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Infection incidence, kernel colonisation, and mycotoxin accumulation in durum wheat inoculated with *Fusarium sporotrichioides*, *F. langsethiae* or *F. poae* at different growth stages

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Abstract A 2-year field experiment was conducted to determine the effects of *Fusarium sporotrichioides*, *F. langsethiae*, or *F. poae* on durum wheat plants artificially inoculated at different growth stages. The percentage of symptomatic kernels was similar among the three species, but incidence of infected kernels was lower for *F. langsethiae*. Kernel colonization was higher when plants were inoculated before and during anthesis for *F. sporotrichioides* and *F. poae*, but unaffected by timing of inoculation for *F. langsethiae*. Production of T-2/HT-2 toxins was higher for *F. sporotrichioides* than for *F. langsethiae*. Significant accumulations of nivalenol were detected for *F. poae*. Across all three species, there was a weak correlation ($r = 0.16$; $P = 0.031$) between the incidences of symptomatic and infected kernels, but a stronger correlation ($r = 0.53$; $P < 0.001$) between infection incidence and the quantity of fungal DNA (species-specific) in kernels. Mycotoxin content was correlated ($r > 0.58$; $P < 0.007$) with infection incidence or fungal DNA in kernels, but only for *F. sporotrichioides* and *F. poae*.

Keywords T-2 · HT-2 · Nivalenol · Small-grain cereals · Fusarium head blight

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Introduction

Fusarium head blight (FHB) is a destructive disease of small grain cereals and is caused by a complex of *Fusarium* species. The best known species of the complex is *F. graminearum* sensu stricto, which is also the most common species worldwide, including Europe. Other species have also been frequently reported, such as *F. avenaceum*, *F. culmorum*, *F. poae*, and *F. sporotrichioides* (Xu et al. 2008). More recently, *F. langsethiae* has been described as a frequent contaminant of wheat kernels in Europe (Torp and Nirenberg

2004; Imathiu et al. 2013a) and Italy (Infantino et al. 2007; Infantino et al. 2015). In addition to reducing wheat production, these *Fusarium* species also produce mycotoxins in the kernels, resulting in reduced quality of the final product. The most common *Fusarium* mycotoxins found in small-grain cereals are trichothecenes, which are classified as type A and type B trichothecenes, as well as zearalenones, and fumonisins (Desjardins et al. 2004). The most important type A trichothecenes are: T-2 and HT-2 toxins and derivatives (T-2 triol, T-2 tetraol), produced mainly by strains of *F. sporotrichioides*, *F. langsethiae* and *F. sibiricum*; diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (MAS), produced by strains of *F. poae*, *F. sporotrichioides, F. langsethiae* and *F. sibiricum*; and neosolaniol (NEO), produced mainly by strains of *F. sporotrichioides* and *F. poae*. The type B trichothecenes include deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives (3-Ac-DON, 15-Ac-DON, and fusarenone X), which are mainly produced by strains of *F. graminearum*, *F. culmorum*, *F. cerealis* (syn. *F. crookwellense*), and *F. poae* (Logrieco et al. 2003; Stępień and Chełkowski 2010; Yli-Mattila and Gagkaeva 2016).

The accumulation of trichothecenes in food and feed causes toxicoses in humans and animals, respectively (Richard 2007). The EU has therefore regulated the occurrence of some of these compounds [e.g., DON (European Commission 2006)]. For T-2 and HT-2 toxins, the European Commission (European Commission 2013) has recommended a maximum level of their sum in wheat kernels. Legal limits for these mycotoxins, however, are being debated (European Commission 2013), considering that these compounds are the most toxic mycotoxins produced by *Fusarium* species (Desjardins 2006).

Some confusion exists regarding which *Fusarium* species produce T-2 and HT-2. Some authors have reported that *F. poae* is able to produce both T-2 and HT-2 (Dinolfo and Stenglein 2014; Kulik and Jestoi 2009; Kurchenko and Tsyganenko 2013; Stenglein 2009; Stępień and Chełkowski 2010; Thrane et al. 2004). Proctor et al. (2009), however, reported that the *Tri16* gene, which is needed for T-2 production, is truncated and therefore most likely non-functional in *F. poae*. Consistent with the latter finding, Somma et al. (2010) reported that samples of wheat kernels heavily contaminated by *F. poae* in Italy were not contaminated by either T-2 or HT-2 and that none of the many *F. poae* strains tested for mycotoxin production were able to produce the two mycotoxins. On the other hand, also Vanheule et al. (2017), who studied genetic divergence and chemotype occurrence in a wide population of *F. poae* isolated worldwide, confirmed the lack of T-2 and HT-2 production in this species. Therefore, the possible accumulation of T-2 and HT-2 in wheat fields could be related to the occurrence of *F. langsethiae* and *F. sporotrichioides*, which are known to produce these mycotoxins (Desjardins et al. 2004). Recently, the novel species *F. sibiricum* has been identified (Yli-Mattila et al. 2011), which is also able to produce T-2 and HT-2 toxins, and an isolate belonging to this species has also been retrieved in Italy by (Infantino et al. 2017). These species are morphologically closely related to *F. poae* (O'Donnell et al. 2013) and often they co-exist in wheat fields. Therefore, a better understanding is needed of the ability of *F. langsethiae*, *F. sporotrichioides*, and *F. poae* to infect wheat plants and produce toxins.

