Population Dynamics Between *Fusarium pseudograminearum* and *Bipolaris sorokiniana* in Wheat Stems Using Real-Time qPCR

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Abstract

Moya-Elizondo, E. A., Jacobsen, B. J., Hogg, A. C., and Dyer, A. T. 2011. Population dynamics between *Fusarium pseudograminearum* and *Bipolaris sorokiniana* in wheat stems using real-time qPCR. Plant Dis. 95:1089-1098.

Fusarium pseudograminearum and *Bipolaris sorokiniana* are causal agents of Fusarium crown rot and common root rot, respectively, of wheat and cause significant losses worldwide. Understanding the population dynamics between these two pathogens at late stages of wheat development is needed. The effect of *F. pseudograminearum* and *B. sorokiniana* inocula applied singly or in mixtures at seeding to spring wheat 'Hank' was measured using seedling stand, grain yield, and pathogen populations in the first internode at heading, milk, and harvest stage of wheat development using real-time quantitative polymerase chain reaction. High and low rates of *F. pseudograminearum* inoculum reduced *B. sorokiniana* populations in field trials but *B.*

sorokiniana inoculations did not affect *F. pseudograminearum* populations. Populations of both pathogens increased from heading until harvest, with *F. pseudograminearum* colonizing lower internodes earlier than *B. sorokiniana*. Neither pathogen prevented infection by the other in the first internode of wheat stems. Inoculations increased incidence of infection and co-infection relative to natural settings observed for both pathogens. At the seedling stage, both fungi, individually or combined, reduced the seedling stands when compared with a non-inoculated control for the three location-years. Grain yield and *F. pseudograminearum* populations were inversely correlated, while *B. sorokiniana* populations were not correlated with yield.

Fusarium crown rot (FCR) and common root rot (CRR) of wheat and other cereals are endemic disease problems that cause significant losses in the Northern Great Plains of North America and other semiarid regions of the world (2,3,35,41,42,44,48,50). In Montana and the Pacific Northwest, FCR of small grains is primarily caused by *Fusarium culmorum* (W. G. Sm.) Sacc. and *F. pseudograminearum* (O'Donnell & T. Aoki; group I) (= *Gibberella coronicola*) (28,30,31,42). In other regions, *F. graminearum*, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh., *F. equiseti* (Corda) Sacc., and *Microdochium nivale* (Fr.) Samuels & I. C. Hallett (= *F. nivale* (Fr.) Sorauer) have been associated with crown rot of cereals (2,10,14,33,43,48). *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur) is the causal agent of CRR and, with FCR, is part of a complex causing dryland seedling, foot, crown, and root rot in cereals crops across the globe (7,9,10,13,14,20,22,31,43,44,50). *Fusarium* spp. noted above and *B. sorokiniana* affect wheat and other cereals at every stage of development from seedling through grain fill (20,31).

Among the *Fusarium* spp. causing crown rot in Montana, *F. culmorum* causes the greatest losses to seedling blight while *F. pseudograminearum* and *F. graminearum* cause more severe late season disease (8). The adoption of conservation tillage practices in wheat-fallow production systems has led to an increase of *F. pseudograminearum* populations in recent years (31,38). *F. pseudograminearum* is strictly a residue-born pathogen that depends on infested late-season tillers for survival between cropping years (32,39). It has been hypothesized that this places strong selection pressure on the pathogen to capture durable plant residues in order to survive the prolonged non-cropping period (crop/fallow system) of Montana (8). In general, *B. sorokiniana* is favored by conventional

tillage in barley (13) and wheat production systems (7,12,26,34,47). However, in one study, researchers found higher infection rates where stubble was retained versus where it was removed (51).

Recent surveys in Montana and the Canadian Prairies have shown a negative correlation between populations of FCR and CRR pathogens (10,28). Given the widespread geographical range and economic importance of FCR and CRR in cereal cropping systems, it is important to understand the potential for antagonism between the causal pathogens because this may have practical control implications. Dual-inoculation studies between FCR and CRR pathogens have been conducted since the 1930s (21) to address potential interaction between these pathogens. Ledingham's work (21) determined an antagonistic effect on seedling emergence of wheat plants simultaneously inoculated with *F. culmorum* and *B. sorokiniana*. Tinline (46) reported that colonization of the internode by *B. sorokiniana* does not prevent subsequent invasion by fusaria; however, prepossession of the internode by fusaria greatly reduces subsequent infection by *B. sorokiniana* in studies of single or combination inoculation of wheat with *B. sorokiniana*, *F. culmorum*, and *F. acuminatum*. Scardaci and Webster (36) identified antagonism between *F. graminearum* and *B. sorokiniana* when they were co-inoculated in barley, resulting in lower levels of seedling blight and root rot. These authors also determined that, when the pathogens were inoculated in sequence, one 21 days before the other, the pathogen inoculated first was reisolated most frequently, indicating the importance of prior colonization and possession of substrate. Fernandez et al. (11) showed that *F. acuminatum* inoculation significantly augmented the effect of *B. sorokiniana* infection when wheat seedling plants were inoculated with both fungi. Although these studies have demonstrated potential antagonism between these pathogens, it is important to understand that they were carried out using autoclaved soil to avoid masking the interaction between the cereal root rot pathogens by other microflora. This does not simulate the environment confronted by these two pathogens in a field situation. In the one study conducted under field conditions, co-inoculations with *F. culmorum* and *B. sorokiniana* did not affect wheat emergence or yield (21) but no data were collected on relative population dynamics or their impact on late-season crown and root rot was not reported.

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Accepted for publication 2 April 2011.

