Amylose-Free (“waxy”) Wheat Colonization by *Fusarium* spp. and Response to *Fusarium* Head Blight

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Abstract

Hexaploid *waxy* wheat (*Triticum aestivum* L.) has null mutations in *Wx* genes and grain lacking amylose with increased digestibility and usability for specialty foods. The *waxy* cultivar Mattern is susceptible to *Fusarium* head blight (FHB) caused by *Fusarium graminearum* species complex, which produces the mycotoxin deoxynivalenol (DON). In experiment 1, conducted during low natural FHB, grain from *waxy* breeding lines, Mattern, and wild-type breeding lines and cultivars were assessed for *Fusarium* infection and DON concentration. Nine *Fusarium* species and species complexes were detected from internally infected (disinfested) grain; *F. graminearum* infections were not different between *waxy* and wild-type. Surface- and internally infected grain

(nondisinfested) had greater numbers of *Fusarium* isolates across *waxy* versus wild-type, but *F. graminearum*-like infections were similar; however, DON levels were higher in *waxy*. In experiment 2, conducted during a timely epidemic, disease severity, *Fusarium*-damaged kernels (FDK), and DON were assessed for *waxy* breeding lines, Mattern, and wild-type cultivars. Disease severity and FDK were not significantly different from wild-type, but DON was higher in *waxy* than wild-type lines. Across both experiments, *waxy* breeding lines, Plant Introductions 677876 and 677877, responded similarly to FHB as moderately resistant wild-type cultivar Overland, showing promise for breeding advanced *waxy* cultivars with reduced FHB susceptibility.

The *waxy* mutation, identified in wheat (*Triticum* spp.), other small grains, and other crops, reduces amylose content of the starch (Graybosch 1998). In wild-type (*Waxy*) plants, starch is made up of amylose and amylopectin: amylose makes up approximately one-fourth of starch, and amylopectin constitutes the remaining portion (Fasahat et al. 2014; Maningat and Seib 2010). In general, the percentage of amylose in the starch is negatively correlated with digestibility (Svihus et al. 2005). *Waxy* (*Wx*) encodes for granule bound starch synthase (GBSS), which is critical for amylose production. Spontaneous or induced null mutations (*wx*) in this gene can

significantly reduce amylose to nearly 0% of the starch (Lin et al. 2017; Morante et al. 2016; Nakamura et al. 1995; Sattler et al. 2009).

In hexaploid wheat, there are three structural genes, one on each of the three genomes (A, B, and D) encoding isoforms of GBSS that control amylose content in the endosperm of the grain (Miura et al. 1994). These enzymes are called GBSSI, to distinguish them from GBSSII, which is encoded by a separate set of genes (*GBSSII*) that control amylose content in other wheat tissues (Vrinten and Nakamura 2000). The presence of one or two *waxy* null alleles (i.e., single or double mutant in one or two genomes, respectively) results in production of reduced-amylose starch in the endosperm, called “partial *waxy*” (Graybosch 1998; Nakamura et al. 1993). Wheats with three null alleles (*waxy* mutations in all three genomes) produce essentially amylose-free starch (Graybosch 1998; Nakamura et al. 1995). Partial *waxy* wheats are sources of flour with optimal quality characteristics for specialty food products, particularly Asian noodles (Guo et al. 2003; Hung et al. 2006; Ramachandran et al. 2016). Amylose-free *waxy* wheats are valuable for production of modified food starches (Graybosch 1998), blending to formulate superior flours to be used for food products, or increased performance of baked goods and Asian salted noodles (Graybosch and Hansen 2016; Hung et al. 2006). Flours made from *waxy* wheat may also be used to extend the shelf life of baked goods (Bhattacharya et al. 2002; Graybosch 1998). Although the *waxy* trait was first reported in maize over 100 years ago (BeMiller 2009), *waxy* wheat development began just over 25 years ago (Nakamura et al. 1993; Seib 2000); currently, there are only three publicly available *waxy* wheat cultivars for use in major wheat-growing areas in the United States (Graybosch et al. 2014, *in press*; Morris and King 2007). The current study describes a set of *waxy* breeding lines adapted to the harsher conditions in the Northern Great Plains and their responses to natural infections by *Fusarium* spp. (Graybosch et al. 2018).

The reduction or lack of amylose in starch granules impacts the constituents of the grain. Total starch and protein concentrations were shown to be similar when comparing wild-type and *waxy* grain, but lipid and β -glucan concentrations were significantly greater in *waxy* wheat grain compared with wild-type or partial *waxy* (Ahuja et al. 2013). Starch of *waxy* grain was shown to have significantly different physicochemical properties than wild-type (Shevkani et al. 2017). These differences included higher thermal transition

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temperatures (onset and peak) for *waxy* starch, higher energy (enthalpy) required for gelatinization, and nearly half the time required to reach peak viscosity compared with wild-type; however, pasting temperatures (the temperature at which starch swelling begins) were similar (Demeke et al. 1999; Graybosch et al. 2000, 2003; Hung et al. 2008; Mangalika et al. 2003). The crystallinity of starch, as determined by X-ray diffraction analysis, was shown to be negatively associated with amylose content (Demeke et al. 1999; Fujita et al. 1998). Additionally, starch from *waxy* grains was shown to have significantly greater *in vitro* enzymatic hydrolysis compared with wild-type starch (Ahuja et al. 2013; Mangalika et al. 2003).

In the 2011 USDA-ARS coordinated Northern Regional Performance Nursery (NRPN), the *waxy* cultivar Mattern (tested experimentally as NX04Y2107), with three null alleles, was among the most susceptible to naturally incited *Fusarium* head blight (FHB). Observations of production fields near Mead, NE, also showed Mattern to be highly susceptible to FHB. A previous report of *waxy* sorghum indicated that it was not more susceptible to *Fusarium* grain pathogens compared with wild-type, whereas a report of reduced-amylose maize indicated increased susceptibility to *Fusarium* spp. (Funnell-Harris et al. 2015; Loesch et al. 1976). Therefore, it is unclear whether the physicochemical alterations in *waxy* starches affect interactions with pathogens in wheat, resulting in grain more susceptible to *F. graminearum* or other *Fusarium* pathogens. Under natural infection, *Fusarium* species can colonize the flowers or developing grain (Atanasoff 1920). Some may produce mycotoxins such as trichothecenes, enniatins, or moniliformin (Covarelli et al. 2015; Hietaniemi et al. 2016). These infections reduce grain quality and result in mycotoxin accumulation during grain development and in storage (Mylona et al. 2012; Osborne and Stein 2007). Grain fungal communities may be competitive or may coexist, thus potentially producing multiple mycotoxins (Czaban et al. 2015; Hietaniemi et al. 2016; Lutz et al. 2003; Nicolaisen et al. 2014; Wagacha et al. 2010).

