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

V. ROSSI¹, V. TERZI², F. MOGGI¹, C. MORCIA², P. FACCIOLI², M. HAIDUKOWSKI³, M. PASCALE³

¹*Istituto di Entomologia e Patologia vegetale, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100-Piacenza, Italy,*

²*C.R.A., Istituto Sperimentale per la Cerealicoltura, Via San Protaso 302, 29017-Fiorenzuola d'Arda (Piacenza), Italy, and*

³*Istituto di Scienze delle Produzioni Alimentari, CNR, Via Amendola, 122/O, 70126-Bari, Italy*

Abstract

*Accuracy of a qPCR assay in quantifying the DNA of trichothecene-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*

Correspondence: V. Rossi, E-mail: vittorio.rossi@unicatt.it

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.

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

19 *Plant and fungal materials*

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21 Three durum and three bread wheat varieties were used, characterised by increasing susceptibility to
22 FHB: Simeto, Duilio and S. Carlo for durum wheat; Bilancia, Centauro and Sagittario for bread
23 wheat. These varieties were selected to ensure different levels of *Fusarium* infection and kernel
24 colonization and therefore to constitute a representative data set for robust results.
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28 Three monosporic strains of *F. culmorum* (named MPVP/69, MPVP/70 and MPVP/71) and two
29 of *F. graminearum* (ITEM 6477 and ITEM 6484) were used. *F. culmorum* strains have been used in
30 previous works (Rossi et al. 2001, 2002) and maintained in our culture collection repository, while
31 *F. graminearum* strains were kept at the Institute of Sciences and Food Production (CNR, Bari,
32 Italy). All fungal strains were able to produce DON *in vitro* when grown on autoclaved wheat.
33 Strains were stored on PDA (Potato Dextrose Agar) at 4°C.
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39 To produce conidia for artificial inoculations, PDA 'starter' plates were inoculated with each
40 fungal strain from stock cultures and incubated for one week at 20°C. Plugs (0.5 cm² wide) were
41 then extracted from the periphery of these colonies with a cork borer, transferred to the centre of a
42 PDA plate (9 cm diameter), and incubated for 15 days at 25°C, with a photoperiod of 12 hours light
43 and 12 hours dark. Fungal colonies were washed two times with 10 ml of sterile water and gently
44 shaken; these suspensions were filtered with a double layer of sterile cheesecloth and suspended
45 again in sterile water, streptomycin sulphate and neomycin sulphate (both at 50 mg per l), and
46 Tween 20 (0.1%), until a concentration of 1x10⁴ conidia/ml. The suspensions used for inoculation
47 were finally prepared by mixing equal amounts of the three suspensions produced for each strain of
48 the same *Fusarium* species.
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58 *Field experiments*

