyrrocidine A had oxidized to a product that was later identified as 17,18-epoxy-pyrrocidine A (11) and tentatively named pyrrocidine C. The dried pyrrocidine A fraction was triturated with acetonitrile and the acetonitrile supernatant solution was set aside for the purification of pyrrocidine C. The remainder was then crystallized from acetonitrile to give 76 mg of pyrrocidine A as white lustrous flakes.

Later separations were performed using a Versaflash system (Supelco, Bellefonte, PA). The ethyl acetate extract from eight freeze-dried 15-day-old cultures of *A. zeae* NRRL 13540 grown on 200 g of rice at 25°C with a total weight of 20 g was separated using a VersaPak 40-by-75-mm silica cartridge (Supelco). The extract was dissolved in 400 ml of hexane, centrifuged at 1,700 rpm, and the hexane decanted. The cartridge was conditioned with 400 ml of hexane and the sample loaded. The column was eluted as above but using 200- and 400-ml increments instead of 50- and 100-ml increments. The pyrrocidine A and B fractions weighed 298 and 376 mg, respectively. The methanol eluate was saved for the purification of the cerebrosides. The pyrrocidine A fraction was triturated twice with acetonitrile and once with hexane, which reduced the weight to 189 mg. Crystallization from acetonitrile gave 99 mg of slightly yellow pyrrocidine A.

Extracts from 20 rice cultures were separated using a VersaPak silica cartridge as described above to give a pyrrocidine B fraction weighing 993 mg. Further separation on a VersaPak 40-by-75-mm C18 cartridge using 150- and 300-ml steps instead of 50- and 100-ml steps, as described above for the tC18 cartridge, reduced the weight of the pyrrocidine B fraction to 541 mg. This was combined with a 660-mg pyrrocidine B fraction obtained in the same way. The sample was dissolved in ethyl acetate and treated with activated charcoal (Darco G-60; EM Science, Gibbstown, NJ). The filtered solution was dried under a filtered air stream and dissolved in boiling chloroform/hexane (1/25). The solution was allowed to cool and stand uncovered in a fume hood until most

Fig. 1. Chemical structures of pyrrocidine A, pyrrocidine B, and dihydroresorcyllide, metabolites produced by *Acremonium zeae*.

TABLE 1. Antimicrobial activity of pyrrocidines A and B produced by *Acremonium zeae* to *Aspergillus flavus* and *Fusarium verticillioides*^a

Isolate	Pyrrocidine A		Pyrrocidine B		Nystatin	
	MIC	GI ₅₀	MIC	GI ₅₀	MIC	GI ₅₀
<i>Aspergillus flavus</i> NRRL 6541	≤5	>1–50	>50	>50	≤10	>3–50
<i>Fusarium verticillioides</i> NRRL 25457	≤10	>2–50	>50	>25–50	≤25	>5–50

^a Inoculum was a conidial suspension filtered through sterile cheese cloth. Concentrations tested were 1, 2, 3, 5, 10, 25, and 50 µg/ml. MIC = minimum inhibitory concentration and GI₅₀ = growth inhibition >50% relative to the control.

but not all of the solvent had evaporated. Pyrrocidine B precipitated as a fine powder. This was resuspended in hexane and, after filtering, gave 237 mg of pyrrocidine B.

Comparison of in vitro activities. A minimal inhibitory concentration (MIC) was assigned to the smallest treatment concentrations for which no fungal or bacterial growth was recorded and another value was designated when fungal or bacterial growth inhibition exceeded 50% of the growth recorded for methanol control wells (GI₅₀). Pyrrocidines A and B were evaluated in 96-well plates with a growth area of 0.32 cm² and volume of 370 µl

(BD Primaria Clear 96-well Microtest Plate No. 353872; Becton Dickinson, Franklin Lakes, NJ.) at concentrations of 1, 2, 3, 5, 10, 25, and 50 µg/ml.

