The Potential for Cereal Rye Cover Crops to Host Corn Seedling Pathogens

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ABSTRACT

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Cover cropping is a prevalent conservation practice that offers substantial benefits to soil and water quality. However, winter cereal cover crops preceding corn may diminish beneficial rotation effects because two grass species are grown in succession. Here, we show that rye cover crops host pathogens capable of causing corn seedling disease. We isolated *Fusarium graminearum*, *F. oxysporum*, *Pythium sylvaticum*, and *P. torulosum* from roots of rye and demonstrate their pathogenicity on

corn seedlings. Over 2 years, we quantified the densities of these organisms in rye roots from several field experiments and at various intervals of time after rye cover crops were terminated. Pathogen load in rye roots differed among fields and among years for particular fields. Each of the four pathogen species increased in density over time on roots of herbicide-terminated rye in at least one field site, suggesting the broad potential for rye cover crops to elevate corn seedling pathogen densities. The radicles of corn seedlings planted following a rye cover crop had higher pathogen densities compared with seedlings following a winter fallow. Management practices that limit seedling disease may be required to allow corn yields to respond positively to improvements in soil quality brought about by cover cropping.

Winter cover cropping is an agricultural management practice in which living cover is established during the otherwise fallow period between fall grain harvest and planting of the subsequent grain crop in the spring. Two of the primary goals for this practice are to improve soil quality and reduce negative environmental impacts of summer annual grain crop production, especially of corn (*Zea mays* L.) and soybean (*Glycine max* L.). For instance, among the problems caused by corn and soybean production are nitrate contamination of surface and groundwater resources (Burkart and James 1999; David et al. 2000), loss of soil to erosion (Pimentel et al. 1995), and degradation of soil quality through the depletion of soil organic matter (Paustian et al. 1997). Integrating cover crops into corn and soybean grain production systems could substantially reduce erosion, decrease losses of nitrogen and phosphorus, and improve soil quality and agroecosystem sustainability (Kaspar and Singer 2011; Kladvik et al. 2014; Weil and Kremen 2007).

Rye (*Secale cereale* L.) is the most common and reliable cover crop in the upper Midwest because it is one of the few cover crops that can successfully establish when planted in the fall after corn or soybean harvest, is winter hardy throughout the region, and accumulates meaningful amounts of biomass before spring planting (Snapp et al. 2005). Seed is also readily available at a relatively low cost. Winter cereal cover crops can be readily integrated into existing corn-soybean production systems, could feasibly be implemented over large areas, and have been highlighted as cost-effective strategies for improving environmental stewardship (Kladvik et al. 2014). As a result, cover cropping has been proposed as one of the primary management practices in state- and regional-scale initiatives to reduce nutrient losses to surface waters. For instance, the Iowa Nutrient

Reduction Strategy (2014) includes example land-use scenarios in which all corn-soybean and continuous-corn acres in the state are planted to cover crops. The United States Department of Agriculture Census of Agriculture reports 4.2 million ha of crop land planted to cover crops across the United States in 2012 (NASS 2012).

Along with their benefits, however, there may be challenges associated with cover cropping. For instance, yield decreases have sometimes been observed in corn following winter cereal cover crops (Dinnes et al. 2002; Johnson et al. 1998; Kaspar and Bakker 2015). This occasional yield penalty has led some farmers to abandon cover crops or never even try them. Understanding the basis for corn population and yield decreases following cereal cover crops is crucial for the development of management strategies and cover crop selection or breeding programs that will reduce or eliminate this effect, opening up the possibility of corn yield increases in response to beneficial effects of cover cropping on soil quality.

Previously, researchers have suggested that allelopathy, reduced water or nitrogen availability, poor planter performance, or increased pest pressure may be possible causes for corn yield decreases following cereal cover crops (Duiker and Curran 2005; Karlen and Doran 1991; Kessavalou and Walters 1997; Munawar et al. 1990; Tollenaar et al. 1993). We propose an alternative hypothesis: winter cereal cover crops may contribute to elevated populations of corn seedling pathogens. Under conducive environmental conditions, this may cause increased incidence of corn seedling diseases and reduced seedling survival, plant growth, and, consequently, yield.

Corn and the cereal grains belong to the same plant family, Poaceae. Given this close phylogenetic similarity, it might be expected that a number of pathogens would be shared (Gilbert et al. 2015). For instance, the ability to infect both rye and corn has been demonstrated for *Fusarium* spp. (Chongo et al. 2001), *Pythium* spp. (Vestberg 1990), and *Rhizoctonia* spp. (Neate 1989). Furthermore, characteristics of the agricultural production systems using cereal cover crops before corn may facilitate pathogen transfer. For instance, in no-till systems, cover crop and main crop roots grow repeatedly through the same persistent macropores year after year, and these macropores contain residues of dead cover crop and main crop roots (Rasse and Smucker 1998; Williams and Weil 2004). Thus, live roots growing

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through these root channels may be exposed to pathogen inoculum associated with dead roots, and pathogen transfer may occur via direct root-to-root contact.

One avenue by which cover crops may facilitate high pathogen densities in soil is by providing pathogens an opportunity for rapid growth and reproduction on weakened or dying host tissue when cover crops are terminated. Typically, in corn production systems, winter cereal cover crops are terminated with glyphosate or another herbicide before planting corn (Clark 2012). Rosenbaum and colleagues (2014) found that rates of root infection by *Fusarium* spp. increased after host plants were terminated with glyphosate. Pathogens such as *Fusarium* and *Pythium* spp. also have the ability to grow and reproduce saprotrophically (Leplat et al. 2013; Martin and Loper 1999), suggesting the possibility of an inoculum build up on fresh cover crop residue.

Second, cover crops may act as a “green bridge”: a living host that offers pathogens an escape from the highly competitive environment of decomposing plant residues in soil. Pathogens that are facultative saprotrophs may initially flourish on a dying host but would be displaced by more strongly competitive saprotrophs as host tissues decompose. Spring-terminated cover crops provide live root refugia throughout the late fall, winter, and early spring, potentially shielding pathogens from the competition they would experience in soil or residue. In a field study, Smiley et al. (1992) observed that planting spring barley soon after terminating volunteer winter cereal plants resulted in greater Rhizoctonia root rot than planting barley 22 days after termination. They hypothesized that a delay in planting barley after spraying volunteer cereal plants with glyphosate allowed for pathogens to be displaced by other microbial colonists of the dead roots. This led to recommendations of minimum intervals between herbicide application and planting to reduce disease severity in the following crop (Babiker et al. 2011). It has been argued that weeds may serve as green bridges and support pathogen population growth on recently killed tissue (Powell and Swanton 2008), and similar effects seem likely where cover crops host pathogens able to infect the following cash crop.

