Comparison of ELISA for Fusarium, Visual Screening, and Deoxynivalenol Analysis of Fusarium Head Blight for Barley Field Nurseries

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ABSTRACT

Breeding for resistance to Fusarium head blight {FHB; caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch]} is complicated because there is no accurate method for quantifying the disease organism. Most breeders rely on visual scoring for FHB and deoxynivalenol (DON) analysis to assess disease severity, but both DON and visual scoring are subject to error. The objective of this study was to compare a *Fusarium*-specific quantifiable enzyme-linked immunosorbent assay (ELISA) to DON and visual assessment of FHB. A doubled-haploid mapping population was grown in two environments and breeding lines in the North American Barley Scab Evaluation Nursery (NABSEN) were grown at four locations. Both experiments used a randomized complete block design and were analyzed for Fusarium by ELISA, DON, and visually scored for FHB. ELISA values for the doubled-haploid lines were consistent over years, and lines that were low in ELISA were also low in DON. Broad sense heritability was greater for ELISA (0.48) than DON (0.19) or visual scores of disease (0.29) in the NABSEN study. Numbers of locations and replications necessary to screen for disease were calculated for ELISA, DON, and FHB. ELISA required one-third to one-fourth as many replications and locations as did DON or FHB. Temperature and osmotic potential had little effect on mycelial antigen used for ELISA in vitro, but both affected DON. ELISA is a practical alternative to combined visual scores and DON analysis for breeders interested in improving FHB resistance.

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Abbreviations: DON, deoxynivalenol; ELISA, enzyme-linked immunosorbent assay; FHB, Fusarium head blight; NABSEN, North American Barley Scab Evaluation Nursery.

Fusarium head blight (FHB) of barley (*Hordeum vulgare* L.) and other small grain cereals is an economically destructive disease caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] and related trichothecene toxin–producing fusaria (Rudd et al., 2001). Screening for FHB involves inoculation of the developing spike followed by visual screening of disease development. Because disease development is dependent on environmental conditions, sequential observations are necessary to assess the disease. Chemical analysis for deoxynivalenol (DON) is also performed, but disease development and toxin production are frequently poorly correlated (de la Pena et al., 1999).

Numerous variables affect fungal colonization of the grain spike which include cultural practices, spike morphology, canopy density, plant height, rainfall, relative humidity, temperature, and host plant genetic resistance (Teich et al., 1987; Rudd et al., 2001;

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Kolb et al., 2001). The number of perithecia growing on corn (*Zea mays* L.) stalk residue (during the sexual stage of the disease cycle) increases with increasing duration of stalk wetness (Dufault et al., 2002a) and the number of days with average daily temperatures between 15 and 25°C (Dufault et al., 2002a, 2002b). Increasing relative humidity also increases perithecia development (Dufault et al., 2002b). Thus, quantity of disease inoculum is controlled by these environmental conditions. Moisture, applied during and after anthesis in the form of misting, also affects disease development and DON production in wheat (Nita et al., 2005). Matric potential also affects growth of *F. graminearum* (Ramirez et al., 2004). The matric potential of media is correlated to mycelial water potential, and spore germination and mycelial growth are greatest when water potential is greater than −1.4 MPa and growth rate is faster at 25°C than at 15°C. Hence, growth of the organism and expression of disease symptoms are confounded by numerous environmental and genetic variables. To further complicate analysis for FHB, asymptomatic kernels may contain significant amounts of DON while symptomatic kernels within the same samples may not (Dowell et al., 1999; Champeil et al., 2004; Liu et al., 1997; Hill et al., 2006; Nita et al., 2005). Thus, a more direct method of measuring *Fusarium* spp. infection would be desirable to (i) reduce the number of analyses necessary to accurately assess *Fusarium* infection, (ii) eliminate apparent pleiotropy between disease symptomology and mycotoxin production, (iii) reduce error associated with environmental effects on disease assessment (i.e., DON) and (iv) reduce error associated with asymptomatic expression of FHB.

Ergosterol has been used with varying success to estimate the colonization of grain by *Fusarium* spp. in controlled experiments (Abramson et al., 1998; Muthomi et al., 2002; Reid et al., 1999). Ergosterol sample preparation and analysis is tedious (Varga et al., 2006), it is a nonspecific measure for fungal mycelia (Nes, 1974), and its abundance relative to mycelial mass varies among fungal species (Pasanen et al., 1999). Therefore it is unlikely that ergosterol will serve as a good chemical marker to quantify fungal mass in samples that are likely to be contaminated with other fungal species. Enzyme-linked immunosorbent assays (ELISA) are another direct measure of *Fusarium* spp. mycelia (Abramson et al., 1998; Gan et al., 1997; Hill et al., 2006; Iyer and Cousin, 2003). Advantages to using ELISA over ergosterol are the specificity of fungal antigens (Hill et al., 1998), ease of sample preparation, low volume and cost of reagents, and use of nonhazardous aqueous buffers in the analysis (Hill et al., 1998). Recently we reported that an ELISA method using antibodies specific to *Fusarium* spp. had lower coefficients of variability than FHB scores or DON analysis in a field experiment (Hill et al., 2006). ELISA values were not highly correlated with DON or visual assessments of FHB in that study, but lines selected for low ELISA had low DON and FHB scores suggesting that modest changes in amount of *Fusarium* quantified by ELISA can have a significant impact on FHB symptoms and DON. The objectives of this study were to (i) assess broad-sense heritability and resource allocation when screening for FHB resistance using visual scores of disease severity, DON, and ELISA as disease indices and (ii) assess environmental and biotic factors affecting abundance of mycelial antigen and DON production by *Fusarium* spp. cultured in vitro.

