Assessment of *Fusarium pseudograminearum* and *F. culmorum* Biomass in Seedlings of Potential Host Cereal Species

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Abstract

Fusarium crown rot is a major disease of wheat and barley worldwide, with the most frequently isolated causal agents being *Fusarium pseudograminearum* and *F. culmorum*. This study has successfully designed a quantitative polymerase chain reaction assay that is specific for *F. culmorum*, which has been used in conjunction with a previously established *F. pseudograminearum*-specific assay to compare the location and extent of infection by each fungus across a range of potential hosts, including six winter and three summer cereal species. All common winter cereals, excluding oat, demonstrated a similar range of visual and fungal biomass results when inoculated with either *F. pseudograminearum* or

F. culmorum. Oat exhibited the lowest visual disease ratings and fungal biomass values of the winter cereals, while the sorghum, maize, and rice cultivars returned the lowest values overall. The ranking of host species according to visual discoloration was strongly correlated for both pathogens. Visual reactions to *F. pseudograminearum* were greater than those caused by *F. culmorum* in all potential hosts trialed; however, fungal biomass results only indicated this trend for barley. These results demonstrate significant variation in the ability of these pathogens to colonize the range of cereal species examined and also suggest differences between the pathogens in their patterns of host colonization.

Fusarium crown rot has been recorded in all areas where wheat is grown, including Australia (Backhouse et al. 2004; Burgess et al. 1975), Europe (Balmas 1994; Cassini 1981; Rossi et al. 1995), North America (particularly the Pacific Northwest region of the United States) (Cook 1968; Moya-Elizondo et al. 2011; Smiley and Patterson 1996), South America (Moya-Elizondo et al. 2015), South Africa (Klaasen et al. 1991; Van Wyk et al. 1987), West Asia (Saremi et al. 2007; Tunali et al. 2008), and North Africa (Gargouri et al. 2001; Mergoum et al. 2000). In Australia, crown rot results in annual estimated losses of \$79 million in spring wheat and \$18 million in barley (Murray and Brennan 2009a,b). In the Pacific Northwest of the United States, crown rot has been estimated to cause a mean yield reduction of 9.5%, reaching as high as 35%, in commercial fields (Smiley et al. 2005).

Fusarium pseudograminearum (O'Donnell & T. Aoki, = *F. graminearum* group I, = *Gibberella coronicola*) and *F. culmorum* (W. G. Sm.) Sacc. are the most common causal agents of crown rot, with environmental conditions affecting the prevalence of each species within regions (Backhouse et al. 2004; Moya-Elizondo et al. 2011; Smiley and Patterson 1996; Tunali et al. 2008). In Queensland and New South Wales, Australia, crown rot is predominantly caused by *F. pseudograminearum* (Backhouse et al. 2004; Burgess et al. 1975; Scott et al. 2004). *F. pseudograminearum* is also the most common species associated with crown rot in Victoria and South Australia; however, *F. culmorum* is also frequently isolated in the high-rainfall areas of these states, where *F. culmorum* can be the dominant pathogen in some fields (Backhouse and Burgess 2002; Backhouse et al. 2004). Cropping systems and rotations have also been reported to affect the occurrence of these two pathogens in the Pacific Northwest of the United States (Smiley et al. 2016).

Both *Fusarium* spp. can be associated with the *Fusarium* crown rot "complex" but have been described as affecting wheat hosts differently. Studies by Liddell (1985) indicated that *F. pseudograminearum* caused severe aboveground symptoms earlier than *F. culmorum*, colonizing the subcrown internode, leaf sheaths, and culm bases and causing marked necrosis. *F. culmorum* caused less severe necrosis of these

tissues and caused a cortical rot of the crown roots. Dyer et al. (2009) also indicated that the disease severity index of wheat culms was lower for *F. culmorum*-inoculated plants than *F. pseudograminearum*; however, *F. culmorum* caused greater stand losses (Dyer et al. 2009; Malalasekera and Colhoun 1968). Examination of these pathogens has also included DNA analyses of field soils prior to planting (Hollaway et al. 2013), which indicated that levels of *F. pseudograminearum* and *F. culmorum* DNA were positively correlated with crown rot expression (visual stem browning and whitehead expression) in durum wheat, spring wheat, and barley.

F. pseudograminearum and *F. culmorum* have been isolated from all major winter cereals. Spring wheat (*Triticum aestivum* L.), durum wheat (*T. turgidum* L. var. *durum*), barley (*Hordeum vulgare* L.), and triticale ($\times$ *Triticosecale* Wittmack) are symptomatic primary hosts for *F. pseudograminearum* (Purss 1966; Quazi et al. 2009; Simmonds 1941). Cereal oat (*Avena sativa* L.) and the grass weed wild oat (*A. fatua* L.) become infected by *F. pseudograminearum* but are considered asymptomatic hosts that are likely to contribute to the maintenance of inoculum (Burgess et al. 2001; Nelson and Burgess 1994, 1995). *F. pseudograminearum* has also been isolated from many other grass genera, including *Agropyron*, *Austrostipa*, *Avena*, *Bromus*, *Danthonia*, *Dicanthium*, *Hordeum*, *Panicum*, *Phalaris*, and *Zea* (Bentley 2007; Burgess 1967; Purss 1969; Simmonds 1966). *F. culmorum* has a similarly wide range of host plants such as wheat, barley, oat, rye (*Secale cereale* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L. Moench), and various grasses (Paterson et al. 2005). This broad host range, which includes grass weeds, allows these *Fusarium* spp. to survive in many environments and produce further inoculum even when host crops have been removed. This ability has major implications for cropping, particularly when planning crop rotations, weed control, and disease management.

