Applying Real-Time Quantitative PCR to Fusarium Crown Rot of Wheat

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ABSTRACT

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Fusarium crown rot (FCR) of wheat is a persistent problem that causes significant losses worldwide. In Montana, FCR is caused primarily by *Fusarium culmorum* and *F. pseudograminearum*. Recently, a real-time quantitative PCR (QPCR) assay was developed for FCR using primers and probes specific for a segment of the trichodiene synthase (*tri5*) gene. The purpose of this study was to determine the utility of QPCR for accessing FCR severity on wheat in field experiments. In 2004 and 2005, plots of spring and durum wheat were inoculated with varying levels of *F. pseudograminearum* oat inoculum and grown under rain-fed conditions. Two weeks prior to harvest, plants were collected from the plots and assessed for FCR severity and analyzed by QPCR for *Fusarium* DNA quantities. Disease severity scores (DSS) and *Fusarium* DNA quantities were positively correlated with each other for all three cultivars in 2004 but for only the durum cultivar in 2005 ($P < 0.05$). In 2004, grain yields for both spring wheat cultivars were negatively correlated with *Fusarium* DNA quantities ($P > 0.05$). When DSS and *Fusarium* DNA quantities negatively correlated with yield, both measurements were comparable in predicting yield reduction ($R = -0.64$ and -0.77 , respectively). Results indicate that this QPCR assay is effective in measuring FCR severity in wheat.

Additional keyword: TaqMan

In Montana (Northern Great Plains) and other semiarid regions of the world, Fusarium crown rot (FCR) of wheat (*Triticum aestivum* L.) is a persistent problem that causes significant losses (8,9,11,16,17). In the Pacific Northwest of United States, winter wheat losses attributed to FCR were estimated at 9% of total yield in 1994 (24,29). Statewide estimates are not available for Montana but in Hill, Judith Basin, and Roosevelt Counties, FCR-related spring wheat losses were estimated at 8% in 2002, or about \$4.45 million (W. Grey, personal communication).

FCR is caused by a complex of fungi including *Fusarium culmorum* (W. G. Sm.) Sacc., *F. pseudograminearum* (O'Donnell & T. Aoki; group I), and *F. graminearum* Schwabe (group II). In other regions, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh., *F. equiseti* (Corda) Sacc., *Bipolaris sorokiniana* (Sacc.) Shoemaker, and *Microdochium nivale* (Fr.) Samuels & I. C. Hallett also have been included in the crown rot disease complex (2,10,11,15,16, 25,30,31,37). In addition to causing crown rot, the members of the FCR complex also are known to cause Fusarium head blight,

or head scab, which has reached epidemic levels in some of the more humid wheat-producing regions of the United States (38).

Infective propagules of *Fusarium* spp. overwinter on crop residues as mycelia or, in the case of *F. culmorum*, in the soil as durable chlamydospores. These propagules infect newly sown plants, eventually colonizing their crowns and lower internodes as they develop (10). Symptoms associated with FCR of wheat include stand reductions and the rotting of root, crown, and stem tissues that take on a distinctive chocolate-brown color. In Montana-grown hard red spring wheat, the browning of stem tissues rarely occurs past the second internode, whereas browning up to the fourth internode has been reported for hard red springs grown in the Pacific Northwest (24,29). Under drought conditions, plant defenses weaken and *Fusarium* spp. invade and obstruct the vascular tissues. Severe infections can result in the abortion of tillers and the production of "white-heads" (8,9,12,17,23,28).

Traditional methods for evaluating FCR severity involve the scoring of mature wheat tillers using a subjective scale that's based on the intensity and distance brown discoloration extends up the tillers. FCR severity scales typically are divided into four or five categories that range from 0 to 100% browning of an internode. After scoring the tillers, the data can be manipulated several ways for analysis. Some examples are to calculate a disease severity

index (equation factoring in incidence and severity; 6), the incidence of stem browning (17), the percentage of tillers in each severity category (24,29), or an average CRR (36). Paulitz et al. (24) and Smiley et al. (29) demonstrated that yield losses correlated best with the percentage of tillers where browning had extended to the fourth internode. White-head incidences and basal browning also have been shown to be good predictors of yield loss (17). Because traditional procedures are labor intensive, time consuming, and vary greatly from evaluator to evaluator, a better system of measuring Fusarium crown rot severity is needed.