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

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3 plots (1 m²) according to a completely randomized design, with three replicates. Seeds were treated
4 with tebuconazol (3 g/q seed) before sowing to prevent foot rots and then cropped using the
5 common practice, with exclusion of fungicide applications.
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8 Plots were inoculated with either *F. graminearum* or *F. culmorum* conidia. Considering that
9 susceptibility of wheat plants to *Fusarium* infection changes with the growth stage (Andersen 1948,
10 Diehl and Fehrmann 1989, Lacey et al. 1999), plots were inoculated at six growth stages to obtain
11 different levels of infection: heading (stage 59 of the BBCH scale; Meier, 2001), beginning of
12 anthesis (stage 61), full anthesis (stage 65), water (stage 71), milk (stage 75) and dough (stage 85)
13 ripening. Inoculations were performed in late afternoon, by distributing 0.6 l of spore suspension
14 per plot by a nebulizer. After inoculation plots were managed in such a way to favour infection: in
15 2003, plants were wetted every 2 to 3 hours during daytime by distributing water with a hand-
16 operated nebulizer, taking care that plants did not drip water; in 2004, a permanent nebulizer was
17 applied over the plants, misting water for 40 sec every 35 min during daytime and every 3 hours in
18 the dark. An uninoculated test was included in the experimental design to determine the natural
19 occurrence of *Fusarium* infection.
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30 One-hundred spikes were collected randomly from the edge of each plot at maturity and
31 threshed. Kernels were split in three sub-samples to determine: i) proportion of infected kernels; ii)
32 amount of *Fusarium* DNA; iii) DON contamination.
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35 To determine *Fusarium* infection, kernels were washed in running tap water for 20 min,
36 sterilised with ethyl alcohol (70%) for 15 sec and then in sodium hypochlorite (2%) for 2.5 min,
37 rinsed three times in sterile water and dried on absorbent paper under a sterile air flow. Kernels
38 were placed in Petri plates on water agar (1.2%) adjusted to pH 5.2. After 6 to 8 days of incubation
39 at 25°C, fungal colonies growing from the kernels were transferred to other Petri plates on PDA
40 with streptomycin sulphate (50 mg per l) and incubated at 25°C, with an artificial day length of 12
41 hours. *Fusarium* species were then identified according to Nelson et al. (1983) and Burgess et al.
42 (1988). When necessary, isolates were transferred onto carnation-leaf agar, as described by Nelson
43 et al. (1983).
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53 *Environment-controlled experiments*

54 In 2006, four wheat varieties (Duilio, S. Carlo, Bilancia, Sagittario) were sown in pots (50 cm in
55 diameter, 120 seed per pot, 4 pots per variety) and grown under environment-controlled conditions.
56 These plants were inoculated with the two *Fusarium* species at full anthesis (65 BBCH).
57 Inoculations were performed by distributing 0.3 l of spore suspension per pot by a nebulizer; an
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3 uninoculated test was included. After inoculation pots were maintained at 25°C, 100% relative
4 humidity, for 72 hours and then put outdoor.
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7 Five spikes per pot were collected before inoculation and after 6, 12, 18 hours, 1, 2, 3, 6, 9 and
8 12 days. Spikes were washed under running tap water using a brush to remove epiphytic fungal
9 parts, and then washed again for 30 min. Afterwards, spikes were dried on absorbent paper, frozen
10 with liquid nitrogen and stored at -80°C until determination of the amount of *Fusarium* DNA.
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13 *Fusarium* DNA analyses

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16 DNA was extracted from lyophilised *Fusarium* mycelium, prepared as reported in Terzi et al.
17 (2007), according to the method described by Al-Samarrai and Schmid (2000).
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23 *DNA extraction from wheat samples.* Samples of wheat grains (field experiment) and of spike
24 tissues (environment-controlled experiment) were respectively milled with Ika M20 Universal Mill
25 and finely ground with mortar and pestle in liquid nitrogen. Three-hundred milligrams of this
26 vegetal material were mixed with 860 µl of extraction buffer (10 mM Tris-HCl pH 8.0, 150 mM
27 NaCl, 2 mM EDTA, 1% SDS) and 100 µl of 5M guanidinium chloride. After incubation at 56-60°C
28 for 3 h, the samples were centrifuged for 10 min at 14000 rpm. Five µl of RNase (500 µg/ µl) were
29 added to 500 µl of the supernat and incubated at 37°C for 10 min, to digest contaminating RNA.
30 The extracted DNAs were purified according to the Wizard protocol and eluted with 50 µl buffer
31 (10 mM Tris-HCl, pH 9.0). DNA concentrations were determined at 260 and 280 nm.
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41 *PCR primers.* GLUDF (GGT TGG CAC CGG AGT TG)/GLUDR (TCA CCG CTG CAT CGA
42 CAT) primers, designed on wheat seed storage protein sequences, have been previously
43 demonstrated to be genus-specific, able to produce a 101 bp amplicon in genotypes belonging to
44 *Triticum* genus (Terzi et al. 2003). This primer pair was used to check the amplifiability of DNAs
45 extracted from all wheat samples. From the *Tri5-Tri6* intergenic sequence (AY134892 accession
46 number), the primer pair 22F (AATATGGAAAACGGAGTTCATCTACA)/122R
47 (ATTGCCGGTGCCTGAAAGT) was designed to amplify a 100 bp fragment of the *Fusarium*
48 genome (Terzi et al. 2007).
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57 *Real time PCR.* Real time PCR reactions were prepared with 6.25 µl of SYBR Green PCR Master
58 Mix (Applied Biosystems, Foster City, CA, USA), 900 nM forward and reverse primers, 25 ng of
59 DNA template and water to 12.5 µl. All PCR samples and controls were prepared in duplicate. The
60 PCR mixture was held at 50°C for 5 min and denatured at 95°C for 10 min. After this initial step,