Quantitative methods for reporting fungal biomass have been used in several studies to examine *F. pseudograminearum* (Hogg et al. 2007; Knight and Sutherland 2013; Knight et al. 2012) or *F. culmorum* (Leišová et al. 2006; Petrisko and Windes 2006) colonization of hosts; however, not comparatively in the same study. These investigations have indicated a strong correlation between visual disease ratings and fungal biomass in wheat and barley, along with host genotypic variation. Genotypic variation in seedling reaction to disease within wheat has been well documented for *F. pseudograminearum* (Knight et al. 2012; Percy et al. 2012; Wildermuth and McNamara 1994) and *F. culmorum* (Dyer et al. 2009; Grey and Mathre 1988); however, a comparison between a

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large range of potential host species has not been performed for either pathogen.

The first objective of this study was to develop a primer/probe-based quantitative polymerase chain reaction (qPCR) assay for specifically detecting and quantifying *F. culmorum* in plant tissue. The assay was designed to require conditions similar to those described by Knight et al. (2012) for quantifying *F. pseudograminearum*, to allow a multiplex qPCR approach. Visual disease symptoms and fungal biomass of *F. pseudograminearum* and *F. culmorum* were then examined across a range of nine potential host species in seedling trials to investigate the response of different cereal species to infection by each pathogen and to compare the infection pathways and disease expression arising from the two pathogens in the same host species.

Materials and Methods

Fungal strains and inoculum preparation. Pathogen strains included the *F. pseudograminearum* isolate A03#24, collected from Tara, Queensland, Australia (Malligan 2009), and *F. culmorum* isolate Fc08-35, collected from Inverleigh, Victoria, Australia in 2008 (supplied by the Department of Primary Industries, Victoria, Australia). A single conidium culture was grown and maintained on spezieller nährstoffarmer agar (Nirenberg 1976). Subcultures were grown on starch nitrate agar (Dodman and Reinke 1982) in the dark at 25°C for 14 days. Conidia were collected by flooding plates with 5 ml of a 0.3% Tween-20 solution before filtering through two layers of cheesecloth with high-purity water (MilliQ; Millipore, Billerica, MA) to a volume of 10 ml. The conidial suspension was quantified using a counting chamber (Weber and Sons, Lancing, United Kingdom) and diluted to a concentration of 10^6 conidia/ml. The final suspension was stored for a maximum of 2 months at -70°C before inoculation.

Cultivars, growth conditions, and inoculation. Fifteen cultivars representing nine cereal species were grown for inoculation tests. Seed of 'EGA Wylie' (*T. aestivum*), 'Livingston' (*T. aestivum*), 'EGA Bellaroi' (*T. turgidum* L. var. *durum*), 'Jandaroi' (*T. turgidum* var. *durum*), 'Tilga' (*Hordeum vulgare*), 'Grout' (*Hordeum vulgare*), 'Hawkeye' ($\times$ *Triticosecale*), and 'Quoll' (*A. sativa*) were sourced from the Australian Winter Cereals Collection; 'Berkshire' ($\times$ *Triticosecale*), 'Rysun' (*Secale cereale*), and 'Vampire' (*S. cereale*) from the University of Sydney; 'MR Buster' (*Sorghum bicolor*) and 'PAC 606' (*Z. mays*) from Pacific Seeds; 'Sherpa' (*Oryza sativa* L.) from Ricegrowers Limited; and 'Taipan' (*A. sativa*) from Agri-Science Queensland. Each seed was planted in a 5-by-5-by-10-cm pot at a depth of 2.5 cm in Premium Hi-Retention growing medium (Power Blend, Narangba, Queensland, Australia). All seedlings were grown in an environmentally controlled glasshouse at 24 and 15°C (day and night, respectively) for 14 days.

Seedlings were inoculated 14 days after planting using a method modified from Mitter et al. (2006) and described by Knight et al. (2012). Potted seedlings were laid horizontally, each coleoptile was gently rubbed 0.5 cm behind the tip with a sterile toothpick, and 6 μ l of the 10^6 conidia/ml inoculum was placed on this area. Control plants were treated with 6 μ l of high-purity water. After inoculation, seedlings were placed in a growth chamber in the dark for

48 h with 2 cycles of 25°C for 14 h followed by 15°C for 10 h at 100% relative humidity. After 48 h, seedlings were placed upright in an environmentally controlled glasshouse and subsequently maintained at 24 and 15°C (day and night, respectively) for 21 days. Two independent replications of the experiment were conducted at different times. Each replicate consisted of five inoculated plants and two noninoculated control plants of each cultivar. Plants were positioned in a randomized block design after removal from the 48-h dark incubation. *F. pseudograminearum* and *F. culmorum* inoculations were in separate randomized blocks which were grown and inoculated simultaneously.