Infection in small-grain cereals by *Fusarium* has traditionally been assessed by isolating fungi from kernels via classical methods. This involves surface sterilization of kernels, plating on selective media, and identification based on morphological observation of the fungal colonies (Pitt and Hocking 2009). Molecular methods, however, can now be used to identify and quantify *Fusarium* species in infected grains or plant tissues (Hill et al. 2008). In particular, quantitative PCR (qPCR) assays are currently available for *F. sporotrichioides*, *F. langsethiae*, and *F. poae* (Fredlund et al. 2010; Halstensen et al. 2006; Nicolaisen et al. 2009; Waalwijk et al. 2004; Yli-Mattila et al. 2008). Such assays measure both living and dead mycelia and therefore account for mycelia that are actively producing toxin and mycelia that formerly produced toxin. The traditional plating method, in contrast, detects only living mycelium in the kernels (Rossi et al. 2007).

The current research had two objectives. The first was to quantify the infection of harvested durum wheat grain by three trichothecene-producing *Fusarium* spp. (i.e., *F. sporotrichioides*, *F. langsethiae*, and *F. poae*). For this objective, two cultivars of durum wheat were inoculated at different growth stages, and *Fusarium* biomass in kernels was quantified with qPCR. The second objective was to determine the relationships between the following response variables: levels of *Fusarium* DNA in kernels, the incidence of symptomatic kernels as determined by visual assessment, the incidence of infected kernels as determined by classical plating methods, mycotoxin contamination, and crop yield.

Materials and methods

Fungal strains and inoculum preparation

Two strains of *F. sporotrichioides*, two strains of *F. langsethiae*, and four strains of *F. poae* were provided by the Institute of Sciences of Food Production (ISPA-CNR, Bari, Italy). The identities of the strains were confirmed by Elongation Factor 1α (Somma et al. 2010). Strains used in the present study are ITEM 692 and ITEM 695 belong to *F. sporotrichioides*; both strains produce T-2, HT-2, and NEO, and were isolated from corn and wheat kernels, respectively, in the USA (Logrieco et al. 1990). Strains ITEM 11020 and ITEM 11025 belong to *F. langsethiae*; they produce T-2 and HT-2 (Nazari et al. 2014) and were isolated from durum wheat kernels in southern Italy. Strains ITEM 9138, ITEM 9211, ITEM 10402, and ITEM 10408 belong to *F. poae* and were isolated from wheat kernels in North Italy. Strain ITEM 9138 produces beauvericin (BEA), fusarenon-X (FX), NEO, and NIV; strain ITEM 9211 produces BEA, DAS, FX, NEO, and NIV (Somma et al. 2010); ITEM 10402 and ITEM 10408 have not been characterized for the production of mycotoxins. The ability of the strains used in the present study to produce mycotoxins other than those stated (i.e. DAS) has not been investigated. The fungal strains were stored at 4 °C in tubes containing water agar.

To obtain inoculum suspensions, plugs (0.5 cm diameter) of each isolate were placed in the centre of Petri plates (9 cm diameter) containing PDA (Himedia, Mumbai, India). The plates were then incubated for 14 days at 30 °C with 12 h of light and 12 h of dark. After incubation, five uniform plates for each isolate were selected; the colonies were gently scraped and washed with 20 ml of sterile water. The resultant suspension was passed through two layers of gauze to remove hyphae; the concentration of conidia in the resulting suspension was determined using a Bürker chamber and was adjusted to 1×10^4 conidia/ml. The suspension used for inoculation of plants was obtained by mixing equal amounts of the conidial suspensions of the strains of the same species and adding Tween 20 (Sigma-Aldrich, MO, USA) (0.1%).

Field experiment

In 2010–2011, a field experiment was conducted in Ravenna (North Italy), in an experimental farm in which *F. sporotrichioides*, *F. langsethiae*, and *F. poae* were not detected (or only occasionally detected) in FHB experiments carried out in previous years. The experiment had a split-split-plot design, with three replicates; cultivar was the main plot, fungal species was the sub-plot, and inoculation time (crop growth stage) was the sub-sub-plot. The plots were 1.5 × 4 m, and each plot was surrounded by a 1-m border. Additional plots were not inoculated and were used to determine the natural occurrence of *Fusarium* infection. The previous crop was wheat. The plots were tilled before wheat was sown. The crops were managed according to the usual practice, with no irrigation or fungicide application. The experiment was repeated in 2011–2012 at a nearby site; in this case, the previous crop was pea.

The cultivars of durum wheat (*Triticum durum* Desf) in the experiment were Iride and Saragolla, which have intermediate and high susceptibility to FHB, respectively (Haidukowski et al. 2012; Lionetti et al. 2015). In 2011, the plots were artificially inoculated with the conidial suspensions of either *F. sporotrichioides*, *F. langsethiae*, or *F. poae* at one of the following growth stages (BBCH scale of Meier 2001): i) end of heading (BBCH59, 04 May); full flowering (BBCH 65, 10 May); end of flowering (BBCH69, 17 May); late milk (BBCH77, 24 May); or soft dough (BBCH85, 30 May). In 2012, the plots were inoculated with the same fungi at one of the following growth stages; and ii) end of heading (BBCH59, 03 May); beginning of flowering (BBCH61, 08 May); full flowering (BBCH 65, 15 May); early milk (BBCH73, 22 May); or late milk (BBCH77, 29 May). In both years, the first inoculation was performed at the end of heading (BBCH59), and the following inoculations were done at weekly intervals. Therefore, differences in the growth stages at the time of inoculation between years were due to year-to-year variation in plant development.

