Soybean Fungal Endophytes *Alternaria* and *Diaporthe* spp. are Differentially Impacted by Fungicide Application

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Abstract

In field trials in Iowa, we investigated the association of a fungicide applied at early pod set to the diversity and composition of foliar endophytic fungi in presenescent soybeans. The main purpose of our study was to determine whether fungicides affect the microbiome of soybean plants during the pod-fill reproductive stage. In a replicated experiment focused on the impact of a fungicide application including a quinone outside inhibitor (QoI) and a pyrazole-carboxamide spanning two growing seasons, healthy stems and leaves near the tops of soybean were sampled for endophytic fungi. The survey yielded 1,791 isolates belonging to 17 putative species, identified by morphology and sequence analysis of the ribosomal DNA internal transcribed spacer region. Taxa were grouped by genus into operational taxonomic units: *Alternaria*, *Colletotrichum*, and *Diaporthe* were the dominant genera isolated. Plant parts

were analyzed separately using a multivariate community analysis of isolate counts per plant. The 14.3% fluxapyroxad and 28.6% pyraclostrobin fungicide spray significantly increased the proportion of *Diaporthe* isolates over no-spray controls, whereas the inverse occurred for foliar *Alternaria* isolates. In addition, seed harvested from fields with shorter-season varieties and sprayed with fungicide showed higher percentages of *Diaporthe* isolates than fields with no fungicide spray. In conclusion, soybean farmers may want to consider that the application of a QoI fungicide in the absence of disease pressure might adversely impact seed quality.

Keywords: *Fusarium*, *Glycine max*, latent infection, maturity groups, microbial communities, molecular identification, *Nigrospora*, strobilurin

Fungal endophytes inhabit plants without causing visible disease symptoms. The designation “endophyte” refers only to the moment of detection, without regard to future interactions (Schulz and Boyle 2005). Endophytic fungi act as mutualists, as pathogens when the host is stressed, or as saprobes when the host dies (Redman et al. 2001; White et al. 2016). Redman et al. (2002) showed that an endophytic *Curvularia* sp. increased host tolerance of *Dichanthelium lanuginosum* (panic grass) to temperatures up to 65°C, thereby improving host fitness. On the other hand, several fungal endophytes produce phytotoxins in culture (Borges and Pupo 2006; de Lima Fávoro et al. 2012; Gao et al. 2011; Suryanarayanan et al. 2012). Schulz and Boyle (2005) suggested that asymptomatic colonization is a momentary balance of antagonisms between host and endophyte, regardless of the life-history strategy of the fungal endophyte. Therefore, crop management strategies that alter the physiology of the plant host may modify its endophytic community.

Transmission of endophytes is a key factor for their classification (Rodríguez et al. 2009). Class I endophytes are obligately related to and systemic within their hosts. For example, the *Epichloë* and *Balansia* endophytes are transmitted through the embryos of their grass and morning glory hosts, respectively (Tadych et al. 2007; White et al. 2016). Nonclavicipitaceous endophytes (classes II to IV) are facultatively related to multiple hosts and are typically vectored on the surfaces of seeds, plant residue, and soil (White et al. 2016). The compositions of communities of nonclavicipitaceous endophytes are influenced by local environment and geography (Arnold 2007; U'Ren et al. 2012). The nonclavicipitaceous endophyte, *Colletotrichum gloeosporioides*, was found in eight different hosts in Thailand, but isolates differed in their ability to cause anthracnose among those hosts (Photita et al. 2005).

Surveys of the soybean (*Glycine max* L. Merrill) foliar fungal endophyte community have been conducted in Minnesota, Brazil, and Argentina (da Costa Stuart et al. 2018; de Souza Leite et al. 2013; Impullitti and Malvick 2013; Larran et al. 2002; Pimentel et al. 2006); these studies established repeatable culture-based methods for performing endophyte isolations. So-called “latent infection,” defined as the association or colonization of tissues by pathogens for a prolonged period without visible symptoms, has long been detected in apparently healthy soybean plants (Sinclair 1991). Yet little is known about how these fungi affect their host as endophytes. There is some evidence that endophytes may reduce plant disease and enhance soybean growth. For instance, drenching with culture filtrate from the endophyte *Cladosporium sphaerospermum* increased soybean plant height (Hamayun et al. 2009). Salinity tolerance in soybean is promoted by a *Penicillium* endophyte (Khan et al. 2011).