Disease assessment. Harvest of leaf sheaths and roots was performed at 21 days after inoculation (dai). Leaf sheath samples were collected as combined leaf sheath 1 to 4 of each individual plant. At harvest, each of the leaf sheaths was rated based on the percentage of brown discoloration of the entire leaf sheath (Wildermuth and McNamara 1994). Briefly, leaf sheaths were rated for disease on a 0-to-4 scale, where 0 = no visible discoloration and 1 = less than 25%, 2 = 25 to 50%, 3 = 51 to 75%, and 4 = greater than 75% discoloration. Visual discoloration was recorded as the average of the ratings of the four leaf sheaths from an individual plant. Noninfected control plants were used to distinguish disease symptoms from background pigmentation present in some of the cultivars tested. The upper 2-cm portions of the primary roots were washed and collected for Tilga, Grout, Livingston, EGA Wylie, Jandaroi, and EGA Bellaroi. All harvested tissues were stored at -70°C until lyophilization.

DNA extraction. Combined leaf sheaths 1 to 4 or the upper 2-cm portion of the primary roots of each individual plant were lyophilized, weighed, and ground with a TissueLyser (Qiagen, Doncaster, Victoria, Australia). Ground tissues were subsampled into 15- to 20-mg samples for extraction. Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Sydney, NSW, Australia) according to the manufacturer's recommended procedure. Samples were eluted into 100 μ l of autoclaved high-purity water and stored at -20°C until required.

***F. culmorum* qPCR assay validation.** A species-specific primer/probe combination (Table 1) was designed for *F. culmorum* from the GenBank accession AY880844.1 (Klemsdal and Elen 2006; Schilling et al. 1996) using Primer3 software (Rozen and Skaletsky 2000). A dual-labeled probe was obtained from Biosearch Technologies (Novato, CA). Primer and probe sequences were assessed by a BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) search of the GenBank nucleic acid database using the Others (nr etc.) database: Nucleotide Collection (nr/nt). DNA of each cereal cultivar and a range of *Fusarium* spp. (Table 2) was used to assess the specificity of the qPCR assay. Five 10-fold serial dilutions of pure genomic *F. culmorum* DNA and *Triticum* DNA were run in triplicate to assess the multiplex assay. Intra- and interassay variation was assessed using a single DNA sample extracted from an *F. culmorum*-inoculated plant sample.

Dual-labeled probe PCR conditions. *F. pseudograminearum* and *Triticum* primer/probe sets were used as described by Knight et al. (2012) and the *F. culmorum* and *Triticum* primer/probe sets were amplified under the same qPCR conditions. Reactions were performed in 20- μ l volumes containing autoclaved high-purity

Table 1. Primer pairs (forward = F and reverse = R) and dual-labeled probes (P) developed for specific quantification of *Fusarium pseudograminearum*, *F. culmorum*, or *Triticum* sp. DNA

Primer/probe	Sequence (5'→3')	Target	Product (bp)	GenBank accession
Fp_TEF1 α 0.2F ^z	ATCATTGGAATCGCTCGACG	<i>F. pseudograminearum</i>	...	...
Fp_TEF1 α 0.2R ^z	AAAAATTACGACAAAGCCGTAAGAA	<i>F. pseudograminearum</i>	82	...
Fp_TEF1 α 0.2P ^z	ACTCGACACGCGCCTGTTACCC	<i>F. pseudograminearum</i>	...	DQ382162
Wh_TEFg.5F ^z	TCCGTGAAGGATTGAGGAA	<i>Triticum</i> sp.	...	...
Wh_TEFg.5R ^z	GTCCTCTTCTAGAGTTCAAGTCACC	<i>Triticum</i> sp.	120	...
Wh_TEFg.5P ^z	TGGCCACGACTCCTGGAACCTATA	<i>Triticum</i> sp.	...	DQ247872
Fc_K13B-P_F	ATGACCGAAGACTCGGAGAA	<i>F. culmorum</i>	...	...
Fc_K13B-P_R	ACCTTGCTGCTGAGCTCGTCT	<i>F. culmorum</i>	112	...
Fc_K13B-P_P	CGAACTCGCAGGCAGAAGCG	<i>F. culmorum</i>	...	AY880844.1

^z Primer/probe sequences published in Knight et al. (2012).

water, 0.5 U of Immolase DNA polymerase, 1× Immobuffer, 2.5 mM MgCl₂, 100 µM dNTP, 150 nM *F. pseudograminearum* or *F. culmorum* CAL Fluor Gold-labeled probe, 150 nM *Triticum* FAM-labeled probe, 0.25 µM forward and reverse primers for *F. pseudograminearum* or *F. culmorum* and *Triticum* spp., and 5 µl of DNA template.