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,

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3 incidence of infected kernels (in %) were transformed using the arcsin of the square root of the
4 percent value.
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7 A preliminary data analysis was performed by calculating summary statistics and by drawing
8 box-plots to explore data variability. Afterwards, a factorial analysis of variance (AOV) was made
9 to test significant effects of the main factors considered (year, *Fusarium* species, wheat growth
10 stages and varieties) as well as their interactions. The Fisher protected least square difference (LSD)
11 was applied at $P=0.05$ to separate means. Effects of wheat species and varieties were not analysed
12 in detail because this was not within aims of the present work.
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17 Pearson's coefficient of correlations were calculated to determine the degree of association
18 between variables and a regression analysis was performed to determine quantitative relationships
19 between *Fusarium* DNA and DON in grain samples, using the nonlinear regression procedure of
20 SPSS.
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24 Also *Fusarium* DNA data obtained with the qPCR assays performed in the second experiment
25 were ln-transformed before applying a factorial AOV and the LSD test at $P=0.05$.
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31 Results

32 *Fusarium* infection, DNA and DON in wheat kernels

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34 Colonisation of kernels by the two *Fusarium* species was higher in 2004 than in 2003, with an
35 overall average of 876 pg of DNA and 46 pg, respectively. DNA of *F. culmorum* was 871 pg on
36 average against 54 pg for *F. graminearum*. Spikes inoculated at anthesis produced more colonised
37 kernels (1012 pg at stage 61 and 1470 pg at stage 65), than these inoculated at heading (214 pg),
38 water (49 pg), milk (24 pg) and dough (4 pg) ripening (Figure 1). AOV showed a significant effect
39 of all the above mentioned factors (at $P<0.001$); year accounted for 21% of total variance, *Fusarium*
40 species for 6% and growth stage for 32%. Wheat variety was also significant (at $P<0.001$) and
41 accounted for 4% of total variance. Interactions 'year x growth stage' (5% of total variance),
42 '*Fusarium* species x growth stage' (4%), and 'year x variety' (8%) were also significant (at
43 $P<0.01$).
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53 Incidence of kernels infected by *F. culmorum* was 1.7 times higher than incidence of *F.*
54 *graminearum* (19.0% and 12.3% on average, respectively), but magnitude of this difference
55 changed depending on different growth stages when spikes have been inoculated (interaction
56 '*Fusarium* species x growth stage' was significant at $P<0.05$) (Figure 1). For inoculations made
57 during ripening differences between the two species were not significant, frequency of infected
58 kernels ranging between 7.0% and 13.2%. At full anthesis (stage 65), 35.4% of kernels were
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3 infected by *F. culmorum* but only 16.6% of them were infected by *F. graminearum*; at early
4 anthesis (stage 61) infection incidences were 26.2% and 17.0% for the two fungi, respectively. For
5 the inoculations made at heading (stage 59) incidences were of 16.7% and 9.8% of kernels,
6 respectively. Maximum incidence of infected kernels was observed when *F. culmorum* was
7 inoculated at full anthesis in 2004 (51.0% of kernels).
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12 DON contamination was higher in kernels that have been inoculated with *F. culmorum* than with
13 *F. graminearum* (1.34 ppm and 0.27 ppm on average respectively); this difference was significant
14 (at $P < 0.001$) and accounted for 5% of total variance. Year and growth stages were also significant,