We performed several experiments to investigate the potential for rye cover crops and their root tissues remaining after termination to host pathogens of corn. We completed Koch's postulates to demonstrate that isolates of *Fusarium graminearum*, *F. oxysporum*, *Pythium sylvaticum*, and *P. torulosum* originating in rye roots were capable of causing disease in corn. *F. graminearum* and *F. oxysporum* can cause corn seed rot, seedling blight, and stalk rot (Broders et al. 2007b; Iowa State University Extension and Outreach 2012; Sparks 2014). *P. sylvaticum* and *P. torulosum* can cause seedling damping-off, and are commonly found on corn in the upper Midwest (Broders et al. 2007a; Matthiesen et al. 2016). We used quantitative polymerase chain reaction (qPCR) to assess the density of these four target corn pathogens in rye roots and in corn seedlings.

MATERIALS AND METHODS

***Fusarium* and *Pythium* strains.** Strains of *F. graminearum*, *F. oxysporum*, *P. sylvaticum*, and *P. torulosum* were isolated from roots of cereal rye that had been grown either in the field or in a controlled-environment chamber (16-h days with 20°C days and 10°C nights) in soil from a corn field, and terminated with glyphosate 7 to 17 days prior to sample collection. Symptomatic sections of rye root were rinsed briefly in 0.6% NaOCl, washed in sterile distilled water, blotted dry, and plated on semiselective media. *F. graminearum* was isolated on Nash-Snyder media (Nash and Snyder 1962). *F. oxysporum* was isolated on water agar containing streptomycin (300 mg liter⁻¹) and metalaxyl (14.2 mg liter⁻¹). *Pythium* spp. were isolated on P₅ARP medium (Jeffers and Martin 1986) plus benomyl at 10 mg liter⁻¹.

Putative *Fusarium* strains were subcultured onto potato dextrose agar. To ensure that isolates were not mixed strains, dilute conidial suspensions were spread over water agar plates and individual germinating macroconidia were transferred to new plates with the

aid of a dissecting microscope and a flame-sterilized wire spatula. For morphological identification, strains were grown on carnation leaf agar under bright light with 12-h days at 25°C (day) and 20°C (night) (Leslie and Summerell 2006).

Putative *Pythium* strains, having coenocytic hyphae, were transferred to dilute V8 agar (40 ml of V8 juice, 0.6 g of CaCO₃, 0.2 g of yeast extract, 1.0 g of sucrose, 0.01 g of cholesterol, and 20 g of agar per liter) with neomycin sulfate at 100 mg liter⁻¹ and chloramphenicol at 10 mg liter⁻¹. *Pythium* strains were provisionally identified through morphological characteristics (Vanderplaats-Niterink 1981; Waterhouse 1967).

For identification by marker gene sequence, *Fusarium* and *Pythium* strains were cultured in potato dextrose broth on a shaker table (25°C, 175 rpm). Mycelium was washed with deionized water, freeze dried, and pulverized by bead beating. The DNeasy Plant Mini Kit (Qiagen) was used to extract DNA. For *Fusarium* strains, the translation elongation factor 1- α (TEF) marker gene was amplified using primers ef1 and ef2 and the method of Geiser et al. (2004). For putative *F. graminearum* strains, we modified the forward primer (ef1_Fgram: 5'-ATG GGT AAG GAG GAS AAG AC-3') to enhance amplification efficiency. For *Pythium* strains, the cytochrome C oxidase subunit I (COI) marker gene was amplified using primers OomCoxI-Levup and OomCoxI-Levlo and the method of Robideau et al. (2011). To facilitate sequencing of amplicons, the M13 primer sequence was appended to the 5' end of both ef2 and OomCoxI-Levlo primers. Sanger sequencing was performed on the resulting amplicons and chromatographs were trimmed manually using FinchTV (Geospiza). *Fusarium* spp. TEF gene sequences were matched against entries in the *Fusarium*-ID database (Geiser et al. 2004). *Pythium* spp. COI gene sequences were compared with those provided by Robideau et al. (2011). Sequence data for isolates used in pathogenicity assays have been deposited in GenBank as accessions KT380027 to KT380030.

Tests of pathogenicity on corn. We tested whether putative pathogens isolated from rye roots were able to cause disease on corn seedlings, using a representative isolate from each of four target pathogen species (i.e., *F. graminearum* 412-135, *F. oxysporum* Foxy12, *P. sylvaticum* 2015-01-15a, and *P. torulosum* Pyth37). These isolates were collected in this work from rye roots, as described above.

To produce macroconidia for inoculum, *F. graminearum* was grown on 1.5% water agar sprinkled with sterile wheat bran under bright light with 12-h days at 25°C (day) and 20°C (night). Conidia were dislodged with a bent glass rod and washed off the plate surface with water, filtering out wheat bran and mycelium with a 4 $\times$ layer of cheesecloth. Spore concentration was assessed by direct microscopic count with a Neubauer Chamber Cell. Macroconidia were incorporated at a rate of 50,000 spores g⁻¹ into a pasteurized substrate (65°C, 24 h) consisting of equal volumes of coarse sand and vermiculite.

Inoculum of *F. oxysporum* was produced as colonized wheat grains. Wheat grains were soaked in tap water overnight, drained, and autoclaved on two consecutive days in a plastic bag with an air-exchange patch. The sterilized substrate was infested with 10 l-cm² pieces of colonized agar medium. Bags were heat sealed and incubated in the dark (25°C, 10 days). On the fifth day, each bag was massaged to distribute the pathogen evenly throughout the substrate. For the control treatment, a bag was set up and incubated in the same way, except that no colonized agar pieces were added. Pots were infested by placing 15 wheat grains in a layer approximately 2.5 cm below the corn seed.