The inhibitory activity of the broad-spectrum antifungal agent nystatin (Sigma-Aldrich, St. Louis) was also evaluated for potential inhibitory activity (MIC and GI₅₀) against this collection of fungal endophytes and pathogens of maize, serving as a positive control. Appropriate dilutions of the test compound in 10 µl of methanol were added to each of eight replicate wells and evaporated to dryness. Eight replicate wells received 10 µl of methanol

TABLE 2. Antimicrobial activity of pyrrocidines A and B to fungal endophytes and pathogens of maize^a

Pathogens and endophytes	Pyrrocidine A		Pyrrocidine B		Nystatin	
	MIC	GI ₅₀	MIC	GI ₅₀	MIC	GI ₅₀
Stalk- and ear-rotting pathogens						
<i>Fusarium graminearum</i>						
NRRL 13188	≤1	<1–50	>50	>25–50	≤10	>1–50
NRRL 31250	≤1	<1–50	>50	>50	≤5	>1–50
<i>Nigrospora oryzae</i>						
NRRL 6414	≤1	<1–50	≤10	>5–50	≤5	≈2–50
NRRL 47510	≤1	<1–50	≤5	>1–50	≤1	>1–50
<i>Rhizoctonia zeae</i>						
NRRL 40186	≤2	<1–50	>50	>10–50	≤10	>3–50
NRRL 40187	≤2	<1–50	nt	nt	nt	nt
<i>Stenocarpella (Diplodia) maydis</i>						
NRRL 13615	≤1	<1–50	≤25	>10–50	≤1	<1–50
NRRL 31249	≤2	<1–50	≤25	>5–50	≤1	<1–50
<i>Trichoderma viride</i>						
NRRL 6418	>50	≈10–50	>50	>50	>50	>10–50
Symptomless endophytes						
<i>Acremonium zeae</i>						
NRRL 6415	>50	>50	>50	>50	≤10	>1–50
NRRL 13540	>50	>50	>50	>10–50	≤3	>1–50
<i>F. oxysporum</i>						
NRRL 37597	>50	≈25–50	>50	>50	≤10	>1–50
<i>F. proliferatum</i>						
NRRL 6413	>50	≈25–50	>50	>25–50	≤25	>3–50
NRRL 13569	>50	>10–50	>50	>50	≤10	≈2–50
NRRL 22058	>50	>50	>50	>50	≤10	>2–50
<i>F. subglutinans</i>						
NRRL 22002	>50	>10–50	>50	>50	≤3	>1–50
NRRL 22034	>50	≤50	>50	>50	≤5	>1–50
<i>F. verticillioides</i>						
NRRL 25457	>50	≈25–50	>50	>25–50	≤10	>3–50
NRRL 13586	>50	>10–50	>50	>25–50	≤10	>2–50
NRRL 20984	>50	>50	>50	>50	≤10	>3–50
Mycoparasites –Fungicolous						
<i>Penicillium funiculosum</i>						
NRRL 6417	>50	≈50	>50	>50	>50	>25–50
NRRL 35431	>50	>50	>50	>50	≤50	>10–50
<i>P. pinophilum</i>						
NRRL 6420	>50	>50	>50	>50	≤50	>10–50
<i>P. variable</i>						
NRRL 6419	>50	>50	>50	>50	>50	>50
<i>Verticillium lecanii</i>						
NRRL 26576	>50	>25–50	>50	>50	>50	>50
Kernel rotting saprotrophs						
<i>Aspergillus flavus</i>						
NRRL 6541	>50	>1–50	>50	≤50	≤10	>3–50
<i>A. niger</i>						
NRRL 6411	>50	>25–50	>50	>50	≤50	>2–50
<i>Eupenicillium ochrosalmoneum</i>						
NRRL 6568	>50	>1–50	>50	>50	≤50	>25–50
<i>P. oxalicum</i>						
NRRL 6416	>50	>25–50	>50	>50	≤5	>1–50
Phylloplane – Foliar diseases						
<i>Alternaria alternata</i>						
NRRL 6410	≤10	>1–50	>50	>10–50	≤5	>1–50
<i>Cladosporium cladosporioides</i>						
NRRL 6421	≤5	>3–50	>50	≈50	≤10	>1–50
<i>Curvularia lunata</i>						
NRRL 6409	>50	>1–50	>50	>50	≤5	>2–50

^a Inoculum was a suspension of conidia or hyphal cells from potato dextrose agar slant cultures grown for 6 days at 25°C. Concentrations tested were 1, 2, 3, 5, 10, 25 and 50 µg/ml. MIC = minimum inhibitory concentration, GI₅₀ = growth inhibition >50% relative to the control, and nt = not tested.

