Can. J. Plant Pathol., 2020
Vol. 42, No. 4, 547–559, https://doi.org/10.1080/07060661.2019.1700169

Tri5 gene expression analysis during postharvest storage of wheat grain from field plots treated with a triazole and a strobilurin fungicide

CARLOS BOLANOS-CARRIEL[1], STEPHEN N. WEGULO[1], P. STEPHEN BAENZIGER[2], KENT M. ESKRIDGE[3], DEANNA FUNNELL-HARRIS[4], NIKI MCMASTER[5], DAVID G. SCHMALE III[5] AND HEATHER E. HALLEN-ADAMS[6]

[1]*Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE, USA*
[2]*Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA*
[3]*Department of Statistics, University of Nebraska-Lincoln, Lincoln, NE, USA*
[4]*United States Department of Agriculture USDA-ARS, Wheat, Sorghum, and Forage Research Unit, Lincoln, NE, USA*
[5]*School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA, USA*
[6]*Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, USA*

(Accepted 28 November 2019)

Abstract: Fusarium head blight (FHB) and the associated mycotoxin deoxynivalenol (DON) negatively impact the wheat industry worldwide. In North America, FHB is mainly caused by *Fusarium graminearum* sensu stricto. The purpose of this study was to evaluate, under storage conditions, the expression of the DON biosynthetic gene trichodiene synthase (*Tri*5) of *F. graminearum* in grain of hard red winter wheat cultivars 'Overley' (FHB-susceptible) and 'Overland' (moderately resistant to FHB) from field plots treated or untreated with the triazole fungicide Prosaro and the strobilurin fungicide Headline. Infected grain was stored and periodically sampled to determine gene expression by qRT-PCR analysis. The *F. graminearum* housekeeping gene *GAPDH* was consistently detected, indicative of metabolically active fungi, and *Tri*5 detection was significantly higher in 'Overley' compared with 'Overland'. *Tri*5 gene expression and DON concentrations showed little to no correlation; consequently, *Tri5* expression levels did not accurately predict DON concentrations. The strobilurin did not significantly reduce *Tri*5 gene expression compared with untreated wheat. In the triazole treatment, a significant reduction in the relative expression of *Tri*5 was detected after 120 days, as well as a downregulation of *Tri*5 from 60 to 120 days of storage in 'Overley'. In grain from strobilurin-treated plots of both cultivars, the expression of *Tri*5 increased from 0 to 30 days after tempering. Genetic expression of *Tri*5 that is necessary for the production of DON can increase during storage of high-moisture grain. *Fusarium* fungi can persist in wheat kernels for several months postharvest and may actively produce toxin during this period.

Keywords: deoxynivalenol, Fusarium head blight, gene expression, strobilurin, triazole, trichodiene synthase, wheat

Résumé: La brûlure de l'épi causée par le fusarium (BEF) et la mycotoxine qui lui est associée, le désoxynivalénol (DON), nuisent à la production de blé à l'échelle de la planète. En Amérique du Nord, la BEF est principalement causée par *Fusarium graminearum, stricto sensu*. Le but de cette étude était d'évaluer, dans des conditions d'entreposage, l'expression du gène biosynthétique du DON, la trichodiène synthase (*Tri5*) de *F. graminearum*, dans les grains de blé de force rouge d'hiver des cultivars 'Overley' (réceptif à l'égard de la BEF) et 'Overland' (moyennement résistant à la BEF) provenant de parcelles traitées avec les fongicides triazole Prosaro et strobilurine Headline, ainsi que de parcelles non traitées. Les grains infectés ont été entreposés et périodiquement échantillonnés pour déterminer l'expression génétique par PCRq en temps réel. *GAPDH*, le gène constitutif de *F. graminearum*, a été systématiquement détecté, ce qui est révélateur de champignons métaboliquement actifs, et la détection de *Tri5* était significativement plus systématique chez 'Overley' que chez 'Overland'.

Correspondence to: Heather E. Hallen-Adams. E-mail: hhallen-adams2@unl.edu

***Present address**: Department of Plant Pathology, 1680 Madison Avenue, Selby Hall, The Ohio State University, Wooster, OH 44691, USA

Published online 17 Jan 2020

L'expression génétique de *Tri5* et les concentrations de DON ont affiché peu ou pas de corrélation; en conséquence, les degrés d'expression de *Tri5* n'ont pu contribuer à prédire avec précision les concentrations de DON. La strobilurine n'a pas significativement réduit l'expression génétique de *Tri5* comparativement au blé non traité. Quant au traitement au triazole, il a permis de détecter, au bout de 120 jours, une réduction significative de l'expression relative de *Tri5*, de même qu'une régulation à la baisse de *Tri5* chez 'Overley' pour un entreposage de 60 à 120 jours. Dans les grains des deux cultivars des parcelles traitées avec la strobilurine, l'expression de *Tri5* s'est accrue de 0 à 30 jours après frasage. L'expression génétique de *Tri5* nécessaire à la production de DON peut s'accroître durant l'entreposage de grains à forte teneur en eau. *Fusarium* peut subsister dans les grains de blé pendant plusieurs mois après la récolte et peut activement produire la toxine durant cette période.

Mots clés: blé, brûlure de l'épi causée par le fusarium, désoxynivalénol, expression génétique strobilurine, triazole, trichodiène synthase

Introduction

Fusarium head blight (FHB) is a major threat to wheat (*Triticum aestivum*) production worldwide. In North America, FHB is mainly caused by *Fusarium graminearum*. FHB causes economic losses due to reduction in yield and accumulation of mycotoxins such as deoxynivalenol (DON). Mycotoxins are considered a global food security issue especially in low-income countries and places with deficient management of cereal grain during postharvest storage (Haubruge et al. 2003).

DON is one of many trichothecene mycotoxins, a group of related sesquiterpenoid compounds produced by a wide range of Sordariomycetes. DON inhibits protein synthesis allowing movement of the fungus from cell-to-cell or from floret to rachis in wheat spikes, in which it serves as a virulence factor (Jansen et al. 2005; Brown et al. 2012). Conjugation of DON to a less toxic glucoside (deoxynivalenol-3-glucoside; D3G) plays an important role in wheat resistance to FHB. Transgenic wheat expressing a UDP-glucosyltransferase for DON detoxification had significantly lower FHB compared with controls (Li et al. 2015).

The trichodiene synthase gene (*Tri*5) catalyses the isomerization and cyclization of farnesyl pyrophosphate to trichodiene as the initial step in the DON production pathway (Hohn and Beremand 1989; Desjardins 2006). Expression of the *Tri*5 gene during wheat infection is related to the inhibition of plant defence responses such as thickening of the cell wall during the colonization process (Jansen et al. 2005; Mudge et al. 2006). In the FHB-susceptible spring wheat cultivar 'Wheaton', strains of *F. graminearum* with a disrupted *Tri*5 gene showed reduced virulence and slow development of FHB symptoms (Proctor et al. 1995).