MATERIALS AND METHODS

Experiment 1: Field Evaluations

Doubled-Haploid Mapping Population

Evaluation of the ELISA method in an inoculated and mist-irrigated field trial used a set of 15 doubled-haploid lines from a 'Zhedar2'/ND9712//'Foster' mapping population that were selected for high, medium, or low ELISA values. The doubled-haploid lines were planted in hill plots spaced 0.30 m apart with 15 seeds per plot at Osnabrock, ND, in May 2003 and May 2004. The entries were planted in a randomized complete block design with two replications. Field inoculation with *F. graminearum* was conducted by infesting autoclaved barley and maize grain with a mixture of five *F. graminearum* isolates (North Dakota isolates 172, 173, 176, 582, and 672) and spreading 50 g of infested grain per square meter in the experimental plots approximately 1 wk before heading and a second time when 50% of the heads in a plot had emerged from the flag leaf. Overhead mist-irrigation for 30 s every half hour from 0400 to 0800 h and again from 1800 to 2000 h was used to promote infection in the nursery. The rate of irrigation was approximately 2300 L ha^{-1} min^{-1}.

Ten spikes from each plot were randomly selected at the soft-dough stage and scored for FHB severity by determining the percentage of the seed expressing damage typical of *Fusarium* infestation. The seedheads were hand-harvested at maturity, the grain threshed, analyzed for DON by gas chromatography—mass spectrometry (Tacke and Casper, 1996) and *Fusarium* proteins by ELISA.

North American Barley Scab Evaluation Nursery (NABSEN)

The second field study utilized plots from four locations that were planted to the NABSEN. The NABSEN format was chosen because it is a uniform test for FHB response for 48 elite barley lines plus resistant, moderately susceptible, and susceptible check lines. Seed from the entries were planted in 1.5 by 6.2 m plots at Fargo, Casselton, and Langdon, ND, and St. Paul, MN, in May 2004. All locations, except Casselton, were grown under mist-irrigated conditions. Field inoculation with *F. graminearum* was conducted at the North Dakota locations as described above. Inoculations were performed in Minnesota using 50 isolates of *F. graminearum*

grown separately under laboratory conditions and mixed to a final concentration of 1×10^5 macroconidia per milliliter of media. The macroconidia were applied using a CO_2 powered backpack sprayer at heading and again 3 d later. Disease assessments were made on 10 to 20 randomly selected spikes of each line at the mid-dough stage of kernel development, approximately 14 to 17 d after inoculation. The severity of FHB was determined at all locations by dividing the total number of infected spikelets by the total number of spikelets (Prom et al., 1997). Plots were harvested at grain maturity using a small plot harvester.

Deoxynivalenol Analysis

Seed harvested from the plots were analyzed for DON using the methods of Tacke and Casper (1996). Five grams of ground seed from each plot were placed into tubes containing a 40-mL 86:16 acetonitrile/water mixture. Samples were extracted by placing the tubes on a shaker for 1 h. Extract (4 mL) was passed through an Alltech C:18 clean-up column (Alltech Associates Inc., Deerfield, IL) and 2 mL of the elution was transferred to a clean test tube and dried under water-free nitrogen at 55°C. The DON was then derivatized with 100 μL of a 10:1 mixture of trimethylsilyimidazole and trimethylchlorosilane for 1 h. A 3% sodium carbonate solution was added to this mixture to neutralize the derivatives. One milliliter of iso-octane, with 0.5 mg L^{-1} mirex (ULTRA Scientific, N. Kingstown, RI), was added to the solution and shaken for 10 min to extract the derivatized DON into the iso-octane layer. The iso-octane solution (1 mL) was injected into a gas chromatograph with an electron capture detector to verify the identity and determine the concentration of the toxin.

Fusarium Quantification by ELISA

Fusarium spp. were quantified on harvested seed using the genus-specific monoclonal antibody from cell line IF8 in an ELISA format (Hill et al., 2006). All extraction and dilution buffers and chromogenic reagents for immunoquantification of *F. graminearum* were generously provided by Agrinostics Ltd. Co. (Watkinsville, GA). *Fusarium* proteins were extracted by placing 5 g of whole grain into 50-mL disposable centrifuge tubes. Thirty milliliters of fungal extraction buffer was added using a Filamatic vial filler (National Instrument Co., Baltimore, MD). The tubes were placed into racks, positioned horizontally on a rotary shaker (Eberbach Labtools, Ann Arbor, MI) at 75 rpm for 1 h. The extraction buffer was decanted from the seed, a 1-mL subsample centrifuged at $10{,}000 \times g$ to remove particulate matter, and then the particle-free subsample was diluted 1:6 in a microtiter plate coating buffer. Fifty microliters of the diluted samples were placed into wells of Dynex Immulon IV (Thermo Labsystems, Franklin, MA) microtiter plates for the ELISA analysis. All samples were analyzed in duplicate.