and served as controls. PDB (200 µl) seeded with each tested fungus or 200 µl of TGY broth seeded with each bacterium (1×10^8 cells ml⁻¹) were added to experimental wells. Immediately before pipetting media seeded with microbes into experimental wells, 10 µl of methanol was added to redissolve the test compound. The 96-well plates containing the fungi were incubated on a shelf for up to 64 h at 25°C and examined at 8- to 16-h intervals using a plate reader at a wave length of 550 nm (Dynatech MR 5000 with BioLinx Version 2.0 Assay Management Software; Dynatech Laboratories, Inc., Chantilly, VA) for evidence of inhibition of fungal growth in the wells, which is a measure of fungistatic activity. Plates containing bacteria were incubated on a shaker at 130 rpm for up to 48 h at 25°C and examined at 8- to 16-h intervals.

RESULTS

The results that follow contrast the sensitivities of common microbial endophytes and pathogens of maize to pyrrocidine antibiotics. In experiments using a filtered conidial inoculum, pyrrocidine A displayed significant in vitro activity against *Aspergillus flavus* (MIC = ≤ 5 µg/ml) and *F. verticillioides* (MIC = ≤ 10 µg/ml), with pyrrocidine A being more active than B (Table 1). All other comparisons of the in vitro antifungal activities of pyrrocidines and nystatin (MIC) were performed using an inoculum consisting of the conidia or hyphal cells produced by individual test fungi in PDA slant cultures (Table 2). The MIC values of pyrrocidine A revealed potent activity of 1 to 2 µg/ml against major stalk and ear rot pathogens of maize that are also associated with pre- or post-emergence seedling blights, including *F. graminearum* (holomorph: *Gibberella zeae*), *Nigrospora oryzae* (holomorph: *Khushia oryzae*), *Rhizoctonia zeae* (holomorph: *Waitea circinata*), and *Stenocarpella maydis* (syn. = *Diplodia maydis*), while inhibiting the growth of *Trichoderma viride* (holomorph: *Hypocrea* sp.) (GI₅₀ ≈ 10 to 50 µg/ml). Among all fungi tested, *N. oryzae* was the most sensitive to pyrrocidine B (MICs = 5 to 10 µg/ml). Pyrrocidine A also exhibited significant activity against dematiaceous hyphomycetes that colonize the phylloplane *Alternaria alternata*, *Cladosporium cladosporioides* (holomorph: *Mycosphaerella tassiana*) (MICs = 5 to 10 µg/ml) and the maize leaf spot pathogen *Curvularia lunata* (holomorph: *Cochliobolus lunatus*) (GI₅₀ = >1 to 50 µg/ml). Kernel-rotting saprotrophs presented a wide range of sensitivity to pyrrocidine A, with *Aspergillus flavus* and *Eupenicillium ochrosalmoneum* (GI₅₀s = >1 to 50 µg/ml) displaying greater sensitivity than *A. niger* and *Penicillium oxalicum* (GI₅₀s = >25 to 50 µg/ml).

Symptomless 'protective' endophytes, including *Acremonium zeae*, *F. oxysporum*, *F. proliferatum* (holomorph: *G. fujikuroi* var. *intermedia*), *F. subglutinans* (holomorph: *G. subglutinans*), and

F. verticillioides (holomorph: *G. moniliformis*) displayed little or no sensitivity to pyrrocidines (GI₅₀s = >10 to 50 µg/ml) yet were sensitive to nystatin (Table 2). At the same time, all of the fungi that were sensitive to pyrrocidine were also sensitive to nystatin. Pyrrocidine A was less active against *F. verticillioides* NRRL 25457 (GI₅₀ ≈ 25 µg/ml) than in tests in which filtered conidia composed the inoculum (Table 1). Mycoparasites, recorded as symptomless endophytes of maize seed or stalks, including *P. funiculosum*, *P. pinophilum*, *P. variabile*, and *V. lecanii*, revealed little sensitivity to pyrrocidines or nystatin (Table 2).

Pyrrocidine A also exhibited potent activity against *Clavibacter michiganense* subsp. *nebraskense*, causal agent of Goss's bacterial wilt of maize (MICs = 1 to 2 µg/ml), as well as *Bacillus mojavensis* (MICs = 1 to 2 µg/ml) and *Pseudomonas fluorescens* (MICs = 1 to 2 µg/ml), maize endophytes applied as biocontrol agents (Table 3). The pyrrocidines were ineffective (MICs = >50 µg/ml) against the wilt-producing bacterium *Pantoea stewartii* subsp. *stewartii* (syn. *Erwinia stewartii*), and *P. agglomerans* (syn. *Enterobacter agglomerans*; *Erwinia herbicola*), an asymptomatic endophyte of maize.