Quantitative reverse transcript PCR (qRT-PCR) can measure the abundance of transcripts of the *Tri*5 gene *in planta* (Mudge et al. 2006) and thereby provides an indirect estimate of DON production capacity. In a greenhouse experiment, Hallen-Adams et al. (2011) detected significant differences in the pattern of expression of DON biosynthetic genes during infection of wheat spikes of FHB-susceptible and -resistant spring wheat cultivars 'Wheaton' and 'Alsen', respectively. Various studies have reported *Tri*5 highly upregulated at 7 days after inoculation of wheat spikes (Mudge et al. 2006), 72 hours after inoculation (Hallen-Adams et al. 2011), and between cell division (growth of head) and cell differentiation (formation of grain) stages in susceptible wheat (Chetouhi et al. 2016), while Brown et al. (2012) detected a peak in *Tri*5 gene expression during initial asymptomatic infection.

In the wheat–*F. graminearum* pathosystem, FHB and DON are commonly managed through the use of moderately resistant cultivars, but also by applying fungicides. However, the selection of the fungicide chemical class and the fungicide application timing are critical for effective management. In the field, strobilurin fungicides can increase DON levels in wheat (Edwards et al. 2001; Simpson et al. 2001; Pirgozliev et al. 2002; Mesterhazy et al. 2003; Ellner 2005; Blandino and Reyneri 2009; Madden et al. 2014). The mechanism underlying this increase is not known. In contrast, triazole fungicides are effective in controlling FHB and DON (Amarasinghe et al. 2013; Edwards et al. 2001; Pirgozliev et al. 2002; Mesterhazy et al. 2003; Wegulo 2012; Wegulo et al. 2015).

Grain mould pathogens can be divided into preharvest or field, and postharvest or storage, according to their prevalence in different phases during development in the grain ecosystem. FHB-associated pathogens are considered as field moulds; however, poor drying and cleaning practices can lead to postharvest FHB-pathogen colonization and mycotoxin accumulation (Aldred and Magan 2004). During storage, one of the critical factors influencing grain quality is moisture

content. High grain moisture content is conducive to mycotoxin accumulation in grain (Comerio et al. 1999; Hope et al. 2005). The dynamics of DON production and persistence during postharvest storage of *F. graminearum*-infected wheat after different field-applied fungicide treatments is unknown. The use of quantitative PCR techniques in wheat treated with fungicides provides a method to discriminate among fungicide efficacies which may not apparently differ based on visual disease assessments (Doohan et al. 1999).

Pre- and postharvest management strategies can significantly influence FHB pathogen abundance in grain in the field and during storage. The abundance of FHB pathogens in grain has been shown to be correlated with DON (Demeke et al. 2010; Horevaj et al. 2011). The correlation between *Tri5* DNA concentration from trichothecene-producing *Fusarium* species and DON in harvested grain has been studied using competitive PCR to determine the efficacy of fungicides applied at anthesis in winter wheat (Edwards et al. 2001). *Tri*5 DNA and DON were at high levels and were positively correlated in inoculated field trials. Additionally, the *Tri*5-PCR assay showed that metconazole and tebuconazole (triazole fungicides) were highly effective in controlling trichothecene-producing *Fusarium*, and that the highest concentration of *Tri*5 DNA (pg ng^{-1} of total DNA) was obtained in grain from azoxystrobin (a strobilurin)-treated plots.

Seed grain provides a good reservoir for FHB pathogens. High loads of *Fusarium* and DON can be readily detected in grain harvested from wheat fields in an FHB epidemic year. Grain inoculated with *F. culmorum* at anthesis showed 16 times higher concentration (pg ng^{-1} of total DNA) of *Fusarium* DNA than uninoculated seed lots (Glynn et al. 2007). The *Tri*5 gene has been used in qRT-PCR to evaluate the relative transcript abundance at different points of kernel colonization. The expression of *Tri*5 never ceased throughout the entire process of kernel colonization (Hallen-Adams et al. 2011). *Tri*5 gene expression and mycelial *in vitro* growth rate of *F. graminearum* were independent and the expression of *Tri*5 remained constant irrespective of the solute stress and incubation temperature (Marin et al. 2010).

This study was conducted to evaluate the expression of the *Tri*5 gene in stored grain of winter wheat cultivars ‘Overley’ (FHB-susceptible) and ‘Overland’ (moderately resistant to FHB) that were treated in the field with the fungicides Headline (pyraclostrobin; a strobilurin) and Prosaro (prothioconazole + tebuconazole; triazoles).

Materials and methods

Field experiments

During the growing seasons in 2015 and 2016, field trials were conducted under dryland and irrigated conditions at the Eastern Nebraska Research and Extension Center (ENREC) near Mead, Nebraska (41.2286°N, 96.4892° W). The irrigation system consisted of a fixed-superficial rectangular framework. Full Circle Impact Sprinklers (Model 30H; Rain Bird, Azusa, CA) were spaced 6.7 m × 4.6 m apart, had a trajectory angle of 27 degrees and operated in a range of 25–80 psi (1.7–5.5 bar). Irrigation cycles were programmed using an ICC-commercial irrigation controller model 800 PL (Hunter Industries, San Marcos, CA). Run time cycles were 5 minutes on and 15 minutes off from 10 a.m. to 5 p.m. seven days a week, amounting to ~15 mm water per day. Irrigated plots received more than twice as much moisture as rainfed plots: 570 mm vs. 270 mm from 30 May to 22 June 2015 and 502 vs. 223 mm from 23 May to 16 June 2016 – timeframes considered critical for *F. graminearum* infection.

Hard red winter wheat cultivars ‘Overley’ (FHB-susceptible) (Fritz et al. 2004; Wegulo et al. 2011) and ‘Overland’ (moderately resistant to FHB) (Baenziger et al. 2008; Jin et al. 2013) were sown during the previous autumn in plots measuring 1.2 m × 6.1 m (autumn 2014; 2015 growing season) or 1.2 m × 4.6 m (autumn 2015; 2016 growing season). During the third week of April (~4 weeks before anthesis) in both years, field plots were inoculated by spreading corn kernels colonized with *F. graminearum* (67 kernels m^{-2}) on the soil surface (Wegulo et al. 2011).

