

# Evidence for alteration of fungal endophyte community assembly by host defense compounds

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## Summary

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- Plant defense compounds are common stressors encountered by endophytes. Fungi readily evolve tolerance to these compounds, yet few studies have addressed the influence of intraspecific variation in defense compound production on endophyte colonization. We compared the influence of defense compound production on the composition of fungal endophyte communities in replicated field experiments.
- Maize (*Zea mays*) produces benzoxazinoids (BXs), compounds with antifungal byproducts persistent in the environment. Fungi were isolated from leaf and root tissue of two maize genotypes that produce BXs, and a natural mutant that does not. Isolates representing the species recovered were tested for tolerance to 2-benzoxazolinone (BOA), a toxic BX byproduct.
- In seedling roots and mature leaves, the community proportion with low BOA tolerance was significantly greater in BX nonproducers than producers. Mean isolation frequency of *Fusarium* species was up to 35 times higher in mature leaves of BX producers than nonproducers.
- Fungal species with relatively high tolerance to BOA are more abundant in BX producing than BX nonproducing maize. Production of BXs may increase colonization by *Fusarium* species in maize, including agents of animal toxicosis and yield-reducing disease in maize. Overall, results indicate that production of defense compounds can significantly alter endophyte community assembly.

## Introduction

Communities of nonmycorrhizal fungal endophytes occupy most plants (Schulz & Boyle, 2005; Arnold, 2007). They can directly affect plant fitness and indirectly influence surrounding plant and arthropod communities (Arnold *et al.*, 2003; Finkes *et al.*, 2006; Rudgers *et al.*, 2007). Fungal endophytes are transmitted in seed (vertically), or from plant to plant via fungal propagules (horizontally). An excellent example of obligate vertical transmission is the well-studied ascomycete, *Neotyphodium*, which forms associations with temperate grasses mediated by molecular signaling dependent on the compatibility of endophyte and host species (reviewed in Schardl *et al.*, 2004). Phylogenetic and molecular genetic evidence indicate co-evolution of some *Neotyphodium* species with their plant hosts (for reviews see Clay & Schardl, 2002; Schardl *et al.*, 2004). By contrast, the majority of fungal endophytes are transmitted horizontally or facultatively by seed. These fungi can form diverse species assemblages within plants, often at high density (Arnold, 2007). There are still

many open questions about the mechanisms that influence colonization and community structure of horizontally transmitted endophytes, but certain abiotic environmental factors and plant defense compounds are known to be important.

Abiotic factors such as fertilizer application can influence the community structure of fungal endophytes in maize (*Zea mays*) (Seghers *et al.*, 2004), and water activity and temperature can significantly influence the outcome of interactions between maize endophytes (Marin *et al.*, 1998). Plant defense compounds can also influence interactions between the plant and its surrounding community of fungi, bacteria, insects, and plants (Niemeyer & Perez, 1995). All plant species studied to date produce defense compounds (Hashimoto & Shudo, 1996; Dixon, 2001). Common tolerance strategies adopted by fungi include activation of membrane transporters that pump toxicants out of cells, and enzymatic detoxification (VanEtten *et al.*, 2001). Detoxification of host compounds can be a virulence factor among pathogens (VanEtten *et al.*, 2001).

Ability to detoxify may also increase competitiveness of root endophytes (Carter *et al.*, 1999). Increase in endophyte competitiveness among tolerant species is likely the result of higher growth rates in the presence of host toxins than less well-adapted species (Arnold *et al.*, 2003; Nicol *et al.*, 2003; Saunders & Kohn, 2008). This fitness benefit would be expected to influence endophyte community assembly. In a comparison of fungal communities in *Avena sativa* (oat) and *Triticum* sp. (wheat) roots, Carter *et al.* (1999) found that the majority of oat-derived isolates tolerated the oat defense compound, avenacin A-1. The spatial scale of influence may increase when plant defense compounds are secreted in soil, affecting colonization by mycorrhizal and soil fungi (Stinson *et al.*, 2006; Broeckling *et al.*, 2008).

Commercial maize has been selectively bred to produce high quantities of defense compounds, the benzoxazinoids (BXs). Several BX byproducts are toxic to microbes, insects and plants (Barry & Darrah, 1991; Niemeyer & Perez, 1995; Hashimoto & Shudo, 1996). The primary BXs found in maize are 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one (DIBOA). These compounds reside in the cell vacuole as biologically inactive beta-glucosides. They are enzymatically converted to toxic benzoxazinoids upon cell disruption (Hashimoto & Shudo, 1996), ultimately degrading to the biologically active and stable benzoxazolinones, 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA). These are formed systemically and secreted from root tissue (Park *et al.*, 2004). They can be produced both constitutively and in response to tissue damage (Cambier *et al.*, 2000; Oikawa *et al.*, 2004).

All commercial maize genotypes produce BXs. Concentrations of DIMBOA have been recorded ranging from 2.86 to 12.90 mm kg<sup>-1</sup> FW (Zuniga *et al.*, 1983; Cambier *et al.*, 2000). The concentrations of BXs and their toxic byproducts in plant tissue can vary with plant age, tissue, genotype and environment (Zuniga *et al.*, 1983; Richardson & Bacon, 1993; Cambier *et al.*, 2000). Uptake of BX byproducts from soil by plants has been reported (Chiapusio *et al.*, 2004). Accumulation of BX byproducts in soil is expected to depend on all of these factors, as well as the interactions between members of the microbial community (Bacon *et al.*, 2007).