DISCUSSION

Healthy maize plants become internally colonized with a diverse assemblage of microorganisms, a large majority of which are established as symptomless endophytic infections. Relatively few of these microbes are able to directly attack healthy maize tissues. A larger number produce disease symptoms in association with different forms of abiotic or biotic stress-related damage to plant tissues, which may cause premature senescence (52). Although some of these organisms are commonly recorded as pathogens of maize wherever it is grown and may also infect other cereals and grasses (Gramineae), others are isolated as pathogens of crops, belonging to different plant families, or are recognized as entomopathogens, mycoparasites, inhabitants of the rhizosphere, or common soil-inhabiting saprotrophs (12,54,61). Maize grown in different climate zones and included in crop rotations developed for soil types within larger natural ecosystems can be expected to host locally adapted assemblages of microorganisms (12,24,34,41). *A. zeae* is reported to be a prevalent fungal endophyte among such microbial assemblages of maize and has been shown to interfere with the in vitro growth of fungal pathogens of maize and produce pyrrocidine antibiotics (56,59).

Pyrrocidines A and B displayed significant antifungal activity against *A. flavus* NRRL 6541 and *F. verticillioides* NRRL 25457 using conventional paper-disc assays in which PDA was seeded with a filtered conidial inoculum (59). In the present study, the in vitro activities (MIC) of pyrrocidines A and B were initially determined using a filtered conidial inoculum produced by the same

TABLE 3. Antimicrobial activity of pyrrocidines A and B to bacterial endophytes and pathogens of maize^a

Pathogens and endophytes	Pyrrocidine A		Pyrrocidine B	
	MIC	GI ₅₀	MIC	GI ₅₀
<i>Bacillus mojavensis</i>				
NRRL B-14818	≤ 2	$>1-50$	≤ 10	$>5-50$
NRRL B-14700	≤ 1	$>1-50$	≤ 5	$>3-50$
<i>Clavibacter michiganense</i> subsp. <i>nebraskense</i>				
NRRL B-41133	≤ 1	$>0.25-50$	≤ 10	$>1-50$
NRRL B-41134	≤ 2	$>0.25-50$	≤ 10	$>1-50$
<i>Enterobacter agglomerans</i>				
NRRL B-14004	>50	>50	>50	>50
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>				
NRRL B-794	>50	>50	>50	>50
<i>Pseudomonas fluorescens</i>				
NRRL B-4290	≤ 1	$>1-50$	≤ 10	$>1-50$
NRRL B-14678	≤ 1	$>1-50$	≤ 10	$>1-50$

^a Concentrations tested were 0.25, 1, 2, 3, 5, 10, 25, and 50 µg/ml. MIC = minimum inhibitory concentration and GI₅₀ = growth inhibition $>50\%$ relative to the control.

fungal strains (Table 1). Pyrrocidine A displayed significant activity against both fungal strains. However, in tests using an inoculum consisting of both hyphal cells and conidia, pyrrocidine A was less active against *F. verticillioides* (Table 2). An example of differential inhibition of spore germination and vegetative growth by antifungal antibiotics was reported for *F. oxysporum* f. sp. *radicis-cucumerinum* (49).

The present results suggest that seed-infecting endophytes and pathogens of maize that display similar disease symptoms or share relevant ecological characteristics (54) can also exhibit a similar pattern of sensitivity to pyrrocidine A. As a group, stalk and ear rot pathogens of maize that are also causal organisms of seedling blights (22,52) (e.g., *S. maydis*, *F. graminearum*, *R. zeae*, and *N. oryzae*) were most sensitive to pyrrocidine A (Table 2). *T. viride* causes a severe ear rot of maize but was less sensitive to pyrrocidine A. *Trichoderma* spp. in general are resistant to antibiotics produced by other microorganisms (16). Although *Trichoderma* spp. are recognized for their mycoparasitic behavior and production of antifungal antibiotics, strains representing a few taxa have been reported to cause diseases of apple, maize, or alfalfa (4). As a colonist of maize, *T. viride* appears to bridge the ecological divide between symptomless endophyte in healthy maize stalks (45), virulent ear-rotting pathogen (52), and presumptive mycoparasite (50).