During anthesis, three different treatments were applied to randomly selected plots. Field treatments were (1) triazole, (2) strobilurin, or (3) untreated control plots. The triazole fungicide Prosaro (prothioconazole + tebuconazole; Bayer CropScience) and the strobilurin fungicide Headline (pyraclostrobin; BASF) were applied to spikes at the rate of 0.467 L ha^{-1} and 0.657 L ha^{-1}, respectively, using a CO_2-powered backpack sprayer (241 kPa) and four tee-jet nozzles spaced 30.5 cm apart on a boom. A non-ionic surfactant (Induce) was added to each fungicide/water mixture at a rate of 0.125% vol/vol. Untreated plots (sprayed with water only) served as controls. Treatments were replicated four times in a randomized complete block design. Twenty-four hours following fungicide treatment, spikes in all plots were spray-inoculated with a spore suspension of *F. graminearum* (1×10^5 spores mL^{-1}) using a hand-pumped backpack sprayer (Wegulo et al. 2011). Plots

were harvested with a small plot combine when grain moisture content dropped below 15%.

Postharvest storage experiments

Two postharvest storage experiments (each repeated once) were conducted using grain from the two field trials. In experiment 1, grain of cultivar 'Overland' from dryland plots in the 2015 growing season and subjected to each of the three treatments (triazole, strobilurin and untreated) was used. In experiment 2, grain of cultivar 'Overley' from irrigated plots in the 2016 growing season and subjected to each of the three treatments was used. Grain of cv. 'Overland' contained high levels of *Fusarium*-damaged kernels (FDK) due to a severe FHB epidemic in 2015. This grain was cleaned to remove FDK using a fractionating aspirator as the uncleaned grain had levels of DON above the range of quantitation, and retaining the harvested quantity of FDK under 16–20% moisture could lead to sufficient *Fusarium* growth to destroy the remaining seed and prevent data collection. Additionally, growers clean visibly contaminated grain prior to storage, so this removal of FDK was consistent with standard practice. In 2016, low levels of FHB developed resulting in low or negligible levels of FDK. Therefore, grain of cv. 'Overley' from the 2016 growing season was not cleaned to remove FDK. Additional studies (data not shown) demonstrated comparable DON levels in cleaned 'Overland' grain from 2015 dryland plots and in uncleaned 'Overley' grain from 2016 irrigated plots, so these conditions were chosen for the postharvest study.

Postharvest storage experiments began with dried grain. Grain samples were stored at 32°C and 65% relative humidity (RH). Hydration curves for both experiments were generated based on preliminary studies. Moisture was measured at the beginning of the storage experiment (day 0) and at every time point thereafter, and the grain was tempered to 16% or 20% moisture by adding water calculated from the following equations: for experiment 1 $Y = 2.11 + 0.12X$, $R^2 = 0.70$, and for experiment 2 $Y = 6.47X - 3.85$, $R^2 = 0.96$; where X is the total millilitres of sterile distilled water to be added to the grain, and Y is the variation between the initial moisture content of the grain and the desired moisture content.

Non-sterile grain (300 g), free of solid impurities, was placed on a plastic tray (50 cm long × 30 cm wide × 3 cm high) and spread evenly over the tray's surface. Grain samples were brought to desired moisture levels, i.e., tempered with sterile distilled water using a hand-held bottle sprayer. After tempering, grain samples were homogenized manually and transferred to a hermetically sealed sterile Microbox® micropropagation container of dimensions 15 cm × 15 cm × 20 cm ($SacO_2$, Veldeken, Belgium) (Supplementary Figure 1, online). Microbox® containers have a filter in the lid for gas exchange which blocks the entrance of external microorganisms. Water activity (a_w) was determined using a Pawkit® water activity meter (Decagon Devices, Pullman, WA). Grain moisture content (%) was determined using a grain moisture seed tester (DICKEY-john Corp., Auburn, IL), model GAC 500-XT. After 14 days of tempering, grain moisture and a_w were determined. Sterile distilled water was added to attain 16% or 20% grain moisture (a_w 0.60 or 0.75, respectively). Grain samples were monitored for changes in the percentage of moisture content and a_w at 30-day intervals. Relative humidity and temperature inside the containers were monitored using a WatchDog® data logger model 1400 (Spectrum Technologies, Thayer Court, IL) (Supplementary Figure 1, online). Samples were stored in a seed storage container (Bally Case & Cooler, Inc., Bally, PA) in the dark at 10°C and 40% RH.

Deoxynivalenol determination

From each of the micropropagation containers at a specified storage time (0, 30, 60, 90 and 120 days), the mass of grain inside the container was mixed through agitation and homogenized, then a random sample of grain was taken and milled using a cyclone sample laboratory mill (UDY Corporation, Fort Collins, CO). DON quantification was performed in an Agilent 6890/5975 system using gas chromatography-mass spectrometry (GC-MS).

RNA extraction and purification, and cDNA synthesis

Samples of grain from cultivars 'Overland' (experiment 1) and 'Overley' (experiment 2), at 16% and 20% moisture, were collected as above, freeze-dried and stored at −80°C until RNA extraction. RNA extraction was performed on a minimum of six biological replicates (two independent samples of grain were taken from three of the field plot replicates) for each treatment using the hot phenol-chloroform and lithium chloride precipitation method according to the specifications of Goswami et al. (2006) with modifications. Freeze-dried grain samples were ground in a mortar and mixed with a heated (80°C) mixture of 1:1 extraction buffer (Tris-LiCl-EDTA-SDS) and phenol. The extract was transferred into 30 mL tubes, and a half volume of chloroform was added to the mixture. The mixture was centrifuged for 30 minutes at 2500 × *g*. An aqueous layer formed at the top of the tube was transferred to a new tube and one third volume of 8M LiCl was added.

The tube was incubated on ice for at least 2 hours. Centrifugation was performed for 5 minutes at 12 000 × *g* and 4°C. The pellet formed at the bottom of the tube was washed with 3 mL of 2M LiCl and 3 mL of 70% ethanol. The supernatant was removed and resuspended in 2 mL ultra-pure DEPC-treated water, followed by the addition of 200 µL of 3M NaOAc, and 5.5 mL of 95% ethanol. The suspension was kept at −80°C for 15 minutes, and then centrifuged for five minutes at 12 000 × *g*. The pellet at the bottom of the tube was washed with 3 mL of 70% ethanol. Centrifugation and washing were repeated twice. The pellet was dissolved in 100 µL ultra-pure DEPC-treated water, and transferred to a 1.5 mL Eppendorf tube. RNA was quantified using an Eppendorf BioPhotometer plus (Eppendorf North America, Hauppauge, NY). For DNase treatment, a mixture of 2 µL (20 U) of Thermo Scientific DNase I (Life Technologies, Carlsbad, CA), 4 µL of 10× incubation buffer, and 88 µL of nucleic acids was incubated at 37°C for 15 minutes, and then incubated further at 75°C for 10 minutes after adding 0.2 M EDTA. <>The sample was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. After RNA purification using the Qiagen kit, the concentration and absorbance at 260/280 nm were taken with the BioPhotometer.