One particularly insidious wheat grain disease is FHB, caused by several *Fusarium* species (Goswami and Kistler 2005; Spanic et al. 2010; Umpiérrez-Failache et al. 2013). Disease symptoms are visible within 3 to 4 days following infection and are manifested as premature whitening or bleaching of one or more spikelets on a head, which can progress to partial or entire whitening of the head. Diseased spikelets are sterile or contain shriveled kernels that appear chalky white or pink, referred to as *Fusarium*-damaged kernels (FDK), scabby kernels, or “tombstones” (McMullen et al. 2012). *Fusarium graminearum* Schwabe 1839 is the most commonly found species causing FHB in North America (Gilbert et al. 2014; Panthi et al. 2014; Vujanovic et al. 2012) and is capable of producing the trichothecene mycotoxin deoxynivalenol (DON) (Goswami and Kistler 2005). Although major occurrences can be sporadic, epidemics have increased since the early 1990s (Del Ponte et al. 2009; Duveiller et al. 2007; Zeller et al. 2003). The pathogen survives in crop debris on which the primary infectious propagules, ascospores, are generated (Gilbert and Fernando 2004; Manstretta et al. 2015). It has been shown that increased surface debris, particularly from wheat or maize crops as a result of reduced-tillage practices, can increase the incidence and severity of FHB (Bateman et al. 2007; Schaafsma et al. 2005). Moderate to warm temperatures and moisture in the form of rain or irrigation before and during anthesis favor FHB and mycotoxin production (Cowger et al. 2009; Osborne and Stein 2007). The disease results in deteriorated and shriveled grains, or asymptomatic grains, with high DON levels, that are essentially unusable for milling and down-stream uses (Cowger and Arrellano 2010; Tkachuk et al. 1991). These grain lots are often discounted or even rejected entirely at elevators, leading to substantial losses in revenue (Dahl and Wilson 2018; McMullen et al. 1997). DON is toxic to humans and animals at moderate to low exposure levels and has effects on gastrointestinal, reproductive, and immune systems (Katika et al. 2012; Raiola et al. 2015). Because DON is not heat labile, the toxin can be found in flour and baked goods (Savi et al. 2016; Trigo-Stockli et al. 1996; Zhang and Wang 2015).

A collection of *waxy* winter wheat breeding lines, most of which have recently been added to the USDA-ARS National Small Grains

Collection (Aberdeen, ID) as Plant Introductions (PI) (Graybosch et al. 2018), were developed to be adapted to the relatively harsh conditions in the Northern U.S. Great Plains (Paulsen and Shroyer 2008). These lines, the *waxy* wheat cultivar Mattern and wild-type breeding lines and cultivars, also adapted to this region, were grown for harvest in 2013, 2014, and 2015 in Nebraska. The *waxy* breeding lines and Mattern have the *waxy* allele in all three genomes. Trace to low FHB disease levels were observed in Nebraska during 2013 and 2014 (Baenziger et al. 2014, 2015), but during 2015 there was a severe FHB epidemic (Lilleboe 2015). Because decreased amylose content in wheat grain may affect interactions with *F. graminearum* and other *Fusarium* spp., these *waxy* breeding lines were assessed for infection by *Fusarium* species (2013 and 2014 grain) and FHB symptoms (2015) and mycotoxin accumulation (2013, 2014, and 2015). These studies were undertaken to determine whether *Fusarium* spp. infection rates and DON levels are similar in *waxy* versus wild-type grain under both low and high disease severities and whether genetic variation exists among *waxy* wheat lines in response to *Fusarium* pathogens.

Materials and Methods

Field plantings. Field studies were seeded as the wheat component of an oats (*Avena sativa* L.)/wheat/soy (*Glycine max* L.) rotation. The 13 *waxy* breeding lines (12 PIs and one unreleased line), the *waxy* cultivar Mattern (*waxy* check), and 18 Nebraska-adapted wild-type cultivars and breeding lines (wild-type checks) were grown during 2013 and 2014 at the University of Nebraska, Eastern Nebraska Research and Extension Center (ENREC) near Mead, NE. The *waxy* breeding lines and Mattern have three *waxy* alleles, and their pedigrees are presented in Table 1. Among the wild-type cultivars and breeding lines (Table 2), Overland has been reported to be moderately resistant to FHB (Jin et al. 2013). Wesley is considered moderately susceptible to FHB (Jin et al. 2013), and Jagalene is susceptible to FHB (Wegulo et al. 2011). In 2015, the breeding lines, Mattern, and a subset of three wild-type checks were grown at ENREC and at University of Nebraska, Havelock Farm, in Lincoln, NE, herein referred to as Havelock. The wild-type checks grown in 2015, Mace, Settler CL, and Overland, were chosen based on superior performance in Nebraska (<https://cropwatch.unl.edu/wheat>), or, in the case of Mace, for resistance to wheat streak mosaic virus. Each year at Mead, 89.6 kg/ha of nitrogen (N) was applied at the fall planting along with Finesse herbicide (chlorsulfuron and metsulfuron methyl), and in the spring, plots were treated with herbicides 2,4-D and Prowl-H₂O (pendimethalin). At Havelock, plots were treated with Finesse and 44.8 kg/ha of N at planting, and in the spring, they were treated with Prowl-H₂O and received another 89.6 kg/ha of N. No fungicides were applied at either location. The *waxy* breeding lines and *waxy* and wild-type checks were grown in three randomized complete blocks each year, in 3-m² plots. Grain from each plot was harvested with a small plot combine.

Details of monthly mean temperatures and relative humidity, and rainfall totals from the heading growth stage (Zadoks growth stage 50 to 59 [Zadoks et al. 1974]) through harvest, are given in Supplementary Table S1; daily means for the period of time wheat was most vulnerable to infection are also listed. Head emergence and anthesis occurred 3 to 5 days apart, depending on the temperature. During 2013 and 2014, FHB was at similarly low levels. In 2015, FHB was at epidemic levels at both locations and stripe rust also was observed at Havelock. Because analyses of plant responses and control cultivars were different, results obtained from 2013 and 2014 plantings were designated as “experiment 1,” and results from 2015 plantings were designated as “experiment 2.”

Analysis of fungi for experiment 1. *Collection and identification of Fusarium spp. from internal infections.* Previous studies of *Fusarium* spp. in air samples taken at Mead and Havelock had indicated that *F. graminearum* and *Fusarium proliferatum* were the dominant species throughout the summer growing season, which included late June and mid-July (Funnell-Harris and Pedersen 2011; Funnell-Harris et al. 2017). Subsamples (5 g) from combined (harvestable) grain from each plot were prepared for determination of fungal

colonization. Grain harvested in 2013 had symptoms similar to black point. Grain harvested in 2014 had no consistent symptoms. There were no noticeable symptoms of FHB in either year. To identify *Fusarium* spp. from internal infections, grains collected from each plot were surface disinfested by placing 15 to 20 randomly chosen whole grains in a 15-ml conical tube and washing first with 95% ethanol for 5 min, followed by 1% sodium hypochlorite with 0.01% Tween (Sigma-Aldrich, St. Louis, MO) for 15 min, by gently inverting the tubes, and then rinsing three times in sterile purified water (Labconco, Kansas City, MO). The grain samples were dried in a laminar-flow hood, and then 10 grains were placed onto pentachloronitrobenzene (PCNB) agar medium (Nash and Snyder 1962). It was previously determined that as few as five grains per plot can lead to detection of the major *Fusarium* pathogens of sorghum, as many as 11 different species/genotypes (Funnell-Harris et al. 2010). The percentage of grains with fungal colonies growing onto the medium for each plot was determined. Individual colonies growing from each grain onto the medium were transferred to one-half strength potato dextrose agar (PDA), amended with 100 μ M ampicillin (Sigma-Aldrich). Rarely (0.1% of grains), two distinctively different colonies were obtained from an individual grain; in this case both isolates were collected and counted within the appropriate species category. To more easily observe conidiophore and conidial structures, colonies grown on PDA with characteristics consistent with *Fusarium* spp. were subcultured onto sporulation medium (1.5% agar with 80 mM potassium chloride; KCl), by aseptically cutting an agar block from the edge of a PDA culture, placing onto the center of KCl medium, and growing 7 to 10 days at room temperature. The combination of colony morphology on PDA and conidiophore and conidial morphology on KCl was used to determine putative species identification of a given isolate, or it was designated as *Fusarium* sp. (Leslie and Summerell 2006). Owing to similarities in morphological characteristics and variability in culture of *Fusarium* spp. (Aoki et al. 2014; Davari et al. 2012), most (over 85%) morphological identifications were confirmed by molecular techniques. To produce single-conidium-purified cultures, conidia produced from a KCl culture were gently dislodged using sterile purified water, and then a conidial suspension was streaked onto peptone glucose agar (1% glucose, 0.5% peptone, 7 mM potassium phosphate monobasic, 2 mM magnesium sulfate heptahydrate, and 2.2% agar) amended with 100 μ g/ml of ampicillin and incubated at room temperature for 24