Conversely, regardless of plant species, there is a scarcity of information on how agronomic practices or external factors shape the fungal or bacterial endophyte community. It has been reported that host genotype shapes the fungal endophyte community, as was noted for *Phaseolus vulgaris* (Parsa et al. 2016) as well as tree species (Hoffman and Arnold 2008; Oono et al. 2015). Insect damage did not impact the endophyte community of the white snakeroot plant (*Ageratina altissima*) (Christian et al. 2016). Pan and May (2009) showed that *Ustilago maydis* (corn smut) affected the assemblage of fungal endophyte communities in *Zea mays*. Fungicide applications on wheat showed a moderate but significant difference in the relative abundance and diversity of nontarget fungi (Karlsson et al. 2014). Kuklinsky-Sobral et al. (2005) found that two soybean cultivars (Foscarin and Cristalina) from fields treated with glyphosate herbicide differed in endophytic bacteria community from plants of the same cultivar without glyphosate treatment. Prior et al. (2017) showed that azoxystrobin had the greatest effect on endophytic community diversity on broad bean and common bean, compared with sulfur and copper, and lasted more than 5 weeks after application. da Costa Stuart et al. (2018) investigated the effect of cropping systems on soybean endophytes and found that conventional practices reduced foliar fungal endophyte diversity by one-third compared with organically grown soybeans.

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Midwestern U.S. soybean farmers have received conflicting advice regarding the advisability of applying quinone outside inhibitor (QoI) fungicides in the absence of disease to increase yields (Kandel et al. 2016; Swoboda and Pedersen 2009; Wise and Mueller 2011). QoI fungicides have been linked to delayed leaf senescence and prolonged photosynthetic activity of green tissue, which both impact yield (Balba 2007; Bradley and Sweets 2008). QoI fungicides include pyraclostrobin, azoxystrobin, and trifloxystrobin and belong to Fungicide Resistance Action Committee (FRAC) Code Group 11 fungicides also known as strobilurins (Mueller 2013).

The purpose of this study was to determine the effect of applying a fungicide spray containing a QoI on the soybean endophyte community. We used a culture-based approach to determine the diversity and incidence of fungal endophytes of leaves and stems. A follow-up investigation was also conducted to determine whether these fungal endophytes colonized the seed crop and whether internal seed colonization was impacted by fungicide spray.

Materials and Methods

Field study. This study was conducted using an ongoing fungicide trial, in which cultivars were provided by private industry in fields that were annually rotated between corn and soybean. Conventionally grown soybean fields were annually rotated with corn and were planted at Iowa State University research farms in 2015 (N 41° 42.26 W 93° 17.1), 2016 (N 41° 59.03 W 93° 38.4), and 2017 (N 42° 23.8 W 93° 38.1) near Ames, Iowa, at a row spacing of 76 cm at a rate of 345,800 seeds/ha. A 0.5-ha area contained three field strips with cultivars of differing maturity groups (MGs). Soybean MGs are based on the day length required to begin the reproductive process; each whole-number MG increase adds approximately 10 days to the onset of flowering (Edwards and Purcell 2005). Fungicide treatments (fungicide spray and no spray) were randomly assigned in four replicated four-row subplots (6.1 × 3 m) per field (Table 1). The fields contained additional fungicide treatment plots not included in this study. At reproductive stage 3 (R3), when pods were beginning to fill (Fehr and Caviness 1977), designated plots were sprayed with a commercial fungicide (292 ml/ha) composed of 14.3% fluxapyroxad and 28.6% pyraclostrobin (FRAC codes 11 and 7) (Priaxor; BASF Corp., Research Triangle Park, NC) mixed with water. In mid-September 2015 and 2016, plant tissue samples

for endophyte isolation were collected from the outer two rows of each four-row subplot, leaving the center rows for harvest. A total of 20 plants per fungicide treatment (fungicide spray and no spray) were collected and pooled across the four subplots (Table 2). Asymptomatic leaves at the fifth node from the top, were designated as “no spray” and “fungicide spray.” The stem and leaf of that same plant was sampled. The reproductive stage of plants in each field was noted on the collection dates (Table 1) as described by Fehr and Caviness (1977). Briefly, reproductive stage 5 (R5) is “beginning seed” when seeds are 3 mm long in the pods located at the uppermost nodes of the main stem, R6 is “full seed” when a green seed fills the pod capacity at one of the four uppermost nodes on the main stem, and R7 is “beginning maturity” when one of the pods on the main stem reaches its mature color.

At harvest, grain weight and moisture content were collected for each subplot ($n = 4$) and grain weight was adjusted to 13% grain moisture (Table 1). Grain moisture measurements were determined as described by the American Society of Agricultural and Biological Engineers (2017). Paired comparisons of moisture and yield were made for fungicide spray and no-spray treatments for 2015, 2016, and 2017 (4 subplots × 3 fields × 3 years × 2 fungicide treatments). Daily soil temperature and precipitation data were obtained from a weather station located within 3 km of the fields (Iowa Environmental Mesonet; <http://mesonet.agron.iastate.edu/agclimate>).