Complementary strand DNA (cDNA) was prepared using the Thermo-Fisher Maxima First Strand cDNA Synthesis Kit for qRT-PCR (Life Technologies, Carlsbad, CA). RNA was adjusted to 1 µg per 20 µL reaction using molecular biology grade water. On ice, 4 µL of 5× reaction mix and 2 µL of reverse transcriptase were mixed together with 14 µL of the RNA-water suspension for a total of 20 µL volume reaction. cDNA synthesis was conducted in a T-100™ thermal cycler (BIO-RAD, Hercules, CA) and the amplification protocol consisted of an initial cycle at 25°C for 10 minutes, followed by incubation at 50°C for 15 minutes, and final cycle at 85°C for 5 minutes.

Quantitative reverse transcript PCR

Quantitative reverse transcript PCR was conducted in an Eppendorf MasterCycler RealPlex (Eppendorf North America, Hauppauge, NY) using SYBR Green I chemistry. Data acquisition and visualization was carried out by the MasterCycler ep RealPlex software. Primer pairs *Tri*5-F (5'–TCT ATG GCC CAA GGA CCT GTT TGA–3') and *Tri*5-R (5'– TGA CCC AAA CCA TCC AGT TCT CCA −3'), and *GAPDH*–F (5'- CTA CAT GCT CAA GTA CGA CTC TTC C – 3') and *GAPDH* – R (5'– GCC GGT CTC GGA CCA CTT G – 3'), amplifying *Tri*5 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; housekeeping gene), respectively (Hallen-Adams et al. 2011) were used in a paired qRT-PCR assay. The housekeeping gene was amplified to normalize the expression of the *Tri*5 gene. Additionally, wheat actin was used as a control for RNA quality (Hallen-Adams et al. 2011; data not shown). qRT-PCR assays were conducted in a 96-well PCR plate. A preliminary assay was conducted using cDNA samples from wheat kernels to determine the optimal primer concentration in the qRT-PCR. Final reaction volume contained: 1 µL of undiluted cDNA product, 2.5 µL (25 pmol) of each *Tri*5 primer (forward and reverse) or 1.5 µL (15 pmol) of each *GAPDH* primer, 12.5 µL of the Thermo Fisher Maxima SYBR Green qPCR Master Mix (Life Technologies, Carlsbad, CA), and water to 25 µL. Amplification consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 68°C for 20 seconds. Final holding temperature was 4°C.

Threshold values from each qRT-PCR assay were adjusted manually. Cycle threshold (Ct) values were determined for *Tri*5 and *GAPDH*. Individual Ct values of the target *Tri*5 gene were compared with that of the housekeeping gene *GAPDH*, and the relative ratio of expression was calculated (cycle threshold [Ct] ratio). Normalization of the *Tri*5 gene expression was done based on the Ct value of the fungal *GAPDH* from each corresponding assay. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008), using the expression of the untreated check plots as calibrators (reference samples).

Experimental design and statistical analysis

In the field, experimental design was a split plot in randomized complete blocks with four replications, with cultivars ('Overland' and 'Overley') as the main plots and fungicide treatments (triazole, strobilurin and untreated) as the subplots. Dryland and irrigated plots were located in different fields and considered as separate experiments. In the postharvest storage experiments, experimental design was a split-split-plot-in-time with three replications and each experiment was repeated once. Whole plots were the field-applied fungicide treatments (triazole, strobilurin or untreated), subplots were the grain moisture treatments (16% or 20% moisture), and sub-subplots were the grain sampling time treatments (0, 30, 60, 90 and 120 days). Results did not differ significantly in experiment 1 between 16% and 20% moisture treatments, so only the 20% moisture treatment

was evaluated in experiment 2. In the *Tri*5 gene expression experiment, each run had three biological reps from each fungicide treatment by postharvest storage time combination. Treatments were arranged in a completely randomized design with a factorial of five storage sampling times × 3 fungicide treatments. Statistical analysis of the ratio of *Tri*5 gene expression relative to *GAPDH* was carried out with SAS software version 9.4 (SAS Inc, Cary, NC) using generalized linear mixed models (PROC GLIMMIX). Experiments were not combined due to the contrasting environmental conditions of the two growing seasons. Analysis of the two replicate runs of each experiment was conducted separately based on the homogeneity of error variances determined by the F-ratio test (Gomez and Gomez 1984). Ratio of expression was analysed as a function of the fungicide treatment, storage time and the interaction fungicide treatment by storage time. The Satterthwaite approximation was used in the model to account for variances not being equal. Random effects were the rep and the fungicide treatment by rep. The least significant difference test (LSD, $\alpha = 0.05$) was used to compare pairs of LS means. Combined LS means by run and standard errors (SE) were used to generate graphs.

Pearson correlation coefficients between LS means of the ratio *GAPDH*/*Tri*5 and DON, and between LS means of Ct *Tri*5 and DON were computed using PROC CORR in SAS. The Chi-square (χ^2) test was used to determine if the threshold at which the *Tri*5 gene was detected differed by cultivar ('Overley' versus 'Overland'). A logit test on the binary response distribution was conducted using PROC GLIMMIX. The binary variable consisted of the presence or absence of the gene of interest and/or the reference gene. Type III test of fixed effects was used to determine if there was an effect of fungicide treatments, postharvest storage time, or the interaction between fungicide treatment and storage time on the detection of the *Tri*5 gene.

Results

In cv. 'Overland' – 2015 (moderately resistant to FHB; experiment 1), FDK levels after cleaning were: 1.5 ± 0.8% in grain from non-treated check plots; 0.8 ± 0.6% in grain from Prosaro-treated plots, and 1.6 ± 1.0% in grain from Headline-treated plots. In cv. 'Overley' – 2016 (susceptible to FHB; experiment 2), low levels of FHB developed resulting in low or negligible levels of FDK.

In experiment 1, *Tri*5 was detected in 74% of the total reactions conducted (300/403) (Table 1). In cv. 'Overley' (susceptible to FHB and DON accumulation; experiment 2), *Tri*5 was detected in 96% of the reactions (353/376). The Chi-square test showed a significant difference ($P < 0.001$) in the frequency of reactions in which *Tri*5 gene expression was detected in cv. 'Overley' versus cv. 'Overland' (Table 1). Descriptive statistics by experiment in each combination of fungicide treatment by postharvest storage time are presented in Table 2. Overall, the coefficients of variation were low in both experiments with 12.8% and 8.2% for Ct *Tri*5 and 13.8% and 12.7% for the ratio of *Tri*5 expression relative to *GAPDH* in experiments 1 and 2, respectively. In most cases the standard deviation in the Ct *GAPDH* was higher than in the Ct *Tri*5 (Table 2). The housekeeping gene *GAPDH* showed high relative expression (Table 2) denoted as lower Ct values than *Tri*5 in both experiments (Table 2).