With the advent of real-time quantitative polymerase chain reaction (QPCR), researchers possess the unprecedented ability to accurately quantify a specific pathogen within a host plant. QPCR involves the monitoring of PCRs using fluorescent dyes. By comparing the amplification pattern of an unknown sample against that of known standards, the quantity of target DNA in the unknown sample can be estimated (14). The two main chemistries employed by QPCR are SYBR Green I and dual-labeled fluorescent probes, or TaqMan probes. SYBR Green I is an intercalating agent that fluoresces when it is bound to any dsDNA, whereas TaqMan probes fluoresce only when the fluorochromes are released by polymerase activity. Where fluorescent probes are employed, QPCR assays are sensitive, highly specific, and can be performed in a relatively short amount of time (approximately 2 h).

Several QPCR assays have been designed to detect the presence of specific *Fusarium* spp. within various plant and food materials (7,13,26,27,32,35). For studying FCR of small grains, Strausbaugh et al. (32) developed a TaqMan-based QPCR assay to quantify the FCR complex in wheat and barley roots. With primers and probe designed from the trichodiene synthase (*tri5*) gene of *F. graminearum*, their assay detects not only *F. graminearum* but also *F. culmorum* and *F. pseudograminearum*. Applying their assay to greenhouse studies, Strausbaugh et al. (32) found significant correlations between percent infected root area and *Fusarium* DNA quantities in *F. culmorum*-inoculated plants. In field studies, they found no correlation between disease severity index and *Fusarium* DNA quantities in roots for plants sampled at Feekes growth stage (GS) 10.1 to 10.5 (late boot to flowering).

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Although demonstrating that their QPCR assay was a promising technique for diseases evaluation of seedlings in controlled environments, they did not address several concerns about their assay. In particular, the relative sensitivity of the assay among *F. culmorum*, *F. graminearum*, and *F. pseudograminearum* was not examined, nor was the relationship between traditional visual disease categories and *Fusarium* DNA quantities. In addition, their research focused on evaluating seedling roots for disease severity, whereas most FCR evaluations are done using mature stem tissues as described above.

The purpose of this study is to build on the work of Strausbaugh et al. (32) in evaluating QPCR for FCR disease assessment. To accomplish this we addressed (i) sampling and DNA isolation protocols for mature stem tissues, (ii) the specificity of the QPCR assay reported by Strausbaugh et al. (32) among three FCR pathogens it detects, and (iii) relationships among traditional visual CRRs, grain yields, and *Fusarium* DNA quantities.

MATERIALS AND METHODS

Culturing. To obtain large quantities of mycelia for DNA isolations, fungal isolates were grown for 7 days at 22°C in 600-ml Roux culture flasks containing 25 ml of clarified liquid V8 media (10% V8 juice and CaCO₃ at 1 g/liter). The flasks were inoculated with four 1-cm² agar plugs from actively growing cultures. Mycelium was collected under vacuum using a Büchner funnel and Whatman no. 4 filter paper. Collected mycelium was washed with sterile distilled water (SDW) three times and then freeze dried. To obtain macroconidia, *Fusarium* isolates were grown on carnation-leaf agar media (CLA) [5 to 10 pieces of sterile carnation leaf [about 5 mm²] per 2 ml of 2% water agar] for 3 weeks, after which sporodochia were harvested with a sterile needle and placed in 1 ml of SDW. The concentration of macroconidia in suspension was determined using a Levy-Hausser counting chamber (C. A. Hausser & Son, Philadelphia).

DNA isolations. DNA from mycelia and macroconidia was isolated using the Fast-DNA kit (QBiogene Inc., Irvine, CA) per the manufacturer's instructions with the following modifications. The extraction buffer consisted of 800 µl of CLS-VF buffer and 200 µl of 25:24:1 phenol:chloroform:isoamyl-alcohol (pH 8.0). Fungal samples were homogenized in the FastPrep FP120 Instrument (QBiogene Inc.) for 30 s on speed 5.0 m/s. Before eluting DNA, the binding matrix was vacuum dried in a SpeedVac SVC100 (Savant, Farmingdale, NY) to remove any residual ethanol. Total DNA was eluted in a final volume of 50 µl of molecular grade water and spectrophotometrically quantified using a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA). Fungal DNA

samples were diluted 1:5 with molecular-grade water for QPCR analysis.

Due to the tough, dry nature of mature stem tissues, a few modifications were made to the fungal DNA isolation procedure to accommodate mature stem tissues. This included the addition of another ceramic ball to the homogenization tubes, the presoftening of samples by leaving them in CLS-VF extraction buffer on ice for 1 h prior to homogenization, and the FastPrep FP120 settings being increased to a speed of 6.0 m/s and a time of 45 s. To enhance the removal of fibrous stem material, the initial centrifugation step was extended to 12 min, and 25 mg of polyvinyl-polypyrrolidone was added to homogenization tubes to remove humic acid. All DNA samples obtained from mature stem tissues were diluted 1:10 with molecular grade water for QPCR analysis.