to 28 h. Conidia were examined under a stereoscopic zoom microscope (25 $\times$) with a high resolution objective (Nikon Instruments, Melville, NY), and an individual germinating conidium was aseptically transferred to PDA using a surface disinfested scalpel; three germings were transferred per isolate. Cultures were incubated at room temperature for 10 to 14 days, at which time the colony morphologies of single-conidium-purified cultures were compared with that of the original isolate. Single-conidium-pure isolates were stored as previously described (Funnell-Harris et al. 2013). Briefly, conidial suspensions prepared in sterile skim milk were stored on sterile silica gel (Windels et al. 1988) at 4°C; conidial suspensions in sterile purified water added to an equal volume of 80% glycerol in cryovials were stored at -80°C; and conidial suspensions were aseptically streaked onto PDA slants, grown 3 to 5 days at room temperature, and then stored at 4°C. Amplification, sequencing, and comparison of sequences in *FUSARIUM*-ID were conducted on DNA from single-conidium-pure isolates. Each isolate was grown in glucose-asparagine liquid medium (VanEtten and Bateman 1971) by shaking (150 rpm) at room temperature 5 to 6 days and then filtered (70 mm no. 2 Whatman qualitative filter paper; Westnet, Canton, MA) gently under vacuum to collect the mycelium. Mycelium was lyophilized and ground, and then DNA was extracted using the protocol of Lee and Taylor (1990). Polymerase chain reaction (PCR) amplification was performed using primers for the 5' region of the *translation elongation factor 1- α* (*TEF*) gene, ef1 (forward primer; 5'-ATGGGT AAGGA(A/G)GACAAGAC-3') and ef2 (reverse primer; 5'-GGA (G/A)GTACCAGT(G/C)ATCATGTT-3'), and the previously published reaction mixture and conditions (Geiser et al. 2004). Amplification products were either column purified or gel purified using the Amicon Ultra-015 Centrifugal Filter Device (Millipore, Billerica, MA) or the E.Z.N.A. Ultra-Sep Gel Extraction Kit (Omega Bio-tek, Norcross, GA), respectively. Purified amplification products were sequenced using the same primers as for amplification (Eton Bioscience, San Diego, CA), and sequences from opposite strands were assembled using Sequencher 5.4.1 (Gene Codes Corp., Ann Arbor, MI), and consensus *TEF* sequences were compared with those in the *FUSARIUM*-ID database (<http://www.fusariumdb.org/index.php>) (Geiser et al. 2004). Sequences were deposited in GenBank, accession numbers MG826853 through MG826912.

Collection and identification of surface and internal infections of grain by Fusarium spp. Grain grown and collected from plots, as described above, was aseptically applied directly to the surface of

Table 1. The waxy breeding lines with Plant Introduction (PI) designations and pedigrees utilized in this study

Line	PI number ^z	Pedigree
NX12Y8186	PI 677868	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8187	PI 677869	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8188	PI 677870	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8190	PI 677872	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8205	PI 677873	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8209	PI 677874	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8210	PI 677875	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8212	PI 677876	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8213	PI 677877	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8214	PI 677878	NX04Y2066/Wesley//99Y1445/ NuHorizon
NX12Y8219	NA	NuDakota//NX04Y2090/NX03Y2115
NX12Y8222	PI 677881	NuDakota//NX04Y2090/NX03Y2115
NX12Y8223	PI 677882	NuDakota//NX04Y2090/NX03Y2115

^z NA = not applicable. In this case, local selection number (line) was utilized in the text and tables.

Table 2. Checks with local name, Plant Introduction (PI) number, and waxy phenotype utilized in this study^y

Local name	PI number	Waxy phenotype
Mattern*	PI 665947	waxy
Alice	PI 644223	Wild-type
Camelot	PI 653832	Wild-type
Goodstreak	PI 632434	Wild-type
Infinity CL	PI 639922	Wild-type
Jagalene	PI 631376	Wild-type
Mace*	PI 651043	Wild-type
McGill	PI 659689	Wild-type
Millennium	PI 613099	Wild-type
NE06545	PI 667038	Wild-type
NI08708	NA ^z	Wild-type
NW07505	NA	Wild-type
Nuplains	PI 605741	Wild-type
Overland*	PI 647959	Wild-type
Pronghorn	PI 593047	Wild-type
Robidoux	PI 659690	Wild-type
Settler CL*	PI 653833	Wild-type
Tam 111	PI 631352	Wild-type
Wesley	PI 605742	Wild-type

^y All checks listed were grown during 2013 and 2014. Checks grown during 2015 are indicated with an asterisk.

^z NA = not applicable. In these cases, local selection number was utilized in the text and tables.

PCNB agar without prior disinfestation. This indicated not only internal infections but those on the surface that could be removed or destroyed by the disinfestation treatment. Ten randomly chosen grains per plot were applied to the medium. Collection of fungi grown from grain on PCNB agar was as described above. Grains with fungal colonies having characteristics indicative of *Fusarium* spp. were enumerated, and the percentage of grains with *Fusarium* contamination per plot was determined. Fungal colonies with characteristics consistent with *F. graminearum* grown on PDA were counted by comparing with known *F. graminearum* isolates, and the percentage of grain with isolates that had characteristics similar to *F. graminearum* per plot was also determined. All other *Fusarium* isolates determined unlikely to be *F. graminearum* were grouped together as “other *Fusarium* spp.,” and the number of grains per plot containing such isolates was determined.

Analyses of disease responses for experiment 2. The waxy breeding lines (Table 1), the waxy cultivar Mattern (check), and three wild-type cultivars (Table 2) were planted at two locations in Mead and Lincoln, NE, during 2015, as previously described. A timely epidemic allowed assessment of response of these lines to FHB under high disease pressure. Because FHB disease severity, DON levels, and percent FDK were high, it was likely that most *Fusarium* isolates would be *F. graminearum* (Markell and Francel 2003; Panthi et al. 2014).

FHB disease severity. Disease severity was assessed on a “per plot” basis and visually scored using a 1 to 10 linear scale of the proportion of bleached spikelets per head, with 1 being no evidence of disease and 10 being highly susceptible (96 to 100%). Plots were scored approximately 27 (Havelock) or 30 (Mead) days after head emergence.