It has been proposed that the ability to detoxify benzoxazolinones enhances colonization success in maize (Glenn *et al.*, 2001). Some detoxifying species, particularly *Fusarium verticillioides*, *Fusarium subglutinans*, *Fusarium proliferatum* and *Fusarium graminearum* cause disease in corn, but are also common endophytes. As endophytes, they can lead to the asymptomatic contamination of grain, in some cases producing toxins that cause mycotoxicosis in animals, and are suspected risk factors for cancers and other human health problems (Ueno *et al.*, 1997; Marasas, 2001; Marasas *et al.*, 2004). Contamination of maize grain by *Fusarium* is estimated to cause millions of dollars of economic loss annually in the USA (Wu, 2007; Wu

& Munkvold, 2008). Breeding programs aimed at deterring infection of maize by *Fusarium* using native resistance mechanisms have been largely unsuccessful (Munkvold, 2003).

Here, we investigated the influence of BX production on the assembly and composition of fungal endophyte communities in maize. Specifically, three hypotheses were tested: (1) BX production increases the proportion of fungi tolerant to the toxic BX byproduct, BOA, in endophyte communities; (2) BX production increases the incidence of *Fusarium* in maize; and (3) BX producing genotypes harbor endophyte communities that are less diverse than genotypes that do not produce BXs. To test these hypotheses, fungal endophyte communities from three maize genotypes were compared: one was a natural mutant deficient in BXs (BX<sup>-</sup>) and the other two were commercial genotypes that produce BXs (BX<sup>+</sup>). The objective was to observe communities in BX<sup>-</sup> versus BX<sup>+</sup> maize. Common species were tested for BOA tolerance in vitro. Community structure was consistent with expectations for BX influence in seedling roots and mature leaves. BX production significantly increased the frequency of *Fusarium* in leaves of mature plants.

## Materials and Methods

### Maize genotypes

Each location was planted with three maize (*Z. mays* L.) lines W22 and B37, two genotypes both producing BXs and commonly used to produce commercial hybrids, and *bxbx*, the only recorded natural mutant lacking the ability to produce BXs (Hamilton, 1962). The three genotypes are Yellow Dent maize, characterized by a genetic background of flint and floury maize and common kernel phenotypes (Smith *et al.*, 2004). Because of the nonlinear dynamics of BX concentration in plants and soil, we chose to assess the influence of BX production, rather than concentration, on endophyte communities.

### Study site and collection times

Maize was planted in two Ontario locations approx. 123 km apart: Ridgetown, with a history of soybean crops from 2001 to 2004, and Harrow, with a history of maize from 1999 to 2004. Within the plot, each genotype was planted in 12 rows, one genotype per row, as a row intercropping design. Assignment of rows within the field was random. The Ridgetown plot was surrounded by soybean, which does not produce BXs, and the Harrow plot by BX-producing maize. Planting was on June 12, 2005, with sampling carried out 2 wk and 9 wk subsequently. Whole plants were collected in paper bags and stored at 4°C.

### Isolation of fungi from plant tissue

Plants were rinsed with distilled water and surface dried at room temperature. From each plant, eight healthy 1.0 × 2.0 cm

segments were taken 0.5 cm from the midrib and above the leaf collar of the second and third leaf blades (2-wk-old plants) or fourth and fifth blades (9-wk-old plants). Plants collected at 2 wk had three emergent leaves (V3 growth stage). Plants collected at 9 wk were entering the R3 stage of growth, midway through kernel development and approx. 3 wk before physiological maturity (Ritchie *et al.*, 1993). Leaf senescence begins at physiological maturity. Our goal was to analyse healthy plant material; tissue was therefore collected before maturity. Healthy root segments 2.0 cm long and 0.2–0.3 cm diameter were taken from the radicle and lateral seminal roots. Tissue segments were surface-sterilized first in 70% ethanol (2 min), then in 0.53% NaOCl (2 min) and finally in sterile double-distilled water (2 min).

Tissue segments were incubated on two growth media. A general, neutral medium, potato dextrose agar (PDA; Difco, Detroit, MI, USA) was used to capture a relatively broad snapshot of the fungal community. A selective medium amended with 1.00 mg ml<sup>-1</sup> BOA (Glenn *et al.*, 2001) was used to determine the mean number of *Fusarium* colonies per plant (subsequently termed, abundance), isolated from 9-wk-old plants; this time-point was initially selected as an indicator of potential infestation of crop debris. Potato dextrose agar is expected to be more favorable than BOA medium to fungi that are BOA-sensitive.

Four leaf and four root segments were incubated on PDA amended with antibiotics (1.00 g l<sup>-1</sup> streptomycin sulfate and 0.25 g l<sup>-1</sup> neomycin sulfate), and four segments of each tissue were incubated on BOA medium. Segments from a total of 144 plants were plated on PDA (12 plants, 1 plant per row × 2 locations × 2 times: 2 wk and 9 wk post-planting × 3 maize genotypes); 9–12 plants per treatment were analyzed. A total of 144 plants were plated on BOA medium (24 plants, 2 plants per row × 2 locations × 1 time: 9 wk post planting × 3 maize genotypes); 16–24 plants (8–12 rows) per treatment were analysed. All tissues were surface sterilized and plated within 96 h of collection. Plates were incubated at room temperature under a 12-h light–12-h dark regime.