Real-time QPCR. The primers and probe used for QPCR were designed as described in Strausbaugh et al. (32). The probe was dual-labeled with the 6-carboxyfluorescein (6-FAM) fluorescent reporter dye and the 6-carboxyl-tetramethylrhodamine (TAMRA) fluorescence quencher. The probe was synthesized by PE Applied Biosystems (Foster City, CA) and all the primers used in this study were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). QPCR assays were performed using the Rotor gene 2000 (Corbett Research Inc. Sydney, Australia), using a two-step protocol with the following thermocycling parameters: 2 min at 55°C, 10 min at 94°C, and 40 cycles of 15 s at 94°C and 60 s at 60°C. QPCR reactions (25 µl) consisted of 12.5 µl of Universal TaqMan Master Mix (PE Applied Biosystems), 2.5 µl of 2 µM TRIS TaqMan probe, 2.25 µl of 1:1 20 µM Gzeae87T forward and reverse primers, 2.75 µl of molecular grade water, and 5 µl of sample DNA. Each real-time run included four 10-fold dilution standards, ranging from 6.6 million copies down to 6,600 copies. The curve produced by plotting the log of the DNA standards concentrations versus the cycle number always had an *R* value ≥0.99. Reactions were carried out to 40 cycles, which gave a detection limit of approximately 50 copies. Both the DNA standards and no-template control (NTC) samples were run in triplicate for each real-time run. Water controls included in each DNA extraction also were run with each experiment to assess the amount of cross-contamination occurring during the DNA isolation process.

The DNA standard used for absolute quantification during QPCR was a cloned fragment of the *tri5* gene from *F. culmorum* isolate 2223 (collected in Loma, MT). The fragment was amplified using primers Fusclone forward (5'-CGGGTC CAGATGTTTGCCAT-3') and Fusclone reverse (5'-ACTGCTCAATCCAGCAT CCC-3') designed from *F. culmorum* se-

quence GenBank accession no. AY102602. The resulting 680-bp fragment was cloned using Invitrogen's TOPO TA Cloning Kit (Carlsbad, CA) per the manufacturer's instructions and colony selections confirmed with PCR using the primers Gzeae87T forward and Fusclone reverse. The cloned DNA was extracted using Qiagen Mini-prep Kit (Valencia, CA) and sequenced at the Washington State University Core Facility (Pullman) using the Fusclone primer set to confirm its identity.

QPCR specificity and sensitivity among FCR species. The QPCR assay was tested on 10 *Fusarium* spp. and 12 other plant pathogens representing 13 genera (Table 1). For each species, 10 ng of DNA was tested via QPCR to check for nonspecific reactions and experiments were repeated once in their entirety. To assess the sensitivity of the QPCR assay for *F. culmorum*, *F. pseudograminearum*, and *F. graminearum*, five single-spored isolates were chosen for each species. These 15 isolates were identified to the species level following the guidelines found in Nelson et al. (21), with the identities of representative isolates being verified at the *Fusarium* Research Institute, University Park, PA. To distinguish between *F. graminearum* and *F. pseudograminearum*, isolates were grown on CLA for 3 weeks at 22°C, under a combination of cool-white and black-light fluorescent lights, with a 12-h light-and-dark cycle. Plates then were examined for the presence of bluish-black perithecia, which are indicative of *F. graminearum*. The morphological identifications of the 15 FCR isolates were confirmed by PCR using the primer sets Fc01 (22), Fg16N (22), and Fp1 (1).

These 15 identified FCR isolates then were used to determine sensitivity differences in the QPCR assay among the three FCR species. For this, genomic DNA from 50 mg of freeze-dried mycelia and from 100,000 macroconidia was examined. In addition, 250 ng of DNA from each isolate also was examined via QPCR in case of cell density differences in the mycelia and, consequently, DNA quantity. These experiments were repeated once in their entirety and the data combined after a test for heterogeneity of variances was performed.