All inoculations were performed in late afternoon and by distributing 100 ml of spore suspension per m^2 of plot with a nebulizer. To limit the drift of the inoculum to neighbouring plots, the inoculum was applied in the absence of wind. In addition, the plot to be inoculated was temporarily isolated from adjacent plots by placing 1.5-m-high plastic panels on the three sides of the plot in the path of the inoculum spray; the panels were removed when the inoculation was completed. After inoculation, an overhead misting system kept the plants moist for 48 h.

At maturity, 100 spikes were collected randomly from each plot and threshed. Kernels were divided into three subsamples to determine the incidence of the symptomatic and infected kernels, the amount of *Fusarium* DNA, and mycotoxin contamination. For mycotoxin analysis, the samples from replicate plots were pooled. The whole plot was harvested to determine grain yield, 1000 grain weight, specific grain weight, and protein content. All weights are adjusted to a kernel moisture content of 14%.

Air temperature, relative humidity, and rainfall were recorded hourly by an automatic weather station (iMeteos OEM Model-1, Pessl Instruments GmbH, Weiz, Austria) located 100 m from the field site.

Re-isolation of fungal pathogens

To determine *Fusarium* infection, kernels were surface sterilized in ethyl alcohol (70%) for 10 s and then in sodium hypochlorite (1% available chlorine) for 2 min; they were subsequently rinsed three times in sterile water and dried on absorbent paper under a sterile air flow. The kernels were placed in Petri plates on DCPA (15 g of peptone, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \times 7H_20$, 1 ml of dichloran solution (0.2% *w*/*v* in ethanol), 100 mg of chloramphenicol, and 10 g of agar per 1 l; after the substrate was autoclaved, 50 mg/l of neomycin and 50 mg/l of streptomycin were added). After 6 to 8 days of incubation at room temperature, fungal colonies growing from the kernels were transferred to other Petri plates containing PDA with streptomycin (50 mg/l) and neomycin (50 mg/l); the plates were incubated at 25 °C with an artificial day length of 12 h. *Fusarium* species were then identified according to Nelson et al. (1983) and Leslie and Summerell (2006). When necessary, isolates were transferred to carnation-leaf agar, as described by Nelson et al. (1983). Incidence of infection was then calculated as the percentage of kernels infected by *F. sporotrichioides*, *F. langsethiae*, or *F. poae*.

Genomic DNA isolation

From fungal mycelium

Each strain was grown in 100 ml of liquid medium (glucose 4%, malt extract 0.3%, yeast extract 0.3%, and peptone 0.5%) in shake culture (160 g) at 25 °C for 48 h. The mycelium was collected on a filter, washed with sterile-distilled water, lyophilized, and ground to a fine powder. DNA was extracted from 25 mg of lyophilized mycelium with the E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) according to the manufacturer's protocol.

From grain samples

After threshing, wheat kernels were ground to a flour for 1 min each sample with a grinder M20 (IKA Works, Wilmington, NC, USA). This flour was carefully homogenised and a 200-mg quantity was 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 5 M guanidinium chloride. After incubation at 60 °C for 3 h, the samples were centrifuged for 10 min at 20,000 g. A 5-μl volume of RNase (500 μg/μml) was added to 500 μl of the supernatant, which was then incubated at 37 °C for 10 min for digestion of the contaminating RNA. The extracted DNAs were purified according to the Wizard protocol by using Wizard® Minicolumns and Wizard® MiniPreps DNA Purification Resin (Promega, Milan, Italy) and were eluted with 55 μl of buffer (10 mM Tris-HCl, pH 9.0).

Genomic DNA was checked for quality and quantity by agarose gel electrophoresis (0.8%) and by comparison with a DNA standard. Both genomic DNA, from fungal mycelium and from wheat kernels, was quantified by using the spectrophotometer NanoDrop ND-1000 (Thermo-Scientific).

The DNA from the mycelium of *Fusarium* species used as reference was used to build the standard curve, the DNA from the grain was used to identify and quantify the fungi in the grain, as described in the following section.

Real-time PCR

The quantity of fungal DNA of each target species in wheat samples was determined with real-time PCR assays with species-specific TaqMan probes. The probes and primers used in this study are listed in Table 3. The primers and probe for *F. poae* DNA detection were from Waalwijk et al. (2004); the primers and probes for *F. sporotrichioides* and *F. langsethiae* were from Köhl et al. (2015). TaqMan probes, labelled at the 5′ end with the fluorescent reporter dye 6-carboxylfluresceine (FAM) and at the 3′ end with black hole quencher, were synthesized by Sigma-Aldrich Biotechnologies (St. Louis, MO, USA).

Different real-time PCR assays were performed to determine the optimal primers and probe concentrations for *F. sporotrichioides*, *F. langsethiae*, and *F. poae*. A standard curve was plotted from six serial dilutions (ranging from 10 ng to 0.1 pg) of purified genomic DNA of a representative strain for each species. Each assay was conducted in triplicate. Wheat DNA extracted from water-inoculated wheat heads was used as a negative control. For an additional negative control, sterile-distilled water was used in place of DNA template. Each 20-μl reaction volume contained 20 ng of genomic DNA isolated from the inoculated wheat heads, 10 μl of PremixExTaq (Takara), 320 nM of each reverse and forward primer for *F. sporotrichioides* or *F. poae* and 200 nM for *F. langsethiae*, 100 nM TaqMan probe, and 0.32 μl of ROX. Each reaction contained also 100 pg of Potato Leaf Roll Virus (PLRV) DNA as amplification control, 83 nM of the related VIC labelled probe and 333 nM of each related primer. Real-time PCR assays were performed in a 7500 Fast Real Time PCR System (Fisher Scientific). The PCR cycling and temperatures were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amount of fungal DNA was expressed as pg of DNA per mg of wheat flour.