Effectiveness of the surface sterilization procedure was tested on PDA by plating out 500 µl of the rinse water from the sterilization procedure, and independently by using the tissue imprint method described by Schulz & Boyle (2005). Approximately 25% of tissue samples processed were tested, and no surface contaminants were detected.

### Identification of fungal isolates

Fungi emerging from plant tissue were isolated and established in axenic culture. Sporulating cultures were identified morphologically. Each *Fusarium* isolate was established in axenic culture from a single spore and identified morphologically when diagnostic characters were evident, or using DNA sequence data when such characters were ambiguous or absent. Isolates of *Fusarium* were grown on Carnation Leaf Agar (Leslie *et al.*,

2006) and PDA for morphological identification (Summerell *et al.*, 2003; Leslie *et al.*, 2006). For each *Fusarium* species that was identified morphologically, the identity of a subset of isolates was verified with DNA sequence data. Nonsporulating fungi were grouped into morphotypes. To maintain consistency, all morphotyping was done by one of the authors (M.S.). Morphotypes were then confirmed in blind tests by the other author (L.M.K.).

Isolates obtained on PDA were used to characterize 24 fungal endophyte communities (2 locations × 2 times × 2 tissue types × 3 maize genotypes). All isolates obtained from PDA were morphotyped, and isolates recovered more than three times were identified taxonomically. Of the isolates obtained on BOA medium, 57 isolates, 18 from leaf tissue and 39 from root tissue, were identified using molecular sequence data.

### DNA isolation, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was isolated using the DNeasy Plant Minikit (Qiagen, Mississauga, ON, Canada). For identification of morphotypes, the nuclear ribosomal internal transcribed spacer region (ITS) was amplified by PCR using primers ITS-1F and NLB-3 (*c.* 700 bp) (Gardes & Bruns, 1993; Martin & Rygielwicz, 2005). For identification of *Fusarium* isolates, the translation elongation factor 1-alpha (TEF) gene was amplified using primers TEF-1 and TEF-2 (*c.* 700 bp) (Geiser *et al.*, 2004).

The final volumes of the PCR mixture (50-µl volume) were 9.75 µl glass-distilled H<sub>2</sub>O, 5.00 µl 10× PCR buffer, 5.00 µl deoxynucleoside triphosphates, 0.50 µl of each primer (50 mM), 4.00 µl of magnesium chloride, 0.25 µl Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), and 25.00 µl of a 50-fold dilution of genomic DNA. A Perkin-Elmer GeneAmp System 9600 or 9700 thermocycler was used to amplify PCR product. The PCR program used was: 1 cycle of 95°C for 8 min, 35 cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min, and 1 cycle of 72°C for 10 min. Sequencing was performed at the Genetic Analysis Core Facility (USDA-ARS-ERRC, Wyndmoor, PA, USA) using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Contigs were assembled and edited in SEQUENCHER 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA), and BLAST searches of the NCBI GenBank database were conducted for tentative identification. A 99% sequence match to a sequence of known origin in the database was counted as a correct species identification. DNA sequences have been deposited in GenBank as FJ496215–FJ496332.

### Assignment of isolates to BOA tolerance class

Isolates were assigned to a tolerance class corresponding to the highest concentration of BOA that supported growth (Table 1). The PDA was amended with BOA (stock solution

**Table 1** 2-Benzoxazolinone (BOA) tolerance thresholds of fungal endophyte species/morphotypes isolated from maize (*Zea mays*) (*bxbx*, B37, W22 genotypes)

Tolerance class	BOA tolerance threshold	Species/morphotype	Order	Number of isolates in tolerance class	Total number of isolates tested
I	0.25 mg ml <sup>-1</sup>	<i>Alternaria alternata</i>	Pleosporales	4	4
		Morphotype no. 14	Sordariales	4	4
		<i>Periconia macrospinoso</i>	Anamorphic ascomycete	3	3
II	0.50 mg ml <sup>-1</sup>	<i>Cladosporium</i> sp.	Capnodiales	2	2
		<i>Epicoccum nigrum</i>	Anamorphic ascomycete	2	2
		<i>Fusarium acuminatum</i>	Hypocreales	1	1
		<i>Fusarium incarnatum-equiseti</i> species complex	Hypocreales	5	5
		<b><i>Fusarium oxysporum</i> species complex</b>	Hypocreales	6	13
		<i>Fusarium proliferatum</i>	Hypocreales	1	1
		<i>Fusarium redolens</i>	Hypocreales	1	1
		<i>Fusarium sporotrichioides</i>	Hypocreales	1	1
		<i>Fusarium tricinctum</i>	Hypocreales	1	1
		<i>Penicillium</i> sp.	Eurotiales	3	3
		<i>Periconia circinatum</i>	Anamorphic ascomycete	3	3
III	0.75 mg ml <sup>-1</sup>	<b><i>Fusarium oxysporum</i> species complex</b>	Hypocreales	7	13
		<i>Fusarium solani</i> species complex	Hypocreales	2	2
		<i>Fusarium verticillioides</i>	Hypocreales	1	1
		Morphotype no. 22	Anamorphic ascomycete	2	2
		Morphotype no. 51	Anamorphic ascomycete	1	1
		<b><i>Nigrospora oryzae</i></b>	Trichosphaerales	2	4
		<b><i>Trichocladium</i> sp.</b>	Sordariales	2	4
		<i>Trichoderma</i> sp.	Hypocreales	2	2
IV	1.00 mg ml <sup>-1</sup>	<i>Fusarium culmorum</i>	Hypocreales	4	4
		<i>Fusarium graminearum</i>	Hypocreales	1	1
		<b><i>Trichocladium</i> sp.</b>	Sordariales	2	4
		<b><i>Nigrospora oryzae</i></b>	Trichosphaerales	2	4
V.	1.10 mg ml <sup>-1</sup>	<i>Rhizopus</i> sp.	Mucorales	3	3