Statistical Analysis

Combined analysis of variance was performed across environments for FHB severity, DON, and ELISA using the PROC MIXED procedure of SAS (SAS Institute, 2002). Germplasm was considered a fixed effect while environments (years or locations) were considered random effects. Mean separation was performed using a Fisher's protected LSD at the 0.05 probability level. Linear regression was conducted for the mean ELISA values and mean DON values using the PROC REG procedure of SAS.

Variance components for FHB severity, DON, and ELISA were calculated within the NABSEN data set for the genotype × location interaction as well as the error term associated with replications within locations. The variance components for FHB severity, DON, and ELISA were used to calculate the number of replications (R) required to detect differences between two genotypes using a modification of the method of Mendenhall and Scheaffer (1973) in Eq. [1]:

$$R \geq 2\left(t_{\alpha/2} + t_{\beta}\right)^2 s^2 \Big/ d^2 \qquad [1]$$

where $t_{\alpha/2}$ is the tabular t value with a probability value of 0.05, t_{β} is the t value associated with accepting a false null hypothesis ($\beta = 0.25$), s^2 the error variance, and d^2 the difference between genotypic means for head blight index (ELISA, FHB, or DON) expressed as a percentage of the mean.

A modification of Eq. [1] was used to determine the number of environments (E) required to show specific differences between treatments when a genotype × environment interaction is present in the experiment (Adcock et al., 1997):

$$E \geq 2\left(t_{\alpha/2} + t_{\beta}\right)^2 \left[s_1{}^2 + \left(s_1{}^2 - s_2{}^2\right)\Big/ R\right] \Big/ d^2 \qquad [2]$$

where $t_{\alpha/2}$ is the tabular t value with a probability value of 0.05; t_{β} is the t value associated with accepting a false null hypothesis ($\beta = 0.25$); $s_1{}^2$ is the residual error variance; $s_2{}^2$ is the error variance associated with replications within a location, R is the number of replications within a location; and d^2 is the difference between genotypic means for head blight index (ELISA, FHB, or DON) expressed as a percentage of the mean.

Data were used to calculate the number of replications and environments necessary when d = 10, 25, and 50% of the mean.

Estimates of the genotype variance (σ^2_G), genotype × environment variance (σ^2_{EG}), and error variance (σ_e) were based on expected mean squares and calculated by the mixed procedure in SAS (SAS Institute, 2002) for the NABSEN study. Broad sense heritability (H) was calculated for ELISA, DON, and FHB from these estimates of variance. If the genotype × environment interaction was significant, H was calculated as

$$H = \sigma^2_G \Big/ \left(\sigma^2_G + \sigma^2_{EG}/R + \sigma_e/RE\right) \qquad [3]$$

where R is the number of replications and E is the number of environments (locations). If the genotype × environment interaction was not significant, H was calculated as

$$H = \sigma_G^2 \Big/ \left(\sigma_G^2 + \sigma_e / RE\right) \quad [4]$$

Experiment 2: Abundance of Mycelial Antigen and Deoxynivalenol in *Fusarium* spp. Cultured in Vitro

Varying Laboratory Environmental Conditions

Corn meal malt agar was osmotically adjusted by the addition of potassium chloride (KCl) to −0.08, −0.27, and −0.77 MPa. Circular pieces of Spectra/Por dialysis tubing (Spectrum Co., Gardena, CA; 120-mm diam.) were placed on the surface of the solidified agar. Tufts of mycelium from *F. graminearum* isolate AEG 00-01 were placed onto the center of the dialysis tubing and the petri dishes placed into matched low-temperature environmental incubators (Precision Scientific, Chicago, IL) set at 15, 25, or 35°C. After 7 d the plates were removed from the incubators and the mycelium was scraped from the surface of the dialysis tubing. The mycelium was freeze-dried, weighed, and ground to pass through a 1-mm mesh screen.

Water potential of the media containing different amounts of salt was measured on a subset of five agar plates for each salt treatment. Solidified agar plugs were sampled from each plate using a 0.5-cm cork borer. The agar plugs were placed into precalibrated end-window thermocouple psychrometers (Model 85-12V, J.R.D. Merrill Specialty Equipment Corp., Logan, UT) within 15 s of sampling to measure water potential. The chambers were placed into an isothermal water bath at 25°C for 4 h before measuring water potential of the agar plugs.

A 10-mg subsample of freeze-dried and ground mycelium was placed into a 1.5-mL microcentrifuge tube and DON was extracted in 1 mL distilled water for 1 h. The mixture was centrifuged at 10,000 × *g* to clarify the extract and then analyzed for DON using the Veratox 5/5 ELISA kit (Neogen Co., East Lansing, MI). *Fusarium* antigen was extracted from the mycelium and analyzed by ELISA as described above.

Statistical Analysis

The experimental design was a split-block design with temperature treatments serving as main plots and the water potential treatments randomly assigned as subplots within the main plots. The experiment was replicated in time (three replications total) by randomly assigning the environmental chambers to the temperature treatments and repeating the experiment exactly as described. All treatment variables were considered fixed effects when analyzing the data using the Proc ANOVA subroutine of SAS (SAS Institute, 2002). Mean separations were performed using a Fisher's protected LSD at the 0.05 probability level.

