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Assessment of *Fusarium* infection in wheat heads using a quantitative PCR assay

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Abstract

Accuracy of a qPCR assay in quantifying the DNA of tricothecene-producing F. culmorum and F. graminearum within harvested wheat grains and head tissue was evaluated in comparison with incidences of infected kernels and deoxynivalenol (DON) levels. In a first experiment six durum and bread wheat varieties were grown in randomized plots for a 2-year period, and inoculated with Fusarium macroconidia at six growth stages between heading and dough ripening, to obtain a wide range of Fusarium head blight (FHB) incidences. There was a close relationship between fungal DNA and amount of DON, and this relationship was consistent over Fusarium species, wheat species and varieties, and over a wide range of FHB infection. In a second experiment potted wheat plants were grown under environment-controlled conditions and inoculated with the two Fusarium species at full flowering; head samples were collected before inoculation and after 6 hours to 12 days, and processed by the qPCR assay. This assay made it possible to detect the dynamic of fungal invasion in planta after infection had occurred, and to single out the presence of infection before onset of the disease symptoms: a robust detection of the infection occurred within 18 to 24 hours for F. culmorum, and within 2 to 9 days for F. graminearum.

Keywords: *Fusarium head blight, real-time qPCR, deoxynivalenol, Triticum, Fusarium culmorum, Fusarium graminearum*

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Introduction

Fusarium head blight (FHB) is a relevant disease of small-grain cereals worldwide (Parry et al. 1995, McMullen et al. 1997). Several causal organisms have been associated with the disease, but *Fusarium avenaceum* (Corda ex Fries) Sacc., *F. graminearum* Schw. (*Gibberella zeae* Petch), *F. culmorum* (Wm. G.) Sacc., *F. poae* (Peck) Wollenw, *F. cerealis* Cooke (syn. *F. crookwellense*), and *Microdochium nivale* (teleomorph, *Monographella nivalis*) are the most common species (Parry et al. 1995).

Damage caused by FHB is multifold: reduced yields, discoloured and shrivelled kernels, reduced grain weight, lowered market grade, reduced seed quality due to reduced germination, seedling blight and poor stand (Bechtel et al. 1985, Tuite et al. 1990). FHB also is of great concern since several species that cause the disease can produce mycotoxins (Bottalico et al. 1989) and particularly trichothecenes, a group of sesquiterpenoid mycotoxins that cause a wide range of acute and chronic effects in humans and animals (IARC 1993, D'Mello et al. 1999).

Deoxynivalenol (DON), also known as vomitoxin, is the predominant trichothecene found in cereal grains in Europe and North America produced by *Fusarium culmorum*, *F. graminearum*, and *F. poae* (Placinta et al. 1999, Bottalico and Perrone 2002). The Scientific Committee for Food (SFC) of the European Commission has recently established a TDI (Tolerable Daily Intake) of 1 µg/kg bodyweight/day for DON in humans. In addition, the Commission of the European Community has recently fixed the maximum levels of DON in cereals and cereal products (EC Regulation No 856/2005). Therefore, accurate methods for early detection of both *Fusarium* infection and DON contamination are needed to be applied along the production chain, from growing plants to raw materials and processed food, to ensure efficient consumer protection.

Methods for quantifying FHB include visual assessments of disease severity and incidence of affected ears, spikelets or damaged kernels at harvest (Hilton et al. 1999, Lacey et al. 1999). All these methods estimate disease symptoms and not pathogen populations. In contrast, methods based on plate counts of kernels infected by *Fusaria* (Henriksen and Elen 2005) determine incidence of the different FHB causing fungi. Identification of the fungal species involved in a FHB epidemic can be determined by subculturing colonies growing from the infected kernels onto agar plates and examining their morphological characters (Nelson et al. 1983); it requires taxonomical expertise and is time expensive.

DNA-based methods for identification of *Fusarium* species have been developed and applied as an alternative to morphological approaches. Assays based on SCAR (sequence-characterized amplified region) markers for the differential detection of *F. culmorum*, *F. graminearum*, *F. avenaceum*, and *F. poae* have been developed (Nicholson et al. 1998, Llorens et al. 2006).