Assessing pathogen population dynamics in wheat tissues has historically been done using isolation and culturing of targeted pathogen species. This method is labor intensive and complicated by many factors such as competition and antagonism from other organisms in plant tissue, needed expertise in identifying species, and media selectivity that may favor isolation of certain fungi over others (16). Given these numerous limitations, Pettitt et al. (33) suggested that pathogen–pathogen interactions within wheat stems would be best assessed by using real-time quantitative polymerase chain reactions (qPCRs). These qPCR methods have already been proven invaluable in other systems where assessments of competition and interactions among ectomycorrhizal fungi and among alfalfa root pathogens have been successfully conducted (17,49). Recently, a number of qPCR assays have been developed to quantify FCR and CRR pathogen populations but have yet to be applied to assess interactions among the species (15,16,28,45). Because both FCR and CRR incidence and severity are increasing under conservation tillage systems, and because negative associations between the causal pathogens have been documented, it is important to assess the potential interaction between these pathogens, particularly at later stages of wheat development under field conditions. To address this need, this study examined the disease and the pathogen community dynamics between aggressive pathogenic isolates of *F. pseudograminearum* and *B. sorokiniana* within inoculated wheat plants at seedling, heading, milk, and harvest stages of wheat development under field conditions using qPCR. These results were then compared with observations made on individual tillers collected at harvest from inoculated spring wheat plots and from naturally infested commercial spring and winter wheat fields.

Materials and Methods

Pathogen isolates and wheat cultivar. Field trials were conducted to assess the population interactions between *F. pseudograminearum* isolate 2228 (Fpg2228) and *B. sorokiniana* isolate 2344 (Bs2344) in the hard red spring wheat (*Triticum aestivum* L.) ‘Hank’ (WestBred, Bozeman, MT). Isolate Fpg2228, collected in Loma, MT (Liberty County), was chosen for the *Fusarium* inoculations because it is an aggressive isolate that reduced yield on wheat (8). Isolate Bs2344, collected in Carter, MT (Chouteau County), was chosen for *B. sorokiniana* inoculations for the same reason as described for Fpg2228 (A. T. Dyer, unpublished data). For both fungi, oat kernel inoculum was produced as described by Mathre and Johnston (27).

Field trials with *F. pseudograminearum* and *B. sorokiniana*. During the summer of 2008 and 2009, three experiments were conducted to assess the population dynamics between *F. pseudograminearum* and *B. sorokiniana* within spring wheat during seedling (Feekes growth stage 1), heading (Feekes growth stages 10.1 to 10.5), milk (Feekes growth stage 10.54), and harvest (Feekes growth stage 11) stages. Trials were conducted at the Montana State University (MSU) Arthur H. Post Research Farm (Bozeman, MT) in 2008 and 2009, and at the MSU Central Agricultural Research Center (Moccasin, MT) in 2008. Soil at the Bozeman and Moccasin sites are Amsterdam silt loam and Judith clay loam, respectively.

The experimental unit was a plot eight rows wide with 30-cm centers seeded at 200 seeds per 3-m row. Plots were sown on 5 May 2008 and 18 May 2009 in Bozeman and 28 April 2008 in Moccasin. Plots were inoculated in-furrow at planting with Fpg2228 and Bs2344 whole-oat-kernel inoculum using three inoculation rates forming a three-by-three factorial design with six replications for a total of 54 plots. In-furrow inoculation rates were 0 g, 1 g (low), and 3 g (high) of colonized oat-kernel inoculum per meter of row for both fungi. Field sites were top dress fertilized before seeding with nitrogen at rates of 67.2 kg/ha in Moccasin and 51.6 kg/ha in Bozeman during 2008, and 78.6 kg/ha in Bozeman in 2009. Weeds were controlled at both locations and years by hand hoeing.

Plant emergence was assessed at the two-leaf growth stage (Feekes growth stage 1) between 22 to 25 days post planting.

Emerged seedlings were counted for rows 2, 4, and 6 on 27 May 2008 and 2 June 2009 in Bozeman and 20 May 2008 in Moccasin. Thirty tillers were randomly chosen from each plot at the heading stage (Feekes growth stages 10.1 to 10.5), milk stage (Feekes growth stage 10.54), and harvest stage (Feekes growth stage 11) for DNA extraction and qPCR analysis. During heading and milk stage sampling times, stems were collected from the second and seventh rows, while rows three through six were left for yield analysis. Stem collection at the heading stage was done on 11 July 2008 and 16 July 2009 in Bozeman and on 8 July 2008 at Moccasin. Milk stage stem collection was conducted on 18 July 2008 and 6 August 2009 in Bozeman and 21 July 2008 in Moccasin. Stems for harvest stage analyses were collected from the middle four rows of the plots after harvest on 27 August 2008 and 16 September 2009 in Bozeman. In Moccasin, stems were collected on 22 August 2008.

The middle four rows of each plot in Moccasin were harvested with a Wintersteiger plot combine (Wintersteiger USA Inc., Salt Lake City, UT), while the four middle rows of each plot in the Bozeman experiments were harvested with a small-bundle rice binder (Mitsubishi Agricultural Machinery Co., Ltd., Tokyo) and threshed using a Vogel thresher (Bill’s Welding, Pullman, WA).

DNA isolation. To isolate pathogen DNA within wheat tillers, the leaf tissue was removed from the 30 collected tillers and 3-mm stems sections were cut from the base of the first internode of each tiller. These tissue samples were then processed following protocols previously described by Hogg et al. (15) using FastDNA kits (QBiogene Inc., Irvine, CA). The resulting DNA samples were diluted 1:10 with molecular-grade water for qPCR analysis.

Quantification of pathogens from first internode infections.

Real-time qPCR quantification of *B. sorokiniana* and *F. pseudograminearum* populations within plant tissues used probes dual-labeled with 6-carboxyfluorescein fluorescent reporter dye and 6-carboxytetramethylrhodamine fluorescence quencher. The primer–probe combination used to quantify *F. pseudograminearum* populations was previously described (15,45). For *B. sorokiniana*, primers and probe were designed using reported sequences of glyceraldehyde-3-phosphate dehydrogenase-like (*gpd*) gene sequence for isolate ND93-1, GenBank accession EF513209.1, as described by Moya et al. (28). All qPCR assays were performed using the Rotor-Gene Q (QBiogene Inc.) and a two-step protocol with the following thermocycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The 25- μ l reaction for qPCR contained 12.5 μ l of Universal TaqMan Master Mix (Applied Biosystems, Carlsbad, CA), 2.5 μ l of 2 μ M TaqMan probe, 2.25 μ l of a 1:1 mixture of 20 μ M forward and reverse primers (Integrated DNA Technologies, Inc. Coralville, IA), 2.75 μ l of molecular-grade water, and 5 μ l of DNA sample. Each run included four 10-fold-dilution standards, ranging from 6.6 million to 6,600 copies. For *F. pseudograminearum* and *B. sorokiniana*, the DNA standards used for absolute quantification during qPCR were cloned fragments of the *tri5* and *gpd* genes taken from *F. culmorum* isolate 2223 (15) and *B. sorokiniana* isolate 2234, respectively. The process of quantification followed the protocol as described by Moya et al. (28). As an internal control, all DNA samples were spiked with 335 pg of plasmid DNA containing the β -actin gene from *Meloidogyne javanica* (29). qPCR reactions for this internal control were run for all DNA samples to determine any inhibition of qPCR reactions. No inhibition of qPCR reactions was detected using this internal control. In this article, DNA copy numbers of the *tri5* gene was considered as the quantified population of *Fusarium* spp. of the FCR complex (*F. culmorum*, *F. pseudograminearum*, and *F. graminearum*), while the amount of DNA copy numbers of the *gpd* gene was considered the quantified population of *B. sorokiniana*.