15 and accounted for 11% and 17% of variance, respectively. Contamination was higher in 2004 (1.44
16 ppm on average) than in 2003 (0.18 ppm). DON was higher in kernels from spikes that have been
17 inoculated at anthesis (1.65 ppm at stage 61 and 2.19 ppm at stage 65) than at heading (0.69 ppm)
18 and especially during ripening (from 0.21 at stage 71 to 0.02 at stage 85). Because a significant
19 ($P < 0.001$) interaction '*Fusarium* species x growth stage', differences between growth stage were
20 higher for *F. culmorum* than for *F. graminearum* (Figure 1).
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29 A significant correlation was observed between *Fusarium* DNA and DON contamination of
30 kernels ($r = 0.797$, $P < 0.0001$, $n = 144$) and this correlation was consistent over years and fungal
31 species: correlation coefficients varied between 0.617 for *F. graminearum* in 2003 and 0.848 for *F.*
32 *culmorum* in 2004 (Table I). When DNA was lower than 54 pg (a value of 4 on the ln-scale) about
33 70% of grain samples had a DON content lower than the detection limit; when DNA was higher
34 than this value 93% of samples contained measurable DON, when DNA was greater than 1096 pg
35 (7 in the ln-scale) all samples were contaminated (Figure 2). Quantitative relationship between the
36 ln-transformed values of these two variable was fitted by a power equation that accounted for 88%
37 of data variability (Figure 3). Back-transformation of the fitted data showed that increases in
38 *Fusarium* DNA resulted in nonlinear increases of DON: DON accumulation per unit of fungal DNA
39 was greater for severely infected grain samples than for the less infected ones.
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49 *Fusarium* DNA was also correlated with incidence of infected kernels, but this correlation was
50 lower and less significant ($r = 0.195$, $P = 0.019$, $n = 144$); furthermore, the coefficient of correlations
51 between these two variables were significant only in 2004 (Table I), when the incidence of infection
52 was higher. In a similar way, infected kernels were also correlated with DON, but less closely than
53 DNA did ($r = 0.411$, $P < 0.001$, $n = 144$) and only in 2004 (Table I).
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58 *Fusarium* DNA detection after infection

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60 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,

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3 at $P < 0.001$; only '*Fusarium* species x variety' interaction was significant at $P = 0.017$. Time elapsed
4 after infection and *Fusarium* species were the more influential factors, accounting for 72% and 12%
5 of total variance, respectively, with their interaction accounting for 7%.
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8 Using the qPCR assay the *Fusarium* DNA was always detected in the inoculated spikes before
9 the appearance of first disease symptoms. Considering a level of 4 pg of DNA (1.61 in the ln-scale)
10 as the minimum content of DNA for robust detection of the fungi in the spike tissue, it was
11 observed that *F. culmorum* in wheat spikes was detected for first time 18 hours after infection in
12 three varieties and after 24 hours also in the bread wheat Bilancia (Table II). After this time amount
13 of DNA progressively increased until 9 days and then did not significantly increased in the
14 following 3 days. First robust detection of *F. graminearum* DNA occurred 48 and 72 hours after
15 inoculation in the durum wheat S. Carlo and Duilio, respectively. In bread wheat Sagittario first
16 detection occurred after 6 days and only after 9 days in Bilancia (Table II). On average of different
17 varieties, the DNA content of spikes progressively increased until the last sample collected 12 days
18 after inoculation.
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32 Discussion