To produce inoculum for *Pythium* spp., equal volumes of coarse sand and vermiculite (1,450 g total) were combined with 50 g of corn meal and 450 ml of water in an autoclavable plastic bag, which was sterilized and incubated as described above. After homogenization, this colonized medium was used directly as the potting medium for corn seedling growth.

Corn seed (DuPont Pioneer hybrid variety PO448; no chemical seed treatments) were surface disinfested (shaking for 2 min in 70%

ethanol and 20 min in 0.6% sodium hypochlorite + detergent, then rinse four times with sterile water) and preimbibed between sheets of moist germination paper for 18 h at room temperature. One seed was planted per pot (3.8 by 21 cm; Stuewe & Sons, Inc.), with 10 replicate pots per treatment. Pots were grown in a randomized block design at a constant 14°C with 12-h days in a controlled-environment chamber. Pots were watered daily with 15 ml of deionized water or fertilizer solution (on the 10th day only), and plants were harvested after 21 days. Roots were washed and root length was measured using WinRhizo Pro (v. 2009c; Regent Instruments, Inc.).

Fusarium and *Pythium* strains were reisolated from root tissue at the leading edge of lesions, using procedures for isolation as described above. Attempts were made to isolate *Fusarium* and *Pythium* spp. from the roots of uninoculated control plants as well. Isolates collected from corn roots were compared morphologically and by marker gene sequence to confirm that the same isolate that was used in inoculation was recovered from the diseased tissue.

Pathogen population dynamics on rye roots under controlled-environment conditions. To assess pathogen population dynamics in the roots of herbicide-treated rye plants, rye was grown in a controlled-environment chamber at 25°C with 12-h days. Pathogen inocula were prepared as described above and used to infest a potting medium consisting of (final proportions) 75% field soil and 25% sand/vermiculite (1:1) mix. For each pathogen, pots were randomly assigned ($n = 7$) (i) to be harvested prior to herbicide application, (ii) to be harvested 2 weeks later with no herbicide application, or (iii) to receive herbicide application and then be harvested 2 weeks later. Preimbibed rye seed were transplanted into infested medium and glyphosate was applied 33 days after planting at simulated field application rates.

Rye roots were washed under a high-pressure stream of water, with a mesh screen to trap and support roots. Washed roots were

freeze dried and pulverized by beating with a tungsten-carbide bead (3 mm in diameter; Qiagen) on a MiniBeadbeater (Biospec Products). DNA was extracted from approximately 25 mg of dry tissue (actual weight was determined for each sample) using the DNeasy Plant Mini Kit (Qiagen).

Assessment of corn pathogen load in roots of field-grown rye and corn. In spring 2014 and 2015, rye roots were collected from field experiments located near Ames, IA. In each field, rye ('Elbon') was grown as a winter cover crop in a corn-soybean rotation system (Table 1). Soil cores (3.18 cm in diameter and 15 cm in depth) were drawn directly over the crown of individual rye plants and were transported on ice to the laboratory, where they were frozen until processing. In 2015, we also collected corn seedling radicle and mesocotyl tissue for assessment of pathogen densities. Six corn plants were dug up per plot. For each plant, we collected the first 2 cm of radicle and 2 cm of mesocotyl extending away from the seed. Sections of radicle or mesocotyl from three plants were bulked into a single DNA extraction, leaving two subsamples per plot. Rye roots and corn tissue samples were washed and processed for DNA extraction, as described above.

qPCR for measurement of pathogen density. DNA extracts from corn tissue or rye roots were used to quantify the abundance of four pathogens of corn via qPCR using an Opticon2 DNA Engine (MJ Research; samples from 2014) or a CFX96 (Bio-Rad; samples from 2015). In each case, the PCR was performed in a 20- μ l volume using Quantitect SYBR Green (Qiagen; 2014 samples) or QuantiFast SYBR Green (Qiagen; 2015 samples), forward and reverse primers at 200 or 500 nM (for *F. oxysporum*) final concentrations, and 5 μ l of template DNA.

Detection and quantification of *F. graminearum* density in rye roots followed the method of Horevay et al. (2011), using primers Tri6-10F and Tri6-4R to amplify a component of the trichothecene

TABLE 1. Summary of field experiments sampled in this work

Year, site	Glyphosate spray dates	Rye root sample dates ^y	Subsamples per plot	Plot-level replicates	Prior crop	Soil series present ^z	Geographical coordinates
2014							
B42	April 22	May 9	1	10	Soybean	Clarion, Nicollet	42.008° N, -93.792° W
B44	May 3	May 9	1	10	Silage corn	Clarion, Nicollet	42.008° N, -93.792° W
B54	April 22	May 9	1	6	Soybean	Clarion, Spill, Coland	42.006° N, -93.789° W
	May 3	May 9	1	6	...	...	...
	May 7	May 9	1	6	...	...	...
	Unsprayed	May 9	1	18	...	...	...
F86	April 22	April 25	1	5	Soybean	Clarion, Nicollet, Webs	42.021° N, -93.765° W
	...	May 9	1	5	...	...	...
	...	May 23	3	5	...	...	...
	May 5	April 25	1	5	...	...	...
	...	May 9	1	5	...	...	...
	...	May 23	3	5	...	...	...
Kelly	April 11	May 9	3	4	Soybean	Canisteo, Nicollet	42.046° N, -93.715° W
	May 3	May 9	3	4	...	...	...
2015							
B54	April 6	May 12	1	6	Soybean	Canisteo, Nicollet	42.006° N, -93.789° W
	April 14	May 12	1	6	...	...	...
	April 23	May 12	1	6	...	...	...
	April 28	May 12	1	6	...	...	...
	May 3	May 12	1	6	...	...	...
	Unsprayed	May 12	1	6	...	...	...
F86	April 14	April 15	2	5	Soybean	Canisteo, Nicollet	42.021° N, -93.765° W
	...	April 29	2	5	...	...	...
	...	May 13	2	5	...	...	...
	...	May 27	2	5	...	...	...
	...	June 8	2	5	...	...	...
	April 28	April 29	2	5	Soybean	Canisteo, Nicollet	42.021° N, -93.765° W
	...	May 13	2	5	...	...	...
	...	May 27	2	5	...	...	...
	...	June 8	2	5	...	...	...
	...	June 23	2	5	...	...	...

^y Multiple glyphosate spray dates at a given site represent distinct experimental treatments, not repeat applications to the same plots.