Advances in the molecular characterization of genes from “toxin clusters” had provided the potential to develop PCR assays based upon the mycotoxin biosynthetic gene sequences for identification and characterization of fungal species and isolates (Edwards et al. 2002, Seifert and Levesque 2004). In particular, the molecular characterization of the *Tri-5* gene cluster, which contains most of the genes involved in trichothecene biosynthesis, has been exploited in the development of several PCR-based assays to target *Fusarium* species and chemotypes (Lee et al. 2001, Ward et al. 2002, Kimura et al. 2003). On these sequences, both generic assays that target all tricothecene producers (Li et al. 2005) as well as species-specific assays have been developed (Demeke et al. 2005). Generic primers designed on *tri5* gene sequence, that amplify all thricothecene producers have been developed by Schnerr et al. (2001), by Doohan et al. (1999) and by Edwards et al. (2001). Sequences from the *Tri3*, *Tri5* and *Tri7* genes have been used to develop multiplex PCR assay for the chemotype identification of several European *Fusarium* isolates (Quarta et al. 2006). Chemotype determination of *Fusarium* isolates from China and Nepal and from England and Wales trough a PCR assays based on *Tri7* and *Tri13* sequences have been reported respectively by Chandler et al. (2003) and Jennings et al. (2004). Primers specific to *Tri6* have been designed and can be used in conjunction with assays for either *Tri5* or *Tri4* as part of a robust detection system for trichothecene producers (Mulè et al. 2005).

Real-time quantitative PCR is a sensitive, rapid and versatile tool that has opened new opportunities for quantitative diagnosis of phytopathogenic fungi (Schena et al. 2004). Waalwijk et al (2004) developed a quantitative assay based on TaqMan technology to quantify and monitor the dynamics of individual species of the complex causing FHB in cereals. Bluhm et al. (2004) developed a 5' fluorogenic real-time PCR assay for group specific detection of trichothecene and fumonisin producing *Fusarium* ssp. using primers and fluorogenic probes designed from genes directly involved in mycotoxin biosynthesis (*tri6* and *fum1*), whereas Sarlin et al. (2006) evaluated the applicability of this technique for quantification of toxigenic *Fusarium* species in barley and malt. In a recent work (Terzi et al. 2007), a qPCR-based analytical method has been developed targeted at the quantification of the biomass of thricothecene producing *F. graminearum* and *F. culmorum* strains, using a pair of primers designed on the *Tri5-Tri6* intergenic sequence, that is involved in DON synthesis. This intergenic sequence has been already used (Bakan et al, 2002; Li et al, 2005), but the analytical approach developed by Terzi et al (2007) is quantitative and can be used also in samples characterized by high level of DNA fragmentation, as those derived from food and feed, because of the small size of the amplicon. For this reason it can be potentially used along the entire production chain.

The objectives of this work were (i) to test accuracy of the qPCR assay to quantify the biomass of tricothecene-producing *F. culmorum* and *F. graminearum* within harvested grains of different bread and durum wheat varieties; (ii) to determine if there is a correlation between the DNA content of *F. culmorum* and *F. graminearum*, incidence of infected kernels as determined by classical plating methods, and DON levels in these grain samples; (iii) to investigate the potential of the qPCR assay for an early *in planta* diagnosis of *Fusarium* infection.

Materials and methods

Plant and fungal materials

Three durum and three bread wheat varieties were used, characterised by increasing susceptibility to FHB: Simeto, Duilio and S. Carlo for durum wheat; Bilancia, Centauro and Sagittario for bread wheat. These varieties were selected to ensure different levels of *Fusarium* infection and kernel colonization and therefore to constitute a representative data set for robust results.

Three monosporic strains of *F. culmorum* (named MPVP/69, MPVP/70 and MPVP/71) and two of *F. graminearum* (ITEM 6477 and ITEM 6484) were used. *F. culmorum* strains have been used in previous works (Rossi et al. 2001, 2002) and maintained in our culture collection repository, while *F. graminearum* strains were kept at the Institute of Sciences and Food Production (CNR, Bari, Italy). All fungal strains were able to produce DON *in vitro* when grown on autoclaved wheat. Strains were stored on PDA (Potato Dextrose Agar) at 4°C.