Trichothecene analysis in the grain samples

Wheat samples inoculated with *F. sporotrichioides* or *F. langsethiae* strains were analysed for the content of T-2 + HT-2 toxins; wheat samples inoculated with *F. poae* strains were also analysed for NIV.

A 10 g quantity of ground kernels was extracted with 50 ml of a CH_3CN/H_2O (84:16; *v/v*) solution; the preparation was stirred for 1 h and then centrifuged at 6000×g for 5 min at 5 °C before the supernatant was passed through a paper filter. A 5 ml volume of the filtrate was evaporated using a centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY, USA); the residue was dissolved in 1 ml of a CH_3OH/H_2O (70:30, *v/v*) solution and centrifuged at 18,000 g for 3 min before LC/MS/MS analysis. Standards for T-2, HT-2, and NIV were purchased from Sigma-Aldrich (Milano, Italy). Acetonitrile, methanol, and water for the LC mobile phase and organic solvents were HPLC grade from Merck (Darmstadt, Germany). Stock solutions of standards were prepared in methanol at 1 mg/ml and were kept at −20 °C in the dark. All working standard solutions were prepared by diluting the stock solution with a methanol/water mixture (70:30, *v/v*).

LC analysis was performed using a system consisting of two Perkin-Elmer series 200 micro pumps (Norwalk, CT, USA). A Gemini 5 μ C18 110A column, 150 × 2.00 mm (Phenomenex, USA) heated to 25 °C was used, and the flow rate was set to 0.2 ml/min, while the injection volume was 20 μl. Mobile phase A consisted of an $H_2O/CH_3OH/CH_3COOH$ mixture (89:10:1, *v/v/v*) containing 5 mM ammonium acetate, and mobile phase B consisted of an $H_2O/CH_3OH/CH_3COOH$ mixture (10:89:1, *v/v/v*) containing 5 mM ammonium acetate. The gradient program was as follows: 55–70% B (3 min), 70–100% B (5 min), constant at 100–30% B (3 min), and finally returning to the initial conditions in 3 min.

MS/MS analyses of trichothecenes were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source. Analyses were performed in MRM (Multiple Reaction Monitoring) using the positive ion mode for T-2 and HT-2 and the negative ion mode for NIV.

The de-clustering potential (DP), focus potential (FP), and the collision energy (CE) were optimized for each compound as it directly infused into the mass spectrometer standard solutions (10 μg/ml) at a constant flow rate of 8 μl/min using a model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). Drying gas (air) was heated to 400 °C, and the capillary voltage (IS) was set to +5000 V in positive ion mode and − 4000 V in negative ion mode. Calibration curves for T-2, HT-2, and NIV were built in the linearity range of 10–500 ng/ml. The detection limits (LOD) for T-2, HT-2, and NIV were 0.5, 1, and 3 μg/kg, respectively, and quantification limits (LOQ) were 2, 3, and 8 μg/kg, respectively.

Data analysis

A factorial analysis of variance (ANOVA) was performed on infection incidence, DNA content, mycotoxin contamination, and yield data; the factors were year, wheat cultivar, and wheat growth stage species. The uninoculated plots were not included in the analysis because the disease and mycotoxin data showed no relevant natural infection. Fungus species was not a factor in the factorial analysis because each fungus was analysed separately. To make variances homogeneous, DNA and mycotoxin data were log-transformed, and percentage data were arcsine-transformed. Pearson

correlation coefficients were calculated to determine relationships between the above variables. All statistical analyses were performed using the SPSS package v. 21 (SPSS Inc., Chicago, IL, USA).

Results

Incidence of symptomatic and infected kernels

Across both cultivars and years, the incidence of kernels with typical FHB symptoms (i.e., discoloration and shrivelling) was similar for *F. sporotrichioides* (11.7 ± 0.6%), *F. langsethiae* (12.4 ± 0.7%), and *F. poae* (13.2 ± 0.9%), while the incidence of infected kernels (as determined by plating and isolation) was 8.2 ± 1.8%, 3.9 ± 0.8%, and 9.6 ± 1.7% for the three species, respectively.

For *F. sporotrichioides* and *F. poae*, infection incidence was significantly influenced by year, wheat growth stage at the time of inoculation, and the year × growth stage interaction, but not by wheat cultivar or corresponding interactions (Table 1). For *F. sporotrichioides*, infection incidence was higher in 2011 than in 2012 (Tables 2 and 3), and was higher in kernels from spikes that were inoculated at full anthesis rather than at other growth stages (Fig. 1A). Similar results were obtained for *F. poae* (Table 2), but infection incidence was also high when plants were inoculated at BBCH 59 in 2011 (Fig. 1B).

For *F. langsethiae*, infection incidence was not significantly affected by growth stage at the time of inoculation or by the growth stage × year interaction but was significantly affected by cultivar and the cultivar × year interaction (Table 2). Infection incidence was irrelevant in 2012 (<1% of infected kernels); in 2011, kernels of Iride were more infected than those of Saragolla (11.3 ± 4.8 and 3.6 ± 1.4% of infected kernels, respectively).