33 This work was planned in such a way to obtain a data set characterised by high variability in both
34 *Fusarium* infection and DON contamination to draw representative and robust results. For this
35 reason, artificial inoculations with *F. culmorum* and *F. graminearum* were made on three durum
36 and three bread wheat varieties characterised by different susceptibility to FHB, at six different
37 growth stages between heading and dough ripening, for a 2-year period.
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42 The degree of *Fusarium* infection was determined as incidence of infected kernels and amount of
43 fungal biomass measured as *Fusarium* DNA, and these variables were correlated with the level of
44 DON contamination. In this work we did not perform a visual disease assessments of FHB, but
45 literature demonstrates that there is no robust association between FHB symptoms and DON
46 contents in grains (Boyacioglu et al. 1992, Lemmens et al. 1997, Liu et al. 1997, Martin and
47 Johnston 1982, Doohan et al. 1999, Edwards et al. 2001, Birzele et al. 2002) because some fungi
48 causing the disease do not produce this mycotoxin. Furthermore, no correlation was found between
49 visual FHB assessments and concentration of trichothecene-producing *F. culmorum* and *F.*
50 *graminearum* measured with a PCR-based assay based on primers derived from *Tri5* (Edwards et
51 al. 2001).
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60 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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3 Similar results had been previously found by other authors (Edwards et al. 2001, Schnerr et al.
4 2002, Waalwijk et al. 2004), but the present work showed that this relationship is consistent over
5 *Fusarium* species, wheat species and varieties, and over a wide range of epidemiological conditions
6 due to different meteorological conditions and times of infection occurrence. In a previous work,
7 different correlations were found between *Fusarium* exoantigens and DON in harvested grain from
8 field samples of hard red spring wheat in Manitoba, Canada, and of soft white winter wheat in
9 Ontario, Canada, indicating that the relationship between amounts of *Fusarium* biomass and DON
10 in harvested grain may be affected by the host and by its interactions with environmental conditions
11 (Abramson et al. 1998). In this study, correlation between *Fusarium* DNA and DON concentrations
12 occurred in experimental fields that have been artificially inoculated with trichothecene-producing
13 strains of *F. culmorum* and *F. graminearum*. This relationship could probably be weaker for field
14 samples, where the mycoflora infecting kernels may differ for the capability to produce DON or, in
15 the case of *F. graminearum*, in the relative amounts of DON and NIV they produce. Nevertheless,
16 good correlations were found with the qPCR assay in field samples showing a high variability in
17 both DNA and DON contents (Terzi et al. 2007).
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21 This work also showed that correlation between *Fusarium* DNA and DON is closer and more
22 consistent than correlation between incidence of *Fusarium*-infected kernels and DON. Significant
23 relationships between the incidence of particular *Fusarium* species and the concentrations of
24 fusariotoxin have been previously found. For instance, Krisinska-Traczyk et al. (2001) found that
25 concentration of *F. culmorum* in total examined grain and grain dust samples collected in Poland
26 was significantly correlated with the concentration of DON, at $P < 0.05$. A significant correlation
27 between the incidence of *F. graminearum* and DON has been also found by Dalcero et al. (1997) in
28 Argentina, while Moreno Contreras et al. (2000) did not show any significant correlation between
29 the presence of *Fusarium* spp. and DON in grain samples collected in Venezuela.