Foliar endophyte collection design. A total of six soybean fields were assessed in both 2015 and 2016. Twenty plant samples collected from each treatment (fungicide spray and no spray) were pooled across four subplots within treatment in each of the six fields. Thus, fields were treated as blocks ($n = 6$). Endophytes from stems and leaves were analyzed separately.

Seed collection design. In 2016 and 2017, three visibly healthy plants from outer rows of each of four subplots per field were flagged in mid-September for postharvest seed collection. Seed samples from each subplot were kept separate to obtain four replicates per field (six fields; $n = 24$).

Leaf and stem endophyte collection. In 2015 and 2016, 20 plants ($n = 5$ per subplot) at R5 stage were collected and pooled across field treatment ($n = 6$). Twelve stems and 20 leaves per field in 2015 and 20 stems and 20 leaves per field in 2016 were sampled in the laboratory. A single leaflet and stem from each collected plant was plated

Table 1. Cultivars, key dates, and harvest data of fields used for testing the impact of a fungicide spray on fungal endophytes

Year	Cultivar	Manufacturer	MG ^a	Planting date	Fungicide treatment ^b	Spray date ^c	Foliar collection date	Reproductive stage ^d	Harvest date	Yield (t/ha)	Seed moisture (%)
2015	AG2431	Asgrow	2.4	19 May	No	–	15 Sep	R6	8 Oct	4.86	12.1
					Yes	3 Aug				5.26	12.3
	31RY45	Pioneer	3.1	19 May	No	–	16 Sep	R5	9 Oct	4.68	15.5
					Yes	3 Aug				4.54	16.3
2016	AG3334	Asgrow	3.3	19 May	No	–	17 Sep	R5	9 Oct	4.57	15.9
					Yes	3 Aug				4.44	16.4
	AG2035	Asgrow	2.0	22 May	No	–	10 Sep	R7	17 Oct	5.09	16.8
					Yes	21 Jul				5.20	16.8
2017	NKS24K2	Syngenta	2.4	22 May	No	–	11 Sep	R6	17 Oct	4.51	11.8
					Yes	21 Jul				4.68	12.2
	39RY43	Dynagrow	3.9	22 May	No	–	12 Sep	R5	24 Oct	4.23	10.5
					Yes	26 Jul				4.28	10.5
2017	AG2035	Asgrow	2.0	30 May	No	–	NA ^e	NA	19 Oct	4.15	12.4
					Yes	31 Jul	NA	NA		3.85	12.6
	AG2733	Asgrow	2.7	30 May	No	–	NA	NA	19 Oct	3.96	11.9
					Yes	31 Jul	NA	NA		4.05	11.9
2017	39RY43	Dynagrow	3.9	30 May	No	–	NA	NA	31 Oct	3.92	9.93
					Yes	2 Aug	NA	NA		3.81	10.0

^a Soybean maturity groups (MGs) are based on the day length required to begin the reproductive process; each whole-number MG increase adds approximately 10 days to the onset of flowering (Edwards and Purcell 2005).

^b Fungicide spray treatment = 292 ml/ha of 14.3% fluxapyroxad and 28.6% pyraclostrobin (Priaxor; BASF Inc.) applied at pod set (reproductive stage 3).

^c Dashes indicate the control treatment where no fungicides were sprayed.

^d Reproductive stage at the time of foliar endophyte sampling performed in 2015 and 2016: R5 = green pod, beginning seed; R6 = green pod, full seed; and R7 = yellow pod (Fehr and Caviness 1977).

^e NA = not applicable, since only seeds were collected in 2017.

for endophyte isolation. Stems cut just below the fifth node from the top of each plant and the associated nonsymptomatic leaves were placed on ice and processed within 6 h of collection. To process, 2-cm-long stem segments centered on the fifth node, and 2-cm² leaf pieces from the base of the center trifoliate leaflet with the main vein centered were excised using a sterile blade. Samples were surface sterilized for 5 min in 8.25% sodium hypochlorite and 30 s in 70% ethanol, rinsed twice in sterile distilled water, and dried in a laminar flow hood on sterile paper towels. To verify the effectiveness of surface sterilization by bleach (Clorox), leaf and stem pieces were imprinted on plates of green bean agar (GBA) (411 g/liter of rinsed pureed cut Green Giant green beans; General Mills, Minneapolis, MN) and 1.5% agar (Difco, Detroit, MI) prior to transfer to a second plate for endophyte isolation. Pieces on the imprinted plates showing external fungal growth after 7 to 30 days were eliminated from the study; the disinfection was considered successful when no fungal growth was observed (Parsa et al. 2016). Isolates associated with leaf and stem samples showing surface growth from preculture imprinting (22 of 192 samples in 2015 and 18 of 240 samples in 2016) were eliminated from the study. Surfaceborne isolates were identified as *Penicillium* sp., *Aspergillus* sp., and *Cladosporium* sp. based on colony and conidia morphology.