Numerous fungal taxa are recognized as colonists of the phylloplane and pathogens causing lesions on maize leaves, with symptoms characterized as leaf spots, leaf streaks, and leaf blights (52). In the present study, each of the three fungal taxa representing this ecological group displayed significant in vitro sensitivity to pyrrocidine A (Table 2). Among these fungi, *Cochliobolus lunata* produces a damaging leaf spot disease in hot, humid maize-growing areas (27). *Alternaria alternata* and *Cladosporium cladosporioides* are common saprophytes and symptomless colonists of the phylloplane of healthy plants, invading the leaves and forming spores as a response to early leaf senescence. *Alternaria* spp. are also reported to cause a leaf blight in the central corn belt while *C. cladosporioides* may produce symptoms of seed rot, although neither disease has been reported to be of economic significance (52).

Among the kernel-rotting saprotrophs, *Aspergillus flavus* and *E. ochrosalmoneum* were more sensitive to pyrrocidine A than *A. niger* or *Penicillium oxalicum* (Table 2). In addition to being known for infesting maize seed and contaminating them with aflatoxins (54), *A. flavus* is also recognized as an entomopathogen (44). *E. ochrosalmoneum* produces citreoviridin in maize grown in the southeastern United States (55) and shows a specificity for sporulating on the conidial heads of *A. flavus* although it has yet to be determined if the fungus is a mycoparasite (19). To our knowledge, there are no reports demonstrating microbial interference with maize seed infection by *A. niger* (47) or *P. oxalicum* (20).

F. proliferatum, *F. subglutinans*, *F. verticillioides*, and *F. oxysporum* are symptomless seedborne endophytes of healthy maize plants and displayed little or no sensitivity to pyrrocidines (Table 2). These fungi are associated with various human infections and shown to be generally resistant to most clinical antifungal agents (25). *F. proliferatum* (26,33) and *F. subglutinans* (21,24), have not been the focus of research on seed transmission and systemic whole-plant infection as with *F. verticillioides* (30,32,60). *F. oxysporum* is not known to cause any characteristic disease symptoms of maize (23,24) yet was the most frequent isolate from rotted mesocotyls 40 to 80 days after planting (36). Interestingly, *F. oxysporum* NRRL 37597 represents the seed borne mycohost of *Arthrobotrys* sp. NRRL 37598, the parasitized mycohost having been isolated from a surface-disinfected white corn seed that was plated with grain sampled at Cerro Gordo, IL. This group of endophytes is associated with *Fusarium* seed rot of maize, especially kernels damaged from insect herbivory (29,31,40), as

well as stalk rot in mature maize plants (9,52). However, none are regarded as aggressive pathogens when compared with fungi recognized as the primary cause of stalk rot, *S. maydis* and *F. graminearum* (52). Seed infected with *F. verticillioides* were less likely to also be infected with other fungal pathogens of maize (7,37,53,57), suggesting that *F. verticillioides* may have a “protective effect” by suppressing the growth of more destructive pathogens *F. graminearum* and *S. maydis* (37). *F. verticillioides* is recognized as producing disease symptoms when responding to exclude primary pathogens from maize tissues severely damaged by insect herbivory (31,39,40) or in response to other forms of environmental stress that lead to premature plant senescence (9, 29,45). It could be expected that *Acremonium zeae* and *Fusarium* endophytes present in healthy stem tissues are also recorded among bacteria and fungi, constituting the “disease complex” in necrotic stem tissues associated with stalk rot (9,45).

Mycoparasites capable of attacking other fungi and used as biocontrol agents showed little or no sensitivity to pyrrocidines (Table 2). Fisher et al. (12) recorded *V. lecanii* as a seedborne endophyte of maize, isolating the fungus from healthy stem core tissues. The fungus is known as both a mycoparasite and a pathogen of insects (1,13,15,28), producing verticillanins which displayed antiinsect activity against the corn earworm *Helicoverpa zeae*, along with 2-decenedioic acid, reported as a nematocidal product from the nematode-trapping fungus *Pleurotus ostreatus* (43). *Penicillium* spp. in the subgenus *Biverticillium* have been observed to parasitize other seed-infecting fungi (54) and may be recorded as seed-rotting pathogens when sporulating heavily on seed initially rotted by *Fusarium* spp. or another suitable mycohost. Among 15 species of *Penicillium* isolated from maize, *Penicillium funiculosum* and *P. oxalicum* were the only species that could invade and colonize maize ears (8).