Field experiments, FCR ratings, and QPCR. During the summers of 2004 and 2005, wheat cvs. Outlook (19), Choteau (18), and Utopia (World Wide Wheat, LLC.) were grown at the Montana State University Arthur H. Post Research Farm (Bozeman), to be analyzed for FCR severity, *Fusarium* DNA quantities, and grain yield. Outlook is a hollow-stemmed hard red spring wheat cultivar that is moderately resistant to FCR, Choteau is a solid-stemmed hard red spring wheat cultivar that is moderately susceptible to FCR, and Utopia is a durum wheat cultivar (*Triticum turgidum*) that is highly susceptible to

FCR. The experiment was planted in a split-plot design with the three cultivars as main plots and the four inoculum rates as sub-plots. Individual plots were four rows wide, seeded at a rate of 200 seed per 3-m row on 30-cm centers using a small-plot seeder. The experiment was replicated six times. Oat inoculum was sown concurrently with the untreated seed at a rate of 0, 4, 8, or 16 g per 3-m row of inoculum to create varying levels of FCR severity. Oat inoculum was produced as described by Mathre and Johnston (20) using *F. pseudograminearum* isolate no. 2228 (collected in Loma, MT).

To assess FCR severity, plants were collected at Feekes GS 11.4 from 15 cm of row at three randomly selected positions in each plot. The positions were limited to the middle rows, with the remaining plants

being harvested for yield. Plants were harvested using a binder and threshed using a large-plot Vogel. From the plants collected, 30 tillers were chosen randomly and rated for FCR severity using a crown rot rating (CRR) scale of 1 to 4. CRRs were based on the presence of brown discolored tissue at the first internode, with a rating of 1 = 0 to 25%, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = 75 to 100% of the internode discolored. The individual CRRs of the 30 stems then were summed to create a disease severity score (DSS) for each plot.

After visually rating the 30 random stems from each plot for FCR severity, 3-mm sections were cut from the stems directly above their crowns to be used for DNA extractions. Because 30 stem sections would overwhelm one 2-ml micro-

centrifuge tube, the stem sections were divided into three subsamples of 10 sections each for grinding purposes. After grinding, the subsamples were centrifuged and 200 µl of supernatant from each of the three subsamples was pooled together to make one sample. DNA then was extracted using the methods described, and samples were analyzed by QPCR. This process was repeated with stem sections taken just above the first and second nodes of the same 30 stems.

Fusarium DNA quantities for CRR groups. To establish *Fusarium* DNA quantities for each of the visual CRR groups (ratings 1 to 4), plants of Choteau and Outlook were taken from the border rows of field plots at Feekes GS 11.4. The susceptible durum wheat check, Utopia, was not analyzed for this set of experiments due to the fact that not enough CRRs 1 and 2 were available. The leaf tissue was removed and the stems were segregated into the four visual CRR groups. From each of the four CRR groups, five groups of 10 representative stems were chosen randomly. For each group of 10 stems, 3 mm of tissue was cut from directly above the crown, first node, and second node of each stem and placed in separate tubes for DNA extractions. In total, this gave five replicates for each of the three stem locations per CRR group. DNA samples were extracted using the methods described and then analyzed by QPCR.

Statistics. Statistical regression analysis was performed using Arc Stat XLISP-PLUS version 3.04 (<http://www.stat.umn.edu/arc/software.html>) and analysis of variance (ANOVA) was carried out using MacAnova (<http://www.stat.umn.edu/macanova/>). Mean comparisons between threshold cycle (C_t) values were made using a protected Fisher's least significant difference at $P < 0.05$. When making comparisons of C_t values between CRR groups, significant differences were evaluated using pair rank-sum tests.

RESULTS

QPCR specificity and performance. The QPCR assay detected all 15 of the FCR isolates it was designed for but failed to detect any of the other fungi tested (Table 1). However, the sensitivity of the QPCR assay was significantly different ($P < 0.05$) among the three FCR species (Table 2). For

Table 1. Quantitative polymerase chain reaction (QPCR) tests conducted with multiple fungal and oomycete genera and species to test the specificity of the assay, which was designed for the *tri5* gene to detect members of the *Fusarium* crown rot complex