Names in bold type indicate species with isolates in two tolerance classes.

of 100 mg ml<sup>-1</sup> in anhydrous ethanol) in each of the following concentrations: 0.00, 0.25, 0.50, 0.75, 1.00, 1.10, 1.20 mg ml<sup>-1</sup>. From a total of 24 species/morphotypes, 61 isolates were assigned to tolerance classes. Two isolates per species/morphotype were assigned when available. All *Fusarium* species and species/morphotypes recovered more than three times were included. Strains were incubated in quadrant Petri dishes for 14 d in the dark at 22°C, and scored for growth or no growth.

### Statistical analyses

For all statistical analyses, each row was considered the unit of replication. For the PDA assays, one plant per row was sampled. For the BOA assay, two plants per row were sampled. The average number of isolates per plant, averaged across each row, was considered a replicate.

**Abundance of *Fusarium* in 9-wk-old plants** One-way ANOVAs were followed by Tukey–Kramer Honestly Significant Difference (HSD) tests for pairwise comparisons between the BX– and BX+ genotypes. The threshold for statistical significance was

$P \leq 0.05$ . Analyses were conducted using JMP in (version 5.1; SAS Institute Inc., Cary, NC, USA).

### Diversity and similarity of fungal endophyte communities

As described earlier in the section Identification of Fungal Isolates, 24 fungal endophyte communities were characterized from isolates obtained on PDA. Diversity was measured using the Simpson's inverse diversity index ( $D$ ) and Fisher's alpha ( $\alpha$ ) (Magurran, 2004). Similarity was determined using the Jaccard's (Magurran, 2004) and Morisita–Horn indices (Chao *et al.*, 2005). All were calculated using ESTIMATES (Colwell, 2000).

### BOA tolerance of fungal endophyte communities

The distribution of isolates in BOA tolerance classes was determined for each community. To test for a difference between the distribution of isolates in BOA tolerance classes in BX+ and BX– plants, a likelihood ratio test was conducted using the JMPin statistical analysis software package. When necessary, results were then adjusted for small expected values using the formula (Gotelli & Ellison, 2004):

$$\chi^2_{\text{adjusted}} = \chi^2 / q_{\min}$$

$$q_{\min} = \frac{1 + \left[ N \sum_{i=1}^n \frac{1}{\sum_{j=1}^m Y_{i,j}} - 1 \right] \times \left[ N \sum_{j=1}^m \frac{1}{\sum_{i=1}^n Y_{i,j}} - 1 \right]}{6vN} \quad \text{Eqn 1}$$

( $n$  is the number of rows,  $m$  is the number of columns,  $N$  is the total sample size,  $v$  is the degrees of freedom and  $Y_{i,j}$  is the frequency of observations in row  $i$ , column  $j$ ).

When global likelihood ratio tests detected a significant difference between the distribution of isolates in BX+ and BX– plants, further tests for a difference between the proportion of fungi in tolerance classes I and II in each community were conducted. Follow-up likelihood ratio tests were conducted on tolerance classes I and II, because a mean of 83% of isolates in each community were in these tolerance classes.

## Results

### Relative abundance and diversity of endophytes from BX– and BX+ maize genotypes in 2- and 9-wk-old plants

Infection density, measured as the proportion of tissue segments yielding isolates, ranged from 54 to 100%, and was higher in August than in June in leaf and root tissue of all maize genotypes (see the Supporting Information, Fig. S1). Of 1495 fungal isolates obtained, 1018 were isolated on PDA, and 477 on BOA. The genus *Fusarium* includes several species complexes. Consequently, the *Fusarium oxysporum* species complex, the *Fusarium incarnatum-equiseti* species complex, and the *Fusarium solani* species complex were each treated as single species in the analyses. A total of 43 species/morphotypes were identified. All of the 13 species BOA were also isolated on PDA. On BOA medium, roots yielded approximately twice the number of isolates as leaves.

Species richness and presence/absence differed between communities from leaf and root tissue on PDA. Leaf and root tissue had 31% (13/42) species/morphotypes in common, root communities had 60% (25/42) species/morphotypes present that were not isolated from leaves, and leaf communities had 9% (4/42) species/morphotypes that were not isolated from roots.

The diversity of endophyte communities was assessed using isolates obtained on PDA. Values of Simpson's  $D$  (inverse) and Fisher's  $\alpha$  were similar across communities from all three maize genotypes, with one exception: in communities isolated from seedling roots in Harrow, BX– plants had higher diversity than BX+ plants (Table S1). In all treatments (2 locations  $\times$  2 collection times  $\times$  2 tissue types), diversity of communities was lower in August than in June.