The effect of the interaction of fungicide treatments by storage time was consistently detected as significant on *Tri*5 gene expression relative to *GAPDH* (Ratio Ct *GAPDH*/Ct *Tri*5) (Table 3). Overall, there was an increase in relative *Tri5* gene expression during storage (Fig. 1). LSD values ($\alpha = 0.05$) over the total reactions at which *Tri*5 was detected in both experiments ($n = 653$) showed a significant difference in *Tri*5 expression at 120 days of storage (Ratio Ct *GAPDH*/Ct *Tri*5 = 0.79) compared with the expression registered at 0 days (0.75) and 30 days of storage (0.76). The trend over postharvest storage time is summarized in Fig. 1 for both experiments. In cv. 'Overland' grain from triazole-treated plots, the Ct ratio of *GAPDH*/*Tri*5 increased from 60 days to 120 days of postharvest storage (Fig. 1). However, *Tri*5 expression in these combinations of fungicide and storage time were not significant compared

Table 1. Count frequency of qRT-PCR reactions for the detection of the *Tri*5 gene in two winter wheat cultivars with different reactions to FHB and DON, 'Overland' (moderately-resistant; experiment 1) and 'Overley' (susceptible; experiment 2) over two runs.

	Overland-2015 Dryland		Overley-2016 Irrigated		
*Tri*5 gene	O[a]	E[b]	O	E	Total
Detected	300	337.82	353	315.18	653
Not-detected	103	65.18	23	60.82	126
Total[c]	403		376		779
χ^2; $P < 0.001$					

[a]O = Observed values represent the total counts of reactions in which *Tri*5 was or was not detected.
[b]E = Expected counts under the independence hypothesis (H_0 = Detection of *Tri*5 is dependent of the cultivar).
[c]Totals are the sum of reactions conducted in two independent runs of each experiment.

Table 2. Summary statistics on the evaluation of the effect of fungicide treatments applied at anthesis and postharvest storage time on gene detection (Ct *GAPDH* and Ct *Tri*5) in grain from the Fusarium head blight (FHB)-moderately resistant cv. 'Overland' and the FHB-susceptible cv. 'Overley' averaged over two runs.

			Total reactions		Mean		Standard Deviation		Minimum		Maximum	
Fungicide treatment	Storage time	Variable	Overland	Overley	Overland	Overley	Overland	Overley	Overland	Overley	Overland	Overley
Check	0	Ct *GAPDH*	25	21	23.37	23.82	3.66	3.56	17.71	18.65	32.71	30.71
	0	Ct *Tri*5	25	20	31.33	32.28	4.15	2.23	24.49	29.14	38.19	36.34
	30	Ct *GAPDH*	19	22	25.76	22.00	4.63	1.56	19.03	18.57	34.91	25.53
	30	Ct *Tri*5	17	21	33.11	31.08	3.73	1.91	25.64	28.19	37.34	35.33
	60	Ct *GAPDH*	23	20	25.64	25.34	4.03	3.43	17.58	20.53	36.12	34.61
	60	Ct *Tri*5	23	20	31.85	32.90	5.77	3.09	18.90	27.99	39.07	39.71
	90	Ct *GAPDH*	18	21	26.79	25.00	3.84	2.99	20.62	21.70	33.97	33.26
	90	Ct *Tri*5	16	21	34.44	31.95	4.12	1.55	24.83	28.54	39.71	34.92
	120	Ct *GAPDH*	25	20	26.40	24.36	3.09	3.70	22.04	19.53	32.44	35.25
	120	Ct *Tri*5	25	21	33.49	30.57	4.05	1.53	25.16	27.19	39.61	32.80
Strobilurin	0	Ct *GAPDH*	24	29	25.29	22.44	3.41	2.60	18.32	18.22	31.40	31.11
	0	Ct *Tri*5	23	29	33.26	30.34	4.52	3.07	25.94	24.95	39.04	36.14
	30	Ct *GAPDH*	31	29	25.53	24.03	3.33	4.02	18.74	18.88	35.13	34.27
	30	Ct *Tri*5	30	29	32.25	30.36	4.58	1.86	24.16	26.77	39.77	33.90
	60	Ct *GAPDH*	28	27	26.52	23.98	3.43	2.79	22.04	18.77	34.35	29.64
	60	Ct *Tri*5	24	27	34.20	31.56	2.69	2.75	28.29	26.70	39.26	36.29
	90	Ct *GAPDH*	27	28	26.04	23.93	3.46	2.96	17.30	18.82	33.92	29.76
	90	Ct *Tri*5	22	26	33.29	31.61	4.47	2.51	16.99	27.23	38.09	36.60
	120	Ct *GAPDH*	26	29	26.43	23.23	3.31	4.58	19.84	17.80	32.62	34.79
	120	Ct *Tri*5	22	28	34.32	30.68	3.48	3.18	28.20	24.92	39.94	37.08
Triazole	0	Ct *GAPDH*	22	25	26.40	24.15	3.57	2.47	17.59	20.74	30.42	29.61
	0	Ct *Tri*5	15	22	32.90	31.62	5.23	3.23	19.38	27.29	38.70	37.96
	30	Ct *GAPDH*	31	25	26.33	24.71	4.60	2.28	17.17	21.34	35.10	28.54
	30	Ct *Tri*5	23	23	33.46	33.10	5.38	2.25	19.19	29.49	39.76	37.59
	60	Ct *GAPDH*	21	24	28.45	23.27	3.24	2.52	23.21	19.51	33.11	31.67
	60	Ct *Tri*5	16	22	34.48	32.13	3.03	2.04	29.60	27.56	39.27	35.61
	90	Ct *GAPDH*	26	25	26.89	24.45	3.51	3.00	20.14	19.87	35.85	31.43
	90	Ct *Tri*5	23	25	31.28	32.45	3.36	2.92	25.69	27.74	38.17	38.87
	120	Ct *GAPDH*	23	25	27.47	23.43	3.31	2.88	22.85	20.19	33.51	31.60
	120	Ct *Tri*5	23	25	32.11	30.93	2.91	2.56	24.71	26.99	37.65	36.50

Table 3. ANOVA summary on the evaluation of fungicide treatments applied at anthesis and storage time on *Tri*5 gene expression relative to *GAPDH* (Ratio Ct *GAPDH*/Ct *Tri*5) in grain from the FHB-moderately resistant cv. 'Overland' and the FHB-susceptible cv. 'Overley'.