Abundance of Antigen in Mycelium from Isolates from Fusarium *spp.*

In order to best estimate the validity of the ELISA analysis, we used isolates of fusaria from the B clade [identified by O'Donnell et al. (2004) as *F. graminearum*, *F. pseudograminearum* T. Aoki and O'Donnell, *F. austroamericanum* T. Aoki, Kistler, Geiser, and O'Donnell, *F. boothii* O'Donnell, T. Aoki, Kistler, and Geiser, *F. asiaticum* O'Donnel, T. Aoki, Kistler, and Geiser, and *F. culmorum* (W.G. Smith) Sacc.] and fusaria from phylogenetically removed species [*F. proliferatum* (T. Matsushima) Nirenberg, *F. solani* (Mart.) Sacc., *F. subglutinans* (Wollenw. and Reinking) Nelson, and *F. verticillioides* (Sacc.) Nirenberg] for this study. The B clade of fusaria are a phylogenetically related group of fungi that, collectively, produce head blight symptoms on cereals. *Fusarium* spp. isolates were inoculated in Shenk-Hildebrandt medium (Sigma-Aldrich, St. Louis, MO) and agitated on a rotary shaker set at 150 rpm. After 5 d of continuous agitation, mycelia were separated from the media using commercial coffee filters and the mycelium was washed with three equivalent volumes of distilled water. The washed mycelium was freeze-dried and weighed. The dried mycelium was ground through a 1-mm wire mesh screen. Ten milligrams of dried mycelium from each *Fusarium* spp. was weighed and placed into 1.5-mL microcentrifuge tubes and 1 mL of 150 mM NaCl extraction buffer was added. The tubes were shaken on an Eberbach EL600 shaker (Ann Arbor, MI) at 150 rpm for 60 min. The tubes were centrifuged at 5000 × *g* and the aqueous fraction serially diluted from 1:10 to 1:5000 in 150 mM NaCl. *Fusarium* antigen was quantified by ELISA as previously described. Dilutions at which the ELISA values were one-half of the maximum were recorded and concentrations per unit of mycelium calculated from standard curves of *Fusarium* protein extracts.

Statistical Analysis

The experiment was arranged in a completely random design with treatments (*Fusarium* species) replicated three times. Dry weight and ELISA data were analyzed using the PROC ANOVA subroutine of SAS version 8.2 (SAS Institute, 2002). Mean weight and antigen concentrations for the *Fusarium* species were separated using a Fisher's protected LSD at the 0.05 probability level.

RESULTS AND DISCUSSION

Experiment 1: Field Evaluations

Doubled-Haploid Mapping Population

The doubled-haploid lines used in this experiment were previously selected for high, medium, or low ELISA values. Given that the antibodies used in the ELISA analysis appear to be specific to *Fusarium* spp., it is assumed that lines with high, medium, and low ELISA values ranged from heavily to lightly colonized lines. Fusarium head

blight visual scores ranged from 3.3 to 32.3% infection with values of 3.3 and 21.8 for the resistant and susceptible checks in the experiment (Table 1). Analysis of variance indicated there was a genotype effect on FHB, but there was no environment (year) or genotype × environment interaction. However, there was significant variability among the FHB data resulting in a high coefficient of variation (CV = 61%) and the error variance represented a large amount of the total variation (0.30). Mean DON values ranged from 4.2 to 53.6 μg g^{-1} seed with values of 4.2 and 30.5 for the resistant and susceptible checks in the experiment. There was a genotype and genotype × environment effect for the DON data. There was significant variability among the DON data, resulting in a high CV (32%) and an error variance representing 10% of the total variation in the experiment. The ELISA data ranged from 42 to 125 μg g^{-1} seed and the resistant and susceptible checks in the population had mean ELISA values of 42 and 108 μg g^{-1} seed. There was a genotype but no environment or genotype × environment interaction for the ELISA data. The ELISA data had the least amount of variation of the three head blight indices as indicated by the low CV (9%) and low proportion of error variance relative to the total variance (0.05). The large amount of variation for the FHB data suggests that lack of a genotype × environment effect was likely due to error. However, low coefficients of variation for the ELISA data indicates that a lack of genotype × year interaction was predicated less on error and more on the genetic potential of the host plant to limit *Fusarium* growth. Therefore broad sense heritabilities were calculated to estimate the how much phenotypic variation in the statistical model was associated with genetic variation. Broad sense heritabilities were 0.91, 0.55, and 0.74 for ELISA, FHB, and DON. Mean ELISA values were regressed with mean DON values. There was a significant linear relationship between ELISA and DON (Fig. 1). Genotypes with low ELISA values were low in DON, genotypes with high ELISA values were high in DON, and genotypes with medium ELISA values tended to be in the medium range of DON values in the plant population. Thus, selection for low ELISA among the doubled haploid population resulted in low DON, and the low ELISA trait was consistent between years.

Less ELISA variation suggests that fewer experimental resources are necessary if evaluation of head blight resistance is conducted by ELISA rather than FHB severity or DON analysis. However, this experiment was limited because (i) only a limited number of plant genotypes were used in the study and (ii) it could be argued that repeating the experiment in the same location is not a true environmental effect. Thus we sought to perform a more robust analysis of head blight assessment using grain harvested from the NABSEN.