GBA was selected as the medium for primary isolation based on preliminary studies that showed that soybean leaf endophyte mycelia were more easily differentiated based on color, texture, and growth pattern than other media (*data not shown*). Cultures were incubated under ambient room temperature (20 to 25°C) and a light/dark regime. At 3 and 6 days, hyphal tips of newly emerged mycelia were transferred to antibiotic-amended one-third-strength potato dextrose agar (PDA) (BD, Franklin Lakes, NJ) in 2015 and 2% water agar with a postautoclave addition of 40 drops of 50% lactic acid in 2016. Primary isolation plates were checked weekly for 3 months and new mycelial growth was subcultured. The colony color and texture of each primary isolate was described and recorded for each leaf or stem piece. Each isolate was assigned a number based on field, fungicide treatment, plant part, plant number, and colony from that primary isolation. A total of 708 and 1,083 isolates were detected on leaf and stem pieces in 2015 and 2016. Data from primary isolates made on GBA were used to estimate the incidence of species per stem or leaf piece. Every purified isolate was photographed and grouped into morphotype based on colony growth rate, texture, and color on acidified PDA (Fig. 1). Purified isolates totaled 505 in 2015 and 753 in 2016, comprising 70% of the total isolates detected from primary growth on GBA. Within these morphotype groups, each isolate was microscopically examined for delineating characteristics, including spores, appressoria, and fructifications, using an Olympus CHBS microscope with an eye piece micrometer; 20 measurements of these structures per isolate (when present) were recorded. Preliminary identifications to genus were based on Roy et al. (2001). Two-

week-old pure cultures ($n = 1,258$) on PDA were placed in 15% glycerol and stored at -80°C .

DNA extraction, amplification, and sequencing. Representative isolates for molecular characterization (195 isolates in 2015 and 189 in 2016) were selected to include every fungal morphology. All sterile cultures and unique fungal morphologies were included; three to five isolates of commonly found fungal morphologies (Fig. 1) were chosen from each field, plant part, and fungicide treatment combination. Template DNA for PCR was extracted from representative cultures by scraping mycelia with a sterile pipette tip and transferring the mycelia into 50 μl of PrepMan Ultra Sample Reagent (Applied Biosystems, Foster City, CA) following the manufacturer's instructions and was then stored at -20°C until used. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA operon was amplified using the universal fungal primer pair ITS1 and ITS4 (White et al. 1990). DNA extraction, PCR reactions, purifications, and sequencing of products were performed as described in Batzer et al. (2005). The closest type material match for 16 species was putatively identified using the nBLAST tool. To verify the BLAST results, all generated sequences were manually aligned with downloaded sequences of well-documented strains or type cultures, when available, using BioEdit (Hall 1999). Alignments of each putative species did not differ by more than three base pairs. Fifteen sequences of representative isolates (as previously described in this paragraph) were deposited in GenBank (MG182675 to MG182675) and 373 additional sequences were subsequently deposited in GenBank as a population study SUB50478 and included accession numbers MK408985 to MK409358 (Table 3). Estimates of the number of endophyte operational taxonomic units (OTUs) isolated from each field sample were based on morphology and associated ITS sequences and grouped to genus.

Seed endophyte assay. The remaining lower portions and the entirety of sampled plants, in 2016 and 2017, respectively, were collected from the outer rows of each plot within 2 days after grain harvest of the center rows, as previously described (Table 1). Collected plants were stored in paper bags in the laboratory until seeds were manually removed from their pods within 4 weeks after harvest. A total of 1,440 seeds (6 fields $\times$ 4 subplots $\times$ 3 plants $\times$ 10 seeds $\times$ 2 fungicide treatments) were assessed over 2 years (Table 1). Seeds were surface sterilized and rinsed as previously described. Single seeds were placed on 5-cm-diameter Petri plates of GBA and incubated as previously described. Seed germination was recorded at 2 weeks and fungal growth was identified to genus based on colony and conidia morphology over a period of 3 months.