The higher infection frequency in 2011 than in 2012 may have been related to weather conditions during the kernel ripening period. Specifically, air temperature was higher and rainfall was lower in June 2012 (mean temperature = 23.5 °C and total rain = 35.1 mm) than in 2011 (mean temperature = 21.9 °C and total rain = 7.2 mm).

The correlation between the incidence of symptomatic kernels and infected kernels was statistically significant but weak ($r = 0.16$, $P = 0.031$, $n = 180$).

Kernel colonisation

For *F. sporotrichioides* and *F. poae*, the fungal biomass in kernels was significantly affected by year, growth stage at the time of inoculation, and the year × growth stage interaction; these sources of variation collectively accounted for 95.8 and 91.7% of the total variance, respectively, for the two species (Table 1). Neither wheat cultivar nor the 2- or 3-way interactions with cultivar had significant effects (Table 1). For *F. langsethiae*, only the year and the year × wheat cultivar × growth stage interaction were significant, with year accounting for more than 95% of the variance.

Kernel colonization by *F. langsethiae* and *F. poae* was higher in 2011 than in 2012, with an overall average of 20.6 ± 1.6 and 1.6 ± 0.2 pg/mg of DNA, respectively, for *F. langsethiae*, and 7.7 ± 2.3 and 2.6 ± 0.6 pg/mg of DNA, respectively, for *F. poae*. Kernel colonization by *F. sporotrichioides* was similar in 2011 and 2012, with 13.2 ± 5.1 and 13.8 ± 5.7 pg/mg of DNA, respectively (Table 2). Apart from *F. langsethiae*, in which inoculation at different growth stages resulted in similar amounts of DNA in kernels, colonization was higher in kernels inoculated before and especially during anthesis than after anthesis, even though the magnitude of these differences depended on the inoculated fungus (*F. sporotrichioides* or *F. poae*) and on the year (Fig. 2).

Across both cultivars and both years, the incidence of infected kernels was correlated with the quantity of fungal DNA ($r = 0.53$, $P < 0.001$, n = 180) with $r = 0.72$ ($P < 0.001$, $n = 60$) for *F. sporotrichioides,* $r = 0.43$ ($P = 0.001$, $n = 60$) for *F. langsethiae*, and $r = 0.56$ ($P < 0.001$, $n = 60$) for *F. poae*.

Mycotoxins

The combined contamination of T-2 and HT-2 was 1.7-times higher in kernels from plants inoculated with *F. sporotrichioides* rather than with *F. langsethiae* (overall average of 252 ± 74 and 145 ± 25 μg/kg, respectively). No contamination by these toxins was detected in kernels from plants inoculated with *F. poae*.

Mycotoxin contamination (T-2 + HT-2) of kernels by *F. langsethiae* was influenced only by year ($P < 0.001$), with 67 ± 7 μg/kg in 2011 and 222 ± 37 μg/kg in 2012 (Table 2). There was no apparent relationship between the content of *F. langsethiae* DNA in kernels and T-2 + HT-2 content, because the higher mycotoxin

Table 1 Results of ANOVA for infection incidence, fungal DNA, and mycotoxin contamination in harvested kernels of two cultivars of durum wheat (Iride and Saragolla), which were inoculated at different growth stages in 2011 and 2012 with *Fusarium sporotrichioides*, *F. langsethiae*, or *F. poae*

Source of variation		*F. sporotrichioides*		*F. langsethiae*		*F. poae*	
	Df[a]	*P* value	% variance[b]	*P* value	% variance	*P* value	% variance
Incidence of infected kernels							
1 Year	1	<0.001	17.6	<0.001	64.7	<0.001	51.8
2 Wheat cultivar	1	0.623	0.3	0.002	13.9	0.109	3.2
3 Wheat growth stage	4	<0.001	39.0	0.841	1.8	0.038	13.2
1 × 2	1	0.365	0.9	0.001	16.6	0.477	0.6
1 × 3	4	<0.001	34.2	0.887	1.4	0.001	25.8
2 × 3	4	0.579	3.2	0.913	1.2	0.820	1.8
1 × 2 × 3	4	0.367	4.9	0.986	0.4	0.555	3.6
Fusarium DNA in kernels							
1 Year	1	0.004	8.5	<0.001	95.2	<0.001	36.0
2 Wheat cultivar	1	0.574	0.3	0.387	0.1	0.185	2.3
3 Wheat growth stage	4	<0.001	61.2	0.455	0.6	<0.001	34.1
1 × 2	1	0.727	0.1	0.543	0.1	0.459	0.7
1 × 3	4	<0.001	26.1	0.080	1.4	0.006	21.6
2 × 3	4	0.845	1.2	0.229	0.9	0.750	2.5
1 × 2 × 3	4	0.583	2.6	0.039	1.7	0.708	2.8
Toxin contamination in kernels[c]							
1 Year	1	0.746	0.2	<0.001	89.1	0.187	5.0
2 Wheat growth stage	4	0.004	50.9	0.976	1.6	0.040	38.0
1 × 2	4	0.005	48.9	0.628	9.3	0.012	57.0

[a] degrees of freedom; [b] % of variance accounted for by the source of variation; [c] for mycotoxin quantification, samples of the replicated plots were pooled, and cultivars were used as replicates in the ANOVA; toxins are T-2 + HT-2 for *F. sporotrichioides* and *F. langsethiae*, and NIV for *F. poae*

contamination in 2012 corresponded to a lower DNA content; the same was true for the incidence of infected kernels.