Evaluation of *Fusarium* spp. and *B. sorokiniana* infections in the first internode of individual wheat tillers. DNA quantification of *Fusarium* spp. and *B. sorokiniana* populations in the first internode of individual tillers was conducted to assess the dynamics between the two fungal species in experimentally inoculated

wheat tillers and tillers collected from naturally infected fields. In 2008, 20 symptomatic tillers were obtained from the spring wheat plots inoculated with FCR and CRR pathogens at Bozeman, MT, a naturally infested spring wheat field in Shonkin, MT, and a winter wheat field in Carter, MT naturally infected with both diseases. During 2009, 20 tillers showing symptoms of the disease were obtained from the inoculated spring wheat plots in Bozeman, MT, naturally infested spring wheat in Cut Bank, MT, and naturally infested winter wheat in Malta, MT. Tillers from the spring wheat field trials were chosen after harvest from the plots inoculated with the high rate of both pathogens (3 g of inoculum per meter of row of each fungus). Tillers from the naturally infested commercial wheat fields were obtained a week before harvest. From each stem, a 3-mm section from the base of the first internode was cut, and the DNA was extracted and then quantified for populations of *Fusarium* spp. and *B. sorokiniana* following the method described above.

Statistical analyses. Multifactor analyses of variance (ANOVAs) considering locations (Moccasin 2008 and Bozeman 2008 and 2009) and inoculation treatments (3 × 3 possible inoculum combinations of Fpg2228 and Bs2344) and interaction between these two factors were conducted for plant emergence, DNA copy numbers of each assessed gene quantified for each date of assessment (heading, milk, and harvest stage), and grain yield. If the factor location was significant ($P < 0.05$) for a variable response, this variable was analyzed separately through a randomized complete block design analysis for each field trial. Multifactor ANOVAs and randomized complete block design analysis were performed by the procedure PROC GLM of the Statistical Analysis System (version 9.2; SAS Institute Inc., Cary, NC) and Fisher's protected least significant difference (LSD) was used to compare means ($P < 0.05$). Prior to analysis, DNA copy numbers quantified through qPCR were $\log(x + 1)$ transformed and their variances were analyzed for homogeneity (Levene test, $P > 0.05$). After the multifactor ANOVAs were conducted, an analysis of covariance was performed on DNA copy number quantified through qPCR in each wheat growth stage by using PROC MIXED with the REPEATED statement for repeated measures (SAS Institute, Inc.), where several covariance matrices were evaluated and the independent structure of covariance between each date of assessment was chosen because it had the lowest Akaike information criterion (AIC) score with the fewest numbers of covariance parameters. This implied that DNA copy numbers quantified at each different stage were independent from one time of assessment to the next. This situation allowed using an univariate analysis split plot in time to assess competition between both pathogen populations in wheat tillers, where the independent variables were different inoculum concentrations of each fungus (none, low, and high), fungal population (*Fusarium* populations and *B. sorokiniana* population), and date of assessment (heading, milk stage, and harvest). A univariate analysis split plot in time for the randomized complete block design was performed by the procedure PROC GLM and an LSD test was used to compare means ($P < 0.05$). Relationships between grain yield and level of infection in each disease assessment date (seedling counts, heading, milk stage, and harvest) were evaluated through correlation and regression analyses by using PROC COR and PROC REG (SAS Institute, Inc.), respectively.

Evaluation of the *Fusarium* spp. and *B. sorokiniana* populations in individual wheat tillers was conducted on the populations obtained from the 40 tillers collected from naturally infected wheat fields and co-inoculated plot fields during the 2 years of research. Populations of each pathogen observed in wheat tillers during each year were grouped to meet requirements of the statistical test. Number of tillers infected by FCR and CRR pathogens, co-infected tillers, and tillers not infected by the pathogens in each group was determined. Comparisons of observed and expected proportions of each described category of infection among fields were conducted through χ^2 tests of homogeneity ($P < 0.05$) and expected proportion of each category within each field was conducted through a χ^2 goodness-of-fit test ($P < 0.05$). Wilcoxon signed rank tests with

continuity correction were used to compare populations of both pathogens as quantified by qPCR combined across the 2 years for the tillers collected from naturally infected wheat fields and co-inoculated plots. The package "Rcmdr" of the R software (www.r-project.org) was used to conduct these nonparametric analyses.

Dependency between *Fusarium* spp. and *B. sorokiniana* populations quantified by qPCR at harvest for plots from each location and for the individual tillers collected from the inoculated and naturally infected fields was determined through correlation analyses using PROC COR (SAS Institute Inc.). Spearman rank correlations were considered to avoid distribution effects of analyzed variables.