North American Barley Scab Evaluation Nursery

The NABSEN was considered an excellent opportunity to evaluate resource allocation for testing using FHB severity, DON, and ELISA. The locations included in this study were selected because they provide known environmental or biological variability under which the methods for head blight assessment could be compared. The nursery was established under rain-fed conditions at Casselton, ND, and under mist-irrigated conditions at all other locations. Fargo is located approximately 20 km east of Casselton and aside from irrigation, rainfall and temperature were essentially identical for the two locations. Langdon is located approximately 250 km northwest of Fargo. It is situated in a valley that is characterized by high humidity

Table 1. Analysis of variance for enzyme-linked immunosorbent assay (ELISA) for *Fusarium*, visual scores for Fusarium head bight (FHB) severity, and deoxynivalenol (DON) in 15 double haploid lines planted at Osnabrock, ND, in 2003 and 2004.

Source of variation	df	ELISA	FHB	DON	Expected mean squares†	
					G × Y significant	G × Y not significant
Environment (E)	1	ns‡	ns	ns		
Rep within E	2	441	299	184		
Genotype (G)	14	1407**	206**	867**	$\sigma^2_e + 2\sigma^2_{GY} + 4\,\sigma^2_G$	$\sigma^2_e + 4\,\sigma^2_G$
G × E	14	ns	ns	187*	$\sigma^2_e + 2\sigma^2_Y$	–
Error	28 [43]§	64	62	85	σ^2_e	σ^2_e
Range of means for lines		42–125 μg g^{-1} seed	3.3–32.3%	4.2–53.6 μg g^{-1} seed		
Foster (susceptible check)		108	21.8	30.5		
Zhedar 2 (resistant check)		42	3.3	4.2		
Coefficient of variation		9	61	32		
Error variance as proportion of total		0.05	0.30	0.10		
Broad sense heritability estimate (SE)		0.91 (0.05)	0.55 (0.19)	0.74 (0.12)		

*Significant at the 0.05 probability level.

**Significance at the 0.01 probability level.

†G × Y, genotype × year interaction.

‡ns, not significant.

§Degrees of freedom in error term when the genotype × environment interaction was not significant.

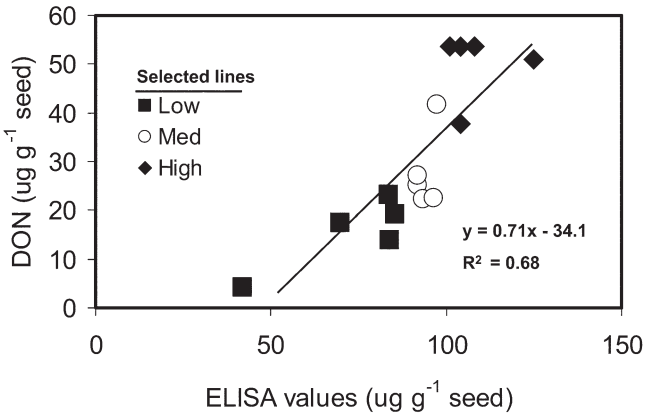

Figure 1. Scattergram of mean enzyme-linked immunosorbent (ELISA) antigen values versus mean deoxynivalenol (DON) values for doubled haploid genotypes of barley grown in Osnabrock, ND. Data represents means of two replications of data from barley grown in 2003 and 2004.

and dew in the early morning hours of the day. Rainfall in Langdon was less and temperature was generally cooler than at Fargo and Casselton throughout the growing season (Table 2). St. Paul had warmer days but cooler nights, and more rainfall, than the other locations. The St. Paul location was also unique in that the *Fusarium* inoculum used was a suspension of macroconidia. The inoculum used at the other locations was laboratory-colonized corn and barley kernels. Thus, the four locations selected for this experiment had diverse experimental conditions.

Analysis of variance indicated there were significant differences among environment (location), genotypes, and genotype × environment interaction for ELISA and DON in the NABSEN study (Table 3). There was also a genotype and environment effect for FHB scores, but no genotype × environment interaction. The range of the genotypic means for the three indices of head blight for the breeding lines within the NABSEN data was less than that in the doubled haploid study. Susceptible, moderately resistant, and resistant check varieties ('Stander', 'Conlon', and 'Chevron') ranged as such for ELISA and DON, but the FHB scores for Stander and Conlon were similar.

Table 2. Environmental conditions for the locations at which the North American Barley Scab Evaluation Nursery plots were planted in 2004.

Location	Month	Temperature			Precipitation
		High	Low	Mean	
		—— °C ——			cm
Fargo† and Casselton, ND	May	16.8	5.8	11.3	14.6
	June	22.9	10.6	16.8	2.4
	July	25.7	14.2	15.0	9.7
	Aug.	22.8	10.7	16.8	4.5
Langdon, ND†	May	13.5	2.3	7.9	12.2
	June	19.8	8.1	14.0	3.1
	July	23.6	11.9	17.3	5.3
	Aug.	19.9	8.4	14.2	7.3
St. Paul, MN†	May	31.1	0.3	15.7	15.8
	June	34.4	6.1	20.3	8.6
	July	34.4	10.6	17.8	12.2
	Aug.	30.0	3.9	17.0	3.7

†Mist irrigated.

It might be expected that both DON and FHB indices of head blight infection would have less experimental variation in the NABSEN study because the breeding lines were selected using these two methods. However, there was more variation for FHB scores and DON than for ELISA. The CVs for FHB and DON were 51 and 54%, while that for ELISA was 26% (Table 3). The error variance as a proportion of the total variance was 0.18, 0.17, and 0.05 for FHB, DON, and ELISA. Broad sense heritabilities were 0.19, 0.29, and 0.46 for FHB, DON, and ELISA. Thus, even though the NABSEN breeding lines were selected based on FHB and DON analyses, ELISA provided data which accounted for more of the phenotypic variation due to the genetic variation of the breeding lines than either FHB or DON.