To produce conidia for artificial inoculations, PDA 'starter' plates were inoculated with each fungal strain from stock cultures and incubated for one week at 20°C. Plugs (0.5 cm² wide) were then extracted from the periphery of these colonies with a cork borer, transferred to the centre of a PDA plate (9 cm diameter), and incubated for 15 days at 25°C, with a photoperiod of 12 hours light and 12 hours dark. Fungal colonies were washed two times with 10 ml of sterile water and gently shaken; these suspensions were filtered with a double layer of sterile cheesecloth and suspended again in sterile water, streptomycin sulphate and neomycin sulphate (both at 50 mg per l), and Tween 20 (0.1%), until a concentration of 1x10⁴ conidia/ml. The suspensions used for inoculation were finally prepared by mixing equal amounts of the three suspensions produced for each strain of the same *Fusarium* species.

Field experiments

In 2003 and 2004, the six wheat varieties were grown in experimental fields located at Fiorenzuola d'Arda (Piacenza, Italy) on clay soils, previously cropped with barley. Varieties were sown in small

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plots (1 m²) according to a completely randomized design, with three replicates. Seeds were treated with tebuconazol (3 g/q seed) before sowing to prevent foot rots and then cropped using the common practice, with exclusion of fungicide applications.

Plots were inoculated with either *F. graminearum* or *F. culmorum* conidia. Considering that susceptibility of wheat plants to *Fusarium* infection changes with the growth stage (Andersen 1948, Diehl and Fehrmann 1989, Lacey et al. 1999), plots were inoculated at six growth stages to obtain different levels of infection: heading (stage 59 of the BBCH scale; Meier, 2001), beginning of anthesis (stage 61), full anthesis (stage 65), water (stage 71), milk (stage 75) and dough (stage 85) ripening. Inoculations were performed in late afternoon, by distributing 0.6 l of spore suspension per plot by a nebulizer. After inoculation plots were managed in such a way to favour infection: in 2003, plants were wetted every 2 to 3 hours during daytime by distributing water with a hand-operated nebulizer, taking care that plants did not drip water; in 2004, a permanent nebulizer was applied over the plants, misting water for 40 sec every 35 min during daytime and every 3 hours in the dark. An uninoculated test was included in the experimental design to determine the natural occurrence of *Fusarium* infection.

One-hundred spikes were collected randomly from the edge of each plot at maturity and threshed. Kernels were split in three sub-samples to determine: i) proportion of infected kernels; ii) amount of *Fusarium* DNA; iii) DON contamination.

To determine *Fusarium* infection, kernels were washed in running tap water for 20 min, sterilised with ethyl alcohol (70%) for 15 sec and then in sodium hypochlorite (2%) for 2.5 min, rinsed three times in sterile water and dried on absorbent paper under a sterile air flow. Kernels were placed in Petri plates on water agar (1.2%) adjusted to pH 5.2. After 6 to 8 days of incubation at 25°C, fungal colonies growing from the kernels were transferred to other Petri plates on PDA with streptomycin sulphate (50 mg per l) and incubated at 25°C, with an artificial day length of 12 hours. *Fusarium* species were then identified according to Nelson et al. (1983) and Burgess et al. (1988). When necessary, isolates were transferred onto carnation-leaf agar, as described by Nelson et al. (1983).

Environment-controlled experiments

In 2006, four wheat varieties (Duilio, S. Carlo, Bilancia, Sagittario) were sown in pots (50 cm in diameter, 120 seed per pot, 4 pots per variety) and grown under environment-controlled conditions. These plants were inoculated with the two *Fusarium* species at full anthesis (65 BBCH). Inoculations were performed by distributing 0.3 l of spore suspension per pot by a nebulizer; an

uninoculated test was included. After inoculation pots were maintained at 25°C, 100% relative humidity, for 72 hours and then put outdoor.

Five spikes per pot were collected before inoculation and after 6, 12, 18 hours, 1, 2, 3, 6, 9 and 12 days. Spikes were washed under running tap water using a brush to remove epiphytic fungal parts, and then washed again for 30 min. Afterwards, spikes were dried on absorbent paper, frozen with liquid nitrogen and stored at -80°C until determination of the amount of *Fusarium* DNA.

Fusarium DNA analyses

DNA was extracted from lyophilised *Fusarium* mycelium, prepared as reported in Terzi et al. (2007), according to the method described by Al-Samarrai and Schmid (2000).