^z Clarion = Clarion loam, Nicollet = Nicollet loam, Spill = Spillville loam, Coland = Coland-Spillville complex, Webs = Webster clay loam, and Canisteo = Canisteo silty clay loam.

biosynthetic cluster. Detection and quantification of *F. oxysporum* density followed the method of Haegi et al. (2013), using primers Fef1F and Fef2R to amplify a portion of the TEF gene. Detection and quantification of *P. sylvaticum* density followed the method of Schroeder et al. (2006), using primers SYL1F and SYL2R to amplify a portion of the internal transcribed spacer (ITS) region of the ribosomal RNA gene. Detection and quantification of *P. torulosum* density followed the method of Asano et al. (2010), using primers AsPyF and AsTOR6 to amplify a portion of the ITS. Nondetects were assigned a small nonzero value, defined as half of the calculated DNA content at a cycle threshold of 40. In all qPCR experiments, each plate included no-template controls and a standard curve of genomic DNA from the target organism, spanning at least five orders of magnitude in template DNA concentration. Technical triplicates were run for all samples and standards. Where multiple subsamples were processed for a given plot, resulting data were averaged to a single mean value per plot prior to statistical analyses. Samples with undetectable levels of all four target pathogens were screened for PCR inhibition by amplifying the plant gene for the large-chain subunit of the ribulose biphosphate carboxylase gene, using primers SI_For and SI_Rev (Kress et al. 2009). Successful amplification was observed in all cases, indicating that failure to detect pathogens was not due to PCR inhibition.

Data processing and statistics. Measured pathogen densities in rye roots from the field were expressed in terms of femtograms of pathogen genomic DNA per milligram of dry rye root tissue and then were scaled by root density in soil to give pathogen density per cubic centimeter of soil. These units were chosen to account for the loss of rye root biomass over time after plant termination with glyphosate; increasing pathogen density per unit of root mass could still result in declining pathogen densities per unit volume of soil because root mass was declining due to decomposition. However, in the growth chamber pot experiment, measured pathogen densities were retained in units of femtograms of pathogen genomic DNA per milligram of dry rye root tissue, because of unnatural root densities in pots and because the object was to observe changes in pathogen density in roots specifically. For both field and pot data, final values were $\log_{10}(x + 1)$ transformed to stabilize variances.

Measured pathogen densities in corn tissue were expressed as femtograms of pathogen genomic DNA per million copies of the corn *tua4* gene, and then were $\log_{10}$ transformed. For the *tua4* qPCR assay (Mideros et al. 2009), a standard curve of known copy number was prepared using a synthesized template. Expressing pathogen DNA abundance relative to host DNA abundance minimizes error

associated with variation in DNA extraction efficiency. Although desirable, this method is not appropriate for dead plant tissues such as the rye roots that we sampled, because host plant DNA is degraded in dead tissues.

For statistical comparison of pathogen densities among treatments within a field (i.e., different intervals between cover crop termination and sampling dates), we used an additive model that incorporated both the experimental block and the treatment as main effects. Where significant differences were evident among treatments, Tukey's honestly significant difference (HSD) was used for post hoc comparisons. For comparison of pathogen densities among fields at a given interval between cover crop termination and sampling dates, a one-way analysis of variance (ANOVA) with Tukey's HSD was used. For comparison of pathogen density within corn tissues grown following a rye cover crop or following a winter fallow, and for comparison of corn seedling root length in pathogen-infested versus noninfested medium, *t* tests were used. Statistical tests were performed in R, version 3.2.2 (R Core Team 2015).

RESULTS

Demonstration of potential for shared pathogens between rye and corn. Isolations from rye roots on semiselective media yielded strains of *F. graminearum*, *F. oxysporum*, *P. sylvaticum*, and *P. torulosum*. Other species, including *F. solani*, *P. irregulare*, and *P. spinosum*, were also isolated but were not investigated further in this work.

Strains of *F. graminearum* were identified on the basis of TEF gene sequence and cultural characteristics, including the formation of perithecia by a single-spored isolate, morphology of macroconidia, and the absence of microconidia. Koch's postulates were completed using *F. graminearum* 412-135, a strain originally isolated from a rye root. Corn grown in a medium that had been infested with this strain had blackened necrotic lesions on the radicle, seminal roots, and mesocotyl (Supplementary Fig. S1). Secondary roots emerging from the radicle and seminal roots were greatly reduced in length, relative to the noninfested control. As a consequence, total root length was significantly lower for corn seedlings grown in a medium infested with *F. graminearum* 412-135 than for seedlings grown in noninfested medium (Fig. 1; *t* test, $P < 0.001$). Corn shoots were also visibly smaller in the presence of *F. graminearum*. Isolations from lesions on corn roots yielded *F. graminearum* strains that had cultural characteristics that were indistinguishable from the inoculated strain and that also had a TEF marker gene sequence identical to that of the inoculated strain (data not shown). Isolations from control plants grown in noninfested medium yielded strains of *F. oxysporum* but no strains of *F. graminearum* (data not shown).

Strains of *F. oxysporum* were identified on the basis of TEF gene sequence and cultural characteristics. Koch's postulates were completed using *F. oxysporum* Foxy12, a strain originally isolated from a rye root. Corn grown in a medium that had been infested with this strain had necrotic lesions that often killed root tips. These lesions were largely limited to the portion of the root system that was in contact with or grew through the inoculum; progression of lesions upward into older tissue was limited. Total root length was significantly reduced for corn seedlings grown in soil infested with *F. oxysporum* compared with seedlings grown in noninfested medium (Fig. 1; *t* test, $P = 0.0013$). Isolations from lesions on corn roots yielded *F. oxysporum* strains that had cultural characteristics that were indistinguishable from the inoculated strain, and that also had a TEF marker gene sequence identical to that of the inoculated strain (data not shown). Isolations from control plants grown in noninfested medium sometimes also yielded strains of *F. oxysporum* but these were morphologically distinct from the inoculated strain and also had polymorphisms in the marker gene sequence relative to the inoculated strain (data not shown). Interestingly, strains of *F. oxysporum* isolated from control plants in both the *F. graminearum*

Fig. 1. Pathogens isolated from rye roots significantly reduced corn seedling root length, as determined in growth-chamber experiments. Root lengths are expressed as a proportion of the control treatment because multiple experimental trials are represented. Within each trial, root length was significantly lower in the presence than in the absence of the pathogen (*t* test, $P < 0.05$; $n = 10$).

and the *F. oxysporum* pathogenicity assays had identical marker gene sequences (data not shown). This may indicate that the contaminating strain was internally seedborne, or a common strain within our plant growth facilities.