The statistical parameters from the NABSEN experiment were used as an estimate of variation for FHB experiments to calculate resource allocation for specific mean separation conditions in which (i) no genotype × environment interactions exist (number of replications within one location) and (ii) the number of locations (environments) needed using different numbers of replications at each location. The NABSEN experiment was chosen because it contained data from four unique environments and had a common set of individuals with greater genetic diversity than the doubled haploid population. The doubled-haploid population was tested under two environments (years) but at only one location. Thus, the errors of analysis for the variables of FHB from the NABSEN study are likely more representative of true error than that of the doubled-haploid study. Equation [1] was used to calculate the optimum number of replications within a single environment, but assumes no genotype × environment interaction as was the case in the doubled-haploid experiment reported herein. The number of replications necessary to detect differences between two individuals with an LSD value representative of 50% of the response means would be impractical with the exception of using the ELISA method for disease assessment (Table 4). However, the genotype × environment interaction was significant in the NABSEN experiment, so the numbers of environments needed to test for specific differences among the genotypes was calculated for varying numbers of replications using Eq. [2]. Increasing replications reduced the number of environments necessary to test for specific differences for FHB severity, DON, and ELISA (Table 5). At least two replications for genotypes planted in two environments are necessary to detect differences equal to 50%,

Table 3. Analysis of variance table showing mean squares for sources of variation of enzyme-linked immunosorbent assay (ELISA), Fusarium head blight scores (FHB), and deoxynivalenol (DON) for the North American Barley Scab Evaluation Nursery study.

Source of variation	df	ELISA	FHB	DON	Expected mean squares	
					G × E significant	G × E not significant
Total	623					
Environment (E)	3	379,556**	6896**	1234**		
Replication within E	6	23,528	393	673		
Genotype (G)	51	1224**	43**	124**	$\sigma^2_e + 3\sigma^2_{EG} + 12\,\sigma^2_G$	$\sigma^2_e + 12\,\sigma^2_G$
G × E	153	614*	ns†	97**	$\sigma^2_e + 3\sigma^2_E$	–
Error (e)	408 [562]‡	350	35	26	σ^2_e	σ^2_e
Range of means for breeding lines		47–91 μg g^{-1} seed	5.7–14.3%	3.5–18.5 μg g^{-1} seed		
Stander (susceptible check)		91	13.7	14.9		
Conlon (moderately resistant)		65.6	14.3	10.7		
Chevron (resistant check)		61	5.8	4.5		
Coefficient of variation		26	51	54		
Error variance as proportion of total		0.05	0.18	0.17		
Broad sense heritability estimate (SE)		0.46 (0.07)	0.19 (0.07)	0.29 (0.06)		

*Significant at the 0.05 probability level.

**Significance at the 0.01 probability level.

†ns, not significant.

‡Degrees of freedom in error term when the genotype × environment interaction was not significant.

and four replications at seven environments to detect differences equal to 25% of the mean for the ELISA method. Calculations for the FHB severity data suggest genotypes planted at seven environments with two replications were necessary to detect differences equal to 50% of the experimental mean. The numbers of environments necessary increased to 25 to detect differences of 25% of the FHB mean. Calculations using the DON data suggest the numbers of locations and replications necessary to detect differences of 25 or 50% of the mean DON values may be an impractical objective.

Despite there being genotype × environment interactions for the DON and ELISA data, the data sets were combined into one data set to generate scattergrams between the mean values of ELISA and DON. While this is a violation of the assumptions of the analysis of variance procedure, the resource allocation calculations indicated multiple locations and replications were necessary to appropriately characterize each response parameter. Scattergrams of ELISA vs. DON values and FHB vs. DON values indicate there is a linear relationship between each (Fig. 2). Regression analysis indicated a better fit of ELISA and DON data ($R^2 = 0.35$) (Fig. 2) than FHB and DON ($R^2 = 0.29$) or ELISA and FHB ($R^2 = 0.11$).

While ELISA was not a perfect predictor of DON content, samples with low ELISA values tended to be low in DON (Fig. 1 and 2). Some highly contaminated grain was low in DON which illustrates the complexity of the environmental and genotypic interactions

Table 4. Number of replications required to show specified treatment differences for Fusarium head blight severity scores (FHB), deoxynivalenol (DON), or enzyme-linked immunosorbent assay (ELISA) for *Fusarium* spp. antigens when the North American Barley Scab Evaluation Nursery data were used as an estimate of population variability.

Disease index	Desired treatment difference (d) as a % of the mean		
	10	25	50
	No. of replications required		
FHB severity	368	59	15
DON	403	65	17
ELISA	94	16	4

Table 5. Number of environments required to show specified treatment differences for Fusarium head blight (FHB) severity scores, deoxynivalenol (DON), or detection of *Fusarium* spp. antigens with enzyme-linked immunosorbent assay (ELISA) when the North American Barley Scab Evaluation Nursery data were used as an estimate of population variability.