DNA extraction from wheat samples. Samples of wheat grains (field experiment) and of spike tissues (environment-controlled experiment) were respectively milled with Ika M20 Universal Mill and finely ground with mortar and pestle in liquid nitrogen. Three-hundred milligrams of this vegetal material were mixed with 860 µl of extraction buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% SDS) and 100 µl of 5M guanidinium chloride. After incubation at 56-60°C for 3 h, the samples were centrifuged for 10 min at 14000 rpm. Five µl of RNase (500 µg/ µl) were added to 500 µl of the supernat and incubated at 37°C for 10 min, to digest contaminating RNA. The extracted DNAs were purified according to the Wizard protocol and eluted with 50 µl buffer (10 mM Tris-HCl, pH 9.0). DNA concentrations were determined at 260 and 280 nm.

PCR primers. GLUDF (GGT TGG CAC CGG AGT TG)/GLUDR (TCA CCG CTG CAT CGA CAT) primers, designed on wheat seed storage protein sequences, have been previously demonstrated to be genus-specific, able to produce a 101 bp amplicon in genotypes belonging to *Triticum* genus (Terzi et al. 2003). This primer pair was used to check the amplifiability of DNAs extracted from all wheat samples. From the *Tri5-Tri6* intergenic sequence (AY134892 accession number), the primer pair 22F (AATATGGAAAACGGAGTTCATCTACA)/122R (ATTGCCGGTGCCTGAAAGT) was designed to amplify a 100 bp fragment of the *Fusarium* genome (Terzi et al. 2007).

Real time PCR. Real time PCR reactions were prepared with 6.25 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM forward and reverse primers, 25 ng of DNA template and water to 12.5 µl. All PCR samples and controls were prepared in duplicate. The PCR mixture was held at 50°C for 5 min and denatured at 95°C for 10 min. After this initial step,

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forty amplification cycles were carried out with the following conditions for each cycle: 95°C for 20 sec, 60°C for 1 min. The PCR reactions were subjected to a heat dissociation protocol and analysed by AB5700 software (Applied Biosystems, Foster City, CA, USA) for melting curve analysis. For the quantification of *Fusarium* DNA, a standard curve was generated by plotting the Ct values vs. the Log₁₀ amount of pure *F. graminearum* or *F. culmorum* DNA (10-fold dilution series). The quantification of fungus DNA in wheat samples was derived from the standard curve run in parallel reactions and the results were expressed as pg of fungal DNA starting from 25 ng of total DNA (of plant and fungal origin) extracted from a sample. A melting curve analysis step was always included in each run to control false positive results that can be arose from primer-dimers hybridization and non-specific amplifications. The sensitivity of the assay, as reported in Terzi et al. (2007) is of 4 pg of fungal DNA, corresponding to about 100 *F. graminearum* haploid genomes (Kullman et al. 2005). The specificity of the assay was previously evaluated on a panel of different *Fusarium* species (Terzi et al, 2007).

DON analyses

DON levels were determined by HPLC/UV after immunoaffinity column clean-up (Haidukowski et al. 2005). Twenty-five g of ground samples were added to polyethylene glycol (PEG-8,000) and extracted with 100 mL water by blending at high speed for 2 min with a Sorvall Omnimixer (Sorvall Instruments, Norwalk, CT, USA). Extracts were filtered through both filter paper (Whatman no. 4) and glass microfibre filter (Whatman GF/A) and cleaned up by DonTest™ HPLC immunoaffinity column (VICAM, Watertown, MA, USA). The toxin was determined by an Agilent 1100 Series HPLC system equipped with a diode-array UV detector set at 220 nm (Agilent Technologies, Palo Alto, CA, USA). The column was a Waters C18 Symmetry Shield, 150 × 4.6 mm, 5 µm (Waters, Milford, MA, USA) preceded by a 0.5 µm Rheodyne guard filter. The mobile phase was a mixture of acetonitrile:water (10:90) eluted at a flow rate of 1.0 ml/min. Appropriate dilutions of sample extracts before loading on immunoaffinity columns were necessary to avoid saturation of the DON-antibody binding sites. The detection limit of the method was 0.05 ppm (signal-to-noise ratio 3:1).

Data analysis

All the statistical analyses of this work were performed using the SPSS package ver. 11.5 (SPSS Inc., Chicago, USA). Data on *Fusarium* DNA (expressed in pg) and of DON (in ppm) collected in the field experiments were transformed to make variances homogeneous, using the natural logarithm in the following way: ln(x+1), where x is value in the original scale. In a similar way,

incidence of infected kernels (in %) were transformed using the arcsin of the square root of the percent value.