	2015-Overland Dryland (*Experiment 1*)						2016-Overley Irrigated (*Experiment 2*)					
	Run 1			*Run 2*			*Run 1*			*Run 2*		
Source	Df[a]	F value	P-value	df	F value	P-value	df	F value	P-value	df	F value	P-value
Fungicide Treatment (*F*)	2	0.29	0.766	2	0.72	0.527	2	1.09	0.395	2	0.53	0.612
Storage Time (*T*)	4	1.11	0.353	4	2.55	0.042	4	0.12	0.974	4	1.92	0.109
*F***T*	8	1.90	0.065	8	2.45	0.016	8	2.46	0.016	8	2.28	0.025
Residual	130			140			167			156		

[a]The number of degrees of freedom was dependent of the total reactions in which *Tri*5 and *GAPDH* were detected.

with strobilurin-treated grain at 30 days and untreated checks at 60 and 120 days of storage (Fisher-LSD; $\alpha = 0.05$). Although a significant increase of *Tri*5 gene expression was detected in cv. Overland grain from strobilurin-treated plots from 60 to 120 days of storage, the detection of the gene of interest was significantly lower ($P = 0.015$; logit test of the binary response distribution) in the grain from triazole-treated (*Tri*5 detected

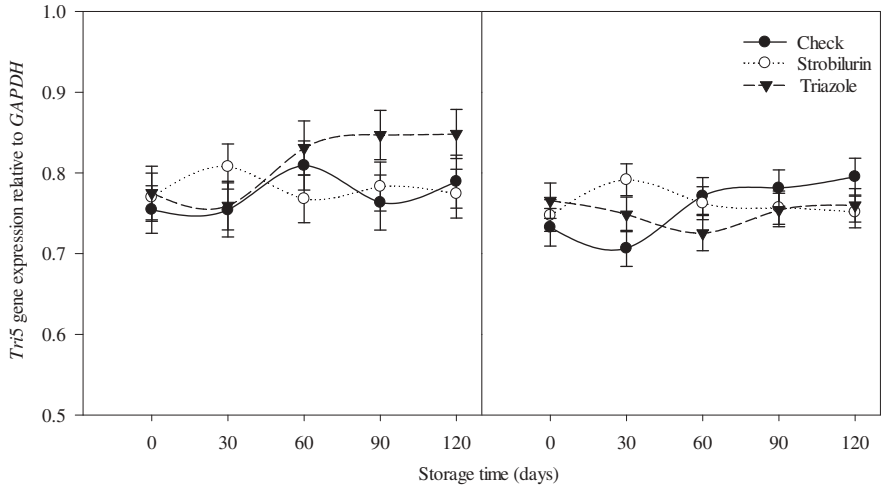

Fig. 1 Effect of postharvest storage time on *Tri*5-gene expression relative to *GAPDH* (Ratio Ct *GAPDH*/Ct *Tri*5) in grain from triazole (Prosaro)- and strobilurin (Headline)-treated plots at anthesis, and non-fungicide sprayed (Check) plots in the 2015 growing season in cv. 'Overland' (moderately resistant to FHB) (experiment 1; left), and in the 2016 growing season in cv. 'Overley' (susceptible to FHB) (experiment 2; right). LS-means are from two independent runs of each experiment. Error bars indicate SE of the LS-means.

in 66% of samples in experiment 1; 94/142) than in the strobilurin-treated (81% *Tri*5 detection; 116/144) and untreated check plots (77% *Tri*5 detection; 90/117).

In cv. 'Overley', *Tri*5 gene expression levels were higher in grain from strobilurin-treated plots (ratio Ct *GAPDH*/Ct *Tri*5 = 0.79) compared with grain from triazole-treated plots (0.73) and untreated check plots (0.75). The strobilurin did not significantly reduce *Tri*5 gene expression compared with the untreated grain (Fig. 1).

In 'Overland' (2015 experiment), samples were tempered to 16% (a_w = 0.60) and 20% (a_w = 0.75) grain moisture content. In this study, DON concentrations as well as *Tri*5 gene expression were similar at 16% and 20% moisture with P-values of 0.878 and 0.435, respectively; therefore, 2016 experiments were conducted using grain tempered at 20%.

At the first evaluation after tempering the grain (30 days of storage), grain from strobilurin-treated plots showed a consistent increase in *Tri*5 gene expression compared with that from untreated check plots (Fig. 2). A 2.6-fold and 4.4-fold change in gene expression was detected in cv. 'Overland' (experiment 1) and cv. 'Overley' (experiment 2), respectively. In both experiments, from 30 to 120 days of storage, fold changes in gene expression were variable without a clear trend and dependent on the expression of the untreated reference sample calibrator instead of a clear fungicide effect (Fig. 2). In grain from cv. 'Overland' (experiment 1) treated with triazole, *Tri*5 was 0.8 times downregulated from 0 to 30 days of storage (Fig. 2).

Fungicide treatments applied at anthesis had a highly significant effect ($P < 0.001$) on DON concentration in both experiments (Fig. 3). Grain from Headline-treated plots showed the highest concentration of DON which was significantly different than the concentration measured in grain from the non-fungicide treated plots, and in grain from Prosaro-treated plots in both experiments. However, in both experiments, Pearson's correlation coefficients were not significant between DON concentration and either the Ct ratio *GAPDH*/*Tri*5 or DON-Ct *Tri*5 (Fig. 4).

Discussion

Under poor storage conditions, the risk of mycotoxin contamination in small grains may increase due to the growth of *F. graminearum* in the grain (Hope et al. 2005; Magan et al. 2014). In this study, qRT-PCR was conducted to track potential changes, under postharvest storage conditions, in gene expression of the *Tri*5 gene in grain from field plots treated with a triazole (Prosaro) or a strobilurin (Headline) fungicide.

Detection of *Tri*5 expression during postharvest grain storage was higher in cv. 'Overley' (FHB-susceptible) than in cv. 'Overland' (moderately resistant) regardless of fungicide treatment. Spread of *Fusarium* through the wheat spike is promoted by DON, and higher *Tri*5 expression (and thus higher DON) in the susceptible cultivar would lead to higher levels of infection and spread of the fungus within the plant (Bai et al. 2001; Jansen et al. 2005; Jiao