Statistical analyses. The difference in the foliar endophyte community composition between fungicide-treated and no-spray control fields was evaluated using a multivariate abundance statistical model (mvabund: Statistical Methods for Analyzing Multivariate

Table 2. Number of plant samples, number of fungal isolates per sample, and genus richness of leaf and stem samples for each field and fungicide treatment

Year	Cultivar	Fungicide treatment ^a	Leaf			Stem		
			Samples (n) ^b	Isolates/leaf (n)	Richness ^c	Samples (n)	Isolates/stem (n)	Richness
2015	AG2431	No	17	5.3	5	11	5.0	6
		Yes	11	5.3	6	10	4.1	6
	31RY45	No	20	3.8	6	12	3.5	7
		Yes	19	3.3	4	12	3.6	5
	AG3334	No	16	6.3	5	11	2.4	5
		Yes	19	2.8	6	12	4.3	6
2016	AG2035	No	20	5.3	7	20	3.7	7
		Yes	20	5.0	6	20	3.8	7
	NKS24K2	No	16	4.4	6	17	4.1	6
		Yes	20	5.9	7	18	3.4	6
	39RY42	No	18	5.8	7	17	5.0	6
		Yes	18	7.2	6	18	3.4	4

^a Fungicide spray = 292 ml/ha of 14.3% fluxapyroxad and 28.6% pyraclostrobin at pod set (reproductive stage 3).

^b Although 20 plants were collected, samples showing inadequate surface sterilization were eliminated from the study.

^c Overall number of genera.

Abundance Data, R package version 3.13.1, <https://cran.r-project.org/web/packages/mvabund/index.html>) (Wang et al. 2018). Preliminary analysis indicated genus composition differences between leaves and stems, so plant part (stems and leaves) was analyzed separately. For each plant part data set, the numbers of isolates

from each of the six predominant genera were counted for each plant, field, and treatment. Fields were treated as blocks and the count per genus was modeled with a negative binomial distribution for overdispersed count data. *P* values were obtained by resampling residuals.

Fig. 1. Primary isolations were observed every 3 days and new mycelial growth was subcultured to acidified potato dextrose agar. **A**, A 2-week-old leaf isolation on green bean agar is shown. Growth was categorized based on colony color and texture and each morphology was recorded for each leaf and stem piece. Frequently observed morphologies were grouped as follows: **B**, olive, felty, thick (genus *Alternaria*); **C**, pale pink or white with submerged dark centers, velvety, even margins (genus *Colletotrichum*); **D**, white, felty, dense (genus *Diaporthe*); **E**, white to pale yellow, smooth, margins feathered (genus *Fusarium*); **F**, deep pink and white, floccose (genus *Fusarium*); **G**, black, narrow white edge, floccose (genus *Nigrospora*); and **H**, golden, with black specks, thin, diffusible yellow pigment (genus *Didymella*-like).

Mean numbers of isolates of each leaf and stem sample and endophyte OTU genus richness were determined. Isolates per sample were compared among plant part, fungicide treatment, and field using an analysis of variance with plant part and fungicide as fixed effects and field and year as random effects (Table 2).

The effect of fungicide treatment ($n = 2$), year ($n = 2$), and cultivar ($n = 6$) on the percentage of seed infection was compared using an analysis of variance in which cultivar and fungicide treatment were fixed effects and replicated subplot ($n = 4$) and year ($n = 2$) were random effects. Analysis was conducted separately for each genus where percent infection (30 seeds) was compared. A Fisher protected least significant difference test ($P \leq 0.05$) was also used to compare *Diaporthe* infection fungicide differences among individual cultivars and among the four MGs (2.0, 2.4, 2.7, and 3.9) with SAS version 9.3 software (SAS, Cary, NC). To determine the strength of the association of fungicide spray impact to MG (x-axis) of the field cultivar on *Diaporthe* seed infection (y-axis), a Pearson correlation was conducted using Microsoft Excel.

Results

Field conditions. Across the 3-year study, fungicide-sprayed treatment fields showed a small (1.8%) but significant increase ($P = 0.0154$) in seed moisture at the time of harvest compared with the no-spray fields, suggesting that the fungicide spray delayed senescence (Table 1). The mean yield for all soybean plots was 4.7 t/ha (Table 1) and was not affected by fungicide treatment ($P = 0.4143$). Symptoms of foliar diseases including brown spot, frogeye leaf spot, Cercospora leaf blight, and sudden death syndrome were observed in the fields at green pod full seed stage (R6); their causal organisms were not detected from any endophyte

samples in this study. Reproductive stages at the dates of foliar collections ranged from R5 to R7; short-season MGs were closer to senescence (Table 1). Total rainfall from planting to harvest dates was 67.7, 61.2, and 25.34 cm in 2015, 2016, and 2017 respectively, differing greatly from the 47.9-cm 30-year average in Ames, Iowa. Soil temperatures showed a comparatively cool growing season in 2015, and warmer growing seasons in 2016 and 2017, although all years were warmer than the 30-year average.