A preliminary data analysis was performed by calculating summary statistics and by drawing box-plots to explore data variability. Afterwards, a factorial analysis of variance (AOV) was made to test significant effects of the main factors considered (year, *Fusarium* species, wheat growth stages and varieties) as well as their interactions. The Fisher protected least square difference (LSD) was applied at $P=0.05$ to separate means. Effects of wheat species and varieties were not analysed in detail because this was not within aims of the present work.

Pearson's coefficient of correlations were calculated to determine the degree of association between variables and a regression analysis was performed to determine quantitative relationships between *Fusarium* DNA and DON in grain samples, using the nonlinear regression procedure of SPSS.

Also *Fusarium* DNA data obtained with the qPCR assays performed in the second experiment were ln-transformed before applying a factorial AOV and the LSD test at $P=0.05$.

Results

Fusarium infection, DNA and DON in wheat kernels

Colonisation of kernels by the two *Fusarium* species was higher in 2004 than in 2003, with an overall average of 876 pg of DNA and 46 pg, respectively. DNA of *F. culmorum* was 871 pg on average against 54 pg for *F. graminearum*. Spikes inoculated at anthesis produced more colonised kernels (1012 pg at stage 61 and 1470 pg at stage 65), than these inoculated at heading (214 pg), water (49 pg), milk (24 pg) and dough (4 pg) ripening (Figure 1). AOV showed a significant effect of all the above mentioned factors (at $P<0.001$); year accounted for 21% of total variance, *Fusarium* species for 6% and growth stage for 32%. Wheat variety was also significant (at $P<0.001$) and accounted for 4% of total variance. Interactions 'year x growth stage' (5% of total variance), '*Fusarium* species x growth stage' (4%), and 'year x variety' (8%) were also significant (at $P<0.01$).

Incidence of kernels infected by *F. culmorum* was 1.7 times higher than incidence of *F. graminearum* (19.0% and 12.3% on average, respectively), but magnitude of this difference changed depending on different growth stages when spikes have been inoculated (interaction '*Fusarium* species x growth stage' was significant at $P<0.05$) (Figure 1). For inoculations made during ripening differences between the two species were not significant, frequency of infected kernels ranging between 7.0% and 13.2%. At full anthesis (stage 65), 35.4% of kernels were

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infected by *F. culmorum* but only 16.6% of them were infected by *F. graminearum*; at early anthesis (stage 61) infection incidences were 26.2% and 17.0% for the two fungi, respectively. For the inoculations made at heading (stage 59) incidences were of 16.7% and 9.8% of kernels, respectively. Maximum incidence of infected kernels was observed when *F. culmorum* was inoculated at full anthesis in 2004 (51.0% of kernels).

DON contamination was higher in kernels that have been inoculated with *F. culmorum* than with *F. graminearum* (1.34 ppm and 0.27 ppm on average respectively); this difference was significant (at $P<0.001$) and accounted for 5% of total variance. Year and growth stages were also significant, and accounted for 11% and 17% of variance, respectively. Contamination was higher in 2004 (1.44 ppm on average) than in 2003 (0.18 ppm). DON was higher in kernels from spikes that have been inoculated at anthesis (1.65 ppm at stage 61 and 2.19 ppm at stage 65) than at heading (0.69 ppm) and especially during ripening (from 0.21 at stage 71 to 0.02 at stage 85). Because a significant ($P<0.001$) interaction '*Fusarium* species x growth stage', differences between growth stage were higher for *F. culmorum* than for *F. graminearum* (Figure 1).

A significant correlation was observed between *Fusarium* DNA and DON contamination of kernels ($r = 0.797$, $P<0.0001$, $n = 144$) and this correlation was consistent over years and fungal species: correlation coefficients varied between 0.617 for *F. graminearum* in 2003 and 0.848 for *F. culmorum* in 2004 (Table I). When DNA was lower than 54 pg (a value of 4 on the ln-scale) about 70% of grain samples had a DON content lower than the detection limit; when DNA was higher than this value 93% of samples contained measurable DON, when DNA was greater than 1096 pg (7 in the ln-scale) all samples were contaminated (Figure 2). Quantitative relationship between the ln-transformed values of these two variable was fitted by a power equation that accounted for 88% of data variability (Figure 3). Back-transformation of the fitted data showed that increases in *Fusarium* DNA resulted in nonlinear increases of DON: DON accumulation per unit of fungal DNA was greater for severely infected grain samples than for the less infected ones.