Based on the similarity indices, the expectation that endophyte communities from BX+ plants would be more similar to one

another than either were to communities from BX– plants was observed in four of eight treatments (Fig. S2). Overall, diversity and similarity indices detected a difference between BX+ and BX– communities in Harrow more often than Ridgetown.

### BOA tolerance levels of endophytic fungi

The BOA tolerance threshold of isolates ranged from 0.25 to 1.10% concentration. No isolates were able to grow at 1.20%. Isolates were categorized in five BOA tolerance classes (Table 1). Twenty-one of 24 species/morphotypes had no intraspecific variation in tolerance level. The remaining three species, *Trichocladium* sp., *Nigrospora oryzae*, and members of the *Fusarium oxysporum* species complex had isolates in two adjacent tolerance classes.

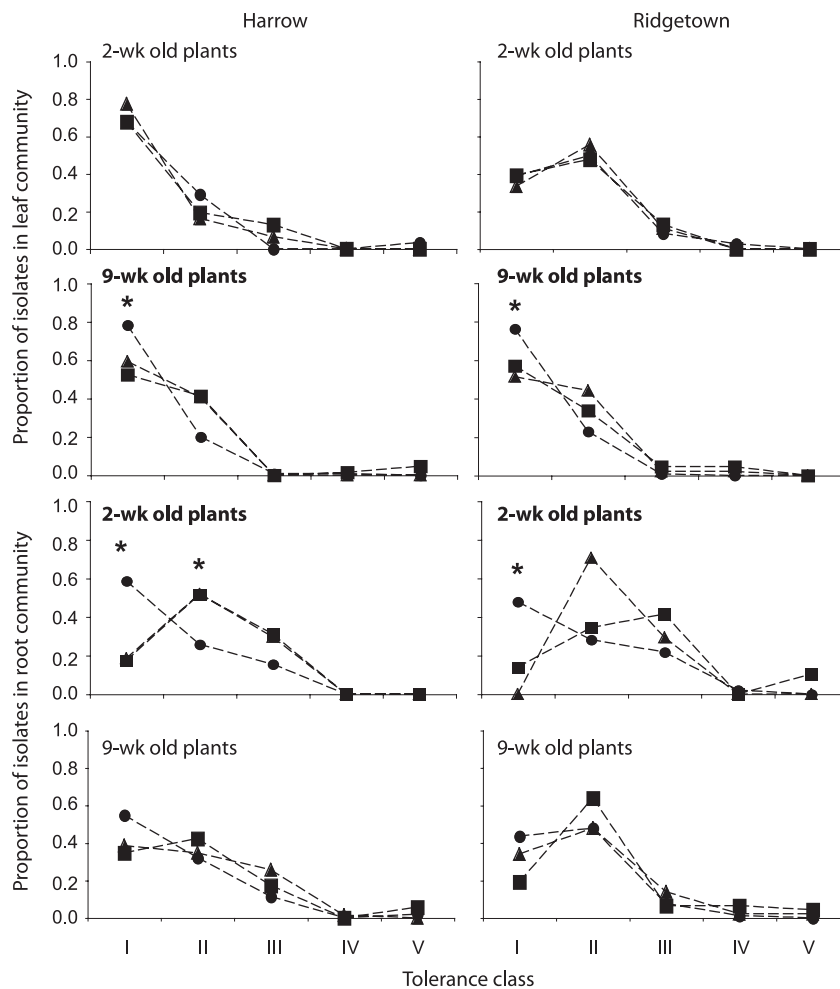
### Partitioning of endophyte communities by BOA tolerance level

The distribution of isolates in BOA tolerance classes was determined for each community characterized from PDA (Fig. 1). Isolates of the species/morphotypes that had intraspecific variation in tolerance level were equally distributed in the adjacent tolerance classes for the analyses.

A significant difference in the tolerance class distribution was seen in four of the eight treatments, in which BX– plants had a significantly greater proportion of isolates in tolerance class I than did BX+ plants (Table 2, Fig. 1). These four treatments were communities from seedling roots in Ridgetown (*bxbx* by W22:  $\chi^2 = 7.64$ ,  $P = 0.0219$ ; *bxbx* by B37:  $\chi^2 = 23.71$ ,  $P = <0.00001$ ) and in Harrow (*bxbx* by W22:  $\chi^2 = 13.64$ ,  $P = 0.0041$ ; *bxbx* by B37:  $\chi^2 = 11.83$ ,  $P = 0.0027$ ), and communities from 9-wk-old leaves in Ridgetown (*bxbx* by W22:  $\chi^2 = 10.95$ ,  $P = 0.012$ ; *bxbx* by B37:  $\chi^2 = 10.21$ ,  $P = 0.0169$ ) and in Harrow (*bxbx* by W22:  $\chi^2 = 12.96$ ,  $P = 0.0047$ ; *bxbx* by B37:  $\chi^2 = 5.94$ ,  $P = 0.0148$ ). In communities from seedling roots in Harrow, BX+ plants had a significantly higher proportion of isolates in tolerance class II compared with BX– plants (Table 2). In seedling roots, BX– plants had dominant species in tolerance class I (*Alternaria alternata* in Harrow and *Periconia macrospinoso* in Ridgetown), while BX+ plants had dominant species in classes II and III (members of the *Fusarium equiseti-incarnatum* species complex and the *F. oxysporum* species complex in both locations). In communities from 9-wk-old leaves, *A. alternata* was dominant in the three plant genotypes, but was isolated less frequently in BX– than in BX+ plants.