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33 The qPCR assay made it possible to detect differences between levels of kernel infection better
34 than fungal isolation from kernels with classical mycological methods. Some reasons can support
35 this result. Kernel infection was determined by plating samples of kernels and by determining
36 percent incidence of grains with developing colonies of *F. culmorum* or *F. graminearum*. This
37 procedure can only distinguish between healthy and infected grains but can't define the biomass of
38 mycelia invading an infected kernel. For instance, a kernel which was infected in an advanced stage
39 of ripening may result as infected using the plating method because a *Fusarium* colony will grow
40 from it, but invasion of grain tissue may have a little extent as well as the amount of DON produced
41 because of a few time will be available between infection and the drop of available water below the
42 minimum for fungal growth and mycotoxin production, respectively (Rossi et al. 2006).
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3 Furthermore, the plating method can only detect living mycelia in *Fusarium*-infected kernels while
4 the qPCR assay provides a measure of biomass taking in account living mycelia actively producing
5 DON but also dead mycelia, which formerly had contributed to the DON content actually found.
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8 The qPCR assay also proved to be an useful tool to detect the dynamic of fungal invasion *in*
9 *planta* after infection had occurred, and to single out the presence of infection before onset of the
10 disease symptoms. A robust detection of the infection occurred within 18 to 24 hours for *F.*
11 *culmorum*, and within 2 to 9 days for *F. graminearum*. This result is very promising for his
12 potential impact on tactical disease management: a detection of infection occurrence within 2 or 3
13 days may result in an efficient application of curative fungicides (Boyacioglu et al. 1992, Homdork
14 et al. 2000, Matthies and Buchenauer 2000, Siranidou and Buchenauer 2001, Suty-Heinze and
15 Dutzmann 2004). It must be considered that this result was obtained by inoculating wheat spikes at
16 full flowering with a high inoculum dose, under optimum environment conditions for infection to
17 occur. It can be then supposed that under these conditions the disease spread rapidly within the
18 spike tissue and that the minimum DNA content for robust *Fusarium* detection (i.e. 4 pg) may have
19 reached early in comparison with natural infections. For this reason, further works will be carried
20 out to confirm possibility to early detect presence of trichothecene-producing *Fusaria* in wheat
21 spikes under field conditions.
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33 The qPCR assay based on *Tri5-Tri6* intergenic sequence (Terzi et al. 2007) provides a valuable
34 tool for detection of trichothecene-producing *Fusaria* both *in planta* and within harvested grains.
35 The assay has many potential uses both for research purposes, such as in selection of wheat
36 cultivars and in testing efficacy of fungicides, and for practical disease management, such as timing
37 fungicide applications and early estimating risk for trichothecene mycotoxins within grains at
38 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 <i>ns</i> ^b	0.836 <0.0001 ^b	0.371 0.0013 ^c
	Inc vs DON	0.189 <i>ns</i>	0.844 <0.0001	0.549 <0.0001
	DNA vs DON	0.799 <0.0001	0.848 <0.0001	0.818 <0.0001
<i>F. graminearum</i>	Inc vs DNA	-0.061 <i>ns</i> ^b	0.608 0.0001 ^b	-0.104 <i>ns</i> ^c
	Inc vs DON	0.019 <i>ns</i>	0.722 <0.0001	0.118 <i>ns</i>
	DNA vs DON	0.617 0.0001	0.816 <0.0001	0.779 <0.0001
<i>F. culmorum</i> + <i>F. graminearum</i>	Inc vs DNA	-0.065 <i>ns</i> ^c	0.774 <0.0001 ^c	0.195 0.019 ^d
	Inc vs DON	0.108 <i>ns</i>	0.832 <0.0001	0.411 <0.0001
	DNA vs DON	0.757 <0.0001	0.828 <0.0001	0.797 <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)