Numbers of foliar endophyte isolates. Paired comparisons showed that more isolates ($P = 0.0111$) were obtained from leaves (5.0 isolates/leaf) than stems (3.8 isolates/stem) (Table 2). The mean number of isolates per plant (4.4) did not differ among year ($P = 0.1545$), fungicide treatment ($P = 0.6150$), or field ($P = 0.2358$). A total of 708 and 1,083 isolates were examined and commonly occurring morphologies were grouped (Fig. 1). Purified isolates ($n = 1,258$) were subsequently identified to genus or species based on colony morphology and microscopic characters (Roy et al. 2001).

Diversity. Most of the isolates were known pathogens on soybean or other plant hosts (Table 3). Based on ITS sequence alignments, a total of 17 species were isolated, with 14 species in 2015 and 15 species in 2016 belonging to 10 genera (Table 3). Based on the morphology of primary isolates, the most frequently isolated species across year and field were *Alternaria destruens* (696 isolates) and *Colletotrichum cliviae* (365 isolates). Alignment of ITS sequences among isolates showed no more than three base pair differences within *A. destruens* and *Colletotrichum cliviae*. The species frequency of every isolate within the genera *Diaporthe*, *Fusarium*, and *Nigrospora* could not be determined based on primary isolate morphology. Consequently, each isolate was placed into an OTU at the genus level

Table 3. Closest matches to soybean endophyte putative species, GenBank accessions, closest BLAST search results to type material (when possible), operational taxonomic unit (OTU) genus group, and lifestyle preferences

Soybean endophyte closest match	Soybean endophyte DMPC ^a	GenBank accession ^b	BLAST match sequence		OTU genus	Lifestyle preference ^c
			Accession	Base pair identity ^b		
<i>Alternaria destruens</i>	KCL7c	MG182675 , MK408985–MK409129	NR_137143	550/550	<i>Alternaria</i>	Saprobic, endophytic, and pathogenic
<i>Colletotrichum cliviae</i>	KCS3a	MG182676 , MK409134–MK409200	NR_137097	496/499	<i>Colletotrichum</i>	Saprobic, endophytic, and pathogenic (anthracnose)
<i>Diaporthe aspalathi</i>	LPS11a	MG409201 –MK409216	KX769840	580/601	<i>Diaporthe</i>	Endophytic and pathogenic (southern stem canker)
<i>Diaporthe actinidiae</i>	KPL4d	MG182679 , MK409227–MK409233	KC145886	549/550	<i>Diaporthe</i>	Endophytic and pathogenic (kiwi stem rot)
<i>Diaporthe caulivora</i>	KCL14d	MG182677 , MK409227–MK409233	NR_111845	566/571	<i>Diaporthe</i>	Endophytic and pathogenic (northern stem canker)
<i>Diaporthe longicolla</i>	KPS7a	MG182678 , MK409217–MK409226	NR_144924	549/551	<i>Diaporthe</i>	Endophytic and pathogenic (soybean seed decay and stem disease)
<i>Diaporthe novem</i>	LPS16b1	MG409234 –MK409239	MH864503	576/592	<i>Diaporthe</i>	Endophytic and pathogenic (soybean seed decay)
<i>Fusarium boothii</i> (<i>F. graminearum</i> complex)	KPS5c	MG182680 , MK409269–MK409305	NR_121203	536/536	<i>Fusarium</i>	Saprobic, endophytic, and pathogenic
<i>F. equiseti</i> (<i>F. graminearum</i> complex)	KCS7e	MG182681 , MK409306–MK409313	NR_121457	532/537	<i>Fusarium</i>	Saprobic, endophytic, and pathogenic
<i>F. keratoplasticum</i> (<i>F. solani</i> group)	KPS8b	MG182682 , MK409317–MK409328	NR_130690	524/533	<i>Fusarium</i>	Saprobic, endophytic, and pathogenic
<i>Nigrospora oryzae</i>	KPS11b	MG182683 , MK409336–MK409341	EU529994	533/533	<i>Nigrospora</i>	Saprobic, endophytic, and pathogenic (leaf spot)
<i>N. sphaerica</i>	KPS10c	MG182684 , MK409329–MK409335	KJ767121	539/539	<i>Nigrospora</i>	Saprobic, endophytic, and pathogenic (leaf spot)
<i>Didymella rosae</i>	NPL2E	MG182685 , MK409240–MK409267	NR_136125	544/567	<i>Didymella</i> -like	Pathogenic (tan spot)
<i>Epicoccum sorghinum</i>	KCS2c	MG182686	KY454467	539/539	NA ^d	Pathogenic (grain mold)
<i>Coniothyrium multiporum</i>	KPL2b	MG182687	NR_111617	422/450	NA	Endophyte
<i>Bipolaris subramanianni</i>	CUL2c	MG182688 , MK409130–MK409133	NR_147496	611/619	NA	Pathogenic (leaf spot)
<i>Plectosphaerella populi</i>	LU85B	MG182689	NR_138002	547/582	NA	Pathogenic (root and collar rots)

^a DMPC = Daren Mueller Personal Collection.