Results

Seedling counts were significantly affected by the inoculations of both fungi at the three trial locations (Table 1; $P < 0.001$), and a significant interaction between inoculation treatment combinations and location was observed ($P < 0.001$). Across locations, *F. pseudograminearum*, *B. sorokiniana*, and combined *F. pseudograminearum* and *B. sorokiniana* inoculations reduced seedling counts by 19, 12, and 27%, respectively. In general, use of inoculum of both Fpg2228 and Bs2344 alone or combined reduced seedling counts for Moccasin by 31%, for Bozeman 2008 by 15%, and for Bozeman 2009 by 20% when compared with the noninoculated plots. Locations showed different numbers of emerged seedling among trials ($P < 0.001$), with the field trial at Bozeman 2008 showing 17 and 36% more emerged seedlings than at the Bozeman 2009 and Moccasin 2008 field trials, respectively. At the Bozeman 2008 and Moccasin trials, the treatment inoculated with high rates of both pathogens had the greatest reduction in seedling counts (25 and 57%, respectively) but this treatment was not significantly different from the combination of low rates of Fpg2228 and high rates of Bs2344 in the Bozeman 2008 trial. In the Bozeman 2009 field trial, the treatments with high rates of Fpg2228 alone or mixed with high or low rates of Bs2344 caused the greatest reductions in seedling counts (33, 30, and 27% reduction, respectively). All field trials showed that treatments inoculated with high rates of Fpg2228 and Bs2344 alone or combined with other rates of both pathogens' inoculum were significantly different than the non-inoculated control ($P < 0.001$).

Table 1. Number of emerged wheat seedlings in 3 m of row at the two-leaf growth stage for different inoculation treatment combinations of *Fusarium pseudograminearum* isolate 2228 (Fpg) and *Bipolaris sorokiniana* isolate 2344 (Bs) observed in field trials conducted at Moccasin, MT 2008 and Bozeman, MT 2008 and 2009.

Treatments ^y	Emerging seedlings in 3 m of row ^x		
	Moccasin 2008	Bozeman 2008	Bozeman 2009
High Fpg	81 d	150 cd	103 e
Low Fpg	114 b	161 b	135 ab
High Fpg–Low Bs	75 d	149 cd	112 cde
Low Fpg–High Bs	81 d	135 ef	123 bcd
High Fpg–High Bs	57 e	131 f	107 de
Low Fpg–Low Bs	95 c	156 bc	130 bc
High Bs	113 b	143 de	133 b
Low Bs	126 a	159 b	137 ab
Noninoculated	134 a	174 a	153 a
Overall mean ^z	97 C	151 A	126 B
$P > F$	<0.001	<0.001	<0.001
LSD	9	12	8

^x Means within a column followed by the same letter were not significantly different according to Fisher's protected least significant difference (LSD, $\alpha = 0.05$). $P > F$ was the probability associated with the F value.

^y Inoculation treatments were 3 g (high), 1 g (low), or 0 g of infested oat seed per meter of row applied in-furrow during seeding.

^z P values obtained from multifactorial analysis of variance assessed location and the interaction between treatment and location had a probability associated with the F value of < 0.001 . Data indicate overall average for each field trial. Means within a row followed by the same letter were not significantly different according to Fisher's protected least significant difference (LSD, $\alpha = 0.05$).

Populations of *Fusarium* spp. and *B. sorokiniana* as expressed by DNA copy numbers of the *tri5* and *gpd* genes, respectively, in the three different late stages of plant growth (heading [Feekes growth stages 10.1 to 10.5], milk [Feekes growth stage 10.54], and harvest [Feekes growth stage 11]) were significantly different ($P < 0.001$). Populations were also significantly different across locations ($P < 0.001$). Therefore, results for each field trial and plant

stage were analyzed individually to fully observe the dynamics involved. The analysis of covariance conducted individually on fungal populations for each field trial showed that the structure of covariance between each date of assessment for all field trials had an independent structure (the lowest AIC). This implied that DNA copy numbers of *Fusarium* spp. and *B. sorokiniana* quantified at each different stage were independent from one time of assessment

Fig. 1. Overall mean copy number for populations of *Fusarium* spp. and *Bipolaris sorokiniana* determined across three growth stages through real-time quantitative polymerase chain reaction of the *tri5* and *gpd* genes, respectively, for no-inoculum, low-inoculum, and high-inoculum rates (0, 1, and 3 g, respectively, of whole-oat inoculum per meter of row) of *F. pseudograminearum* isolate 2228 (Fpg) in field trials conducted in **A**, Moccasin, MT in 2008 and Bozeman, MT in **B**, 2008 and **C**, 2009. Means followed by the same letter on bars of the same color were not significantly different according to Fisher's protected least significant difference ($\alpha = 0.05$). Bars on the top of each column represent the standard error.

Fig. 2. Overall mean copy number for populations of *Fusarium* spp. and *Bipolaris sorokiniana* determined across three growth stages through real-time quantitative polymerase chain reaction of the *tri5* and *gpd* genes, respectively, for no-inoculum, low-inoculum, and high-inoculum rates (0, 1, and 3 g, respectively, of whole-oat inoculum per meter of row) of *B. sorokiniana* isolate 2344 (Bs) in field trials conducted in **A**, Moccasin, MT in 2008 and Bozeman, MT in **B**, 2008 and **C**, 2009. Means followed by the same letter on bars of the same color were not significantly different according to Fisher's protected least significant difference ($\alpha = 0.05$). Bars on the top of each column represent the standard error.

to the next; then, this situation allowed the use of a univariate analysis split plot in time to assess competition between both pathogen populations in wheat tillers (Figs. 1–3). For Moccasin, low and high rates of Fpg2228 inoculum had significantly increased *Fusarium* spp. populations relative to the noninoculated plots ($P < 0.001$; Fig. 1A). Populations of *Fusarium* spp. were not affected by either rate of Bs2234 inoculum (Fig. 2A). The high rate of Fpg2228 inoculum resulted in a reduced population of *B. sorokiniana* in the first internode of tillers ($P < 0.001$; Fig. 1A). The high and low rates of *B. sorokiniana* populations were not different from the noninoculated plots (Fig. 2A). The populations of *F. pseudograminearum* and *B. sorokiniana* at the Moccasin location were different at the three late stages of wheat development ($P < 0.001$; Fig. 2), with the lowest level of *Fusarium* spp. populations observed during heading (125,346 DNA copies of the *tri5* gene), and highest level of this pathogen observed at harvest (3,168,844 DNA copies of the *tri5* gene) (Fig. 3; Table 2). *B. sorokiniana* populations were similar during heading (5,889 DNA copies of the *gpd* gene) and milk stage (11,012 DNA copies of the *gpd* gene) but increased markedly at harvest (192,642 DNA copies of the *gpd* gene). Also, significant reductions of *B. sorokiniana* populations were observed in plots single inoculated with Fpg2228 measured at the milk and harvest stages, when means comparison among individual inoculum treatments were analyzed (Table 2).