Table 2. *Fusarium* and cereal species assessed using the *Fusarium culmorum* and *Triticum* sp. primer/probe sets

Species	Isolates tested (n)	<i>F. culmorum</i> primers ^w	<i>Triticum</i> primers ^w
<i>F. compactum</i> ^x	2	–	–
<i>F. crookwellense</i> ^x	2	–	–
<i>F. culmorum</i> ^y	9	+	–
<i>F. equiseti</i> ^x	2	–	–
<i>F. poae</i> ^x	2	–	–
<i>F. proliferatum</i> ^x	2	–	–
<i>F. scirpi</i> ^x	2	–	–
<i>F. semitectum</i> ^x	2	–	–
<i>F. pseudograminearum</i> ^z	17	–	–
<i>F. graminearum</i> ^x	5	–	–
<i>Triticum aestivum</i>	5	–	+
<i>Triticum turgidum durum</i>	2	–	+
<i>Hordeum vulgare</i>	2	–	–
<i>Avena sativa</i>	2	–	–
× <i>Triticosecale</i>	2	–	+
<i>Secale cereale</i>	2	–	+
<i>Sorghum bicolor</i>	1	–	–
<i>Zea mays</i>	1	–	–
<i>Oryza sativa</i>	1	–	–

^w Symbols + and – indicate a positive and negative polymerase chain reaction result, respectively, for the primer/probe set. All genotypes within each species gave similar responses.

^x *Fusarium* spp. supplied by the University of Sydney, New South Wales, Australia.

^y *F. culmorum* included two isolates supplied by the University of Sydney; two isolates from the Leslie Research Centre, Queensland Department of Agriculture and Fisheries, Queensland, Australia; and five isolates from the Department of Primary Industries, Victoria, Australia.

^z *F. pseudograminearum* included five isolates supplied by the University of Sydney; nine isolates from the Leslie Research Centre, Queensland Department of Agriculture and Fisheries; and three isolates from the Department of Primary Industries, Victoria.

Multiplex real-time qPCR was performed using 100-µl tubes in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). Thermal cycling conditions consisted of 10 min at 95°C followed by 35 cycles of 95°C for 15 s and 64°C for 1 min. The Rotor-Gene 6000 series software collected fluorescence data for both reporter dyes every 0.15 s from each tube.

PCR controls in every assay included no-template controls and genomic DNA standards (positive or negative) for *F. pseudograminearum*, *F. culmorum*, and *Triticum* spp. Each PCR included a 10-fold serial dilution series of pure genomic *Triticum* DNA and *F. pseudograminearum* DNA or *F. culmorum* DNA run in duplicate. DNA concentrations of standards were determined by measuring optical density at 260 nm using a nanophotometer (Implen, Munich, Germany). Standard dilution series included four 10-fold serial dilutions for *F. pseudograminearum* and *F. culmorum* DNA (10 ng to 1 pg) and three 10-fold serial dilutions for *Triticum* DNA (100 ng to 100 pg). Standard curves were generated by plotting known DNA quantities against the threshold cycle calculated by the Rotor-Gene 6000 software in order to produce a regression equation in each assay. Normalization of qPCR results was performed by dividing the *Fusarium* DNA quantity by the weight of extracted tissue (nanograms of *Fusarium* DNA per dry weight grams of host tissue) to produce the values used to describe fungal biomass.

Statistics. For analysis, the ordinal visual discoloration ratings from the four leaf sheaths of an individual plant were averaged. Spearman's nonparametric rank correlation coefficient was used to examine interrelationships between visual discoloration ratings and *Fusarium* biomass values. Visual discoloration ratings were converted to ranks using the Kruskal-Wallis nonparametric procedure. *Fusarium* biomass values were fourth-root transformed. Visual discoloration ranks and *Fusarium* biomass values were analyzed separately for each *Fusarium* sp. using a nonparametric analysis of variance, with cultivar and replicate as fixed and random effects, respectively. Variance parameters were estimated using restricted maximum-likelihood estimation (Patterson and Thompson 1971). Multiple comparisons were performed using Fisher's protected least significant difference test when there was a significant cultivar effect.

Visual discoloration ratings and *Fusarium* biomass values for both *Fusarium* spp. were analyzed together, with cultivar and replicate block fitted as random effects. An unstructured variance model was applied to the genetic variance, allowing for the estimation of a genetic variance for and genetic covariance between each *Fusarium* sp. for the visual discoloration ratings and *Fusarium* biomass values. Estimates of genetic

Table 3. Comparisons of 15 cereal cultivars using leaf sheath visual discoloration (VisD) ratings and fungal biomass (nanograms of DNA per gram of extracted tissue) after inoculation with *Fusarium pseudograminearum* or *F. culmorum*^x

Cultivar	Species	<i>F. pseudograminearum</i>		<i>F. culmorum</i>	
		VisD ^y	Biomass (ng/g) ^z	VisD ^y	Biomass (ng/g) ^z
Taipan	<i>Avena sativa</i>	0.1 d	18 fe	0.2 efg	24 c
PAC 606	<i>Zea mays</i>	0.1 d	0 h	0.1 fg	0 e
MR Buster	<i>Sorghum bicolor</i>	0.1 d	5 g	0.0 g	2 d
Sherpa	<i>Oryza sativa</i>	0.2 d	12 f	0.1 fg	26 c
Quoll	<i>Avena sativa</i>	0.3 d	56 ed	0.2 def	40 c
Rysun	<i>Secale cereale</i>	1.2 c	63 e	0.3 de	652 a
Vampire	<i>Secale cereale</i>	1.3 c	764 cb	0.5 cd	765 a
Berkshire	× <i>Triticosecale</i>	1.6 bc	123 d	0.5 cd	163 b
EGA Wylie	<i>Triticum aestivum</i>	2.2 ab	600 cb	0.8 abc	378 a
Tilga	<i>Hordeum vulgare</i>	2.3 ab	583 b	0.7 abc	91 b
EGA Bellaroi	<i>Triticum turgidum</i> var. <i>durum</i>	2.3 ab	571 cb	0.9 a	466 a
Jandaroi	<i>Triticum turgidum</i> var. <i>durum</i>	2.4 ab	411 cb	0.8 ab	467 a
Livingston	<i>Triticum aestivum</i>	2.5 ab	502 cb	1.1 a	465 a
Hawkeye	× <i>Triticosecale</i>	2.5 ab	471 c	0.8 abc	608 a
Grout	<i>Hordeum vulgare</i>	3.0 a	1,797 a	0.6 bc	122 b