^b Isolates in bold were used to perform BLAST searches. Additional sequences were deposited as a population study SUB50478 and included accession numbers MK408985 to MK409358.

^c Lifestyle preference assignments were based on source information of the closest BLAST accession and on Hartman et al. (2015).

^d NA = not assigned.

based on morphological characteristics of each isolate and ITS sequences of representative isolates.

Endophyte community structure. A multivariate test was conducted separately for leaf and stem plant parts. “Community” was defined as the percentage of each fungal endophyte genus across the isolates sampled from each field ($n = 6$) and fungicide treatment. Since the analysis was within field, the differences between years or reproductive stages were ignored. When considering all six genera, fungicide treatment impacted the leaf ($P = 0.020$) and the stem ($P = 0.007$) endophyte community. Further paired analysis within fields testing whether the fungicide spray treatment and the no-spray control have the same community showed that genus *Diaporthe* was increased in the fungicide spray treatment for both stems and leaves and genus *Alternaria* was decreased in the fungicide spray treatment (Fig. 2).

Seed endophyte assay. Genera *Alternaria* (14%) and *Diaporthe* (12.5%) were the most commonly isolated endophytes from surface-sterilized seeds collected after harvest in 2016 and 2017. *Alternaria* infection rarely killed the seed (2%), whereas nearly all *Diaporthe*-infected seeds failed to germinate (98%) at 2 weeks. *Colletotrichum*, *Fusarium*, and *Didymella*-like endophytes were detected in low numbers (0.3, 1.2, and 1.3%, respectively) and were not affected by fungicide treatment; seed death was not observed in seed infected with these fungi, although necrosis was often observed on developing roots. The percentage of *Diaporthe* seed infection was greatly impacted by cultivar and fungicide treatment ($P < 0.0001$) but these factors did not affect other fungal genera ($P > 0.05$). Further analysis on the percentage of *Diaporthe* seed infection detected an interaction of cultivar and fungicide treatment ($P = 0.0008$), which suggested that the effect of fungicide was not

constant across soybean cultivar. When we grouped cultivar into MGs (Table 1), we found that the treatment effect (fungicide spray) was greater for shorter-season MGs 2.0 and 2.4 ($P = 0.0021$ and $P = 0.0488$), but fungicide spray did not impact *Diaporthe* infection for longer-season MGs 2.7 and 3.9 ($P = 0.4065$ and $P = 0.0998$) (Fig. 3). A Pearson correlation of MG (x) versus the difference between the *Diaporthe* infection percentage of fungicide spray and no-spray control treatment (y) showed a negative relationship ($r = -0.5303$).

Discussion

This is the first report that a foliar fungicide spray increased the incidence and predominance of endophytic *Diaporthe* infection in stems, leaves, and seed. *Diaporthe* species are widely recognized endophytes in many plant species (Gomes et al. 2013; Huang et al. 2015; Murali et al. 2006; Rajamani et al. 2018; Suryanarayanan et al. 2018) and are also the cause of soybean diseases including stem canker, pod and seed decay, and *Phomopsis* seed decay (Wrather et al. 2003). The *Diaporthe* soybean disease community has an extended latent phase, and presumably these organisms become pathogenic when plants become stressed or senesce (Sinclair 1991). Our ITS sequences suggested that several *Diaporthe* species may be soybean endophytes. Additional sequences, including beta tubulin, are required to delineate *Diaporthe* species (Gomes et al. 2013; Huang et al. 2015) and this research is being conducted.

Conversely, we showed that a foliar fungicide decreased *Alternaria* endophytes in leaves and did not affect *Colletotrichum* endophytes. Genera *Alternaria* and *Colletotrichum* were the most frequently isolated foliar endophytes in our study and were commonly found in Argentina and Brazil soybeans (Larran et al. 2002). *Colletotrichum* endophytes are abundant in

Fig. 2. Community analysis showing the percent differences in the predominant endophyte community during pod fill (reproductive stage 5) in leaves and stems between a fungicide spray at early pod set (reproductive stage 3) and no-spray control treatment for six fields. The table values below each genus on the x-axis show the plotted mean percent differences, the standard error, and the statistical results (T-statistic and P value) of testing for treatment differences for each plant part.