Disease index	No. of replications	Desired treatment difference (d) as a % of the mean		
		10	25	50
		No. of environments required		
FHB severity	2	154	25	7
	3	149	24	6
	4	147	24	6
DON	2	309	51	13
	3	256	42	11
	4	230	38	10
ELISA	2	52	9	2
	3	47	8	2
	4	44	7	2

of the disease complex on phenotypic expression of DON. Similarly, low FHB severity scores tended to be low in DON, but some samples with low FHB were in the medium range of DON, and some with medium FHB scores were among the samples with the highest DON content. This affirms research findings of others (Champeil et al., 2004; Liu et al., 1997; Hill et al., 2006; Nita et al., 2005) that grain can be highly infected with *Fusarium*, produce DON, yet remain asymptomatic.

As to which index (DON, FHB severity, or ELISA) of disease is the most appropriate to use for breeding is subject to debate. The joint Food and Agriculture Organization of the United States (FAO) and the World Health Organization (WHO) discuss the limitations of using FHB scores as a predictor of DON at length (http://www.scabusa.org/pdfs/02-03_CODEX_DON.pdf). Since DON may be present on grain that is asymptomatic of the disease and symptomatic kernels may not contain DON (Champeil et al., 2004; Liu et al., 1997; Hill et al., 2006; Nita et al., 2005), the reliability of these parameters are suspect when predicting disease infestation. On the other hand, the ELISA method used herein is a direct quantification of *Fusarium* spp. and is likely to be a more reliable assessment of the disease.

Experiment 2: Abundance of Mycelial Antigen and Deoxynivalenol in *Fusarium* spp. Cultured in Vitro

Varying Laboratory Environmental Conditions

Moisture and temperature have a significant impact on head blight disease development and DON expression. Experiments investigating the role of environmental parameters on disease epidemiology and DON expression focus on exposure to wet conditions before and immediately after anthesis. The number of perithecia growing on corn stalk residue increases with increasing duration of stalk wetness (Dufault et al., 2002a) and as number of days with an average daily temperature between 15 and 25°C increases (Dufault et al., 2002a, 2002b). Increasing relative humidity also increases perithecia development (Dufault et al., 2002b). Thus, quantity of disease inoculum is controlled by moisture and temperature. Moisture, applied during and after anthesis in the form of misting, also affects disease development and DON production in wheat (Nita et al., 2005). However, the effects are not consistent and yearly variation can occur even if misting is kept constant. They found cool summer months were conducive to disease development and DON in grain but when warm conditions prevailed DON was not as prevalent (http://climate.met.psu.edu/data/city/dlysums). Furthermore, they found sound kernels with high (16μg g^{-1}) levels of DON. Therefore, there is an interaction between moisture and temperature for both disease and DON expression, and lack of disease expression does not necessarily portend low DON. For these reasons we investigated the stability of the expression of the ELISA-specific antigen in *F. graminearum* under different environmental conditions to determine whether it was more consistently expressed than DON, and if it is independently expressed relative to fungal growth. The mycelial mass of *F. graminearum* isolate AEG-001 increased with temperature, but was not affected by osmotic potential in the media. Both temperature and osmotic potential influenced the concentration of DON in the mycelium of the isolate (Fig. 3). Deoxynivalenol content was low regardless of osmotic potential when the temperature was 15°C, but was present when the temperature was 25°C or greater. However, increasing osmotic potential of the media reduced DON content of the mycelium to where DON was virtually absent at −0.77 mPa. Thus, temperature had a positive influence on growth and DON production by *F. graminearum*, but osmotic potential masked the temperature effect on DON. Antigen abundance in the mycelium of AEG-001 did not vary regardless of the environmental conditions in which the fungus was grown (Fig. 4).

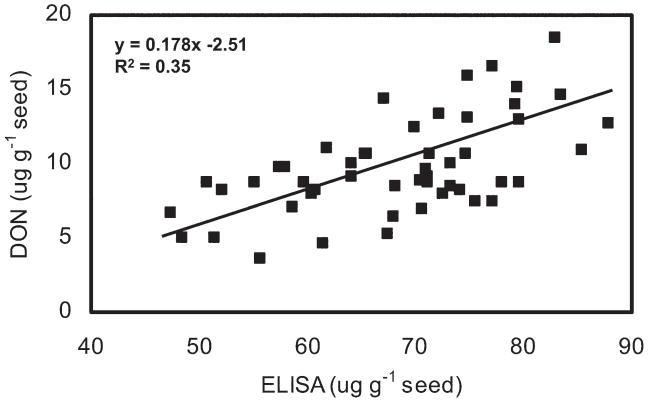

Figure 2. Scattergram of mean enzyme-linked immunosorbent (ELISA) antigen values versus mean deoxynivalenol (DON) values for genotypes of barley in the North American Barley Scab Evaluation Nursery (NABSEN) test. Genotypic means of the barley samples are from a combined data set across four locations.