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

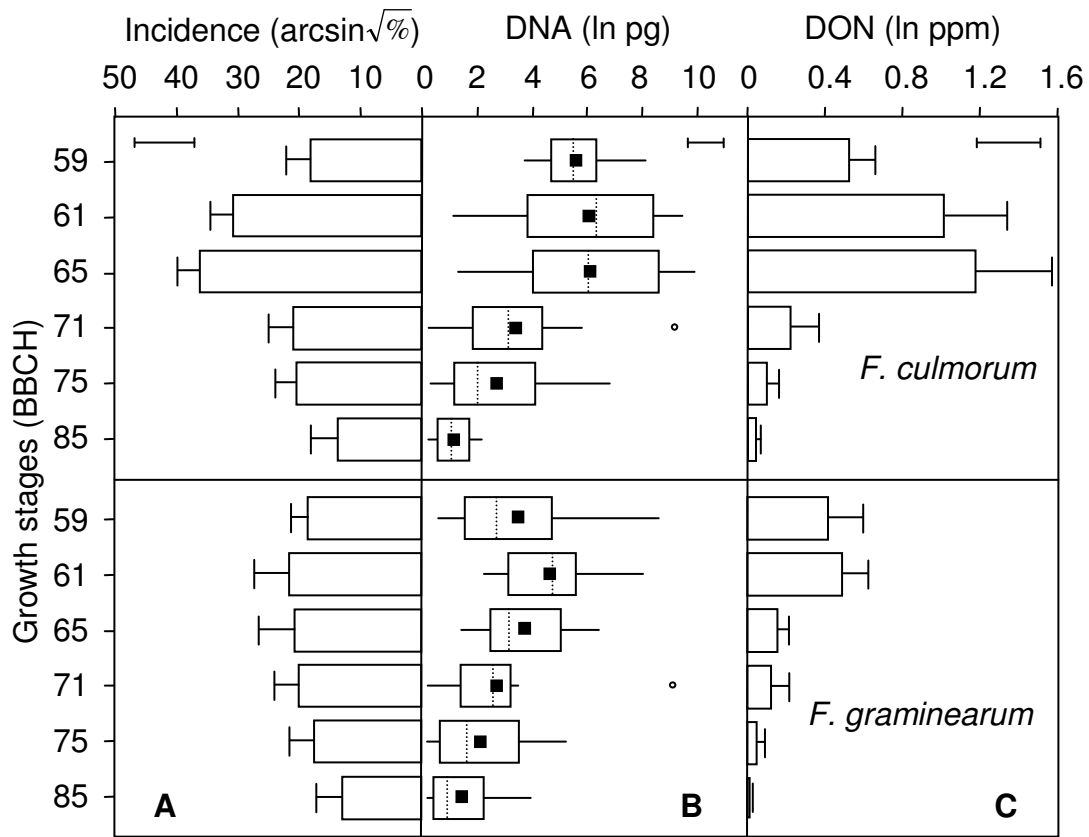
^d Underlining indicates first value when natural logarithm is ≥ 1.61 (i.e. ≥ 4 pg of *Fusarium* DNA over 25 ng of total DNA extracted)

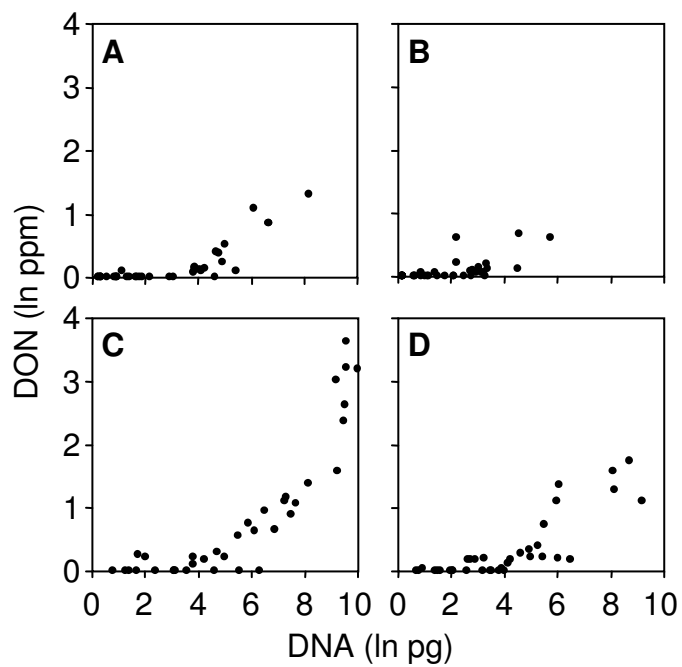
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Figure 1. Incidence of *Fusarium* infection (expressed as $\arcsin\sqrt{\%}$), *Fusarium* DNA (as natural logarithm of pg over 25 ng of total DNA) and content of deoxynivalenol (as natural logarithm of DON in ppm) in wheat kernels that have been inoculated 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 --- 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.





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