Pathogen populations in the 2008 field trial at Bozeman showed patterns similar to those described for Moccasin. Plots inoculated with Fpg2228 increased the population of the pathogen relative to the noninoculated plots, and this increase was associated with reduction of *B. sorokiniana* populations (Fig. 1B). Populations of *B. sorokiniana* in noninoculated plots were not different from the high

and low inoculated rates of Bs2344 ($P = 0.104$) and populations of *F. pseudograminearum* were not affected by the presence of *B. sorokiniana* inoculum (Fig. 2B). Comparison among wheat development stages showed the same level of *F. pseudograminearum* infection between heading and milk stages and significantly higher populations at harvest (Fig. 3; Table 2). *B. sorokiniana* had low populations during heading stage, as observed in Moccasin, but high populations occurred during milk stage. The effects of Fpg2228 inoculum on *F. pseudograminearum* populations were most dramatic in Bozeman 2008 plots and were much more modest in Moccasin and at Bozeman in 2009. For example, populations of *F. pseudograminearum* in Bozeman 2008 were, on average, 15 times greater as indicated by DNA copies of *tri5* gene than at Moccasin at heading (Table 2). Plots inoculated only with high rates of Fpg2238 showed a clear reduction of *B. sorokiniana* population during heading and harvest, whereas this phenomenon was observed only at milk and later harvest stages in Moccasin.

Although the population dynamics displayed in Bozeman in 2009 were less dramatic than in the other two trials, they followed similar patterns. *F. pseudograminearum* inoculations increased infestation with *F. pseudograminearum* populations and those populations reduced the *B. sorokiniana* populations (Fig. 1C). *B. sorokiniana* inoculations did not increase its populations significantly and did not increase or reduce *F. pseudograminearum* populations (Fig. 2C). For 2009 in general, *F. pseudograminearum* and *B. sorokiniana* populations were moderate, never attaining the levels observed in the 2008 trial (Table 2). Populations of *F. pseudograminearum* were lower at heading and similar at milk stage and harvest stage, whereas *B. sorokiniana* populations increased from heading to harvest (Fig. 3). Significant differences among

Fig. 3. Mean copy number for populations of *Fusarium* spp. and *Bipolaris sorokiniana* determined through real-time quantitative polymerase chain reaction of the *tri5* (for *Fusarium* spp.) and *gpd* (for *B. sorokiniana*) genes for three wheat growth stages in Moccasin, MT 2008 and Bozeman, MT 2008 and 2009 field trials inoculated with *F. pseudograminearum* isolate 2228 and *B. sorokiniana* isolate 2344. For each species–location value within an inoculum type, means followed by the same letter were not significantly different according to Fisher's protected least significant difference ($\alpha = 0.05$). Bars on the top of each column represent standard error.

inoculated treatments and the noninoculated control were not observed for the Bozeman 2009 site (Table 2).

F. pseudograminearum colonized wheat tillers earlier than *B. sorokiniana*. Progression of the fungal populations in the first internode in the field trial showed that, at heading, *F. pseudograminearum* had attained a population that was, on average, 37% of the final population observed at harvest; meanwhile, *B. sorokiniana* populations at heading averaged only 5% of the final population observed at harvest. For *F. pseudograminearum*, populations at heading were 4, 65, and 105% of the final population observed at harvest in the field trials in Moccasin and Bozeman 2008 and 2009, whereas *B. sorokiniana* populations were 3, 8, and 4%, respectively.

Grain yield over the three location-years showed significant differences for the different inoculum treatment combinations used for both fungi ($P < 0.001$). Average grain yields were 1,163 kg/ha in Moccasin, 1,843 kg/ha in Bozeman 2008, and 3,346 kg/ha in Bozeman 2009. For the Bozeman 2008 field trial, plots inoculated with *F. pseudograminearum* had significantly lower grain yields and those yields were significantly different from both the noninoculated control and the treatments inoculated only with *B. sorokiniana* ($P < 0.001$). Inoculation with high or low rates of *B. sorokiniana* inoculum alone did not reduce grain yield compared

with the noninoculated plots. These patterns were not observed in Moccasin and Bozeman 2009 field trials, where grain yields were not significantly different among the inoculum treatments. During 2008, the field plots in Bozeman were affected by an unusual hail-storm during grain fill (early milk stage, Feekes growth stage 10.54).

The relationships between seedling establishment and grain yield was only significant for the Bozeman 2009 site ($r = 0.385$; $P = 0.004$), while fungal population–grain yield relationships were only negatively correlated and significant for *F. pseudograminearum* populations in the Bozeman 2008 and the Moccasin trials ($r = -0.624$, $P = <0.001$ and $r = -0.384$, $P = 0.004$, respectively). *F. pseudograminearum* populations and grain yield were not significantly correlated for the Bozeman 2009 field trial. *B. sorokiniana* populations did not affect yield in the three field trials (Moccasin, $P = 0.738$; Bozeman 2008, $P = 0.382$; and Bozeman 2009, $P = 0.544$). In 2008, spring weather was cool and wet and was followed by a relatively warm and dry summer in Bozeman compared with the long-term averages registered in that location. Precipitation was 84, 68, 33, and 18 mm and average temperatures were 13, 20, 27, and 26°C for May, June, July, and August, respectively. Weather conditions in Moccasin were cooler compared with Bozeman and were wet at the beginning of the trial and relatively drier

Table 2. Average gene copy number ($\times 1,000$) for populations of *Fusarium* spp. and *Bipolaris sorokiniana* observed within different inoculation treatment combinations of *Fusarium pseudograminearum* isolate 2228 (Fpg) and *B. sorokiniana* isolate 2344 (Bs) at three growth stages in field trials conducted at Moccasin, MT 2008 and Bozeman, MT 2008 and 2009 as determined through real-time quantitative polymerase chain reaction^a