Mycotoxin contamination (T-2 + HT-2) of kernels by *F. sporotrichioides* was not significantly affected by year ($P = 0.746$) but was significantly affected by growth stage at the time of inoculation ($P = 0.004$) and the growth stage × year interaction ($P = 0.005$), which accounted for 50.9 and 48.9% of the total variance, respectively. Mycotoxin content in kernels was highest in plants inoculated at anthesis and was especially high for plants inoculated at full anthesis in 2011 (Fig. 3A). For *F. sporotrichioides*, T-2 + HT-2 content was correlated with the incidence of infected kernels ($r = 0.88$, $P < 0.001$, $n = 20$) and with the quantity of *F. sporotrichioides* DNA in kernels ($r = 0.81$, $P < 0.001$, $n = 20$) but not with the incidence of symptomatic kernels ($r = 0.01$, $P = 0.957$, $n = 20$).

Mycotoxin contamination (NIV) of kernels by *F. poae* was significantly affected by wheat growth stage at inoculation and by the growth stage × year interaction but not by year alone (Table 1). NIV contamination was highest in plants inoculated at full anthesis in 2011 (Fig. 3B). For *F. poae*, NIV content was correlated with the quantity of *F. poae* DNA in kernels ($r = 0.58$, $P = 0.008$, $n = 20$) and with the incidence of infected kernels ($r = 0.58$, $P = 0.007$, $n = 20$) but not with the incidence of symptomatic kernels ($r = 0.10$, $P = 0.662$, $n = 20$).

Grain yield

Grain yield was not affected by wheat cultivar but was higher in 2011 than in 2012 averaged across both cultivars (7.96 ± 0.12 Mg/ha in 2011 vs. 5.71 ± 0.19 Mg/ha in 2012). Specific grain weight, 1000 grain weight, and protein content were

Table 2 Incidence of infected kernel (%), *Fusarium* DNA in kernels (pg DNA/mg flour), and toxin content (μg/kg) in kernels of two durum wheat cultivars (Iride and Saragolla) in 2011 and 2012

	2011[a]			2012		
Fusarium species	Iride	Saragolla	average	Iride	Saragolla	average
Incidence of infected kernels						
F. sporotrichioides	12.4 *3.2*[b]	16.7 *5.6*	12.5 *3.2*	4.4 *2.8*	3.2 *1.0*	3.8 *1.5*
F. langsethiae	11.6 *1.9*	3.6 *1.4*	7.6 *1.4*	0.1 *0.1*	0.3 *0.2*	0.2 *0.1*
F. poae	18.8 *4.1*	13.2 *3.1*	16.0 *2.6*	4.5 *2.8*	1.7 *1.1*	3.1 *1.5*
Fusarium DNA in kernels						
F. sporotrichioides	12.56 *5.35*	13.89 *8.79*	13.23 *5.06*	11.93 *9.83*	15.69 *4.75*	13.80 *5.67*
F. langsethiae	18.40 *1.39*	22.78 *2.86*	20.59 *1.61*	1.63 *0.23*	1.69 *0.22*	1.66 *0.16*
F. poae	7.42 *1.48*	8.02 *2.11*	7.72 *2.27*	3.27 *0.94*	1.99 *0.80*	2.63 *0.61*
Toxin contamination in kernels[c]						
F. sporotrichioides	336 *222*	296 *211*	316 *144*	169 *55*	207 *50*	188 *36*
F. langsethiae	79 *10*	55 *8*	67 *7*	209 *60*	235 *49*	222 *37*
F. poae	26 *7*	20 *7*	23 *5*	19 *7*	16 *6*	17 *4*

[a] Years of field experiments, in bold; [b] standard error, in Italics; [c] toxins are T-2 + HT-2 for *F. sporotrichioides* and *F. langsethiae*, and NIV for *F. poae*

unaffected by cultivar or year. In both years, inoculation with *F. sporotrichioides, F. langsethiae*, or *F. poae* at any growth stage did not significantly influence the yield variables.

Discussion

In this work, classical plating and molecular methods (qPCR) were used to study infection of wheat by three

Table 3 TaqMan primers and probes used in real-time PCR assays

Primer or probe	Primer sequence (5′–3′)
F. poae-probe[a]	[6FAM]CAAAATCACCCAACCGACCCTTTC [BHQ1]
F. langsethiae- probe[b]	[6FAM]CACACC[+C]ATA[+C]CTA[+C]GTGTAA[BHQ1][c]
F. sporotrichioides-probe[b]	[6FAM]CCACACCC[+A]TAG[+T]TAC[+G]TGTAA[BHQ1][c]
F. poae F[a]	AAATCGGCGTATAGGGTTGAGATA
F. poae R[a]	GCTCACACAGAGTAACCGAAACCT
F. langsethiae F[b]	GTTGGCGTCTCACTTATTATTC
F. langsethiae R[b]	TGACATTGTTCAGATAGTAGTCC
F. sporotrichioides F[b]	GGTTGGCGTCTCACTTATAC
F. sporotrichioides R[b]	AATTTCTGATTCGCTAAAGTGG

[a] (Waalwijk et al. 2004)

[b] (Köhl et al. 2015)

[c] letters in parentheses are LNA (Locked Nucleic Acid) modified bases making probes more specific