^x Mean separations were calculated on transformed VisD rating and fungal biomass values using Fisher's protected least significant difference test ($P > 0.05$). In each column, cultivar scores with the same letter are not significantly different ($n = 10$, 0.05 significance level). Cultivars are ordered by their *F. pseudograminearum* visual discoloration rating.

^y Mean of individual rating scores for first four leaf sheaths.

^z Fungal biomass in combined tissues of the first four leaf sheaths.

variance and genetic covariance were then used to quantify the genetic correlations between species and disease measurements. Estimates of cultivar effects were generated from this model as empirical best linear unbiased predictions.

All analyses were performed in Genstat (18th edition; VSN International Limited, Hemel Hempstead, UK). All tests used a significance level of 0.05. Mean separations, calculated by least significant difference values, were produced from transformed visual discoloration rating and *Fusarium* biomass values.

Results

***F. culmorum* qPCR assay validation.** The *F. culmorum* primer/probe set derived from GenBank accession AY880844.1 specifically amplified DNA of *F. culmorum* isolates only (Table 2). The *F. culmorum* primer/probe set and *Triticum* primer/probe set were compatible during multiplex qPCR assessment, utilizing the PCR conditions reported by Knight et al. (2012). *F. culmorum* DNA was quantifiable from 0.001 to 100 ng and wheat was quantifiable from 0.05 to 500 ng. The linear regression correlations between the threshold cycle and known DNA quantities were strong for both *F. culmorum* ($R^2 > 0.99$, $y = -3.4x + 22.96$, $E = 0.96$) and *Triticum* ($R^2 > 0.99$, $y = -3.2x + 30.0$, $E = 1.03$). The respective mean values and coefficient of variation (CV) for the intra-assay ($n = 10$) and inter-assay ($n = 8$) variability of the threshold cycle of a single sample containing both *F. culmorum* and *Triticum* DNA were 23.6/23.93 *F. culmorum*/*Triticum* (CV 1.8/1.8%) and 23.2/22.7 *F. culmorum*/*Triticum* (CV 4.9/3.6%), respectively. Restricted maximum-likelihood estimation analysis of the two independent replications of this experiment indicated that the data from both could be pooled to form a single data set in which there were 10 data points per variable for each individual host cultivar–pathogen interaction.

Visual discoloration of host cultivars. Visual discoloration of each host cultivar due to *F. pseudograminearum* or *F. culmorum* at 21 dai indicated a broad range of responses (Table 3). In general, visual discoloration was significantly less in Sherpa rice, PAC 606

maize, MR Buster sorghum, and Taipan and Quoll oat than the other cultivars and species. The genetic correlation for visual discoloration between *F. pseudograminearum* and *F. culmorum* was 0.98, where the genetic correlation indicates the agreement in cultivar rankings between the two *Fusarium* spp.

***Fusarium* biomass in leaf sheath tissues.** *F. pseudograminearum* and *F. culmorum* biomass encompassed a wide range of values across the host species (Table 3). The maize (with no detectable *Fusarium* DNA) and sorghum cultivars contained the least *Fusarium* biomass in their leaf sheath tissues, followed by Sherpa rice. Taipan and Quoll oat also reported low levels of *Fusarium* biomass compared with the other winter cereals. The genetic correlation for *Fusarium* biomass between *F. pseudograminearum* and *F. culmorum* was 0.76, where the genetic correlation indicates the agreement in cultivar rankings between the two *Fusarium* spp. tested.

Correlation of visual discoloration and *Fusarium* biomass in leaf sheath tissues. A significant association was detected between visual discoloration and *Fusarium* biomass for both *F. pseudograminearum* ($\rho = 0.72$; $P < 0.001$) and *F. culmorum* ($\rho = 0.69$; $P < 0.001$).

***F. pseudograminearum* and *F. culmorum* biomass in root tissues.** *F. pseudograminearum* and *F. culmorum* DNA was detected in root samples from each of the six cultivars tested (Table 4). Tilga barley had significantly less *F. pseudograminearum* DNA than Jandaroi and EGA Bellaroi durum wheat and Livingston spring wheat. In contrast, there were no significant differences across the cultivars for *F. culmorum* biomass.