Treatments ^y	Heading (Feekes growth stages 10.1-10.5)		Milk stage (Feekes growth stage 10.54)		Harvest (Feekes growth stage 11.0)	
	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>
Moccasin 2008						
High Fpg	181 ab	5.2 ab	363 ab	0.2 c	3,665 a	71.2 d
Low Fpg	201 a	11.0 ab	1,210 ab	13.0 ab	3,590 a	106.6 cd
High Fpg-Low Bs	180 ab	0.6 bc	1,118 ab	8.5 ab	3,611 ab	71.5 cd
Low Fpg-High Bs	133 a	1.7 bc	1,113 ab	14.7 ab	2,965 ab	139.1 bc
High Fpg-High Bs	147 ab	4.5 c	690 ab	5.7 b	4,217 ab	72.0 cd
Low Fpg-Low Bs	97 ab	2.8 ab	2,036 a	1.8 ab	3,382 ab	127.4 bc
High Bs	60 ab	14.5 a	716 b	29.5 ab	2,708 ab	412.6 a
Low Bs	96 ab	9.6 ab	421 ab	12.2 a	1,936 b	338.5 abc
Noninoculated	33 c	3.1 ab	304 ab	13.5 ab	2,445 ab	395.0 ab
Overall mean	125	5.9	885	11.0	3,169	192.6
$P > F^z$	0.026	0.026	0.376	0.0023	0.364	0.0011
Bozeman 2008						
High Fpg	1,685 a	1.4 b	1,848 a	30.2	4,557 a	18.6 d
Low Fpg	1,977 a	3.8 ab	1,521 a	30.5	2,462 a	41.6 bc
High Fpg-Low Bs	4,032 a	2.0 ab	2,024 a	73.4	3,538 a	53.0 cd
Low Fpg-High Bs	1,582 a	10.2 ab	828 a	51.9	3,845 a	62.2 bc
High Fpg-High Bs	2,922 a	4.5 ab	2,923 a	39.5	3,891 a	57.8 bc
Low Fpg-Low Bs	4,412 a	2.1 a	2,770 a	109.6	6,669 a	75.4 bc
High Bs	95 b	15.1 a	17 b	64.8	309 b	172.5 a
Low Bs	31 b	12.3 a	43 b	95.6	489 b	172.5 a
Noninoculated	113 b	9.8 a	31 b	97.4	220 b	110.4 ab
Overall mean	1,872	6.8	1,334	65.9	2,887	84.9
$P > F^z$	<0.0001	0.0261	<0.0001	0.2296	<0.0001	<0.0001
Bozeman 2009						
High Fpg	560	0.7	205	8.4	384	19.7
Low Fpg	383	0.1	286	6.3	249	12.5
High Fpg-Low Bs	501	0.1	282	6.9	431	24.6
Low Fpg-High Bs	355	1.9	241	8.1	336	21.6
High Fpg-High Bs	374	0.8	270	6.8	414	18.1
Low Fpg-Low Bs	272	1.0	205	7.7	331	18.0
High Bs	335	1.0	144	15.1	330	22.3
Low Bs	133	0.8	221	6.8	320	27.4
Noninoculated	248	1.0	188	8.2	203	25.0
Overall mean	351	0.8	227	6.2	333	21.0
$P > F^z$	0.1249	0.5008	0.5832	0.6641	0.1698	0.0988

^a *Fusarium* spp. = populations of *F. culmorum*, *F. pseudograminearum*, or *F. graminearum* that were quantified based on the amount of DNA copy number of the *tri5* gene. Populations of *B. sorokiniana* were quantified based on the amount of DNA copy numbers of the *gpd* gene. For both populations, Mean copy number in table should be multiplied by 1,000 for actual number. Means followed by the same letter in each column were not significantly different according to Fisher's protected least significant difference ($\alpha = 0.05$). Mean separations were performed on copy numbers $\log(x + 1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

^y Inoculation treatments were 3 g (high), 1 g (low), or 0 g of infested oat seed per meter of row applied in-furrow during seeding.

^z $P > F$ was the probability value calculated for copy numbers $\log(x + 1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

during summer months. Precipitation was 110, 75, 11, and 23 mm and average temperatures were 10, 14, 19, and 19°C for May, June, July, and August, respectively. In comparison with 2008, weather in Bozeman in 2009 was warmer and drier in May and was followed by a relatively wetter and cooler summer. Precipitation was 41, 67, 71, and 38 mm and average temperatures were 17, 18, 24, and 24°C for May, June, July, and August, respectively.

Evaluation of *Fusarium* spp. and *B. sorokiniana* infections in the first internode of individual wheat tillers. High co-infection of the individual lower internodes by *Fusarium* spp. and *B. sorokiniana* was observed in co-inoculated tillers collected in 2008 and 2009 from the experimental sites in Bozeman compared with individual tillers collected from naturally infested fields (Fig. 4). The proportion of lower internodes single infected, co-infected, and noninfected by *F. pseudograminearum* and *B. sorokiniana* as detected by qPCR were similar for the naturally infested spring and winter wheat ($\chi^2 = 2.177$, $P = 0.537$). When naturally infested lower internodes of spring or winter wheat were compared with the inoculated spring wheat, the χ^2 showed a significant effect of inoculation (spring wheat versus co-inoculated, $\chi^2 = 17.135$, $P < 0.001$; winter wheat versus co-inoculated, $\chi^2 = 26.876$, $P < 0.001$). In the inoculated plots at Bozeman, *F. pseudograminearum* was detected in 95% of the lower internodes examined compared with naturally infested fields, where infections were detected in 60% of spring wheat and 45% of winter wheat lower internodes. For *B. sorokiniana*, the inoculated plots at Bozeman showed that this fungus was identified from 75% of the lower internodes compared with 53% of spring wheat and 55% of winter wheat lower internodes from naturally infested fields. The Wilcoxon signed-rank test with continuity correction showed that populations of *Fusarium* spp. as expressed by DNA copy numbers of the *tri5* gene observed in individual lower internodes for tillers collected from the co-inoculated plots in Bozeman (mean = 188,210 DNA copy