### Abundance of *Fusarium* in 9-wk-old plants

To compare the difference in relative abundance of *Fusarium* isolates per plant, the mean number of colonies isolated on BOA medium from the BX+ genotypes was compared with that from the BX– plants. A significant difference was detected in



**Fig. 1** Distribution of fungal endophyte community members isolated on potato dextrose agar (PDA) into Tolerance classes I–V. Tolerance class (TC) I = 0.25% 2-benzoxazolinone (BOA) tolerance threshold, TC II = 0.50% threshold, TC III = 0.75% threshold, TC IV = 1.00% threshold, TC V = 1.10% threshold. Leaf and root endophyte communities from *bxbx* (BX–, circles), W22 (BX+, squares) and B37 (BX+, triangles) were characterized in 2-wk-old and 9-wk-old maize (*Zea mays*) plants. Bold type indicates treatments indicated by global  $\chi^2$  tests to have communities distributed differently among TCs in BX+ and BX– plants. Asterisks show where follow-up  $\chi^2$  square tests indicate that the proportion of isolates was significantly different between BX– and both BX+ genotypes. The dashed lines emphasize trends; they do not indicate a series of measurements over time.

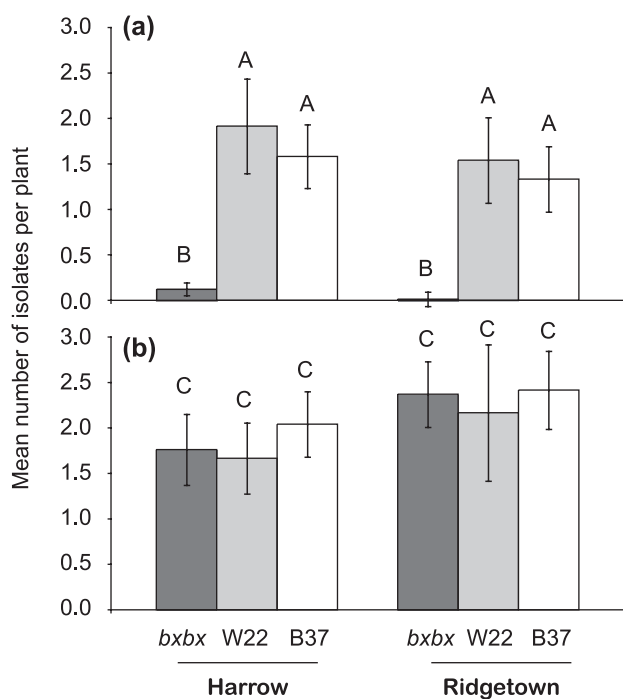
**Table 2** Results of  $\chi^2$  tests for a difference between proportion of isolates in Tolerance Classes I (0.25% 2-benzoxazolinone (BOA) tolerance threshold) and II (0.50% BOA tolerance threshold) in endophyte communities from maize (*Zea mays*) *bxbx* (BX– genotype), W22 and B37 (BX+ genotypes)

Tissue	Plant age	Location	Tolerance class	Genotype comparison	$\chi^2$	<i>P</i> -value
Leaf	9 wk	Harrow	I	<i>bxbx</i> and W22	<b>12.72</b>	<b>0.0004</b>
			II	<i>bxbx</i> and W22	<b>11.42</b>	<b>0.0007</b>
		Ridgetown	I	<i>bxbx</i> and B37	<b>5.17</b>	<b>0.023</b>
			II	<i>bxbx</i> and B37	<b>5.04</b>	<b>0.0248</b>
			I	<i>bxbx</i> and W22	<b>8.38</b>	<b>0.0038</b>
			II	<i>bxbx</i> and W22	0.43	0.513
Root	2 wk	Harrow	I	<i>bxbx</i> and B37	<b>23.43</b>	< <b>0.0001</b>
			II	<i>bxbx</i> and B37	<b>10.78</b>	<b>0.001</b>
			I	<i>bxbx</i> and W22	<b>9.29</b>	<b>0.0023</b>
			II	<i>bxbx</i> and W22	<b>5.24</b>	<b>0.022</b>
		Ridgetown	I	<i>bxbx</i> and W22	6.64	0.01
			II	<i>bxbx</i> and W22	0.84	0.3584
			I	<i>bxbx</i> and B37	<b>5.25</b>	<b>0.022</b>
			II	<i>bxbx</i> and B37	<b>8.52</b>	<b>0.0035</b>
			II	<i>bxbx</i> and W22	1.99	0.1586
				<i>bxbx</i> and B37	<b>6.71</b>	<b>0.0096</b>

\*Tests were conducted following global  $\chi^2$  tests that detected significant difference between communities from BX+ and BX– plants in 2-wk-old roots and 9-wk-old leaves. *P*-values < 0.05 are indicated by bold type.