Isolate no. ^y	Fungi tested	QPCR reaction ^z
2103	<i>Fusarium culmorum</i>	+
2223	<i>F. culmorum</i>	+
2230	<i>F. culmorum</i>	+
2279	<i>F. culmorum</i>	+
2286	<i>F. culmorum</i>	+
423	<i>F. graminearum</i>	+
468	<i>F. graminearum</i>	+
2225	<i>F. graminearum</i>	+
2318	<i>F. graminearum</i>	+
2319	<i>F. graminearum</i>	+
2100	<i>F. pseudograminearum</i>	+
2228	<i>F. pseudograminearum</i>	+
2234	<i>F. pseudograminearum</i>	+
2278	<i>F. pseudograminearum</i>	+
2317	<i>F. pseudograminearum</i>	+
391	<i>F. sambucinum</i>	ND
2277	<i>F. avenaceum</i>	ND
2281	<i>F. acuminatum</i>	ND
2308	<i>F. solani</i>	ND
2269	<i>F. oxysporum</i>	ND
387	<i>F. moniliforme</i>	ND
2312	<i>F. equiseti</i>	ND
797	<i>Verticillium dahliae</i>	ND
781	<i>Thielaviopsis basicola</i>	ND
796	<i>Cephalosporium</i> sp.	ND
558	<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	ND
2121	<i>Pythium ultimum</i>	ND
566	<i>Phialophora</i> spp.	ND
1083	<i>Sclerotinia sclerotium</i>	ND
C2	<i>Aphanomyces cochlioides</i>	ND
Unknown	<i>Penicillium claviforme</i>	ND
2313	<i>Tapesia yulliae</i>	ND
481	<i>Rhizoctonia solani</i>	ND
2287	<i>Bipolaris sporokiniana</i>	ND

^y Isolate numbers reference cultures from the Soil Disease Lab collection at Montana State University.

^z ND = no detection after 40 PCR cycles.

Table 2. Relative sensitivity of the quantitative polymerase chain reaction assay for detecting *Fusarium graminearum*, *F. pseudograminearum*, and *F. culmorum* using DNA from 50 mg of freeze-dried mycelia, 100,000 macroconidia, and a standardized amount (250 ng)^z

Species	50 of mg mycelia		100,000 Macroconidia		250 ng of DNA	
	C_t	Copy no.	C_t	Copy no.	C_t	Copy no.
<i>F. graminearum</i>	16.57 a	112,989,406	31.24 a	9,691	16.14 a	84,444,820
<i>F. pseudograminearum</i>	18.22 b	29,979,512	31.63 a	6,508	17.18 b	42,980,166
<i>F. culmorum</i>	20.75 c	8,525,476	34.66 b	425	19.11 c	12,811,916
LSD	0.93	...	1.77	...	0.70	...

^z Mean threshold cycle (C_t) and copy values represent five isolates per species combined over two experiments. C_t values followed by different letters are significantly different from each other at $P < 0.05$ using Fisher's protected least significant difference (LSD).

mycelia, macroconidia, and standardized DNA samples, *F. graminearum* isolates always had the highest sensitivity (i.e., had the lowest C_t value). The sensitivity of the QPCR assay diminished as one went from *F. graminearum* to *F. pseudograminearum* and from *F. pseudograminearum* to *F. culmorum*. For instance, the sensitivity of the assay for *F. pseudograminearum* was about half (0.51) that of *F. graminearum* for the same amount of standardized DNA. For *F. culmorum*, the assays sensitivity was even lower, at about one-seventh (0.15) that of *F. graminearum*. The pattern in sensitivity differences was not significantly different if one analyzed DNA yields from macroconidia or mycelia, instead of a standardized concentration of DNA from the three species ($P < 0.05$).

CRRs and *Fusarium* DNA quantities. For the spring wheat cv. Outlook in 2004, *Fusarium* DNA quantities in crown tissues of CRR 2 samples were significantly less than those in CRR 3 samples, and *Fusarium* DNA quantities in CRR 3 samples were significantly less than those in CRR 4 samples ($P < 0.01$). Similar results were obtained for both node 1 and node 2 tissues. However, significant differences between CRR 1 samples and CRR 2 samples existed only for crown tissues ($P < 0.05$), not node 1 or node 2 tissues (Fig. 1A). In 2005, similar results were obtained for Outlook except that CRR 1 samples

and CRR 2 samples were significantly different from each other only for node 2 tissue ($P < 0.01$), not crown or node 1 tissues. In addition, CRR 2 samples were significantly different only from CRR 3 samples for crown and node 1 tissues, not node 2 tissues (Fig. 1B).

For the spring wheat cv. Choteau in 2004, *Fusarium* DNA quantities in crown tissues were significantly lower in CRR 1 samples than those in CRR 2 samples, and *Fusarium* DNA quantities in CRR 3 samples were significantly less than those in CRR 4 samples ($P < 0.05$). This also was true for both node 1 and node 2 tissues. CRR 2 samples had significantly less *Fusarium* DNA than CRR 3 samples for node 1 tissues ($P < 0.05$), but not for the crown or node 2 tissues (Fig. 1C). In 2005, similar results were obtained for Choteau, except that CRR 1 and CRR 2 samples were not significantly different from each other at crown and node 1 tissues, just node 2 tissue (Fig. 1D). Also, CRR 2 samples were not significantly different from CRR 3 samples for any of the tissues.