Fusarium DNA was also correlated with incidence of infected kernels, but this correlation was lower and less significant ($r = 0.195$, $P=0.019$, $n = 144$); furthermore, the coefficient of correlations between these two variables were significant only in 2004 (Table I), when the incidence of infection was higher. In a similar way, infected kernels were also correlated with DON, but less closely than DNA did ($r = 0.411$, $P<0.001$, $n = 144$) and only in 2004 (Table I).

Fusarium DNA detection after infection

AOV made on *Fusarium* DNA contents detected in spike tissues of four wheat varieties at different times after inoculation showed a significant effect for all the factor tested and for their interactions,

at $P < 0.001$; only '*Fusarium* species x variety' interaction was significant at $P = 0.017$. Time elapsed after infection and *Fusarium* species were the more influential factors, accounting for 72% and 12% of total variance, respectively, with their interaction accounting for 7%.

Using the qPCR assay the *Fusarium* DNA was always detected in the inoculated spikes before the appearance of first disease symptoms. Considering a level of 4 pg of DNA (1.61 in the ln-scale) as the minimum content of DNA for robust detection of the fungi in the spike tissue, it was observed that *F. culmorum* in wheat spikes was detected for first time 18 hours after infection in three varieties and after 24 hours also in the bread wheat Bilancia (Table II). After this time amount of DNA progressively increased until 9 days and then did not significantly increased in the following 3 days. First robust detection of *F. graminearum* DNA occurred 48 and 72 hours after inoculation in the durum wheat S. Carlo and Duilio, respectively. In bread wheat Sagittario first detection occurred after 6 days and only after 9 days in Bilancia (Table II). On average of different varieties, the DNA content of spikes progressively increased until the last sample collected 12 days after inoculation.

Discussion

This work was planned in such a way to obtain a data set characterised by high variability in both *Fusarium* infection and DON contamination to draw representative and robust results. For this reason, artificial inoculations with *F. culmorum* and *F. graminearum* were made on three durum and three bread wheat varieties characterised by different susceptibility to FHB, at six different growth stages between heading and dough ripening, for a 2-year period.

The degree of *Fusarium* infection was determined as incidence of infected kernels and amount of fungal biomass measured as *Fusarium* DNA, and these variables were correlated with the level of DON contamination. In this work we did not perform a visual disease assessments of FHB, but literature demonstrates that there is no robust association between FHB symptoms and DON contents in grains (Boyacioglu et al. 1992, Lemmens et al. 1997, Liu et al. 1997, Martin and Johnston 1982, Doohan et al. 1999, Edwards et al. 2001, Birzele et al. 2002) because some fungi causing the disease do not produce this mycotoxin. Furthermore, no correlation was found between visual FHB assessments and concentration of trichothecene-producing *F. culmorum* and *F. graminearum* measured with a PCR-based assay based on primers derived from *Tri5* (Edwards et al. 2001).

Correlation and regression analyses of trichothecene-producing *Fusarium* DNA and DON in harvested grain showed a close correlation between biomass and the amount of DON produced.

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Similar results had been previously found by other authors (Edwards et al. 2001, Schnerr et al. 2002, Waalwijk et al. 2004), but the present work showed that this relationship is consistent over *Fusarium* species, wheat species and varieties, and over a wide range of epidemiological conditions due to different meteorological conditions and times of infection occurrence. In a previous work, different correlations were found between *Fusarium* exoantigens and DON in harvested grain from field samples of hard red spring wheat in Manitoba, Canada, and of soft white winter wheat in Ontario, Canada, indicating that the relationship between amounts of *Fusarium* biomass and DON in harvested grain may be affected by the host and by its interactions with environmental conditions (Abramson et al. 1998). In this study, correlation between *Fusarium* DNA and DON concentrations occurred in experimental fields that have been artificially inoculated with trichothecene-producing strains of *F. culmorum* and *F. graminearum*. This relationship could probably be weaker for field samples, where the mycoflora infecting kernels may differ for the capability to produce DON or, in the case of *F. graminearum*, in the relative amounts of DON and NIV they produce. Nevertheless, good correlations were found with the qPCR assay in field samples showing a high variability in both DNA and DON contents (Terzi et al. 2007).