**Table 3** Identity of a subset of isolates obtained from 9-wk-old maize (*Zea mays*) tissue plated on 2-benzoxazolinone (BOA) medium

Taxon	Total identified	Total from leaves	Total from roots
<i>Fusarium oxysporum</i> species complex	27	5	22
<i>Fusarium incarnatum-equiseti</i> species complex	8	4	4
<i>Fusarium subglutinans</i>	4	3	1
<i>Fusarium graminearum</i>	4	1	3
<i>Fusarium culmorum</i>	3	0	3
<i>Fusarium sporotrichioides</i>	4	3	1
<i>Fusarium proliferatum</i>	2	0	2
<i>Fusarium verticillioides</i>	2	1	1
<i>Fusarium solani</i>	1	0	1
<i>Nigrospora oryzae</i>	1	1	0
<i>Trichocladium</i> sp.	1	0	1

**Fig. 2** Mean number of isolates per plant obtained on 2-benzoxazolinone (BOA)-medium from leaf (a) and root (b) tissue of 9-wk-old maize (*Zea mays*) plants in Harrow and Ridgeway, Ontario, Canada. Based on identification of a sample of these isolates, approx. 96.5% of isolates obtained on BOA medium were *Fusarium* species. Tukey–Kramer HSD tests were conducted to compare mean number of isolates per plant obtained from *bxbx* (BX–), W22 (BX+), and B37 (BX+) genotypes. Analysis of leaf and root tissue was conducted separately. The same letter above two columns indicates no significant difference between means. Vertical bars,  $\pm$  SE.

leaves (Harrow  $F = 7.03$ ,  $P = 0.0046$ ; Ridgeway  $F = 9.12$ ,  $P = 0.0014$ ), but not in roots (Harrow  $F = 0.29$ ,  $P = 0.7501$ ; Ridgeway  $F = 0.08$ ,  $P = 0.921$ ). In leaf tissue, averaging values for the two BX+ genotypes, BOA-tolerant fungi were 14 times (Harrow) to 35 times (Ridgeway) more abundant in BX+ genotypes than in the BX– genotype (Fig. 2). From the isolates obtained on BOA medium that were identified,

96.5% were *Fusarium* species (Table 3). A total of nine *Fusarium* species, one isolate of *Nigrospora oryzae* and one isolate of *Trichocladium* sp. were identified. Of these species, six were isolated from leaf and root tissue, four from roots but not from leaves, and one only from leaf tissue. The most abundant species were members of the *F. oxysporum* species complex and the *F. equiseti-incarnatum* species complex.

## Discussion

### Presence of BXs influences endophyte community structure

These results suggest that BX production contributes to the structuring of endophyte communities in seedling roots and 9-wk-old leaves (Fig. 1). The differences over time in these tissues could arise from temporal changes in BX byproduct concentrations and allocation within tissue. Concentrations of constitutive DIMBOA and related compounds have been demonstrated to decrease with shoot and root age (Cambier *et al.*, 2000). This would explain why there were more BOA-tolerant fungi in BX+ than in BX– roots in the seedling, but not in 9-wk-old plants.

Fungal-mediated changes in MBOA concentration may be a factor in the relatively high abundance of BOA sensitive isolates in leaves of 9-wk-old BX– plants compared with BX+ plants. Oikawa *et al.* (2004) observed that inoculation of *A. alternata* in mature maize leaves induced production of HDMBOA-Glc, a proposed precursor to MBOA. Tolerance of fungi to MBOA is positively correlated with tolerance to BOA (Glenn *et al.*, 2001). In the present study, *A. alternata* (Tolerance class I) was isolated more frequently from BX– leaves than BX+ leaves in 9-wk-old plants. Induction of HDMBOA-Glc production by *A. alternata* could result in release and accumulation of MBOA, which would be self-inhibitory, allowing colonization by other species with higher BOA/MBOA tolerance.

Genotypes of a host species can vary significantly in their influence on community organization (Whitham *et al.*, 2006).

Assessing the influence of *Ustilago maydis* resistance on endophyte community assembly in maize, Pan *et al.* (2008) found that community structure was not correlated with resistance, but rather with host genotype. Consistent with this, our results demonstrate differences in endophyte communities among all three genotypes, but with striking differences in tolerance of host toxins among community members between the BX<sup>-</sup> mutant and the BX<sup>+</sup> genotypes.

McGill *et al.* (2006) propose that the most direct route to understanding the mechanisms underlying assembly of speciose communities is through study of functional trait variation across environmental gradients. Here, we have compared the extremes of the BX concentration gradient. Traditional species diversity measures did not detect a difference between endophyte communities from BX<sup>+</sup> and BX<sup>-</sup> genotypes. When the distribution of functional traits in communities was analysed, clear differences between the communities of BX<sup>+</sup> and BX<sup>-</sup> genotypes were apparent. Such a shift in functional diversity, despite no change in species diversity, has frequently been noted in plant communities (for review of plant functional types in ecology see Duckworth *et al.*, 2000).