numbers of the *tri 5* gene, standard deviation [SD] = 235,124, range = 0 to 909,154) were not different from the FCR populations observed in naturally infested tillers collected from spring wheat fields ($V = 279$, $P = 0.123$) but different from those collected from winter wheat fields ($V = 85$, $P < 0.001$), whereas the spring wheat tillers showed higher FCR populations than the winter wheat tillers ($V = 357$, $P = 0.011$ [spring wheat fields: mean = 108,575 DNA copy numbers of the *tri 5* gene, SD = 165,185, range = 0 to 729,407; winter wheat fields: mean = 24,121 DNA copy numbers of the *tri 5* gene, SD = 62,779, range = 0 to 285,228]). Populations of *B. sorokiniana* as expressed by DNA copy numbers of the *gpd* gene were not different between spring and winter wheat naturally infested fields ($V = 229$, $P = 0.717$ [spring wheat fields: mean = 518 DNA copy numbers of the *gpd* gene, SD = 1,663, range = 0 to 8,625; winter wheat fields: mean = 404 DNA copy numbers of the *gpd* gene, SD = 1,096, range = 0 to 6,294]), while populations of *B. sorokiniana* in the Bozeman experimental sites (mean = 6,562 DNA copy numbers of the *gpd* gene, SD = 13,492, range = 0 to 25,868) were different from the *B. sorokiniana* populations quantified for spring wheat ($V = 81$, $P < 0.001$) and winter wheat fields ($V = 118$, $P = 0.001$).

Dependency between copy numbers of *Fusarium* spp. of the FCR complex and *B. sorokiniana* in individual tillers showed a significant negative correlation for the tillers collected from the co-inoculated plots (Spearman rank coefficient: $r = -0.713$, $P < 0.001$) and for the tillers showing infection by one or both diseases collected from the naturally infested fields (Spearman rank coefficient: $r = -0.507$, $P < 0.001$). Infected tillers collected from both spring wheat and winter wheat naturally infested fields had a negative correlation (spring wheat Spearman rank coefficient: $r = -0.480$, $P = 0.011$; winter wheat Spearman rank coefficient: $r = -0.613$, $P < 0.001$). The same was observed for co-inoculated tillers collected from the experimental site in Bozeman but the

Fig. 4. Proportion of 40 individual lower internodes infected by *Fusarium* spp. (*Fusarium culmorum*, *F. pseudograminearum*, and *F. graminearum*) or *Bipolaris sorokiniana*, co-infected with both *Fusarium* spp. and *B. sorokiniana*, or not infected with either for stems collected from naturally infested spring wheat and winter wheat fields and from the co-inoculated plots, as determined by real-time quantitative polymerase chain reaction. For naturally infested fields, 20 symptomatic tillers each were collected from spring wheat fields in Shonkin, MT in 2008 and Cut Bank, MT in 2009 and winter wheat fields in Carter, MT in 2008 and Malta, MT in 2009. For co-inoculated tillers, 20 symptomatic tillers were collected from plots co-inoculated with high rates (oat inoculum at 3 g/m of row) of both *F. pseudograminearum* isolate 2228 and *B. sorokiniana* isolate 2344 at Bozeman MT in 2008 and 2009. Results were combined across year due to similarity of results and compared using a χ^2 goodness-of-fit test ($P < 0.05$).

effect was more noticeable during the 2008 crop season (Spearman rank coefficient: $r = -0.886$, $P < 0.001$) compared with the 2009 crop season (Spearman rank coefficient: $r = -0.388$, $P = 0.091$).

Discussion

In this research, increasing rates of *F. pseudograminearum* inoculum were associated with increasing populations of *F. pseudograminearum* and a corresponding decrease in *B. sorokiniana* populations within the lowest internode of wheat stems. Because of the high indigenous populations of *B. sorokiniana*, it was not possible to evaluate the reciprocal association. The practical implication of this implied antagonism between these pathogens may be that developing control for FCR may not result in the yield gains that one might expect because reduction in FCR-related losses brought about by control of FCR would be partially compensated for by increasing losses due to CRR. For example, development of cultivars resistant to FCR may be associated with increasing losses due to CRR or vice versa. From data collected under natural field situations for this project, almost 50% of the crown rot samples displayed co-infection in which control of the FCR would likely be replaced by an increase in CRR damage. Whether the reciprocal compensating dynamic occurs is an important question that needs to be addressed. Past studies suggest that *B. sorokiniana* may not have a reciprocal effect on *F. pseudograminearum* populations (36,46) and, therefore, suppression of CRR may yield results more directly proportional to this disease's importance in the wheat agroecosystem.

How *F. pseudograminearum* populations suppress *B. sorokiniana* in the lower internode is unknown. Direct antagonism mediated by antibiosis between *B. sorokiniana* and *F. pseudograminearum* mycelium has been not observed under in vitro conditions (38). Nevertheless, Ledingham (21) reported inhibited germination of *B. sorokiniana* spores by the closely related species *F. culmorum*. Other reports have linked the mycotoxin deoxynivalenol (DON) with reduction of gene expression in *Trichoderma atroviride* (25). However, in preliminary in vitro experiments using filter discs amended with DON (at 100 µg/µl), we could not show reduced growth of *B. sorokiniana* isolate 2234 (data not shown). Within wheat tissue, both Tinline (46) and Scardacci and Webster (36) reported that *F. culmorum* and *F. graminearum* infection, respectively, prevented later *B. sorokiniana* infections. If this exclusion dynamic dominates these interactions under natural field conditions, one would expect a deficit in co-infections under field conditions. This was not observed in the trials reported here but more study of its potential appears warranted. Another explanation for the dynamics observed would be that resource competition was involved. Inverse dependence between both pathogen populations was observed at harvest in the plot in Bozeman 2008 and for tillers collected from naturally infected and co-inoculated sites, which suggested a competition dynamic between the pathogens for the first internode. This dynamic for both pathogen populations was observed occurring at tiller and plot levels in this Montana study, and has been observed at regional levels by Fernandez and Jefferson (10) in the Canadian Prairies and observed by us in Montana (28). If resource competition is involved, one would expect classic zero-sum dynamics to be involved, with associated prediction of community stability and compensatory population dynamics. These were not tested within these trials but should be examined in the future. High colonization of lower internodes by *F. pseudograminearum* populations at the heading or milk stage was demonstrated in these studies, whereas *B. sorokiniana* populations were not seen increasing within lower internode until harvest stage. These results are in concordance with the observation of Tinline (46) and Scardacci and Webster (36), who determined that other *Fusarium* spp. preempt *B. sorokiniana* infection. This phenomenon could be suggesting a "priority effect", where an early colonist negatively affects the performance of later arrivals through preemption of shared resources (1,37). This phenomenon has been described recently playing a major role in the dynamics

of early colonization of pine seedlings by ectomycorrhizal fungi (18,19).