FDK. Seeds with obvious symptoms similar to *F. graminearum* infection (white, chalky, shriveled, and sometimes with a pink tint owing to the fungus) were separated from apparently healthy kernels by flotation in water (Johnson and Beemer 1977). This separation method relies on the fact that FDK, less dense than healthy seed, will, when immersed in water, float to the surface, whereas healthy seeds will sink. Additional separation by hand was performed in a few samples when smaller healthy seeds floated to the surface. To perform this separation technique, a tub was fitted with a colander and, within the colander, a plastic cylinder. The tub and cylinder were filled with purified water, and the seeds from one plot were placed within the cylinder. The seeds were agitated using a glass stir rod to ensure they were well separated. A mesh filter screen was used to catch the FDK and place them on paper toweling in a tray. The healthy seeds were lifted from the water within the colander and placed on a separate paper towel-lined tray. The seeds were dried at 50°C and then checked, and further hand separation was performed as needed. The seeds were placed into separate envelopes, and the drying was continued at 40°C overnight. The dry seeds were counted using an electronic seed counter. Total seeds ranging from 1,686 to 3,027 were counted for each of 102 plots. The proportion of seeds from each plot with evidence of apparent *F. graminearum* damage was determined.

Analysis of mycotoxins. Grain subsamples from each plot (20 g) were ground using the Cyclone Lab Sample Mill (Udy Corporation, Fort Collins, CO), individually packaged, and then sent to the North Dakota State University Diagnostic Laboratory (<https://www.vdl.ndsu.edu/>) for analysis of the mycotoxins DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and nivale-nol using gas chromatography with electron capture detection. Plots from 2013 and 2014 had grain with low but detectable levels (≥ 0.50 $\mu\text{g/g}$) of DON, whereas DON and 15-ADON were detected in 2015 grain. For samples with undetectable amounts (below 0.50 $\mu\text{g/g}$), the value 0.49 was used for calculations.

Statistical analyses. Analyses of *Fusarium* spp. from internal infections of grain (experiment 1). To calculate mean percentages of surface-disinfested grain with internal infections, there were two plant phenotypes (14 waxy lines and 18 wild-type lines), 10 grains per plot, and three randomized complete blocks per year for 2 years (2013 and 2014). Total numbers of waxy grains with *Fusarium* spp. was compared with total wild-type grains using a χ^2 test using the PROC FREQ procedure of SAS/STAT software (SAS 2013). The ratios of isolates from different species and species complexes were

compared across all waxy lines and all wild-type lines using a χ^2 test with the ratios of different *Fusarium* species and species complexes in the wild-type considered as “expected.” Pairwise comparisons of numbers of individual *Fusarium* spp. or species complexes collected from waxy grain with that from wild-type were also conducted with χ^2 . In this case, the EXACT statement in PROC FREQ was used to alleviate concerns about asymptotic assumptions not being met. For each *Fusarium* species and species complex detected in waxy grain, a comparison was made with those obtained from wild-type grain, which was considered “expected.” Additionally, for each species within the *Fusarium incarnatum*–*F. equiseti* species complex (FIESC), the proportion of isolates that were from each plant phenotype (waxy and wild-type) was determined and χ^2 was again performed considering wild-type as “expected.” As described before, pairwise comparisons were made by comparing the number in each species or species complex within FIESC detected in waxy grain with the number in wild-type using χ^2 . Additionally, χ^2 was used to compare total numbers of *Fusarium* spp. in each line with the mean from all wild-type lines, which was considered “expected.”

Analysis of surface and internal infections of grain by *Fusarium* spp. from 2013 and 2014 plots (experiment 1). Mean percentages of contaminated grain between waxy and wild-type lines were analyzed with χ^2 tests as previously described. Percentages of these grain isolates that had characteristics similar to *F. graminearum* for each plant phenotype (waxy and wild-type), and those that were other *Fusarium* spp., were determined, and χ^2 was again performed, with percentages of each for wild-type grain considered “expected.” Also using χ^2 , comparisons of mean grain contaminated with *Fusarium* spp. with colony characteristics similar to *F. graminearum*, or all other *Fusarium* spp. from each individual line (waxy and wild-type), were compared with means obtained from all wild-type lines (“expected”). The mean numbers from each individual line contaminated with *F. graminearum*-like or other *Fusarium* spp. were also compared with the mean from the known moderately resistant wild-type line, Overland, again, using χ^2 . Correlations of grains infected by colonies with morphological characteristics similar to *F. graminearum* and total *Fusarium* spp. or other *Fusarium* spp. were compared using PROC CORR of SAS/STAT software (SAS 2013).

Analysis of FHB severity and FDK from experiment 2. Comparisons of mean FHB severity ratings and mean proportions of FDK were conducted using PROC MIXED of SAS/STAT software (SAS 2013). There were three randomized complete blocks at each location, two locations, 14 waxy lines, and three wild-type lines. Means across each plant phenotype (waxy or wild-type) or means for each plant line (17) were compared. Replication and location were considered random effects, whereas grain phenotype was the sole fixed effect. In the MODEL statement, the DDFM = KR option was specified to calculate denominator degrees of freedom using the Kenward–Roger method (Kenward and Roger 1997). In the text and tables, means are expressed as least squares means followed by the standard error.

Analyses of mycotoxins. To compare mean DON (2013 and 2014, or 2015) and 15-ADON (2015 only) levels between the two plant phenotypes (wild-type and waxy) for the combined data, PROC MIXED of SAS/STAT software was utilized (SAS 2013). A combined analysis of DON for years 2013 and 2014 was conducted with 18 wild-type and 14 waxy lines, in three replicate blocks in each of the two years. DON and 15-ADON analyses for year 2015 were conducted separately, with three wild-type and 14 waxy lines, three replicate blocks per location, and two locations. Replication and, in the case of 2015 location, were considered random effects. Grain phenotype (for 2013 and 2014, and 2015) and year (for 2013 and 2014) were treated as fixed effects. In the MODEL statement, the DDFM = KR option was specified to calculate denominator degrees of freedom using the Kenward–Roger method (Kenward and Roger 1997). In the text and tables, means are expressed as least squares means followed by the standard error. DON (2013 and 2014, and 2015) and 15-ADON (2015 only) measurements for each individual line also were compared using PROC MIXED. Correlations of numbers of internal and surface infections by *Fusarium* spp. with colony characteristics consistent with *F. graminearum* or DON levels (2013

and 2104) and correlations of disease ratings with FDK or DON or 15-ADON levels (2015) were conducted using PROC CORR of SAS/STAT software (SAS 2013).