This work also showed that correlation between *Fusarium* DNA and DON is closer and more consistent than correlation between incidence of *Fusarium*-infected kernels and DON. Significant relationships between the incidence of particular *Fusarium* species and the concentrations of fusariotoxin have been previously found. For instance, Krisinska-Traczyk et al. (2001) found that concentration of *F. culmorum* in total examined grain and grain dust samples collected in Poland was significantly correlated with the concentration of DON, at $P<0.05$. A significant correlation between the incidence of *F. graminearum* and DON has been also found by Dalcero et al. (1997) in Argentina, while Moreno Contreras et al. (2000) did not show any significant correlation between the presence of *Fusarium* spp. and DON in grain samples collected in Venezuela.

The qPCR assay made it possible to detect differences between levels of kernel infection better than fungal isolation from kernels with classical mycological methods. Some reasons can support this result. Kernel infection was determined by plating samples of kernels and by determining percent incidence of grains with developing colonies of *F. culmorum* or *F. graminearum*. This procedure can only distinguish between healthy and infected grains but can't define the biomass of mycelia invading an infected kernel. For instance, a kernel which was infected in an advanced stage of ripening may result as infected using the plating method because a *Fusarium* colony will grow from it, but invasion of grain tissue may have a little extent as well as the amount of DON produced because of a few time will be available between infection and the drop of available water below the minimum for fungal growth and mycotoxin production, respectively (Rossi et al. 2006).

Furthermore, the plating method can only detect living mycelia in *Fusarium*-infected kernels while the qPCR assay provides a measure of biomass taking in account living mycelia actively producing DON but also dead mycelia, which formerly had contributed to the DON content actually found.

The qPCR assay also proved to be an useful tool to detect the dynamic of fungal invasion *in planta* after infection had occurred, and to single out the presence of infection before onset of the disease symptoms. A robust detection of the infection occurred within 18 to 24 hours for *F. culmorum*, and within 2 to 9 days for *F. graminearum*. This result is very promising for his potential impact on tactical disease management: a detection of infection occurrence within 2 or 3 days may result in an efficient application of curative fungicides (Boyacioglu et al. 1992, Homdork et al. 2000, Matthies and Buchenauer 2000, Siranidou and Buchenauer 2001, Suty-Heinze and Dutzmann 2004). It must be considered that this result was obtained by inoculating wheat spikes at full flowering with a high inoculum dose, under optimum environment conditions for infection to occur. It can be then supposed that under these conditions the disease spread rapidly within the spike tissue and that the minimum DNA content for robust *Fusarium* detection (i.e. 4 pg) may have reached early in comparison with natural infections. For this reason, further works will be carried out to confirm possibility to early detect presence of trichothecene-producing *Fusaria* in wheat spikes under field conditions.

The qPCR assay based on *Tri5-Tri6* intergenic sequence (Terzi et al. 2007) provides a valuable tool for detection of trichothecene-producing *Fusaria* both *in planta* and within harvested grains. The assay has many potential uses both for research purposes, such as in selection of wheat cultivars and in testing efficacy of fungicides, and for practical disease management, such as timing fungicide applications and early estimating risk for trichothecene mycotoxins within grains at harvest.

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Table I. Pearson's coefficient of correlation between incidence of *Fusarium* infection, amount of *Fusarium* DNA and content of deoxynivalenol (DON) in wheat kernels which have been inoculated with *F. culmorum* and *F. graminearum* in two years.

<i>Fusarium</i> species	Variables ^a	Years		
		2003	2004	Both
<i>F. culmorum</i>	Inc vs DNA	- 0.042	0.836	0.371
		<i>ns</i> ^b	<0.0001 ^b	0.0013 ^c
	Inc vs DON	0.189	0.844	0.549
		<i>ns</i>	<0.0001	<0.0001
	DNA vs DON	0.799	0.848	0.818
		<0.0001	<0.0001	<0.0001
<i>F. graminearum</i>	Inc vs DNA	- 0.061	0.608	-0.104
		<i>ns</i> ^b	0.0001 ^b	<i>ns</i> ^c
	Inc vs DON	0.019	0.722	0.118
		<i>ns</i>	<0.0001	<i>ns</i>
	DNA vs DON	0.617	0.816	0.779
		0.0001	<0.0001	<0.0001
<i>F. culmorum</i> + <i>F. graminearum</i>	Inc vs DNA	- 0.065	0.774	0.195
		<i>ns</i> ^c	<0.0001 ^c	0.019 ^d
	Inc vs DON	0.108	0.832	0.411
		<i>ns</i>	<0.0001	<0.0001
	DNA vs DON	0.757	0.828	0.797
		<0.0001	<0.0001	<0.0001