Comparison of *F. pseudograminearum* and *F. culmorum* visual discoloration levels. Visual discoloration ratings due to *F. pseudograminearum* were significantly greater than those due to *F. culmorum* in the majority of cereal cultivars tested (Table 5). Only Vampire rye, Taipan and Quoll oat, PAC 606 maize, and Sherpa rice did not differ significantly for discoloration caused by either pathogen. The same values are presented in Table 5 as in Tables 3 and 4; however, in Table 5, these have been analyzed to compare the two *Fusarium* spp. on each cultivar. Examination of ratings separated into leaf sheaths 1, 2, 3, and 4 (Table 6) indicated differences between *Fusarium* spp. similar to those described using combined ratings of discoloration. Discoloration due to *F. culmorum* appeared at comparatively low levels in leaf sheath 2, was at low levels or absent in leaf sheath 3, and was absent in leaf sheath 4. In contrast, visual discoloration was apparent in all four leaf sheaths following *F. pseudograminearum* infection in all but the five least susceptible cultivars.

Comparison of *F. pseudograminearum* and *F. culmorum* biomass. The leaf sheaths of Tilga and Grout barley indicated significantly higher levels of *F. pseudograminearum* DNA than *F. culmorum* DNA (Table 5). In contrast, Rysun rye contained significantly more *F. culmorum* DNA than *F. pseudograminearum* DNA. No other cultivars exhibited significantly different levels of pathogen DNA. No significant differences in pathogen DNA were observed in the roots of the spring wheat, durum wheat, and barley cultivars examined (Table 5).

Discussion

This study presents a novel qPCR assay for *F. culmorum*, based on specific sequences reported by Schilling et al. (1996) and Klemsdal

Table 4. Comparisons of barley, spring wheat, and durum wheat cultivars using *Fusarium pseudograminearum* and *F. culmorum* biomass (nanograms of DNA per gram of extracted tissue) in primary root tissues^z

Cultivar	Biomass (ng/g)	
	<i>F. pseudograminearum</i>	<i>F. culmorum</i>
Tilga	25 c	49 a
EGA Wylie	52 bc	41 a
Grout	85 abc	120 a
Jandaroi	104 ab	111 a
EGA Bellaroi	123 ab	126 a
Livingston	120 a	122 a

^z In each column, cultivar scores with the same letter are not significantly different ($n = 10$, 0.05 significance level). Mean separations were calculated on transformed fungal biomass values using Fisher's protected least significant difference test ($P > 0.05$).

Table 5. Comparisons of *Fusarium pseudograminearum* and *F. culmorum* visual discoloration ratings and biomass (nanograms of DNA per gram of extracted tissue) in leaf sheath (LS; one to four) tissues and primary root tissues of 15 cereal cultivars^z

Comparisons	Grout	Tilga	Hawkeye	Berkshire	Livingston	EGA Wylie	Jandaroi	EGA Bellaroi	Vampire	Rysun	Quoll	Taipan	Sherpa	MR Buster	PAC 606
Visual discoloration															
<i>F. pseudograminearum</i>	3.0 a	2.3 a	2.5 a	1.6 a	2.5 a	2.2 a	2.4 a	2.3 a	1.3 a	1.2 a	0.3 a	0.1 a	0.2 a	0.1 a	0.1 a
<i>F. culmorum</i>	0.6 b	0.7 b	0.8 b	0.5 b	1.1 b	0.8 b	0.8 b	0.9 b	0.5 a	0.3 b	0.2 a	0.2 a	0.1 a	0.0 b	0.1 a
DNA in LS (ng/g)															
<i>F. pseudograminearum</i>	1,797 a	583 a	471 a	123 a	502 a	600 a	411 a	571 a	764 a	63 a	56 a	18 a	12 a	5 a	0 a
<i>F. culmorum</i>	122 b	91 b	608 a	163 a	465 a	378 a	467 a	466 a	765 a	652 b	40 a	24 a	26 a	2 a	0 a
DNA in roots (ng/g)															
<i>F. pseudograminearum</i>	85 a	25 a	...	...	120 a	52 a	104 a	123 a	...	...	...	...	...	...	...
<i>F. culmorum</i>	120 a	49 a	...	...	122 a	41 a	111 a	126 a	...	...	...	...	...	...	...

^z In each column, cultivar scores with the same letter are not significantly different for their reaction to *F. pseudograminearum* and *F. culmorum* ($n = 10$, 0.05 significance level). Mean separations were calculated on transformed visual discoloration rating and fungal biomass values using Fisher's protected least significant difference test ($P > 0.05$).

and Elen (2006). This assay has been used along with a previously reported *F. pseudograminearum* qPCR assay (Knight et al. 2012) to compare quantities of pathogen biomass and visual symptoms of infection across a range of potential host species. To our knowledge, this is the first comparative description of crown rot development across a broad range of cereal species and the results provide important information on the relationship between the level of fungal infection and the expression of disease symptoms for the two most prevalent crown rot pathogens of cereals worldwide.