Results

Experiment 1. Internal infections by *Fusarium* spp. from waxy and wild-type grain as determined by surface disinfestation of grain. In 2013 and 2014, trace to low field levels of FHB were

observed. Neither field disease severity ratings nor determination of FDK to assess infection levels would have been informative (Koch et al. 2006), and external FHB infection was not apparent. Therefore, grain was assessed for total infection levels by *Fusarium* spp., including *F. graminearum*. Across all waxy and wild-type lines, there was no significant difference in the percent of waxy and wild-type grain with internal contamination with *Fusarium* spp. (Fig. 1A). When comparing the mean of each individual line (waxy or wild-type),

Fig. 1. *Fusarium* spp. grown from surface-disinfested waxy and wild-type grain onto a semiselective medium, indicating internal infection. **A**, Percent of waxy and wild-type grain infected by *Fusarium* species. χ^2 analysis compared means of percent from waxy grain with wild-type ("expected"). **B**, Numbers of isolates from waxy and wild-type grain for each *Fusarium* species or species complex identified using morphological and molecular techniques. Members of the *F. incarnatum*–*F. equiseti* species complex are denoted as "FIESC"; members of the *Fusarium fujikuroi* species complex unable to be identified to species are denoted as "FFSC"; other isolates unable to be identified to species are denoted as "*Fusarium* sp."; and species each only detected once (wild-type grain: *F. pseudonygamai*; waxy grain: *F. chlamydosporum*, *F. lateritium*, and *F. scirpi*) were grouped together and denoted as "Other." Proportions of isolates in each *Fusarium* group from waxy grain were compared with those from wild-type ("expected") by χ^2 . **C**, Numbers of isolates identified in each FIESC genotype isolated from waxy and wild-type grain. χ^2 was performed to compare proportions of each FIESC genotype from waxy with those detected in wild-type ("expected").

waxy cultivar Mattern and wild-type cultivar Wesley each had mean infection rates of 10.00%, which was significantly higher than the mean of all wild-type lines ($X = 3.94\%$; $P = 0.02$). The waxy breeding line PI 677876 and the wild-type cultivars Camelot and Millennium

Table 3. Percent of all *Fusarium* spp., *Fusarium graminearum*-like, and all other *Fusarium* spp. contaminating non-surface-disinfested grains from naturally infected waxy breeding lines, the waxy cultivar Mattern, and wild-type lines^z

Fungal category	Mean percent infected grain		χ^2	P
	waxy	Wild-type		
Total <i>Fusarium</i> spp.	54.1	47.0	3.7	<0.01
<i>F. graminearum</i> -like	4.5	3.4	904.7	0.08
Other <i>Fusarium</i> spp.	50.0	42.5	23.4	<0.01

^z Grain was aseptically placed onto semiselective medium for *Fusarium* spp.

also had relatively large proportions of grain with internal infections by *Fusarium* species (8.33%; $P = 0.08$), although not significantly different from the mean of all wild-type lines. All other lines (waxy and wild-type) had mean percentages of infected grain in the range of 0.00 to 6.67%, also not significantly different from the mean across all wild-type (Supplementary Table S2). Seventy-seven *Fusarium* isolates were collected and identified to species using morphological and/or molecular techniques. The χ^2 analysis, using the ratios of species found in wild-type lines as “expected,” showed that the proportions of isolates from each species or species complex found in the waxy grains was significantly different (Fig. 1B), indicating that the ratios of different fungi within waxy grain were different from those in the wild-type cultivars chosen for this study. However, pairwise comparison of each species or species complex in waxy versus wild-type grain did not indicate one or a few *Fusarium* species that had significantly different infection rates of waxy or wild-type grain. Specifically, there was no significant difference between *F. graminearum*-infected waxy and wild-type grain ($P = 0.73$), and total

Table 4. Mean percent of grain infected by *Fusarium graminearum* or other *Fusarium* species as a result of superficial and internal infections occurring during 2013 and 2014 and mean levels of deoxynivalenol (DON) ($\mu\text{g/g}$) in nondisinfested grain from individual waxy breeding lines, the waxy check, and wild-type checks^x

Line	Infected grain (%) ^y						DON (μg/g) ^z
	<i>F. gram.</i>	<i>P</i> _{WT}	<i>P</i> _{OV}	Other <i>Fus. spp.</i>	<i>P</i> _{WT}	<i>P</i> _{OV}	
waxy breeding lines							
PI 677868	5.0	0.48	0.74	53.3	0.09	0.84	0.813 ab
PI 677869	5.0	0.48	0.74	50.0	0.24	0.76	0.508 e
PI 677870	0.0	0.15	0.05	61.7	<0.01	0.13	0.895 a
PI 677872	8.3	0.03	0.45	36.7	0.36	0.02	0.645 cde
PI 677873	3.3	0.99	0.38	56.7	0.03	0.47	0.566 de
PI 677874	11.7	<0.01	0.06	41.7	0.90	0.11	0.508 e
PI 677875	3.3	0.99	0.38	45.0	0.70	0.28	0.543 cde
PI 677876	1.7	0.46	0.16	71.7	<0.01	<0.01	0.510 e
PI 677877	1.7	0.46	0.16	73.3	<0.01	<0.01	0.490 e
PI 677878	2.0	0.59	0.23	44.0	0.83	0.26	0.525 de
NX12Y8219	7.0	0.19	0.79	47.5	0.52	0.57	0.520 de
PI 677881	10.0	<0.01	0.23	40.0	0.72	0.09	0.578 cde
PI 677882	3.3	0.99	0.38	40.0	0.70	0.06	0.597 cde
waxy check							
Mattern	1.7	0.46	0.16	35.0	0.24	<0.01	0.678 bcd
Wild-type checks							
Alice	8.3	0.03	0.45	46.7	0.51	0.41	0.490 e
Camelot	0.0	0.15	0.05	45.0	0.70	0.28	0.508 e
Goodstreak	0.0	0.15	0.05	38.3	0.51	0.03	0.490 e
Infinity CL	5.0	0.48	0.74	33.3	0.15	<0.01	0.490 e
Jagalene	6.7	0.16	0.83	40.0	0.70	0.06	0.542 de
Mace	6.0	0.30	1.00	30.0	0.07	<0.01	0.490 e
McGill	0.0	0.19	0.07	30.0	0.07	<0.01	0.490 e
Millennium	1.7	0.46	0.16	46.7	0.51	0.41	0.490 e
NI08708	6.7	0.16	0.83	63.3	<0.01	0.08	0.710 bc
NW07505	2.0	0.59	0.23	56.0	0.05	0.57	0.490 e
Nuplains	3.3	0.99	0.38	40.0	0.70	0.06	0.645 cde
Overland	6.0	0.30	...	52.0	0.17	...	0.490 e
PI 631352	1.7	0.46	0.16	35.0	0.24	<0.01	0.508 e
PI 667038	6.7	0.16	0.83	43.3	0.90	0.18	0.492 e
Pronghorn	0.0	0.15	0.05	33.3	0.15	<0.01	0.490 e
Robidoux	0.0	0.15	0.05	50.0	0.24	0.76	0.528 de
Settler CL	3.3	0.99	0.38	33.3	0.15	<0.01	0.490 e
Wesley	3.3	0.99	0.38	48.3	0.36	0.57	0.577 cde

^x Breeding lines and checks were grown during 2013 and 2014, three replications per year. Subsamples of harvested grain from each plot were made for determination of fungal colonization and for mycotoxin analysis. DON was the only mycotoxin detected during both years.

^y *Fusarium* spp. colonization was determined by directly applying grain onto a semiselective medium for *Fusarium* spp. Percent of seeds that were infected by *F. graminearum* (*F. gram.*) and by all other *Fusarium* spp. (other *Fus. spp.*) were determined for each plot. Means were determined for each line, and for all wild-type lines, and then χ^2 tests were conducted using PROC FREQ (SAS 2013), comparing each line back to the mean ($x = 0.337$) of all wild-type lines (P_{WT}). Additionally, means from each line were also compared with the mean of the *Fusarium* head blight moderately resistant cultivar, Overland (P_{OV}).