^a Inc = percent incidence of infected kernels ($\arcsin \sqrt{\%}$); DNA (ln pg); DON (ln ppm)

^b n = 36 (6 wheat varieties x 6 times of *Fusarium* inoculation, between heading and ripening)

^c n = 72 (6 varieties x 6 times x 2 years or 2 fungi)

^d n = 144 (6 varieties x 6 times x 2 years x 2 fungi)

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Table II. Biomass (expressed as natural logarithm of DNA in pg) of *F. culmorum* and *F. graminearum* within spike tissue of two bread (Bilancia and Sagittario) and two durum (Duilio and S. Carlo) wheat varieties at different times after inoculation.

Hours after inoculation	Wheat varieties				Average
	Bilancia	Sagittario	Duilio	S.Carlo	
<i>F. culmorum</i>					
0	0.00 ^a	0.21	0.00	0.00	0.05 ^c
6	0.35	1.59	1.55	1.08	1.14
12	0.79	1.43	1.55	0.96	1.18
18	1.51	<u>1.67</u>	<u>2.71</u>	<u>2.81</u>	2.17
24	<u>1.64</u> ^d	2.44	2.24	2.04	2.09
48	2.57	3.03	1.64	3.48	2.68
72	4.02	4.92	3.33	5.65	4.48
144	5.68	6.83	7.39	7.53	6.86
216	7.37	8.10	8.80	8.81	8.27
288	6.84	7.10	8.04	8.07	7.51
Average	3.08 ^b	3.73	3.72	4.04	3.64
<i>F. graminearum</i>					
0	0.00 ^a	0.00	0.023	0.00	0.06 ^c
6	0.24	0.00	0.00	0.00	0.06
12	0.00	0.52	0.79	0.52	0.46
18	0.27	1.42	1.06	0.76	0.88
24	0.00	1.58	1.52	0.96	1.02
48	0.00	1.12	0.64	<u>2.78</u>	1.13
72	1.52	1.59	<u>2.31</u>	2.88	2.07
144	1.59	<u>2.71</u>	4.18	3.45	2.98
216	<u>1.96</u>	4.14	5.16	5.01	4.07
288	4.86	5.74	5.34	5.37	5.33
Average	1.04 ^b	1.88	2.12	2.17	1.81

^a LSD_{0.05} = 0.815 for variety x fungus x hour interaction
^b LSD_{0.05} = 0.258 for variety x fungus interaction
^c LSD_{0.05} = 0.408 for fungus x hour interaction
^d Underlining indicates first value when natural logarithm is ≥ 1.61 (i.e. ≥ 4 pg of *Fusarium* DNA over 25 ng of total DNA extracted)

Figure captions

Figure 1. Incidence of *Fusarium* infection (expressed as $\arcsin\sqrt{\%}$), *Fusarium* DNA (as natural logarithm of pg over 25 ng of total DNA) and content of deoxynivalenol (as natural logarithm of DON in ppm) in wheat kernels that have been inoculated with *F. culmorum* and *F. graminearum* at six growth stages (59 = heading, 61 = beginning of anthesis; 65 = full anthesis; 71 = water; 75 = milk; 85 = dough ripening; BBCH scale). The symbol $\pm$ represents the LSD at $P=0.05$ to separate means. In A and C, bars are means of six wheat varieties and two years, while their whiskers represent standard errors. In B, means are represented by the symbol $\blacksquare$, while the box plots show data distribution (the boxes include 50% of data, $\cdots$ is the median, $—$ extend to minimum and maximum values, $\circ$ are outliers).

Figure 2. Relationships between *Fusarium* DNA (as natural logarithm of pg over 25 ng of total DNA) and deoxynivalenol (as natural logarithm of DON in ppm) found in wheat kernels that have been inoculated with *F. culmorum* and *F. graminearum* at six growth stages in 2003 (A and B, respectively) and in 2004 (C and D, respectively). Coefficients of correlation are shown in Table I.

Figure 3. Relationships between *Fusarium* DNA (as natural logarithm of pg over 25 ng of total DNA) and deoxynivalenol (as natural logarithm of DON in ppm) found in wheat kernels that have been inoculated with *F. culmorum* and *F. graminearum* at six growth stages in 2003 and 2004; ---- represents the regression line.