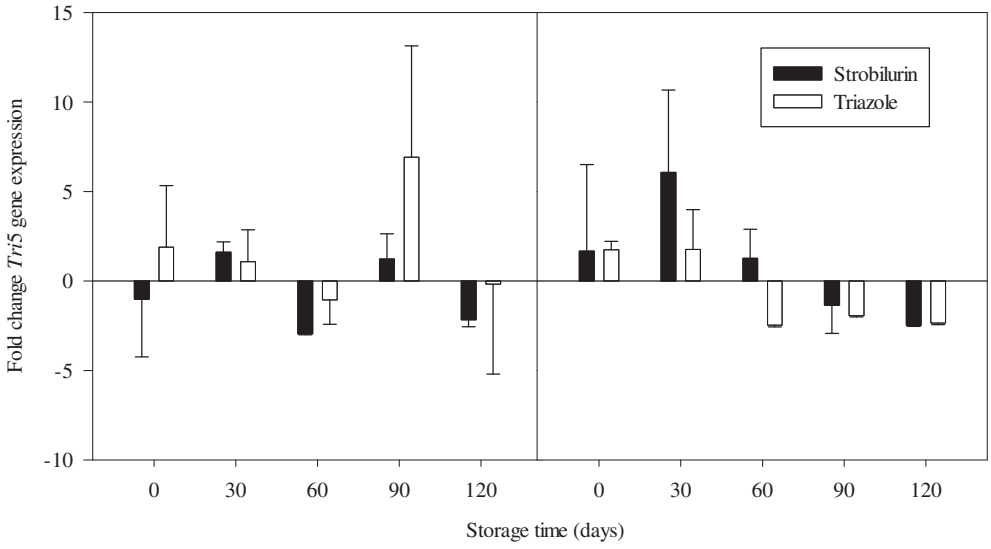

Fig. 2 Fold change in *Tri5* gene expression relative to the *GAPDH* in wheat grain from triazole (Prosaro)- and strobilurin(Headline)-treated plots of cv. 'Overland' (moderately resistant to FHB) (experiment 1; left) and cv. 'Overley' (susceptible to FHB) (experiment 2; right). Non-fungicide treated plots were used as calibrators (reference samples). Data for $2^{-\Delta\Delta Ct}$ calculation correspond to LS-means averaged over two independent runs of each experiment. Error bars indicate SE of the LS-means.

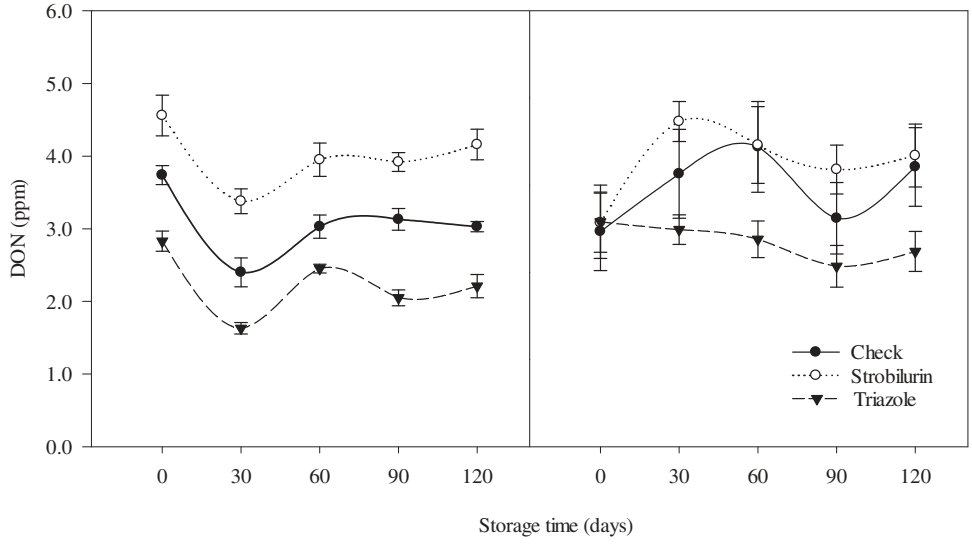

Fig. 3 DON concentration in grain of cv. 'Overland' (moderately resistant to FHB) (experiment 1), and cv. 'Overley' (susceptible to FHB) (experiment 2) for the combinations of fungicide treatments by postharvest storage time. LS-means are from two independent runs of each experiment. Error bars indicate SE of the LS-means.

et al. 2008; Kumar et al. 2015). Our results showed significantly higher (χ^2; $P < 0.001$) frequency of *Tri5* detection in the FHB-susceptible ('Overley') compared with the FHB-moderately resistant ('Overland') cultivar. Jansen et al. (2005) reported that the progress of infection in an FHB-moderately resistant barley (*Hordeum vulgare*) cultivar was slower than in FHB-susceptible cultivars. In addition, Hallen-Adams et al. (2011) found that spring wheat carrying the FHB1 QTL for FHB resistance showed minimal detection of *Tri5* and significant deviation in *Tri5* gene expression compared with an FHB-susceptible cultivar (lacking FHB1).

Results from this study suggest that *F. graminearum* is capable of activating *Tri5* gene expression and promoting

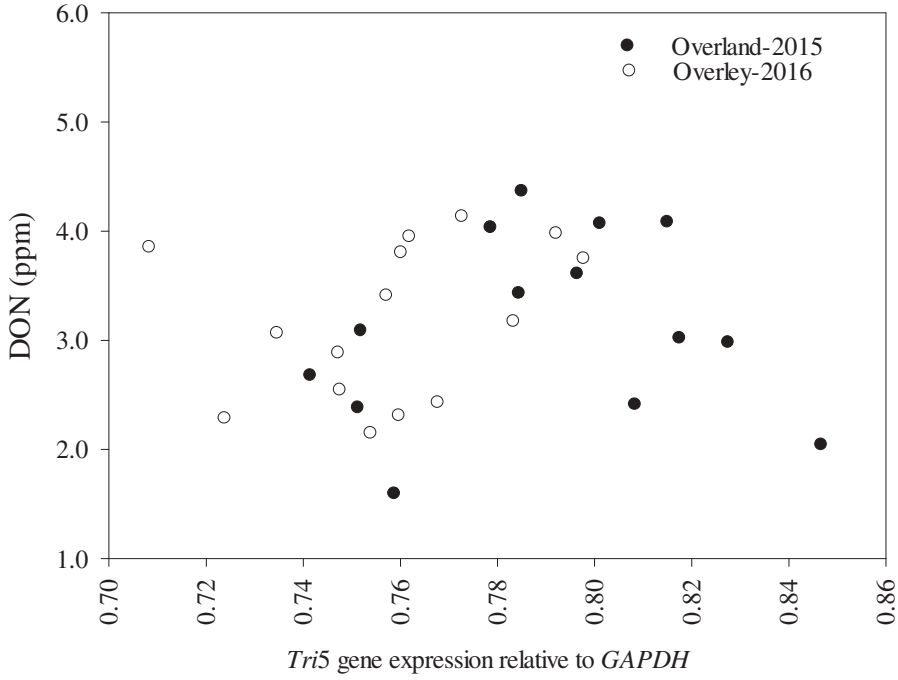

Fig. 4 Scatter plot showing the amount of DON and *Tri*5 gene expression relative to *GAPDH* in grain of cv. 'Overland' (moderately resistant to FHB) (experiment 1), and cv. 'Overley' (susceptible to FHB) (experiment 2) for the combinations of fungicide treatments by postharvest storage time (n = 15). LS-means are from two independent runs of each experiment.