^z Ground grain subsamples were analyzed for mycotoxins at North Dakota State University Diagnostic Laboratory (<https://www.vdl.ndsu.edu/>). For samples with undetectable amounts, the value 0.49 was used for calculations. To compare mean DON levels between all lines, PROC MIXED of SAS/STAT software was utilized (SAS 2013). Values with different letters are significantly different at $P \leq 0.05$. Standard errors for nearly all lines were 0.057 except for PI 677870, PI 677875, NX12Y8219, Mace, McGill, and Settler CL, which were 0.069.

internal infection by *F. graminearum* was only 10.4% of the total *Fusarium* spp. obtained from surface-disinfested grain. The FIESC was the largest group (24.7%) among all the isolates (Fig. 1B), with five known genotypes and two isolates from wild-type grain with unknown genotypes. The ratios of isolates in each FIESC genotype, or the two unknown FIESC genotypes considered together, for isolates in waxy grain, were subjected to χ^2 analyses using ratios of wild-type grain as “expected.” There was a significant difference in the ratios of the different FIESC genotypes isolated from waxy grain (Fig. 1C). Pairwise comparisons within each FIESC genotype did not show significant differences between waxy and wild-type.

Surface and internal Fusarium spp. colonization on waxy and wild-type grain. The percentage of grain with *Fusarium*-like colonies growing onto the surface of the medium was significantly greater for waxy than for wild-type (Table 3). These colonies were subcultured onto PDA and, based on morphological colony characteristics, were assigned to one of two groups: those similar to *F. graminearum* and all other *Fusarium* spp. The percent of waxy grain with *F. graminearum*-like colonies was similar to that of the wild-type, whereas waxy grains with all other *Fusarium* spp., as determined by selection on PCNB medium, were significantly greater than wild-type (Table 3). For surface and internal infections, as determined in non-disinfested grain, individual lines were compared with the mean of all wild-type lines using χ^2 . Among the waxy breeding lines, only three had *F. graminearum* colonization of nondisinfested grain significantly greater than the mean of the wild-type lines during the 2013 and 2014 growing seasons: PI 677872, PI 677874, and PI 677881 (Table 4). All other waxy breeding lines, and the waxy check (Mattern), had levels of *F. graminearum*-like infection not significantly different from the mean of the wild-type lines. Among the wild-type lines, only the cultivar Alice had a significantly greater percent of grain infected with *F. graminearum*-like isolates than the mean of the wild-type lines. Among the waxy breeding lines, four (PI 677870, PI 677873, PI 677876, and PI 677877) had infections by other *Fusarium* spp. significantly greater than the mean across all wild-type (Table 4), whereas two wild-type lines, NI08708 and NW07505, had higher levels than the wild-type mean. Means of each line also were compared with the known FHB moderately resistant wild-type cultivar, Overland. The waxy breeding line PI 677870 and the wild-type cultivars Camelot, Goodstreak, Pronghorn, and Robidoux had percent *F. graminearum*-like grains significantly less than that of Overland (Table 4). All other waxy breeding lines, Mattern, and wild-type lines had percent *F. graminearum*-like infected grain similar to Overland. For percent of grains infected with all other *Fusarium* spp., one waxy breeding line, PI 677872, and six wild-type cultivars (Infinity CL, Mace, McGill, PI 631352, Pronghorn, and Settler CL) had means significantly less than that of Overland (Table 4). Two waxy breeding lines, PI 677876 and PI 677877, had mean percent of grains infected with other *Fusarium* spp. significantly greater than that of Overland. All other lines had levels similar to that of Overland. Correlation analysis demonstrated there was no correlation between the total numbers of *F. graminearum*-like infected grain and total *Fusarium* spp. or other *Fusarium* spp. ($P \geq 0.67$).

DON levels in nondisinfested grain from waxy and wild-type lines. There was a significant difference in mean DON levels across all lines between the two years 2013 (0.614 ± 0.021 $\mu\text{g/g}$) and 2014 (0.493 ± 0.002 $\mu\text{g/g}$) ($P < 0.01$); the mean DON level for 2014

was in the undetectable range. Mean DON levels across years were significantly different for waxy (0.591 ± 0.019) and wild-type (0.524 ± 0.017) grain ($P = 0.01$). Interaction of grain phenotype and year also was significant ($P = 0.03$). Mean DON levels for waxy and wild-type grains are shown in Table 5; for each year, the mean DON level for waxy grain was significantly greater than that for wild-type ($P = 0.02$). Correlation analyses of numbers of internal and surface colonization by *F. graminearum*-like isolates with DON showed no significant association ($r = -0.23$; $P = 0.43$). The highest mean DON level across both years was found in the waxy breeding line PI 677870; this was significantly greater than all the checks, including Mattern, and all the other waxy breeding lines, except PI 677868 (Table 4). Most of the wild-type lines (Alice, Goodstreak, Infinity CL, Mace, McGill, Millennium, NW07505, Overland, PI 667038, and Settler CL) and the waxy breeding line PI 677877 had mean DON levels in the undetectable range.

Experiment 2. FHB severity during the 2015 epidemic. There was no significant difference in mean disease ratings when comparing across all waxy (7.25 ± 1.61) and wild-type (7.78 ± 1.60) lines ($P = 0.09$). Comparison of individual lines showed that the waxy cultivar Mattern and the waxy breeding lines PI 677869, PI 677870, PI 677881, and PI 677882 had similarly high levels of FHB severity, in the range of 8.5 to 9.3 (Table 6). On the other hand, seven waxy breeding lines had FHB severities significantly less than that of Mattern and not significantly different from that of the moderately resistant wild-type cultivar, Overland.

FDK resulting from the 2015 FHB epidemic. There were no significant differences between waxy (0.184) and wild-type (0.190) (SE = 0.04; $P = 0.75$) with respect to proportions of FDK. Seven waxy breeding lines, and the wild-type cultivars Mace and Settler CL, had FDK similar to that of Mattern (Table 6). Six waxy breeding lines had FDK significantly less than that of Mattern, and among them, PI 677873, PI 677874, PI 677876, PI 677877, and PI 677878 all had FDK similar to the moderately resistant wild-type cultivar, Overland ($P \geq 0.09$). Correlation of FHB severity and FDK was significant ($r = 0.74$; $P < 0.01$).

DON and 15-ADON levels in grain from waxy and wild-type lines resulting from the 2015 FHB epidemic. Of the four mycotoxins (DON, 3-ADON, 15-ADON, and nivalenol) evaluated in the 2015 grain, DON and 15-ADON were detected in 100 and 81.6% of the plots, respectively. Comparing the means across all waxy lines with those across wild-type lines showed there were significant differences in the levels of DON and 15-ADON in the waxy lines (Table 7). The mean DON level in Mattern grain was significantly greater than all other lines in the study ($P < 0.01$), whereas Mattern, PI 677868, PI 677869, and PI 677880 all had similarly high levels of 15-ADON (Table 6). The waxy breeding lines PI 677873, PI 677874, PI 677876, PI 677877, and PI 677878, and the wild-type cultivars Mace and Settler CL, all had DON levels not significantly different from the moderately resistant wild-type cultivar, Overland ($P \geq 0.07$), and these, along with PI 677872 and PI 677882, had 15-ADON levels not significantly different from that of Overland ($P \geq 0.06$). The 15-ADON level in Overland grain was in the undetectable range (Table 6). Correlation of FHB severity with DON ($r = 0.49$; $P < 0.01$) or 15-ADON ($r = 0.45$; $P < 0.01$) was significant.