This is also the first direct comparison of *F. pseudograminearum* and *F. culmorum*-inoculated plants using qPCR, and there is potential to utilize these assays to assess diseased plants in a range of situations, including glasshouse and field trials. This method adds a level of specificity which has not been available in previous reports based on primers amplifying a *tri5* gene, which were unable to distinguish between the presence of *F. pseudograminearum*, *F. culmorum*, or *F. graminearum* in the crown rot complex (Hogg et al. 2007; Strausbaugh et al. 2005). Use of the *tri5* gene allows field samples with mixed colonization to have their total *Fusarium* biomass measured in a single reaction, which is important for regions where multiple *Fusarium* spp. contribute to crown rot symptoms (Hogg et al. 2007; Tunali et al. 2008). The species-specific primers described in this study now allow the relative contributions of individual *Fusarium* spp. to be determined in glasshouse and field samples.

Crown rot research in Australia is most frequently performed using *F. pseudograminearum* due to it being the prevalent crown rot pathogen (Backhouse et al. 2004; Burgess et al. 1975); however, some investigations in Australia and the United States have included *F. culmorum* (Dyer et al. 2009; Evans et al. 2010; Hollaway et al. 2013). Wheat reactions to *F. culmorum* or *F. pseudograminearum* result in a similar trend in disease reactions between cultivars (Wallwork et al. 2004), with overall levels of disease generally depending on isolate aggressiveness. The current study also indicated that visible disease reaction and fungal biomass rankings across the range of hosts showed a significant correlation for the two *Fusarium* spp. Although the host reactions to each pathogen follow similar trends, several distinctions have been reported. A comparison of *F. pseudograminearum* and *F. culmorum* in Montana using spring and durum wheat indicated that *F. pseudograminearum* caused greater crown rot disease scores and a greater reduction in yield than *F. culmorum*, whereas *F. culmorum* caused greater seedling blight (Dyer et al. 2009). The *F. pseudograminearum* isolate in this study caused significantly greater visual symptoms than the

F. culmorum isolate in the barley, spring wheat, durum wheat, triticale, and one rye cultivar; however, this only translated into significant differences in fungal biomass for the barley cultivars. Liddell (1985) also described an increased level of visual symptoms in wheat caused by *F. pseudograminearum* compared with *F. culmorum*; however, no pathogen biomass data were available. This may indicate that *F. pseudograminearum* hyphae colonize individual leaf sheaths to a greater extent and further into adjacent leaf sheaths, producing more visual symptoms, whereas *F. culmorum* produces a similar amount of hyphae which does not colonize leaf sheaths as far distant from the inoculation point or as far into adjacent leaf sheaths. Alternatively, host responses to *F. pseudograminearum* may produce higher levels of tissue discoloration than responses to an equivalent biomass of *F. culmorum*. The limitation of *F. culmorum* growth in barley compared with *F. pseudograminearum* indicates that some level of species specificity may exist, which could be investigated further using multiple isolates of these two *Fusarium* spp.

The common host species have had considerable research focus on their crown rot reactions. Genotypic variation (partial resistance) in host response to *F. pseudograminearum* infections has been described in spring wheat (Klein et al. 1989; Percy et al. 2012; Wallwork et al. 2004; Wildermuth and McNamara 1994) and barley (Li et al. 2009; Nelson and Burgess 1994). In durum wheat cultivars, which are typically highly susceptible to crown rot, recent work to transfer resistance across from spring wheat is promising (Martin et al. 2013). Comparative assessment of multiple cereal species for crown rot disease reactions have been limited to date. Klein et al. (1989) reported the incidence of basal browning across 13 spring wheat cultivars, a single cultivar of durum wheat, barley, and triticale in northern New South Wales. Nelson and Burgess (1994) compared the reactions of multiple spring wheat, oat, and barley cultivars to crown rot in glasshouse and field conditions in New South Wales. Similar incidences of disease were recorded for both spring wheat and barley, with genotypic variation being observed within both species. Crown rot symptoms were not observed in any cultivar of oat. An early description by Purss (1969) similarly observed low levels of symptom development in field-grown oat, with disease rarely extending into the culm. Observations in the current study and by Percy et al. (2012) have also indicated very low levels of visual discoloration on oat leaf sheaths. These previous studies, taken together with the low levels of fungal biomass measured in infected oat seedlings in this current study, indicate that oat is a poor host for both of these *Fusarium* spp. and may serve a useful role as a rotation crop in some wheat-growing environments. This proposition requires further investigation in field trials involving the testing of adult plants and postharvest crop stubble for fungal load.

Crown rot reactions for a range of triticale cultivars have not been published, with the reaction of one reported cultivar being of a similar value to spring wheat cultivars (Klein et al. 1989). The triticale reaction to disease and *Fusarium* biomass quantities in the current study were similar to the spring and durum wheat reactions and generally greater than the rye reactions; however, some genotypic variation was observed. Rye has also had limited attention given to its reaction to crown rot disease. Höxter et al. (1992) evaluated a range of rye genotypes in glasshouse and field trials in Germany for their "foot rot" reaction to *F. culmorum* and *F. graminearum*. Genetic variance was observed between genotypes for their disease reactions, with a strong correlation between reactions to *F. culmorum* and *F. graminearum*. Rye in the current study showed less visual discoloration than barley, durum wheat, spring wheat, and triticale. A difference in *F. pseudograminearum* biomass was also recorded between the two rye cultivars, which suggests that further assessment of this species is required to determine whether partial resistance is present. This may act to inform the selection of parents when creating triticale cultivars.