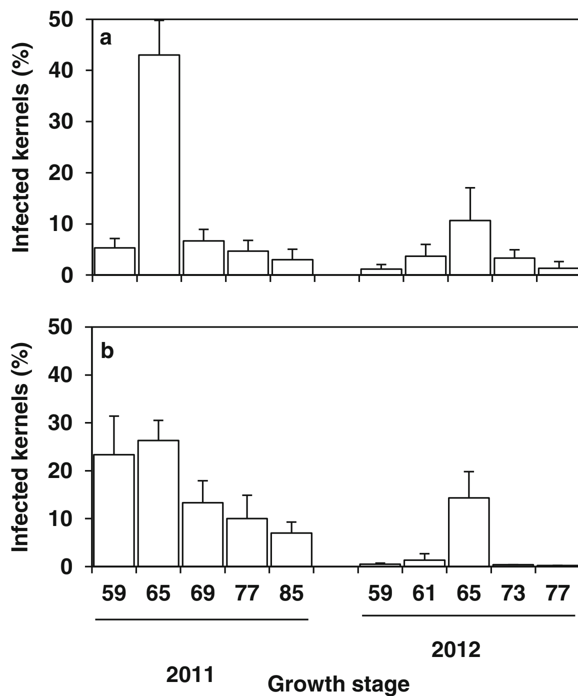

Fig. 1 Incidence of *Fusarium sporotrichioides* (**a**, average of 8.2 + 1.8%) and *F. poae* (**b**, average of 9.6 + 1.7%) in harvested durum wheat kernels produced by plants that were inoculated at one of the following growth stages in 2011 and 2012: 59, end of heading; 61, beginning of flowering; 65, full flowering; 69, end of flowering; 73, early milk; 77, late milk; or 85, soft dough. Stages are according to the BBCH scale. Values are means + SE of two wheat cultivars and three replicate plots. Values are not reported for *F. langsethiae* because growth stage at inoculation was not statistically significant for that species (average of 3.9 + 0.8%). Average infection incidence in uninoculated kernels was <1.1% for all the fungal species

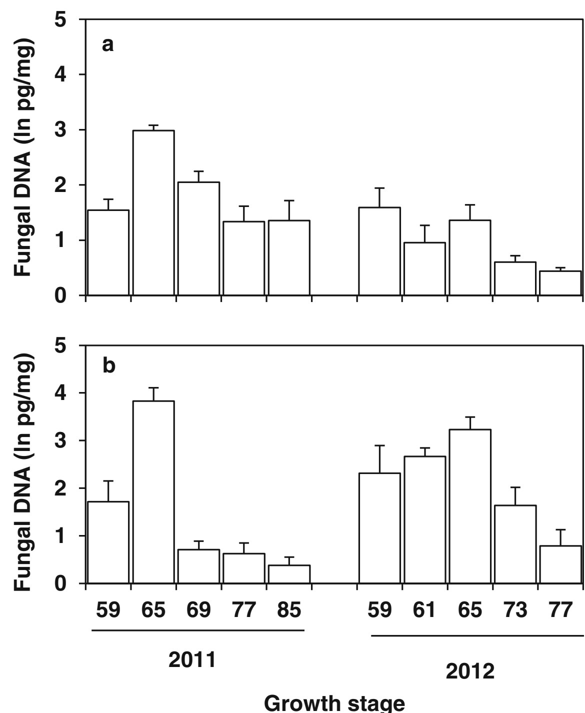

Fig. 2 Content of *Fusarium* DNA (ln of pg DNA per mg of flour) in harvested durum wheat kernels produced by plants that were inoculated with *Fusarium sporotrichioides* (**a**, average of 1.51 + 0.16 ln of pg DNA) or *F. poae* (**b**, average of 1.31 + 0.10 ln of pg DNA) at one of the following growth stages in 2011 and 2012: 59, end of heading; 61, beginning of flowering; 65, full flowering; 69, end of flowering; 73, early milk; 77, late milk; or 85, soft dough. Stages are according to the BBCH scale. Values are means + SE of two wheat cultivars and three replicate plots. Values are not reported for *F. langsethiae* because growth stage at inoculation was not a statistically significant factor for that species (average of 1.77 + 0.12 ln of pg DNA). Average DNA content in uninoculated kernels was <0.25 ln of pg DNA for all the fungal species

Fusarium species. The classical plating method requires the cultivation of fungi and morphological examination, which are time consuming; in addition, morphological identification requires substantial expertise. Another limitation of the classical method is that it does not detect dead hyphae that may have previously produced mycotoxins; hyphal viability in kernels may decline after harvest. Yet another potential problem with the classical method is that kernels may be infected by more than one species, and faster growing species can mask the presence of slower growing species. Because different *Fusarium* species can produce different mycotoxins, the accurate determination of the *Fusarium* species is critical for predicting the potential risk for mycotoxin contamination. Molecular methods are useful for identification at the species level, and they also enable the quantification of the pathogen in the examined matrix. The quantification of individual species is important for studying population dynamics, disease development, and mycotoxin production (Fredlund et al. 2010; Jurado et al. 2006; Kulik 2008; Waalwijk et al. 2004). The use of molecular methods was reviewed by Morcia et al. (2013). Molecular analysis is particularly important for the diagnosis and quantification of a species like *F. langsethiae*, which is difficult to isolate from plant material and which does not produce clear symptoms upon infection of cereals (Imathiu et al. 2013b). TaqMans used in the present study were developed for *F. sporotrichioides*, *F. poae* and *F. langsethiae*. Since *F. sibiricum* has one SNP (Single-Nucleotide Polymorphism) with *F. sporotrichioides* and it does not involve any of the primers of the TaqMan for *F. sporotrichioides*, cross-reaction cannot be excluded.