Strains of *P. sylvaticum* were identified to genus by morphology and to species on the basis of COI marker gene sequence. Koch's postulates were completed using *P. sylvaticum* 2015-01-15a, a strain originally isolated from a rye root. Corn grown in a medium that had been infested with this strain had visible disease lesions, although these were small and often limited to root tips. Despite the absence of widespread necrosis, the radicles of plants grown in an infested medium were shortened and there was extensive root pruning, with few secondary roots and often no seminal roots present. Total root length was significantly lower for corn seedlings grown in soil infested with *P. sylvaticum* than for seedlings grown in noninfested soil (Fig. 1; *t* test, $P < 0.001$). Corn shoots were also visibly smaller in the presence of *P. sylvaticum*. Isolations from lesions on corn roots yielded *P. sylvaticum* strains that had cultural characteristics that were indistinguishable from the inoculated strain, and that also had a COI marker gene sequence identical to that of the inoculated strain (data not shown). Attempted isolations from control plants grown in noninfested medium did not yield any *Pythium* isolates.

Strains of *P. torulosum* were identified to genus by morphology and to species on the basis of COI marker gene sequence. Koch's postulates were completed using *P. torulosum* Pyth37, a strain originally isolated from a rye root. Corn grown in a medium that had been infested with this strain had visible disease lesions, although these were small. Despite the small size of lesions, root pruning was evident; secondary roots were fewer and shorter on the radicle and seminal roots compared with control plants grown in noninfested medium. Root pruning was not as severe as for *P. sylvaticum*. However, total root length was significantly lower for corn seedlings grown in soil infested with *P. torulosum* than for seedlings grown in noninfested medium (Fig. 1; *t* test, $P = 0.0011$). Isolations from lesions on corn roots yielded *P. torulosum* strains that had cultural characteristics that were indistinguishable from the inoculated strain, and that also had a COI marker gene sequence identical to that of the inoculated strain (data not shown). Attempted isolations from control plants grown in noninfested medium did not yield any *Pythium* isolates.

In sum, these isolations and pathogenicity assays, with completion of Koch's postulates, demonstrate conclusively the potential for rye roots to host organisms that are true pathogens of corn.

Pathogen population dynamics in roots of herbicide-terminated rye in a controlled environment. We performed a controlled-environment-chamber experiment to test the potential for pathogens to increase in density in roots of herbicide-treated rye. For each of the four target pathogens, densities increased significantly in rye roots over the 2-week period following herbicide application (Fig. 2; ANOVA with Tukey contrasts, $P < 0.05$). This confirms our hypothesis that these pathogens could increase in density in the roots of rye cover crops as they are terminated prior to corn planting. For *F. graminearum*, pathogen density was not significantly higher in glyphosate-treated than in non-glyphosate-treated plants at the same time point (Fig. 2), indicating that this pathogen was able to increase in density even on living rye plants.

Load of potential corn pathogens in roots of rye cover crops in the field. Each of the four target pathogens was routinely detected over 2 years in roots of rye grown as a winter cover crop prior to corn planting (Table 2). At least one of these four organisms had a detectable presence in rye roots from nearly every plot that was tested. However, the four target pathogens were not equally common or abundant in rye roots.

In 2014, *F. graminearum* was detected in rye roots in 26%, *F. oxysporum* in 97%, *P. sylvaticum* in 85%, and *P. torulosum* in 62% of samples. Among samples in which they were detected, densities of *F. graminearum* were lower than for *F. oxysporum*

(DNA at 1.82 versus 3.58 log fg cm⁻³ of soil), and densities of *P. sylvaticum* were higher than for *P. torulosum* (DNA at 2.47 versus 0.52 log fg cm⁻³ of soil). Thus, the most common of these pathogens (i.e., *F. oxysporum* and *P. sylvaticum*) were also present at the highest densities in rye roots. However, comparisons among species for relative abundance based on DNA contents are complicated by differing genome sizes, gene copy numbers, and aggressiveness as pathogens and may not reflect their relative effect or importance to disease expression.

In 2015, all four pathogens were detected in >90% of rye root samples tested. Mean densities, where present, were DNA at 5.22, 5.95, 3.64, and 2.66 log fg cm⁻³ of soil for *F. graminearum*, *F. oxysporum*, *P. sylvaticum*, and *P. torulosum*, respectively. This increase in pathogen incidence and density compared with 2014 may have been due to more favorable environmental conditions for pathogen growth.

Pathogen density in roots of cereal rye was variable across fields, and this was true even for rye roots with a similar interval of time after the application of herbicide. For instance, in 2014, the field site B54 had the highest contents of *P. sylvaticum* and *P. torulosum* in rye roots at both 6 and 17 days post-spraying (DPS), compared with other field sites which were sampled at similar intervals after herbicide application (Fig. 3).

Several of our field sites included treatments in which rye cover crops were terminated at different dates. At one site (F86), we returned repeatedly to the same plots to sample rye roots at increasing intervals after herbicide application (Table 1). Thus, our sampling of rye roots covered a progression from live, unsprayed plants to roots of plants which had been terminated several weeks prior to sample collection. In 2014, densities of the two *Pythium* spp. increased with time in roots of herbicide-terminated rye plants at the B54 field site (Table 2). Densities of *F. oxysporum* also increased with time in roots of herbicide-terminated rye plants at the F86 field site, whereas densities of *F. graminearum* decreased with time at that same site (Table 2). In many cases, pathogen densities did not change significantly with time after herbicide application.

In 2015, significant changes over time in pathogen density in roots of herbicide-terminated rye cover crops were again observed. In general, the *Fusarium* spp. tended to increase in density over time through the duration of sampling (Table 2). In contrast, the *Pythium* spp. tended to decline in density in rye roots within several weeks of herbicide application, or to increase to a peak and then decline in density (Table 2).