In addition to microbe-microbe interactions, the reduction in the population of *B. sorokiniana* by *F. pseudograminearum* populations could have been affected through indirect antagonistic interactions mediated by the plant, as was suggested for some ectomycorrhizal interactions (18). Several studies have revealed evidence of pathogenesis-related proteins induced by *F. pseudograminearum* and *B. sorokiniana* in wheat plants (4-6,23,24). The reduction of *B. sorokiniana* populations could be caused by elicitation of a resistance response by the fungal populations in the wheat plant, which presumably do not affect *F. pseudograminearum* but are capable of attenuating *B. sorokiniana* colonization of roots, crowns, or culms. Recently, this model was suggested to explain the interaction between *Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell and *Aphanomyces euteiches* Drechsler in alfalfa (49).

The results from individual tiller evaluations showed that the use of pathogen inoculations resulted in greater incidence of infection by *F. pseudograminearum* and *B. sorokiniana* and, therefore, greater incidence of co-infection than that observed in natural settings. This also suggests that the competitive effects witnessed in the field trials are less common under natural field conditions. Differences observed between inoculated trials and naturally co-infested fields provides us with a warning that these studies must examine interaction dynamics in the natural setting in order to place the dynamics into their proper agroecological perspective. From the agronomic point of view, these results showed that a high level of inoculum in field trials would overestimate the incidence and level of antagonism present in naturally infected fields.

During the establishment of the crop, co-inoculations reduced seedling stands more than single inoculations with either pathogen. In addition, an increase in inoculum levels was associated with greater reduction in the emergence for the three trials. These results were in accordance with the results obtained by the classical mixed experiments conducted by Hynes (9,21), who demonstrated that disease expression at seedling stage was considerably enhanced by co-inoculation with *B. sorokiniana* and *F. culmorum*. However, these results were different from the results of Ledingham's (21) field experiments, where combined inoculums of *B. sorokiniana* and *F. culmorum* did not lower seedling emergence as much as the more aggressive of the single pathogen inoculations. Statistical interactions between concentration of inoculum from Fpg2228 and Bs2344 were not observed (data not shown). This situation suggests that the reduction in seedling counts by a combination of inoculum would be associated with the increased inoculum levels represented by the combined inoculums and not due to filling complementary niches. In contrast to seedling stand, yield was only reduced by inoculation with *F. pseudograminearum* in 2008. Inoculation with *B. sorokiniana* did not reduce yield significantly over the noninoculated control, and co-inoculations did not show any additional effect on yield above that seen with individual inoculation with *F. pseudograminearum*. According to these results, *Fusarium* spp. populations could be considered a more aggressive late-season pathogen and better colonizers of lower internodes than *B. sorokiniana* on wheat. Unfortunately, late-season populations of *B. sorokiniana* were only marginally increased by inoculations and, therefore, their relative effect on *F. pseudograminearum* populations and other parameters such as yield cannot be ascertained by the results of this study.

This study only examined pathogen population dynamics at the base of the first stem internode, and the competitive relationship elsewhere in the plant was not addressed. Infection by *B. sorokiniana* has long been associated with infections of the subcrown internode and culm bases (9,36,46), and this has been associated with yield losses (22). For this reason, the assessment of rot at the subcrown internode has been commonly used to define CRR damage (10,12,13,43,44,50-52). If *B. sorokiniana* has some adaptation favoring its colonization of subcrown internodes, sampling of the first stem internode could bias the population dynamics observed

by this study toward *F. pseudograminearum*. Whether the dynamic between these two pathogens would be reversed if the focus of population studies was on the subcrown internodes is something worthy of further study.

The effect of environmental factors on population dynamics of *F. pseudograminearum* and *B. sorokiniana* was observed by this study. Prevailing environmental conditions accelerated or delayed the progression of *F. pseudograminearum* and *B. sorokiniana* across field trials. This was observed at the Bozeman 2009 site, where inoculation did not result in significant increases in pathogen populations due to uncommonly high summer rainfall and lower temperatures that did not favor disease development. In contrast, warmer and drier conditions observed during summer months for both locations in 2008 favored FCR (3,31,40) and CRR development (50), inoculations were more effective, and, as a result, population dynamics were more easily observed.

In conclusion, the competition dynamics between both *F. pseudograminearum* and *B. sorokiniana* showed that rates of *F. pseudograminearum* inoculum were associated with reduction of *B. sorokiniana* populations in the first internode in the field trials. This may have implications for disease control strategies in the future because control of FCR may lead to replacement by CRR. The reciprocal dynamics are unknown. Populations of *B. sorokiniana* may or may not reduce *F. pseudograminearum* populations because high native populations of *B. sorokiniana* obscured dynamics in these experiments and prevented a conclusion being drawn. Both *F. pseudograminearum* and *B. sorokiniana* populations in the wheat stems increased from heading through harvest, with *F. pseudograminearum* preemptively colonizing lower internodes relative to *B. sorokiniana*. Despite effects on their community dynamics, *F. pseudograminearum* and *B. sorokiniana* did not prevent infection by the other in the first internode of wheat plants. Inoculation significantly increased incidence of infection and co-infection relative to natural settings and may have increased the levels of competition observed relative to natural setting. These trials demonstrated the utility of qPCR technique to examine the population dynamics of multiple pathogens in mixed-plant infections under field conditions and showed the importance of comparing the level of infection developed in inoculated experimental trials with real-world agricultural systems managed by growers. Additional studies are warranted to understand this interplay within different disease systems and how variability within species such as *F. pseudograminearum* and *B. sorokiniana* affect their interspecies competition.

Acknowledgments

This research was supported in part by funds from the Montana Wheat and Barley Committee and the Montana Agricultural Experiment Station. We thank B. Johnston, D. Wichman, and J. Johnston for their support and assistance during the experimental work.

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