In 2005, *Fusarium* DNA quantities measured for the four CRR groups for both Choteau and Outlook were significantly lower ($P < 0.05$), in most cases, than those calculated for 2004 samples (Fig. 1). On average, *Fusarium* DNA quantities for similar CRR groups in 2005 Outlook samples were 30% of those detected in 2004

samples. For Choteau 2005 samples, *Fusarium* DNA quantities for similar CRR groups were, on average, 46% of those detected in 2004.

Field plots and regression analysis. In 2004, *Fusarium* DNA quantities obtained from above the crown, first node, and second node significantly positively correlated ($P \leq 0.001$) with DSS for cv. Choteau (Fig. 2; Table 3). *Fusarium* DNA quantities obtained from Choteau crown samples were significantly ($P < 0.05$) negatively correlated with grain yields, whereas no correlation was found for node 1 or node 2 samples (Fig. 3; Table 3). For Choteau, there was no correlation between DSS and grain yield (Table 3).

For Outlook in 2004, crown, node 1, and node 2 samples had *Fusarium* DNA quantities that positively correlated ($P < 0.01$) with DSS (Fig. 2; Table 3). Outlook *Fusarium* DNA quantities also were significantly negatively associated ($P < 0.05$) with grain yields for all three stem locations (Fig. 3; Table 3). The relationship between *Fusarium* DNA quantities and yield for Outlook (i.e., the slope of the line) was not significantly different from that obtained for Choteau ($P = 0.10$). Also, the relationship between *Fusarium* DNA quantities and DSS was similar for Outlook and Choteau for crown, node 1, and node 2 tissues ($P = 0.27, 0.30$, and 0.94 , respectively). For Outlook, DSS negatively

Fig. 1. *Fusarium* DNA quantities (copy number) at the crown, first node, and second node for *Fusarium* crown rot rating groups 1 to 4. Two spring wheat cultivars, **A** and **B**, Outlook and **C** and **D**, Choteau, were analyzed in 2004 and 2005. Error bars represent standard errors.

correlated ($P < 0.001$) with grain yields (Table 3).

For Utopia in 2004, all three stem locations had *Fusarium* DNA quantities that positively correlated ($P < 0.05$) with DSS (Fig. 2; Table 3). The relationship between *Fusarium* DNA quantities and DSS for crown and node 1 tissues was significantly different from that obtained for Choteau and Outlook ($P < 0.01$). The relationship between *Fusarium* DNA quantities and DSS at node 2 for Utopia was not significantly different from that for Choteau and Outlook ($P = 0.10$). No significant associations were observed when Utopia *Fusarium* DNA quantities were regressed against grain yields. Associations between Utopia DSS and grain yields also were not detected.

In 2005, significant positive correlations between DSS and *Fusarium* DNA quantities at the crown, node 1, and node 2 ($P < 0.01$) tissues existed for the susceptible cv. Utopia (Table 3). However, the relationship between *Fusarium* DNA quantities and DSS for crown, node 1, and node 2 tissues for Utopia in 2005 was significantly different from those obtained for Utopia in 2004 ($P < 0.05$). No significant correlations between DSS and *Fusarium* DNA quantities were observed for Choteau or Outlook at any stem location ($P < 0.05$). Choteau DSS was the only variable that significantly correlated with grain yield losses in 2005 ($P = 0.017$). Average DSS for Choteau ($P = 0.022$), Outlook ($P < 0.001$), and Utopia ($P < 0.001$) were significantly lower in 2005 than 2004 by 38, 80, and 70 units, respectively (Fig. 4A). The average *Fusarium* copy number for Choteau, Outlook, and Utopia also was significantly lower ($P < 0.001$) by approximately 1.1 million, 1 million, and 2.8 million copies, respectively, in 2005 (Fig. 4B). Grain yields in 2005 were significantly higher ($P < 0.001$) for Choteau (+10.6%), Outlook (+10.9%), and Utopia (+20.0%) than those in 2004.

DISCUSSION

Our studies represent the first field studies correlating *Fusarium* DNA quantities determined by QPCR with traditional FCR disease assessments and yield reductions. Using the same QPCR assay, Strausbaugh et al. (32) were unable to detect relationships between traditional *Fusarium* root-rot severities and *Fusarium* DNA quantities in field samples. This likely was due to a combination of reasons, one of the major reasons being that they were sampling root tissue. By sampling roots, one must assess larger amounts of plant material to accurately determine pathogen populations and disease levels. Also, by sampling roots, soil is introduced into the DNA extraction process which, if improperly handled, is known to inhibit downstream applications. Another potential reason may be that the organism they were targeting, *F. cul-*

morum, has a lower detection level with this QPCR assay, as we have demonstrated. In other studies utilizing quantitative competitive PCR techniques, re-

searchers also had difficulties in finding significant associations between crown rot severity and pathogen DNA quantities (5,22,34). We believe we succeeded be-

Fig. 2. Relationships between *Fusarium* DNA quantities (copy number) at node 1 and *Fusarium* crown rot disease severity scores for two spring wheat cultivars, Choteau and Outlook, and a durum wheat cultivar, Utopia, in 2004.