legumes (Gonzaga et al. 2015) and other hosts (Photita et al. 2005; Suryanarayanan et al. 2018). Many of the same genera we detected were also reported as soybean endophytes in Minnesota, Brazil, or Argentina (da Costa Stuart et al. 2018; Impullitti and Malvick 2013; Larran et al. 2002; Pimentel et al. 2006). These fungi, in addition to *Fusarium* and *Nigrospora* fungi, have been routinely isolated as endophytes; all four of these fungi are known as weak parasites (Larran et al. 2002; Pan and May 2009; Roy et al. 2001; Suryanarayanan et al. 2018) and as plant decomposers (Dighton 2016). *Fusarium* sp. endophytes have shown potential as beneficial endophytes against nematodes (Vu et al. 2006). In a separate ongoing study, we are using isolates collected in this study to determine the virulence of soybean endophytes on germinating seeds.

Our findings suggest that many commonly found endophytes can be vertically transmitted through the seed and may explain why we found similar endophyte composition in our 2015 and 2016 fields, which were a few kilometers apart, and why these genera are reported in most soybean producing locations in the western hemisphere. Seed transmission by genus *Alternaria* has been reported (Hodgson et al. 2014).

We have shown that a fungicide application at pod set had lasting effects on the endophytic community of late-season plants through harvest, as was previously found with common and broad bean (Prior et al. 2017). We also found that the increase in *Diaporthe* seed infection mostly occurred for early maturing cultivars, but less for later-maturing soybeans. This suggests that the QoI fungicide-induced maturity delay, known as the “greening affect” (Balba 2007), extended the window of opportunity for vertical transmission of *Diaporthe* seed during periods of warm temperatures. Increased seed rot is associated with short-season cultivars, in which the maturation period is more often accompanied by higher temperatures and humidity than for long season cultivars (Balducci and McGee 1987), as we observed. Our results support the finding of Wrather et al. (2004) that the QoI fungicide azoxystrobin increased the incidence of *D. longicolla* seed infection. In contrast, Berkland (2011) reported that an application of the QoI fungicide pyraclostrobin at growth stage pod set reduced *D. longicolla* seed infection by 1.9%. The slight but significant increase of seed moisture at harvest in the fungicide-sprayed plots across all cultivars may be related to the higher incidence of seed rot and fungal growth.

Another factor that may account for the impact of fungicide treatment on the foliar endophytic community and the increased *Diaporthe* seed infection is QoI resistance. The single mode of action of QoI fungicides makes these compounds vulnerable to selective pressure by fungi that have mutations that allows them to service in the presence of QoI fungicides (Miles et al. 2012). Our culture collection is a valuable resource for assessing fungicide sensitivity of endophytes.

Fig. 3. Percentage of *Diaporthe* sp. seed infection from soybean cultivars with differing maturity groups that were sprayed with a fungicide at early pod set (reproductive stage 3) versus no spray. Error bars represent the standard deviation of each subplot cultivar combination within each maturity group.

The relatively low diversity of endophytes detected in the above-ground tissues (17 species) in this study was surprising considering the hyperdiverse fungal endophyte community associated with tropical and boreal trees and herbaceous plants (Arnold 2007; Rodriguez et al. 2009). Possible reasons for this reduced diversity may be related to the agronomic conditions of the soybean crops that were not grown in their native location; rather they were grown in rotated annual cropping systems in temperate latitudes, with strict weed and insect control, in fields that were tilled to reduce crop residue after harvest, and from commercially produced seed. da Costa Stuart et al. (2018) reported that diversity, richness, and evenness decreased in soybeans grown with conventional practices compared with organically grown soybeans. In that study, a 30% reduction of genera was attributed to pesticide use and the precipitous reduction of fungi recovered as the season progressed. Although the culture-dependent methods that we used in this study provided a similar OTU richness reported in other soybean foliar endophyte surveys (Impullitti and Malvick 2013; Larran et al. 2002; Pimentel et al. 2006), direct sequencing would certainly yield a much higher fungal richness than culture-dependent methods (Bakker et al. 2017; Bonito et al. 2016; de Souza Leite et al. 2013). Furthermore, our imprint method prior to culturing reduced the diversity we obtained but assured us that those isolates were truly endophytic, which could not be certain using PCR because DNA from dead fungi on surfaced sterilized tissue can be amplified (Siddique et al. 2017).

Knowledge of easily culturable species present in asymptomatic plants and our extensive culture collection will allow us to examine the impact of these prevalent endophytes on crop hosts and the microbial community, as well as to determine the influence of environment on these fungi. In conclusion, soybean producers may want to consider that the application of a QoI fungicide in the absence of disease pressure might adversely affect seed quality.

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