In all tests, with bacteria or fungi, pyrrocidine A was more active than B, as was earlier shown in tests with *Candida albicans* and gram-positive bacteria, including drug-resistant strains (18). Natural infection of germinated maize seed with pyrrocidine-producing genotypes of *A. zeae* may protect maize seedlings from *Clavibacter michiganense* subsp. *nebraskense*, which can be transmitted in maize seed (5) and produces a systemic vascular wilt of maize plants (51). *A. zeae* might also interfere with the ability of *B. mojavensis* to suppress *F. verticillioides* infection of maize seedlings (2,3) or with the natural establishment of *Pseudomonas fluorescens* in healthy maize plants, thereby reducing the efficacy of biocontrol strains (12,14,48).

The present results have shown that pyrrocidine A exhibited potent activity against *S. maydis*, *F. graminearum*, and *C. michiganense* subsp. *nebraskense*, each of which are seedborne pathogens that can cause severe seedling blights and vascular wilts of maize. Maize seedlings grown from pathogen-infected seed or attacked by soilborne pathogens may not survive to produce mature plants bearing seeds (42). During this period of seedling vulnerability, pyrrocidine antibiotics, as acquired chemical defenses, could contribute to endophyte defense as *A. zeae* spreads within imbibed and germinated seed and establishes itself within the mesocotyl and root crown of emerging seedlings (45). How might this work? A hypothesis is offered in which abiotic or biotic forms of damage to plant tissues trigger a xylem-blocking response in those vascular bundles which are most vulnerable to pathogen ingress. Mucilage, gels, or gum-like substances secreted into xylem vessels provide a preferred substrate for *A. zeae* growth and pyrrocidine production. Microscopic observations revealed, that whenever *A. zeae* was found to be present in xylem vessels, it was growing “vigorously” on these gum-like substances and associated pentose sugars (17). In this example, pyrrocidines would be concentrated within individual vascular bundles, providing an efficient means of delivering a robust and targeted antimicrobial chemical defense. This hypothesis assumes that plants are grown from healthy seed infected with *A. zeae*. The

fungus is most commonly found in the pedicel and abscission layer of seed but may also infect the germ and endosperm, particularly when seed were first soaked for 40 h before dissection (45). Growing from germinated seed, *A. zeae* invades seedling roots, the mesocotyl, and the root crown, also forming single celled propagules which move within the xylem sap of healthy maize tissues and are capable of increasing their numbers through microcyclic conidiation (D. T. Wicklow, *unpublished data*). This results in *A. zeae* being 'prepositioned' in the root crown as well as the lower nodes and internodes of healthy stem tissues, where the fungus can effectively respond to abiotic or biotic forms of damage to maize tissues that enable pathogen establishment. The root crown represents a critical conduit for pathogen access to the vascular bundles of the stem and an opportunity to rapidly spread throughout the plant. Maize responds to mechanical wounding, even in the absence of microbes, by initiating a xylem-blocking response in which mucilage and phenolics are secreted into xylem vessels (10). These authors considered the gels and gums that formed in xylem vessels of maize stems that were wound inoculated with *A. zeae* (46) as being "protective" and a part of the plant defense response. Furthermore, the release of gels and gum-like substances into vascular bundles has also been associated with hereditary factors and physiological stress influenced by environmental conditions such as drought or imbalanced fertility (17). The potential role of host-sensitive cerebroside elicitors produced by *A. zeae* (58) in activating this maize plant defense response also needs to be explored.

In the present study, we have shown that endophytes and pathogens of maize can differ substantially in their in vitro sensitivities to pyrrocidine antibiotics. The organisms found to be most sensitive to these antibiotics are well-established pathogens of maize. Pyrrocidine-producing *A. zeae* endophytes may reduce the diversity and abundance of maize pathogen assemblages, as has been shown for *Neotyphodium* endophytes of grasses (38). At the same time, pyrrocidines were largely ineffective against protective endophytes, including mycoparasites, which produce symptomless infections and have been shown to interfere with more virulent maize pathogens. Insensitivity to pyrrocidines could facilitate the coexistence of *A. zeae* with other protective endophytes and mycoparasites, thereby extending the diversity and range of acquired endophyte defenses.

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