Discussion

Breeding lines that are waxy had been developed to increase the genetic variation for waxy cultivation in the Great Plains and to broaden the usability of wheat overall for modified food starches and specialty baked goods (Graybosch 1998; Graybosch et al. 2018; Kowalski et al. 2017). This region of the United States experiences moderate to severe FHB during intermittent growing seasons (McMullen et al. 2012; Panthi et al. 2014; Shah et al. 2013). Observations of the waxy cultivar Mattern (designated as NX04Y2107) during the USDA-ARS coordinated NRPN, and our observations during other years when grown at Mead, NE, indicated that Mattern appeared to be highly susceptible to this and other grain diseases; therefore, it was imperative to assess responses of the waxy breeding lines to naturally occurring *Fusarium* infections, including by

Table 5. Deoxynivalenol (DON) levels ($\mu\text{g/g}$) in waxy and wild-type grain grown in 2013 and 2014^z

Year	DON ($\mu\text{g/g}$)	
	waxy	Wild-type
2013	$0.667^* \pm 0.036$	0.559 ± 0.032
2014	$0.500^* \pm 0.003$	0.490 ± 0.003

^z To compare mean DON levels between the two plant phenotypes (wild-type and waxy), PROC MIXED of SAS/STAT software was utilized (SAS 2013). Means and standard errors are shown. Asterisks indicate that levels from waxy grain were significantly greater ($P = 0.02$) than those for wild-type.

F. graminearum. Previous studies of *Fusarium* species from air samples taken over research fields at Mead, NE, that included wheat and other grains indicated that *F. graminearum* could be commonly found at critical times during the growing season (Funnell-Harris and Pedersen 2011; Funnell-Harris et al. 2017) depending on environmental conditions conducive to increased sporulation and infection (Duveiller et al. 2007; Leplat et al. 2013). For the three years in which the *waxy* breeding lines were grown, FHB was observed at trace and low levels (2013 and 2014) and at profoundly high, epidemic levels (2015), allowing for assessment of the reaction of these lines to various natural infection levels by *Fusarium* species and *F. graminearum*.

During experiment 1 conducted in the years 2013 and 2014, internal infections by *Fusarium* species, as indicated by fungal growth from surface disinfested grain, were not significantly different (Fig. 1A). However, total external and internal infections by *Fusarium* species, as indicated by direct plating of nondisinfested grain, provided evidence that the *waxy* grains across different lines had higher levels of *Fusarium* species infections than wild-type grains (Table 3). However, mean levels of *F. graminearum* or *F. graminearum*-like infections were not significantly different across *waxy* and wild-type lines when considering trace to low levels of disease observed in the field. Taken together, these results indicated that in 2013 and 2014, differences in internal and external infections by *Fusarium* species in *waxy* and wild-type grain were not owing to *F. graminearum*. Nonetheless, DON levels were significantly higher across *waxy* compared with the wild-type lines.

For experiment 2, the severe levels of FHB observed in 2015 allowed assessment of the response of the *waxy* breeding lines, as well as Mattern and wild-type cultivars, to FHB under high disease intensity during natural infection. In this case, the *waxy* lines had levels of FHB severity and FDK similar to those of the wild-type lines. However, across all *waxy* lines as compared with all wild-type, and across both locations, DON levels were higher in the *waxy* lines. The DON values observed (Tables 6 and 7) were similar to or even

higher than those in previous studies in which high FHB disease severity was observed, whether artificially inoculated (Kharbikar et al. 2015) or resulting from natural infection (Audenaert et al. 2009).

For a meta-analysis reported in 2005 (Paul et al. 2005) of all available studies (published and unpublished) of FHB in naturally or artificially infested fields, individual studies could show strong positive correlations of disease severity (reported as “index” or IND) or FDK with DON, or no significant correlation, or even negative correlation. Across all studies, the strongest significant and positive correlation was between FDK and DON, but disease severity significantly correlated with DON as well. Since that time, some studies with artificial inoculation reported highly significant correlations between FHB disease severity or FDK and DON (Bonin and Kolb 2009; Burlakoti et al. 2007; Kubo et al. 2014), but not all observed this association (Malbrán et al. 2014), suggesting that a given environment can affect DON production. In the current study, experiment 2 data indicated significant correlations between disease severity and FDK or DON levels following a season of natural infections, but there were no significant differences between *waxy* and wild-type

Table 7. Mean deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) levels (μg/g) in grain of *waxy* (breeding lines and check) and wild-type (checks) lines resulting from an epidemic level of *Fusarium graminearum* infection^z

Plant phenotype	DON (μg/g)	15-ADON (μg/g)
Wild-type	15.54 ± 0.76	0.56 ± 0.02
<i>waxy</i>	21.47 ± 1.07	0.76 ± 0.03
<i>P</i>	<0.01	<0.01

^z Grain samples from each plot were analyzed for the mycotoxins DON and 15-ADON at North Dakota State University Diagnostic Laboratory (<https://www.vdl.ndsu.edu/>). The differences of least squares means (LSMs) were determined using the PROC MIXED procedure of SAS/STAT software (SAS 2013). LSMs and standard errors are shown.

Table 6. Mean *Fusarium* head blight (FHB) severity ratings, proportion of *Fusarium*-damaged kernels (FDK), and deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) levels (μg/g) from *waxy* breeding lines and *waxy* and wild-type checks as a result of a severe FHB epidemic during 2015 at two locations in eastern Nebraska^w

Line	FHB severity ^x	FDK ^y	Mycotoxins (µg/g) ^z	
			DON	15-ADON
waxy breeding lines				
PI 677868	7.7 cd	0.174 ± 0.031 bc	25.90 bcd	0.98 ab
PI 677869	8.5 abc	0.206 ± 0.031 ab	26.87 bc	0.93 abc
PI 677870	9.0 abc	0.242 ± 0.035 a	28.93 b	0.93 abc
PI 677872	7.5 cd	0.257 ± 0.042 a	19.73 de	0.62 def
PI 677873	4.8 e	0.116 ± 0.031 ef	15.62 ef	0.51 f
PI 677874	5.5 e	0.140 ± 0.032 cdef	13.20 f	0.54 f
PI 677875	8.2 bc	0.176 ± 0.038 abcde	25.48 bcd	0.83 bcd
PI 677876	5.2 e	0.128 ± 0.036 cdef	12.92 f	0.53 f
PI 677877	5.2 e	0.119 ± 0.030 ef	13.25 f	0.51 f
PI 677878	5.2 e	0.104 ± 0.032 f	16.47 ef	0.63 def
NX12Y8219	8.2 bc	0.235 ± 0.041 ab	21.77 cde	0.90 bc
PI 677881	9.2 a	0.239 ± 0.038 a	19.80 de	0.78 bcde
PI 677882	8.8 ab	0.228 ± 0.039 ab	18.80 e	0.72 cdef
Checks				
waxy				
Mattern	9.3 a	0.230 ± 0.035 a	41.78 a	1.15 a
Wild-type				
Mace	8.2 abc	0.230 ± 0.038 ab	15.75 ef	0.56 ef
Overland	6.2 de	0.134 ± 0.030 def	12.53 f	0.49 f
Settler CL	8.3 abc	0.199 ± 0.039 abcd	18.33 ef	0.62 def

^w Lines were planted in three randomized complete blocks at each location. Disease severity was recorded for each plot, based on a 1 to 10 linear scale. Throughout this table, PROC MIXED of SAS/STAT software (SAS 2013) was used to compare least squares means between lines. Values with different letters are significantly different at $P \leq 0.05$.