Abundance of Antigen in Mycelia from Isolates from Fusarium *spp.*

ELISA quantification of *Fusarium* in barley has consistently provided coefficients of variation that are lower than either that of DON or FHB visual assessments (Hill et al., 2006; data herein). Symptoms of FHB may exist without the presence of DON and, conversely, DON may be present when the grain is asymptomatic (Champeil et al., 2004; Liu et al., 1997; Hill et al., 2006). One explanation for variation of DON and disease symptomology could be variation among distinct species of *Fusarium* that are associated with the disease (O'Donnell et al., 2004) as well as variation in trichothecene toxin chemotype diversity among isolates within those species (Ward et al., 2002). However, ELISA

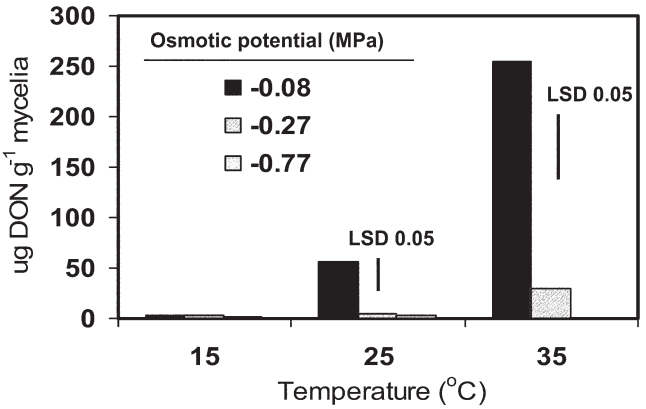

Figure 3. The effect of osmotic potential and temperature on production of deoxynivalenol (DON) in *Fusarium graminearum* isolate AEG-001 grown on corn meal malt agar.

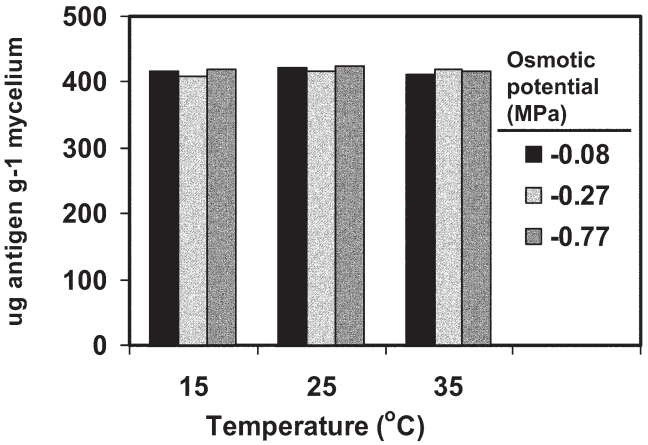

Figure 4. The effect of osmotic potential and temperature on antigen concentration in mycelium of *Fusarium graminearum* isolate AEG-001 grown in vitro.

analysis resulting in lower CVs than DON or FHB data can only be precluded by a robust antibody that quantified the various *Fusarium* species and isolates with consistency over a range of environmental conditions. We tested this hypothesis by examining antigen abundance from various *Fusarium* species in the B clade. Shenk–Hildebrandt media was used to grow the *Fusarium* isolates because it contains amino acids but no proteins. Thus, synthesis of proteins by the fungi is limited by the availability of the amino acids and not metabolism of a protein source. Under these conditions, the species of *Fusarium* tested varied in their ability to grow in the Shenk–Hildebrandt media and had masses ranging from 59 mg for *F. graminearum* to 484 mg for *F. verticillioides* (Table 6). Despite an eightfold difference in the mass of mycelium among the *Fusarium* isolates tested, the abundance of antigen in the mycelium was surprisingly consistent. Hence, the antigen for which antibody cell line IF8 is specific appears to be a highly conserved protein among *Fusarium* species.

The antigen for which monoclonal antibody IF8 is specific is consistent among the *Fusarium* species and across environmental conditions tested, suggesting that ELISA detection using this antibody is likely to be a true quantification of the organism in grain samples. Therefore, variation associated with the ELISA detection system is associated with colonization of the sample and laboratory error.

CONCLUSIONS

If the consistency of antigen expression under laboratory conditions is typical of that when *Fusarium* grows under field conditions, then ELISA quantification of *Fusarium* spp. is likely to be an accurate assessment of fungal infestation. The presence of samples with moderate FHB severity and high DON indicates some seeds in heavily infested samples were asymptomatic of the disease. ELISA analysis was capable of detecting asymptomatic disease-infested samples since samples with low FHB but high in DON had high ELISA values (Fig. 2). Individuals selected for low, medium, and high ELISA values had low, medium, or high DON values as well and were consistent over years in the doubled-haploid study (Table 1) and broad sense heritabilities were higher for ELISA than FHB or DON in both field studies. ELISA was less variable than DON or FHB and the genotypic variance for ELISA accounted for a greater portion of the total variance than did DON or FHB. Resource allocation necessary to test for FHB was less when using ELISA than FHB or DON. Thus, all useful metrics to determine robustness of the three indices of FHB in these studies indicated that ELISA analysis for Fusarium antigens is a practical, and possibly superior, alternative to combined FHB and DON testing for breeders interested in reducing FHB.

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Table 6. Mycelial mass and abundance of antigen in *Fusarium* spp. when grown in liquid media under laboratory conditions.

Fusarium species	Mass	Antigen
	mg	μg g^{-1} mycelium
F. pseudograminearum	303	469
F. graminearum	59	473
F. culmorum	289	453
F. asiaticum	267	485
F. boothii	241	419
F. proliferatum	228	408
F. austroamericanum	223	446
F. subglutinans	171	464
F. solani	112	483
F. verticillioides	484	490
LSD (0.05)	173	ns†

†Not different at the 0.05 probability level.

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