Between the two time series at the F86 field site (early-termination versus late-termination treatments), pathogen densities often differed among rye roots that had passed similar time intervals after herbicide

Fig. 2. Density of four corn seedling pathogens in roots of rye (mean $\pm$ standard error, $n = 7$) grown under controlled-environment conditions in infested growth media. Rye plants were harvested prior to glyphosate application or 2 weeks later, with either glyphosate or no glyphosate applied. Among treatments within each pathogen, means marked with the same letter are not significantly different (analysis of variance with Tukey contrasts, $P > 0.05$).

application. In 2014, densities of *F. graminearum* were higher in samples from the early-termination treatment at 3 DPS than in samples from the late-termination treatment at 4 DPS (*t* test, $P = 0.0004$) (Table 2). The opposite was true for *F. oxysporum*; samples from the early-termination treatment at 3 DPS had lower densities of *F. oxysporum* than samples from the late-termination treatment at 4 DPS (*t* test, $P = 0.026$). These contrasts may be explained by influences of environmental conditions such as soil temperature and moisture on pathogen population dynamics in roots of cereal rye. In the 5 days following herbicide application, mean daily air temperature was 12.5°C for the early-termination treatment but 15.9°C for the late-termination treatment (site IA0200) (Iowa Environmental Mesonet 2015), suggesting that the herbicide may have killed the rye plants more quickly in the late-termination treatment. Optimum temperature for growth may have differed among these pathogen species, and differences in rye physiology or tissue chemistry due to additional time for development may have influenced pathogen densities in roots at later time points.

In 2015, timing of cover crop termination also affected pathogen density at particular intervals after herbicide application. Where differences were significant, the *Pythium* spp. were present at higher densities in the early-termination treatment than in the late-termination treatment (*t* test, $P \leq 0.05$ for *P. sylvaticum* at 29 and at 43 DPS and for *P. torulosum* at 1, 29, and 55 DPS) (Table 2). Density of *F. oxysporum* differed significantly with cover crop termination timing in only one case (*t* test, $P = 0.008$; higher density at 15 DPS in the late-termination treatment). In 2015, mean daily air temperature in the 5 days following

herbicide application did not vary substantially with termination timing (15.7°C for early versus 15.4°C for late termination) (Iowa Environmental Mesonet 2015). Differences in soil temperature or moisture may have influenced pathogen dynamics but we did not measure these variables directly.

Pathogen load in corn seedlings in the field, in response to the presence of a rye cover crop. Radicles of corn seedlings grown following a rye cover crop typically had higher pathogen densities compared with corn seedlings grown following a winter fallow, although statistically significant differences were not observed in all cases. In particular, the rye cover crop significantly increased the density of *F. oxysporum* in corn radicles in both the early- and late-termination treatments at field F86 (Fig. 4; *t* test, $P \leq 0.05$). The rye cover crop also significantly increased the density of *P. torulosum* in corn radicles in field B52 and in the late-termination treatment at field F86 (Fig. 4; *t* test, $P \leq 0.05$). There was a marginal increase in the density of *F. graminearum* in corn radicles in response to rye cover crop presence in the late-termination treatment at field F86 (Fig. 4; *t* test, $P = 0.065$). Pathogen densities in corn mesocotyls generally mirrored those in the radicles but were slightly lower and had fewer significant differences (data not shown).

DISCUSSION

We used the common cover crop system of winter rye grown between soybean harvest and corn planting to assess the potential for cover cropping to increase inoculum of corn seedling pathogens. Our results demonstrate that a number of corn seedling pathogens

TABLE 2. Pathogen densities (mean $\pm$ standard error) observed at each field location and herbicide application treatment^z