Maize and sorghum are not considered typical hosts for *F. pseudograminearum* or *F. culmorum*, with sorghum being recommended as a useful rotation crop in crown-rot-infested fields (Burgess et al. 1996; Monds et al. 2005). Rice has not previously been assessed for its crown rot reaction to *F. pseudograminearum* or *F. culmorum*; however, as a major food source worldwide, it lends itself to being

Table 6. Comparisons of *Fusarium pseudograminearum* and *F. culmorum* visual discoloration ratings in leaf sheath (LS)1, -2, -3, and -4 tissues of 15 cereal cultivars^z

Cultivar	<i>F. pseudograminearum</i>				<i>F. culmorum</i>			
	LS1	LS2	LS3	LS4	LS1	LS2	LS3	LS4
Grout	4.0 a	3.6 a	2.8 a	1.6 a	2.0 b	0.4 b	0.0 b	0.0 b
Tilga	3.7 a	2.9 a	2.0 a	0.7 a	2.1 b	0.7 b	0.1 b	0.0 b
Hawkeye	3.3 a	3.0 a	2.4 a	1.3 a	2.4 a	0.7 b	0.0 b	0.0 b
Berkshire	3.6 a	2.2 a	0.5 a	0.1 a	1.4 b	0.6 b	0.0 b	0.0 a
Livingston	3.6 a	3.1 a	2.2 a	0.9 a	2.9 a	1.3 b	0.3 b	0.0 b
EGA Wylie	3.5 a	3.0 a	1.7 a	0.4 a	2.4 b	0.6 b	0.0 b	0.0 b
Jandaroi	3.9 a	3.0 a	1.9 a	0.8 a	2.4 b	0.7 b	0.0 b	0.0 a
EGA Bellaroi	3.7 a	3.1 a	1.7 a	0.8 a	2.7 b	0.9 b	0.1 b	0.0 a
Vampire	1.9 a	1.5 a	1.3 a	0.4 a	1.2 a	0.6 a	0.3 b	0.0 a
Rysun	1.8 a	1.5 a	1.0 a	0.6 a	1.1 a	0.1 b	0.0 b	0.0 a
Quoll	0.8 a	0.2 a	0.0 a	0.0 a	0.8 a	0.0 a	0.0 a	0.0 a
Taipan	0.3 a	0.0 a	0.0 a	0.0 a	0.6 a	0.0 a	0.0 a	0.0 a
Sherpa	0.4 a	0.2 a	0.0 a	0.0 a	0.2 a	0.0 a	0.0 a	0.0 a
MR Buster	0.5 a	0.0 a	0.0 a	0.0 a	0.0 b	0.0 a	0.0 a	0.0 a
PAC 606	0.3 a	0.0 a	0.0 a	0.0 a	0.2 a	0.0 a	0.0 a	0.0 a

^z For each leaf sheath in each cultivar, *F. pseudograminearum* and *F. culmorum* scores with the same letter are not significantly different ($n = 10$, 0.05 significance level). Mean separations were calculated on transformed visual discoloration rating values using Fisher's protected least significant difference test ($P > 0.05$).

examined and has potential for further investigation considering its position as an experimental model for functional and evolutionary genomics of cereals (Paterson et al. 2005). These three species produced the lowest visual ratings for disease and the sorghum and maize supported the lowest quantities of detectable pathogen DNA.

Colonization of primary roots has been reported as minor compared with leaf sheath tissues during seedling experiments (Knight and Sutherland 2013). *F. culmorum* is generally described as causing more disease on the primary roots (Scherer et al. 2013) compared with *F. pseudograminearum*; however, limited comparative investigations have been reported. The comparison of the primary roots of barley, spring wheat, and durum wheat in this study indicated similar levels of colonization by *F. pseudograminearum* and *F. culmorum* (Table 5). The only significant difference between cultivars for *F. pseudograminearum* colonization in roots was observed in Tilga barley, which developed less pathogen biomass than Jandaro and Livingston (Table 4). Research on root physiology during crown rot disease may prove informative; however, root assessments are unlikely to be useful for routine crown rot disease screening due to the challenges of root extraction from soil and the relatively low levels of root discoloration observed, except in the immediate vicinity of an infected crown or subcrown internode, until the plant is in an advanced stage of the disease (Purss 1969).

This research has presented a qPCR assay for *F. culmorum* and the first direct comparison of *F. pseudograminearum* and *F. culmorum* colonization across a wide selection of potential host species, which has indicated a diverse range of disease responses. Differences in cereal species reactions may reveal potential sources of resistance and inform strategies for crop rotations. Further research is warranted into direct comparisons of multiple isolates of *F. pseudograminearum* and *F. culmorum* for their visual effects and resulting biomass in winter cereals, particularly in field situations. The successful design of *F. pseudograminearum*- and *F. culmorum*-specific qPCR assays allows studies of the interactions between these two pathogens in coinoculation experiments and assessment of the level of competition between them in the field. Comparison of crown rot disease caused by *F. pseudograminearum* and *F. culmorum* is a direction of research which will lead to greater understanding of the potential impacts of these pathogens, particularly in environments where both pathogens are present, and will begin to tease apart the crown rot complex.

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