Table 3. Results of regression analyses between *Fusarium* DNA quantities (at crown, node 1, and node 2), disease severity scores (DSSs), and grain yields for spring wheat cvs. Outlook and Choteau and durum wheat cv. Utopia for years 2004 and 2005

Cultivar, regression ^y	2004		2005	
	R	P	R	P
Outlook				
Yield:Crown	-0.77	<0.001	0.07	0.510
Yield:Node 1	-0.78	<0.001	0.20	0.504
Yield:Node 2	-0.42	0.039	0.02	0.932
Yield:DSS	-0.64	<0.001	-0.15	0.487
DSS:Crown	0.83	<0.001	0.30	0.183
DSS:Node 1	0.84	<0.001	0.01	0.958
DSS:Node 2	0.63	<0.001	0.24	0.264
Choteau				
Yield:Crown	-0.41	0.045	0.02	0.935
Yield:Node 1	-0.38	0.066	-0.10	0.652
Yield:Node 2	-0.17	0.433	0.14	0.528
Yield:DSS	-0.18	0.536	-0.48	0.017
DSS:Crown	0.64	<0.001	-0.07	0.759
DSS:Node 1	0.80	<0.001	0.10	0.628
DSS:Node 2	0.85	<0.001	-0.20	0.339
Utopia				
Yield:Crown	-0.05	0.822	-0.14	0.511
Yield:Node 1	-0.05 ^z	0.829	-0.36	0.082
Yield:Node 2	0.05	0.801	-0.21	0.330
Yield:DSS	0.18	0.382	-0.22	0.472
DSS:Crown	0.42	0.041	0.63	0.003
DSS:Node 1	0.63 ^z	0.001	0.86	<0.001
DSS:Node 2	0.70	<0.001	0.79	<0.001

^y Crown, Node 1, Node 2 = *Fusarium* DNA copy numbers at said stem location and DSS = disease severity scores for experimental plots.

^z Two outliers were removed from the regression analysis.

cause we focused on specific areas where *Fusarium* populations and FCR symptoms were most relevant to yield reductions. We also used *F. pseudograminearum* as an inoculum source, which allowed us greater sensitivity with this QPCR assay. In some instances, the invariable levels of disease preclude the detection of relationships. This occurred in our work and also may have affected these other studies.

Paulitz et al. (24) and Smiley et al. (29) indicated that disease measures taken at the fourth internode correlated best with yield reductions in the Pacific Northwest. In Montana, progression of symptoms to the fourth internode is very rare; therefore, we did not sample that high on the stem. Nevertheless, we did not find that *Fusarium* DNA quantities obtained from one stem location were superior to another at predicting DSS or yield losses based on *R* values from regression analysis. In some instances, *Fusarium* DNA quantities in crown tissues related best to DSS and yield loss (when applicable) whereas, in other cases, *Fusarium* DNA quantities from node 1 or node 2 tissue showed the strongest relationships. Over the 2 years, we found that DSS and *Fusarium* DNA quantities were comparable in predicting yield reductions through regression analysis. When both *Fusarium* DNA quantities and DSS were correlated with yield reductions for Outlook in 2004, *Fusarium* DNA quantities from the crown correlated marginally better than DSS. However, in 2005, the only data that significantly correlated with a yield reduction was DSS for Choteau plots.

When comparing disease evaluations across years, the relationship between *Fusarium* DNA quantities and traditional FCR disease measures was unstable. This is illustrated most clearly by differences in *Fusarium* DNA quantities for CRR groups 1 to 4 in 2004 versus those in 2005. For both Choteau and Outlook, CRR groups 2, 3, and 4 had significantly less *Fusarium* DNA quantities in crown tissues in 2005 than those in 2004. These differences also were evident in field plots. For all three cultivars in 2005, *Fusarium* DNA quantities were roughly one-seventh the amount predicted based on 2004's data. We tested whether these differences might be a result of changes in the QPCR process by running several samples from both years in tandem and obtained similar results. Although we cannot be certain, we suspect that the relatively low disease intensity in 2005 led to the unintentional inflation of our visual disease ratings for that year. Other confounding factors, such as environmental conditions or the presence of other wheat pathogens, also could account for some of the instability in the relationship between DSS and *Fusarium* DNA quantities. These factors may enhance symptom expression or inhibit *Fusarium* population growth in infested tissues.