Year, field site	DPS	Pathogen density in rye roots (DNA at log fg cm ⁻³ of soil)			
		<i>Fusarium graminearum</i>	<i>F. oxysporum</i>	<i>Pythium sylvaticum</i>	<i>P. torulosum</i>
2014					
B42	17	0.70 $\pm$ 0.12	2.98 $\pm$ 0.20	1.71 $\pm$ 0.30	0.48 $\pm$ 0.15
B44	6	1.46 $\pm$ 0.27	3.22 $\pm$ 0.31	1.26 $\pm$ 0.49	0.02 $\pm$ 0.01
B54	Prespray	0.79 $\pm$ 0.04 ns	4.03 $\pm$ 0.16 ns	2.05 $\pm$ 0.17 C	0.15 $\pm$ 0.04 B
	2	0.77 $\pm$ 0.07	3.71 $\pm$ 0.30	2.08 $\pm$ 0.34 BC	0.69 $\pm$ 0.25 AB
	6	0.89 $\pm$ 0.14	3.82 $\pm$ 0.14	4.03 $\pm$ 0.82 A	1.04 $\pm$ 0.34 A
	17	1.25 $\pm$ 0.36	4.00 $\pm$ 0.14	3.67 $\pm$ 0.35 AB	1.44 $\pm$ 0.46 A
F86					
Early termination	3	2.00 $\pm$ 0.06 a	2.55 $\pm$ 0.25 b	1.64 $\pm$ 0.46 ns	0.00 $\pm$ 0.00 ns
	17	1.30 $\pm$ 0.23 ab	3.71 $\pm$ 0.15 a	2.47 $\pm$ 0.34	0.03 $\pm$ 0.02
	31	1.09 $\pm$ 0.26 b	3.92 $\pm$ 0.17 a	3.02 $\pm$ 0.26	0.04 $\pm$ 0.03
Late termination	Prespray	2.10 $\pm$ 0.08 A	2.85 $\pm$ 0.12 B	2.15 $\pm$ 0.37 ns	0.00 $\pm$ 0.00 ns
	4	1.07 $\pm$ 0.15 B	4.27 $\pm$ 0.50 A	2.61 $\pm$ 0.24	0.01 $\pm$ 0.00
	18	1.69 $\pm$ 0.11 A	3.65 $\pm$ 0.11 AB	3.33 $\pm$ 0.20	0.36 $\pm$ 0.22
Kelly	6	0.92 $\pm$ 0.04 ns	3.09 $\pm$ 0.33 ns	0.74 $\pm$ 0.39 ns	0.01 $\pm$ 0.01 ns
	28	0.74 $\pm$ 0.09	2.89 $\pm$ 0.29	0.75 $\pm$ 0.58	0.38 $\pm$ 0.28
2015					
B54	Prespray	3.43 $\pm$ 0.25 BC	4.52 $\pm$ 0.18 B	2.64 $\pm$ 0.22 ns	1.43 $\pm$ 0.29 AB
	9	3.92 $\pm$ 0.43 BC	4.98 $\pm$ 0.11 AB	2.79 $\pm$ 0.46	1.72 $\pm$ 0.33 AB
	14	4.07 $\pm$ 0.46 ABC	5.08 $\pm$ 0.23 AB	2.66 $\pm$ 0.55	1.46 $\pm$ 0.22 AB
	19	4.70 $\pm$ 0.13 ABC	5.19 $\pm$ 0.20 A	2.84 $\pm$ 0.50	2.09 $\pm$ 0.16 AB
	28	4.82 $\pm$ 0.11 AB	5.20 $\pm$ 0.12 A	2.88 $\pm$ 0.35	2.33 $\pm$ 0.13 A
	36	5.37 $\pm$ 0.24 A	4.77 $\pm$ 0.12 AB	3.27 $\pm$ 0.13	1.21 $\pm$ 0.29 B
F86					
Early termination	1	4.92 $\pm$ 0.34 ns	5.09 $\pm$ 0.19 c	4.50 $\pm$ 0.13 ab	4.23 $\pm$ 0.20 a
	15	5.34 $\pm$ 0.44	6.32 $\pm$ 0.21 b	5.35 $\pm$ 0.40 a	4.01 $\pm$ 0.12 a
	29	6.18 $\pm$ 0.51	7.04 $\pm$ 0.16 a	4.49 $\pm$ 0.26 ab	3.80 $\pm$ 0.11 a
	43	6.03 $\pm$ 0.43	7.15 $\pm$ 0.12 a	4.12 $\pm$ 0.21 b	3.20 $\pm$ 0.17 b
	55	5.53 $\pm$ 0.35	6.96 $\pm$ 0.09 a	3.85 $\pm$ 0.25 b	3.99 $\pm$ 0.12 a
Late termination	1	4.96 $\pm$ 0.17 ns	5.19 $\pm$ 0.24 B	4.49 $\pm$ 0.25 AB	2.84 $\pm$ 0.09 B
	15	5.83 $\pm$ 0.27	7.11 $\pm$ 0.08 A	5.07 $\pm$ 0.48 A	3.77 $\pm$ 0.29 A
	29	5.98 $\pm$ 0.25	7.24 $\pm$ 0.14 A	3.63 $\pm$ 0.24 B	2.69 $\pm$ 0.09 B
	41	5.56 $\pm$ 0.37	7.13 $\pm$ 0.12 A	3.48 $\pm$ 0.15 B	3.06 $\pm$ 0.16 B
	56	5.98 $\pm$ 0.28	6.96 $\pm$ 0.16 A	3.32 $\pm$ 0.17 B	2.68 $\pm$ 0.11 B

^z DPS = days post-spraying. Within a field site and for each pathogen, differences between values marked with the same letter are not significantly different (analysis of variance with Tukey contrasts; $P > 0.05$); ns = no significant differences.

do inhabit rye roots. In particular, we have demonstrated that *F. graminearum*, *F. oxysporum*, *P. sylvaticum*, and *P. torulosum* isolated from rye roots are able to cause root disease on corn seedlings. We investigated only four of the many species of corn seedling pathogens yet were able to detect one or more of these species in most samples of rye roots collected from a variety of field locations. This indicates that the hosting of corn seedling pathogens in rye cover crop roots may be quite widespread in production fields.

In some cropping systems, cover crops seem to afford a beneficial rotation effect that includes reductions in disease severity in the subsequent crops, particularly if the cover crop residue is incorporated

(Himmelstein et al. 2014; Zhou and Everts 2007). In other cases, cover crops have been shown to impede the progress of disease epidemics through physical barriers to pathogen transport and other epidemiological mechanisms (Ristaino et al. 1997). However, insufficient attention has been given to the opposite possibility that cover crops may contribute to elevated disease pressure in subsequent crops because they are sometimes hosts to the same pathogens as the following cash crop or because they alter soil environmental conditions in ways that are favorable for disease progression. When shared pathogens are possible, management will need to explicitly account for increased pathogen loads following cover

Fig. 3. Densities of four corn seedling pathogens in roots of rye cover crops (mean $\pm$ standard error) in 2014. For each pathogen and within groupings of similar days post-spraying (DPS) of herbicide for cover crop termination, means marked with the same letter are not significantly different (analysis of variance with Tukey contrasts, $P > 0.05$); ns = no significant differences. X-axis labels refer to field sites. These data are a subset of those reported in Table 2, presented here to facilitate a different comparison.

cropping, particularly when environmental conditions are conducive for disease development.

A relationship in which a cover crop elevates disease pressure for the subsequent grain crop has been demonstrated previously for sorghum following crimson clover (Dabney et al. 1996). Ikley et al. (2015) recently reported on the potential for some cover crop species to host a foliar pathogen of corn, *Clavibacter michiganensis* subsp. *nebraskensis*. It has also been argued previously that one of the mechanisms by which fresh residues reduce weed stands is via stimulating pathogen activity, which affects weed emergence and survival (Mohler et al. 2012). However, shared pathogens or elevated potential for disease are not common topics in the current conversation about cover crops. We do not consider impacts on disease risk to be an insurmountable problem or a reason to discourage cover cropping. Rather, identifying the potential for shared pathogens should enable improved management practices that minimize disease risk, while maximizing the likelihood of beneficial outcomes from cover cropping.

A common management recommendation for this cropping system is that rye cover crops should be terminated 2 weeks prior to corn planting (Ball Coelho et al. 2005; Johnson 2014; Munawar et al. 1990; Raimbult et al. 1991; Singer et al. 2005). Our data from both field and controlled-environment settings indicate that, in some cases, pathogen population densities continue to increase for several weeks after rye cover crops are terminated with herbicides.

However, pathogen transfer probably requires close contact between corn seed or roots and infected rye roots. After corn is planted, it may take additional time (10 to 14 days) before corn seeds germinate and roots grow out to contact pathogen inoculum in dead rye roots. This interval would be even longer if crops were precisely planted, such that rye plants were positioned some distance from the future corn rows.