^x Disease severity was assessed on a “per plot” basis and visually scored using a 1 to 10 linear scale of the proportion of bleached spikelets per head, with 1 being no evidence of disease and 10 being highly susceptible (96 to 100%). Standard errors (SEs) for PI 677870, PI 677872, PI 677873, PI 677876, PI 677878, Mattern, and Overland were 1.6; all other SEs were 1.5.

^y The proportion of grain in each plot with evidence of *F. graminearum* damage was determined.

^z Ground grain subsamples were analyzed for mycotoxins at North Dakota State University Diagnostic Laboratory (<https://www.vdl.ndsu.edu/>). For samples with undetectable amounts, the value 0.49 was used for calculations. DON SE = 3.17; 15-ADON SE = 0.10.

for disease severity or FDK. However, in both experiments 1 and 2, DON levels were significantly higher in *waxy* grain compared with wild-type (Tables 5 and 7). These seemingly contradictory results could be explained by a number of factors. For example, the fungal populations detected during experiment 1 indicated that those infecting the *waxy* PIs and Mattern were different from those in the wild-type cultivars chosen for these studies. *Fusarium* spp. other than *F. graminearum* also can produce DON, including those detected as internal infections in experiment 1, such as *F. acuminatum*, *F. oxysporum*, *F. poae*, *F. proliferatum*, and members of the FIESC (Hubert et al. 2014; Lincy et al. 2011; Marín et al. 2012; Richard et al. 2007); isolates of some of these species may have been included with the “other *Fusarium* spp.” from the internal and external infection screen, a category that was significantly larger in *waxy* grain than in wild-type (Fig. 1, Table 3). Another possible explanation is the previously observed translocation of DON from crown infections to asymptomatic heads of wheat by *F. graminearum* (Beccari et al. 2018; Covarelli et al. 2012); this explanation would assume that infection or translocation was greater or more efficient, respectively, among *waxy* lines than wild-type. Although there were no observed seedling diseases during the three years of this study, this phenomenon can occur during asymptomatic infections (Obanor and Chakraborty 2014).

Another possible explanation is that because the basic starch structure is modified in *waxy* grains, the fungus may be able to utilize additional components for greater DON production. The *waxy* trait is known to affect structure of the endosperm and of starch granules (Demeke et al. 1999; Kang et al. 2006; Sattler et al. 2009). Because physicochemical, morphological, and structural characteristics are affected in *waxy* wheat, amylose content is generally inversely proportional to starch digestibility (Shevkani et al. 2017). Partial *waxy* wheat grains have been shown to have significantly increased levels of mono- and polysaccharides such as sucrose, glucose, maltose, and β -glucans (Nakamura et al. 2006; Yasui and Ashida 2011). Genes of the *F. graminearum* trichothecene (*Tri*) biosynthesis pathway that encode for enzymes that produce DON and its acetylated derivatives 3-ADON and 15-ADON, as well as nivalenol and its acetylated derivative 4-acetyl nivalenol, can be induced in vitro using different carbon sources. For example, it was shown that *Tri5* (encoding for trichodiene synthase) and *Tri4* (encoding for a cytochrome P450 mono-oxygenase that hydroxylates trichodiene) are upregulated by sucrose but not glucose (Jiao et al. 2008; Tokai et al. 2007). Because in vitro culture of *Fusarium* spp. production of DON and other mycotoxins is conducted in media rich in carbohydrates (Jiao et al. 2008; Varga et al. 2015; Vasavada and Hsieh 1987), it is possible that increased availability of sugars, as a result of increased digestibility of *waxy* starch, could induce greater colonization and DON production by *F. graminearum* in *waxy* grains than in wild-type. It is likely that multiple factors, perhaps one or more discussed herein, are involved in the relatively high DON levels observed in some *waxy* lines during high FHB severity.

Nonetheless, this work strongly indicated that among these *waxy* breeding lines were individual lines having desirable agronomic and quality traits, such as adequate grain and flour protein amounts and no apparent grain yield drag (Graybosch et al. 2018), with responses to *F. graminearum* infection (infection rates and DON levels) similar to elite wild-type cultivars. For experiment 1, with trace and low levels of FHB (2013 and 2014), only three of the 13 lines had significantly higher levels of *F. graminearum*-like contamination, and only two lines had significantly higher levels of DON than most of the wild-type lines (Table 4). All the *waxy* breeding lines had *F. graminearum*-like infection levels the same or less (PI 677870) than that of the moderately resistant wild-type cultivar, Overland, and most lines (except PI 677870 and PI 677868) had DON levels similar to Overland (Table 4). For experiment 2, with high FHB intensity, five (PI 677873, PI 677874, PI 677876, PI 677877, and PI 677878) of the *waxy* breeding lines had severity ratings, percent FDK, and DON and 15-ADON levels significantly lower than means observed in cultivar Mattern, and not significantly different from those of Overland (Table 6). These results indicated

that these five lines have great potential for breeding *waxy* lines with reduced susceptibility to FHB under both low and high disease severity.

Understandably, much attention and effort have been placed on FHB pathogens, but there has been little emphasis on other wheat-infecting *Fusarium* species that may also impact the response of wheat to FHB (Kosiak et al. 2004; Muthomi et al. 2008). In the present study the wide range of *Fusarium* spp. other than *F. graminearum* that were detected causing internal infections of the grain (Fig. 1) and the large numbers of other *Fusarium* spp. recovered from the surface, as well as internally (Tables 3 and 4), suggests that toxins besides DON may be present in wheat samples, such as moniliformin, HT-2, T-2, and diacetoxyscirpenol (Covarelli et al. 2015; Tittlemier et al. 2013). Early infections by other fungi could conceivably reduce subsequent infections by *F. graminearum* (Aureli et al. 2015; Jensen et al. 2016; Kosiak et al. 2004), although an inverse correlation of these infections was not observed in the present work. An unexpected result of the current study was that internal infection ratios of *Fusarium* species or species complexes were significantly different between the *waxy* lines and the wild-type lines chosen for this study. Although the *waxy* trait may have caused differential infection by *Fusarium* spp., the genetic backgrounds of the *waxy* lines used, as well as wild-type lines, may also have affected the distribution of *Fusarium* species in the grain (Funnell and Pedersen 2006; Stack and Pedersen 2003).

In summary, the screening of *waxy* breeding lines under trace to low levels of FHB allowed for determining response to internal and superficial infections by *Fusarium* species, including *F. graminearum* (experiment 1). Using surface disinfection before plating and direct plating of nondisinfested grain onto *Fusarium*-selective medium allowed determination that lines PI 677876 and PI 677877 were the most resistant to infections by *F. graminearum* and *Fusarium* spp. similar to *F. graminearum* and had the lowest levels of DON accumulation among the *waxy* lines tested under these conditions (Fig. 1, Table 4). Analysis of disease response parameters during an FHB epidemic year (experiment 2) confirmed that the same two lines, as well as three more lines (PI 677873, PI 677874, and PI 677878) had responses similar to the moderately resistant wild-type cultivar, Overland. These results provided strong evidence that the *waxy* PIs would be good candidates for breeding *waxy* lines with wild-type levels of infection by *Fusarium* spp. and tolerance to *F. graminearum*. Techniques described in this study, such as the combination of direct plating of grain onto PCNB with assessing DON levels, may have great potential for use in breeding programs for screening materials for reduced FHB disease even under low levels of *F. graminearum* infection. This could be especially important in the Great Plains wheat belt, because natural FHB infestations are intermittent but can be devastating, as in 2015, when they occur (McMullen et al. 2012).

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