#### BX<sup>+</sup> plants have a higher incidence of *Fusarium* than BX<sup>-</sup> plants

*Fusarium* was isolated 14 to 35 times more frequently from BX<sup>+</sup> leaves than from BX<sup>-</sup> leaves in 9-wk-old plants. This suggests that *Fusarium* species are more competitive in the presence of BXs. Successive cropping of maize or other BX-producing plants in the same locality may increase the dominance of *Fusarium* in the endophyte community. Carry-over across seasons would be expected through crop residue, which is considered to be the most important source of inoculum for endophyte infection (Sutton, 1992). Our data suggest that crop residue of BX<sup>+</sup> genotypes will have more *Fusarium* inoculum than the BX<sup>-</sup> genotype. If BX concentrations are maintained in crop residue and soil, our data suggest that *Fusarium* is likely to accumulate. Contamination of maize with *Fusarium* species can lead to human disease, yield loss and livestock toxicosis (Ueno *et al.*, 1997; Marasas, 2001; Marasas *et al.*, 2004; Wu, 2007). Reducing inoculum is the most direct approach to reducing fungal infection (Jouany, 2007).

Commercial maize has been selectively bred to produce elevated levels of BXs. Results from our study suggest that presence of BXs can significantly enhance the competitive ability of *Fusarium* species. Given that BXs are general phyto-protectants, commercial cultivation of BX<sup>-</sup> maize is not realistic. Investigation of a relationship between BX concentration and abundance of *Fusarium* in maize tissue could inform crop management strategies. It is possible that there is a threshold concentration of BXs that is high enough to provide insecticidal benefits to the plant, but low enough to obviate any colonization benefit to *Fusarium* species.

#### Commonalities between endophyte community ecology in agricultural and naturally occurring plants

There is an assumption that endophyte communities of agricultural plants and plants in their natural habitat are fundamentally different. Likely this stems from emphasis on individual fungal species as pathogens in agricultural crops, versus a more holistic approach to understanding endophyte communities of wild plants. However, we see four major commonalities between endophyte communities in cultivated and naturally occurring plants. First, the present study found that frequency of endophyte infection increases with plant age, with infection density of leaves reaching 100% in 9-wk-old leaves. Second, we found that in all treatments, diversity decreased over time (Table S1). Both of these trends have also been observed in endophyte communities of tropical plants (Herre *et al.*, 2007). Third, results presented here indicate that above- and below-ground tissue harbor distinct endophyte assemblages, a pattern observed in a diversity of plants (e.g. Kumar & Hyde, 2004). Finally, plant residue is considered the most important source of fungal inoculum in the life histories of both agricultural and wild endophytes (Sutton, 1992; Herre *et al.*, 2007).

Another potential similarity between endophyte communities of plants in agricultural and natural environments is the role of interspecific interactions between fungi in mediating community structure. Arnold *et al.* (2003) proposed that interspecific competition mediated by leaf chemistry is a common mechanism shaping endophyte communities. Our results are consistent with this hypothesis. *Fusarium* was significantly more abundant in BX<sup>+</sup> plants compared with BX<sup>-</sup> plants, indicating that BXs provide a competitive advantage to *Fusarium*. Observational data from the present study indicate that interspecific facilitation may also influence endophyte communities. When isolating fungi from leaf tissue on BOA medium, colonies of *A. alternaria* and *P. macrospinoso* occasionally emerged from the colony center of BOA detoxifying *Fusarium* species. All of the *A. alternaria* and *P. macrospinoso* tested, including isolates obtained on BOA medium (1.00% concentration), could not grow above a 0.25% concentration of BOA. This may indicate that BOA detoxifying *Fusarium* species can facilitate the growth of less tolerant species. A previous *in vitro* study found that maize endophytes able to tolerate 1.00% and 1.10% concentrations of BOA facilitated growth of maize endophytes unable to grow above a 0.25% and 0.50% concentration (Saunders & Kohn, 2008). These commonalities between agricultural and wild endophyte communities may represent general mechanisms in endophyte community assembly, and would therefore be constructive areas for future research.

#### Conclusions

We found that plant defense compounds are a significant factor in structuring fungal endophyte communities of maize.

Glenn *et al.* (2001) proposed that BX production by maize enhances the ecological success of *Fusarium* species in maize; our results support this hypothesis. Further, we found that non-*Fusarium* species with intermediate BOA tolerance levels had a colonization advantage over BOA-sensitive fungi in BX-producing plants, indicating that a large proportion of the fungal community is influenced by defense compound production.

Our data point to the possibility that breeding for elevated concentrations of BXs in maize has unintentionally allowed for increased colonization by *Fusarium* and the possibility of increased inoculum load in plant residue and in soil. These results are preliminary to the next logical step of breeding isogenic corn lines that only differ in quantitative production of BXs. The caveat to this approach will be that concentrations of BX are not static *in planta*, and are also likely to be dynamic in the surrounding soil and crop residue.

Future experiments on endophyte communities of other host species are needed to test the possibility that host defense compounds are a general mechanism in structuring communities. Studies incorporating measurements of multiple factors, such as competition and facilitation between microbes, species composition in the soil-borne 'spore-bank' and microbial niche overlap will help to identify key fungal traits in colonization. From this, we will be better able to form hypotheses about evolution of host colonization strategies within a community context.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Collection data and community bar graphs of isolates obtained from root (S1a) and leaf (S1b) tissue of maize (*Zea mays*) on potato dextrose agar (PDA).

**Fig. S2** Similarity coefficients of fungal endophyte communities from leaf and root tissue of maize (*Zea mays*) grown in Harrow and Ridgetown, Ontario, Canada.

**Table S1** Diversity of fungal endophyte communities in maize (*Zea mays*)

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