Pathogen density in rye cover crop roots can be viewed as a risk factor for corn seedling diseases that have the potential to adversely affect corn performance. However, actual yield losses to disease resulting from pathogen transfer from rye cover crops to corn seedlings will depend on complex interactions and environmental conditions at cover crop termination and later in the season. For instance, air temperature, soil temperature, and soil water content interact strongly with herbicide effectiveness, rates at which rye roots die and decompose, pathogen population growth and competitiveness, susceptibility of corn to pathogen attack, and rate and timing of corn germination and growth. Characteristics of the soil microbiome may also contribute to varying intensities of pathogen suppression (Bakker et al. 2012).

Under some circumstances, elevated rates of corn seedling infection may not lead to significant differences in corn yield. For instance, other factors later in the season, unrelated to the effect of seedling diseases, may impose larger constraints on yield. There are examples in the literature in which corn was planted within a short

Fig. 4. Density of four pathogens in radicles of corn seedlings grown in 2015, either following a winter fallow or following a rye cover crop. An asterisk (*) indicates a significant difference in pathogen density (*t* test, *P* < 0.05).

interval after rye cover crop termination, yet corn grain yield was not significantly affected (Eckert 1988; Kaspar and Bakker 2015). Such examples support the hypothesis that corn performance is most likely to be affected by rye cover crops only when conditions are conducive for disease development and contact of corn roots with dead rye roots in the soil is synchronized with elevated pathogen populations.

We observed significant field-to-field variation in pathogen load in roots of rye cover crops. Spatial variability both between and within fields is expected for soilborne microbial populations, because an organism's presence and population density among locations is subject to random or variable processes such as dispersal limitation, environmental conditions at the time of immigration, and local scale variation in soil edaphic characteristics and moisture regimes. This variability was well illustrated by LeBlanc and colleagues (2015), who found wide variation in the relative abundance of *F. oxysporum* within *Fusarium* communities across hundreds of meters of distance. It is not surprising, therefore, that we found differences in pathogen load and dynamics among samples of cereal rye roots collected from different fields.

There are management implications associated with spatial variability in pathogen load and conduciveness toward disease development. In some locations (i.e., fields or portions of fields), corn may not experience any negative effects from a preceding rye cover crop, because pathogen load is low or disease does not easily progress due to unsuitable environmental conditions or due to strong antagonistic interactions with other soil microorganisms (Kinkel et al. 2011). Corn performance issues or yield reductions following rye cover crops may be most probable or severe in locations with high background pathogen loads or that otherwise tend to be conducive toward disease. However, it is also possible that, under sufficiently high disease pressure, the presence of a rye cover crop may not increase disease risk or losses to disease. That is, if pathogen loads are already very high and environmental conditions are very conducive to infection, then damaging levels of disease may be experienced regardless of the presence of a rye cover crop.

Among locations, pathogen dynamics in rye roots were least pronounced at the Kelly field site, where none of the four target pathogens differed significantly in density with time after herbicide application (Table 2). This field site also had low pathogen loads compared with some other field sites (Fig. 3). Rye roots from the B54 field site experienced the most active pathogen population dynamics, with all four pathogens differing significantly in density in rye roots among herbicide termination timing treatments in at least one of the years sampled. Pathogen loads at the B54 site were also higher than for some other field sites (Fig. 3). Interestingly, the Kelly site has had a long history of cereal rye cover crops (>12 years), whereas plots sampled at the B54 site were all in their first season of cover crops. Other field sites with a long history of cover cropping included B42 and B44, both of which also tended to have low pathogen loads (Fig. 3). Future work should investigate the potential for repeated cover cropping over many years to drive microbial community changes that may limit pathogen establishment, growth, or reproduction.

There is a great deal of phenotypic variation within species of plant pathogens such as *F. oxysporum* and *P. sylvaticum*, and it is likely that not all of the genotypes detectable by qPCR would be equally aggressive in causing disease on corn seedlings; there is often variation in aggressiveness among isolates of a given pathogen species (Broders et al. 2007b; Matthiesen et al. 2016). Nevertheless, our results highlight the potential for dead and dying rye roots to host organisms that may cause root disease in subsequently planted corn seedlings. Additional isolate-based studies to characterize the virulence and aggressiveness of pathogen isolates collected from roots of cereal rye on corn seedlings would be valuable. Although rye roots become increasingly difficult to sample as decomposition proceeds, future work should also track pathogen population dynamics over longer time series, as well as

elucidating the fate of inoculum produced from dead rye roots, including its mobility in soil and the potential for transfer to roots of the subsequent crop.

It is notable that the pathogens we targeted are likely able to cause disease on soybean as well (Arias et al. 2013a,b; Broders et al. 2007a), yet soybean does not seem to suffer yield penalties following rye cover crops as frequently as corn does (Iowa Learning Farms and Practical Farmers of Iowa 2014). One explanation for this apparent discrepancy is that soybean crops are often planted later in the spring than corn, when warmer and drier soils may alleviate conduciveness toward disease. Another factor is that soybean plants are able to compensate quite readily for reductions in population, because of high initial seeding rates (approximately five times greater than corn) and through plastic phenotypic responses such as increased branching or number of pods (Pedersen and Licht 2014). Thus, there may be no negative yield impact even if pathogen transfer from cover crops does reduce the soybean population somewhat. In contrast, corn grain yield is more dependent on population size or number of ears (Nafziger 1994, 2002), making corn yields more sensitive to population reductions or increases in the number of barren plants stemming from early-season disease.

This work highlights the potential for winter cereal cover crops, and the root residue remaining after their termination, to host pathogens of corn. If we are to maximize the benefits of cover crops, cover crop management practices should minimize risks associated with elevated pathogen densities. For instance, basing recommendations for the interval between rye cover crop spraying and corn planting on soil temperature and thermal time rather than on calendar days may be more effective in reducing risk of corn seedling disease. Similarly, positioning rye cover crops in the interrow of the future corn rows would increase the time interval until corn roots intercept decomposing rye roots and become exposed to their resident pathogens. Finally, a priority for cover crop research should be the development of alternative cover crop species or winter cereal cultivars that overwinter in the upper Midwest and provide the desirable benefits to soil and water quality while sharing fewer pathogens with corn, thus allowing for a strong rotation effect.

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