disease in cv. 'Overley', and that this activation is diminished or delayed in cv. 'Overland'. Levels of expression of the *Tri*5 gene by *F. graminearum* may be related to the activation of genes of resistance or susceptibility by the host. Gene expression is highly variable between an FHB-susceptible and an FHB-moderately resistant cultivar. In a microarray study conducted by Bernardo et al. (2007), as many as 86% of the wheat genes differentially expressed in response to *F. graminearum* came from the FHB-susceptible cultivar ('Clark'), and 14% of the genes came from the FHB-moderately resistant cultivar (Ning 7840). Products of genes specific to the FHB-susceptible cultivar could trigger the expression of *Tri*5 in *F. graminearum*, and therefore increase *Tri*5 detection in the FHB-susceptible compared with the FHB-moderately resistant cultivar.

Fungicides applied to control FHB pathogens may reduce the amount of trichothecene-producing *Fusarium* present in grain and, indirectly, DON concentrations (Pirgozliev et al. 2002). In the FHB-susceptible cultivar ('Overley'), the *Tri*5 gene expression levels were lower in grain that came from triazole-treated plots than either non-fungicide treated check or strobilurin-treated plots. Furthermore, only in grain from triazole-treated plots was there a significant reduction in the relative expression of the *Tri*5 detected, while in the grain from the non-sprayed check plots and strobilurin-treated plots, an increase in the relative expression of the *Tri*5 was detected at the end of the postharvest experiments. Therefore, the population of *F. graminearum* actively producing DON was apparently diminished by triazole. Conversely, in grain from strobilurin-treated plots the expression of *Tri*5 increased after tempering the grain (30 days) in both experiments (Fig. 1). *Tri*5 gene expression decreased in wheat treated with triazole. In addition, *Tri*5 was significantly downregulated from 60 to 120 days of storage. However, DON was still being produced in stored grain 120 days after harvest. These findings emphasize the value of triazole fungicide usage (and the fact that treating wheat for foliar diseases with strobilurins will not protect against head blight).

Storage conditions, especially grain moisture, have a significant impact on DON. This study demonstrates that the expression of the *Tri*5 gene (necessary for the production of DON) can increase postharvest due to the presence of a transcriptionally active mass of fungi under conditions of high grain moisture content. In grain of both cultivars, the high and constant expression of the housekeeping gene *GAPDH* indicated the presence of living postharvest fungi (Table 2). There is potential for

metabolically active fungi to grow and produce toxin during several months of storage. Transcriptional activity of the *Tri*5 gene from *F. graminearum* was detectable in grain with high grain moisture content (20%, $a_w = 0.75$) after 120 days of postharvest storage. Hallen-Adams et al. (2011) detected *Tri*5 activity in senescent tissue of a susceptible cultivar. The authors suggested the ability of the fungus to resume DON biosynthesis in dried infected grain. This study corroborates that suggestion, demonstrating increases of transcriptional activity of *F. graminearum-Tri*5 from 0 to 120 days after tempering the grain. In dried grain (<14% moisture content), *F. graminearum* can physiologically adapt to the stress and escape dry conditions. *Fusarium graminearum* has an extraordinary survival ability, even growing after treatments of −70°C for 2 hours and 65°C for one hour (Vujanovic et al. 2012).

*Tri*5 gene expression was not a good predictor of deoxynivalenol grain concentration, as DON was not correlated with *Tri*5 relative expression. Similarly, Bernáldez et al. (2017) found that the expression of the *afl*R aflatoxin regulatory gene in *Aspergillus flavus* was not a good indicator of aflatoxin B1 production. These results are in agreement with Hallen-Adams et al. (2011) who detected inconsistent results of *Tri*5 expression relative to fungal *GAPDH* and DON concentration. Regulation of the trichothecene production is complex, with unknown positive and negative factors affecting the expression of the toxin gene *Tri*5 (Hallen-Adams et al. 2011) and other genes in the *Tri* cluster (Jiao et al. 2008; Schmidt-Heydt et al. 2011). Early induction of *Tri* genes in asymptotic tissue has been detected in several studies (Mudge et al. 2006; Hallen-Adams et al. 2011; Brown et al. 2012; Chetouhi et al. 2016). Lee et al. (2014) hypothesized that a high level of *Tri* transcript accumulation is necessary before initiating DON biosynthesis. *Tri* gene expression has been shown to be maximal during symptomless infection. If DON biosynthesis is dependent on an initial *Tri* accumulation, then direct temporal correlations between DON and gene expression of members of the *Tri* cluster are unlikely.

In conclusion, DON can be present during postharvest storage even if *F. graminearum* is not actively transcribing *Tri*5. Cultivar resistance to FHB and field-applied fungicide chemical class seem to affect expression and, consequently, detection of the *Tri*5 gene. If conditions are favourable for mould growth, *F. graminearum* can express *Tri*5 and lead to a higher accumulation of DON in grain storage. Management strategies, in particular cultivar resistance (Šíp et al. 2010; Mesterhazy 2014) and field-applied fungicide chemical class (Blandino and Reyneri 2009; Wegulo 2012; Willyerd et al. 2012) can significantly impact the quality of grain during storage. The results from this study suggest the integration of cultivar resistance with triazole fungicides applied at anthesis to reduce postharvest *F. graminearum* growth and DON production in grain during storage.

Acknowledgements

The first author was supported by a scholarship awarded by The Secretariat of Higher Education, Science, Technology and Innovation of Ecuador. The authors are indebted to the Nebraska Wheat Board for partial funding of this research. This work was supported in part by grants to S.N. Wegulo (Agreement No. 59-0790-7-080) and D.G. Schmale III (VT PANs #422288, #422533) from the U.S. Wheat and Barley Scab Initiative. This work was supported in part by the Nebraska Agricultural Experiment Station with funding from the Hatch Multistate Research Capacity Funding Program (Accession Number 1006715). This material is based upon work supported by the U.S. Department of Agriculture. This is a cooperative project with the U.S. Wheat & Barley Scab Initiative. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

Funding

This work was supported by the U.S. Department of Agriculture; The Secretariat of Higher Education, Science, Technology and Innovation of Ecuador; Nebraska Wheat Board; U.S. Wheat and Barley Scab Initiative [59-0790-7-080; #422288; #422533]; Hatch Multistate Research Capacity Funding Program [1006715].

Supplemental material

Supplemental data for this article can be accessed online here: https://doi.org/10.1080/07060661.2019.1700169.

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