The four visual CRR groups could not always be statistically distinguished from one another based on *Fusarium* DNA quantities. Because nonparametric analyses were used, we believe the inability to consistently differentiate the four CRR groups was not the result of large variances

but rather the difficulty in visually distinguishing between the disease categories. Most of the problems occurred when trying to separate CRRs 2 and 3. The results show that *Fusarium* DNA quantities tended to increase exponentially when moving up the visual disease scale. In other words, our visual disease assessments only discriminate between large differences in pathogen populations. These results support consolidating the four CRR categories into three categories representing low, medium, and high disease levels. Obviously, relationships between visual disease assessments and pathogen populations are not perfect and the performance of a three-category system should be compared with the four-category system in terms of predicting yield losses.

Due to genetic similarities, the QPCR assay developed by Strausbaugh et al. (32) detects three of the *Fusarium* spp. known to cause FCR in Montana: *F. graminearum*, *F. pseudograminearum*, and *F. culmorum*. Our analysis of these species demonstrated that the sensitivity of the QPCR assay is not equal among them and diminishes as one goes from *F. graminearum* to *F. pseudograminearum* to *F. culmorum*. This is true whether one uses standardized amounts of DNA, mycelia, or macroconidia. The difference in the QPCR

Fig. 3. Relationships between *Fusarium* DNA quantities (copy number) at the crown and grain yields for two spring wheat cultivars, Choteau and Outlook, in 2004.

Fig. 4. **A**, Average *Fusarium* crown rot disease severity scores and **B**, *Fusarium* DNA quantities (copy number) at the crown for two spring wheat cultivars, Choteau and Outlook, and a durum wheat cultivar, Utopia, in 2004 and 2005. Columns with different letters are significantly different at $P < 0.001$, except Choteau disease severity scores, where $P = 0.022$. Error bars represent standard errors.

assays sensitivity does not appear to be completely related to sequence homology. Basic local alignment search tool (BLAST) searches of the NCBI database using the 63-bp amplicon revealed a greater sequence homology in *F. culmorum* entries than *F. pseudograminearum* entries. This may be due to the location and type of base mismatches involved rather than in the total number of mismatches. In addition to the three FCR species included in this study, a BLAST search revealed other *Fusarium* spp. that might be detected with this assay, such as *F. lunulosporum* Gerlach, *F. cerealis*, *F. sporotrichioides* Sherb., and *F. poae* (Peck) Wollenw. These other *Fusarium* spp. are not commonly isolated in Montana, but this should be taken into consideration when using this assay.

In some instances, there was no correlation between *Fusarium* DNA quantities and DSS or *Fusarium* DNA quantities and yield reductions. In the case of Utopia in 2004, the high susceptibility of the cultivar led to uniform high disease severity and, in turn, a uniform yield loss. For the cases involving Choteau and Outlook in 2005, the lack of correlation was likely the result of unfavorable FCR conditions and low disease intensity. The climatic conditions during the 2005 growing season were considerably wetter and cooler than those in 2004. The wetter, cooler weather is not conducive for the development of FCR (6,8,10,12,23,28,29) and, therefore, there were lower disease levels, lower *Fusarium* DNA quantities, and correspondingly little to no impact of disease on yield.

This research has demonstrated that the estimation of *Fusarium* DNA quantity via QPCR can be an effective method for assessing FCR in mature wheat stems under certain conditions. When environmental stress is high and targeted pathogens are present in moderate numbers, QPCR performed as well as, if not better than, traditional disease assessment methods for evaluating field plots. However, when pathogen populations were low and plants were not stressed, QPCR was not an improvement over traditional methods. QPCR has helped revolutionize the field of diagnostic plant pathology by allowing investigators to accurately quantify a specific pathogen within a myriad of host tissues and environmental samples. Due to the increasing adoption of NTC practices, there is potential for an increase in residue-borne *Fusarium* populations (3,4,33). Monitoring *Fusarium* populations in decaying stem tissue using traditional plating methods is difficult and imprecise because of the plethora of microorganisms present. In addition to assessing plants for FCR severity, the QPCR assay described here could be used in the future to monitor *Fusarium* populations living on wheat residues and help understand the dynamics of their survival

and their importance in disease development.

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