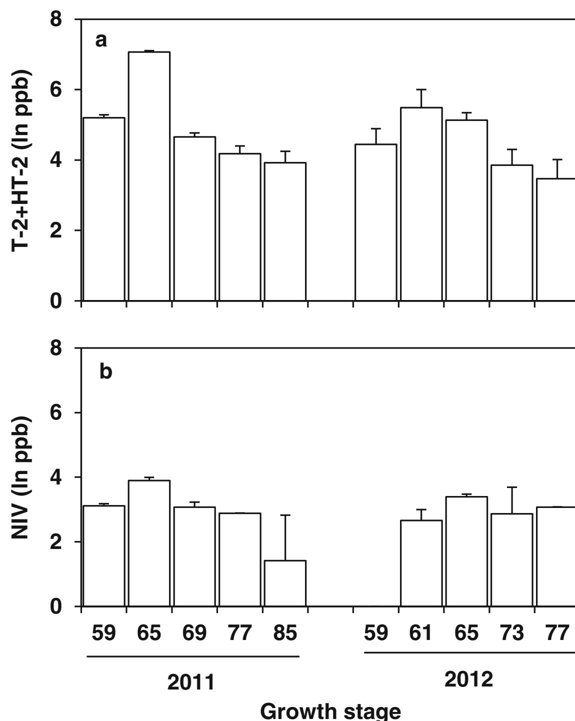

Fig. 3 Toxin content (ln of μg/kg) in harvested durum wheat kernels produced by plants that were inoculated at one of the following growth stages in 2011 and 2012: 59, end of heading; 61, beginning of flowering; 65, full flowering; 69, end of flowering; 73, early milk; 77, late milk; or 85, soft dough, Stages are according to the BBCH scale. Values are means + SE of two cultivars and three replicate plots. A) T-2 + HT-2 content of kernels from *Fusarium sporotrichioides*-inoculated plants; average T-2 + HT-2 content was 5.01 + 0.19 and < 1.2 ln of μg/kg in inoculated and uninoculated kernels, respectively. B) Nivalenol content of kernels from *F. poae*-inoculated plants; average nivalenol content was 2.46 + 0.24 and < 0 .32 ln of μg/kg in inoculated and uninoculated kernels, respectively. Values are not reported for *F. langsethiae* because growth stage at inoculation was not a statistically significant factor for that species; average T-2 + HT-2 content was 4.75 + 0.15 and < 1.1 ln of μg/kg in inoculated and uninoculated kernels, respectively

However, the occurrence of *F. sibiricum* in the wheat samples of this work in numbers able to interfere with the results is unlikely, since no reports of *F. sibiricum* in Italy exist, with the exception of one strain recently reclassified as *F. sibiricum* by Infantino et al. (2015) using mVOCs (microbial Volatile Organic Compounds).

Several studies have reported a correlation between the DNA content of single mycotoxigenic species of *Fusarium* and their corresponding mycotoxins (Fredlund et al. 2008; Waalwijk et al. 2004; Yli-Mattila et al. 2008). In the present work, the contents of T-2 + HT-2 toxins in kernels was correlated with the amount of *F. sporotrichioides* DNA in kernels, but this correlation was not significant for *F. langsethiae*. The present findings for *F. sporotrichioides* agree with those of Nazari et al. (2014), who reported a close correlation between *F. sporotrichioides* DNA and T-2 + HT-2 toxins in artificially inoculated wheat plants growing in pots. Jestoi et al. (2008) also reported correlation between *F. sporotrichioides* /*F. langsethiae* DNA and T-2 toxin in field trials. On the other hand, several studies have reported strong relationships between the quantities of toxins and *F. langsethiae* DNA (Edwards et al. 2012; Fredlund et al. 2010; Halstensen et al. 2006; Nazari et al. 2014; Nielsen et al. 2011; Yli-Mattila et al. 2008), which was not confirmed by the present study. The quantity of *F. poae* DNA was correlated with the NIV content in kernels in the current study. This finding agrees with previous studies by Yli-Mattila et al. (2008, 2009), Vogelgsang et al. (2008), and Kulik and Pszczólkowska (2011).

Correlations between *Fusarium* DNA and mycotoxins have also been reported for other *Fusarium* species, i.e., for *F. graminearum* or *F. culmorum* and DON (Rossi et al. 2007; Schnerr et al. 2002; Terzi et al. 2007; Waalwijk et al. 2004; Yli-Mattila et al. 2008). When the amount of fungus in the kernels was measured in terms of ergosterol content in a previous study, *F. culmorum* abundance was correlated with DON content (Snijders and Krechting 1992).

In the present work, no correlation was found between the percentage of symptomatic kernels and mycotoxin content. This agrees with the findings of Nazari et al. (2014) for *F. sporotrichioides* and *F. langsethiae*. Symptomless kernels can be infected by *F. poae*, as reported by Kulik and Jestoi (2009), who detected the fungal DNA in apparently healthy grains, and by Stenglein et al. (2012), who reported that the number of grains infected with *F. poae* was significantly higher than the number with observable disease symptoms. Our results also agree with previous studies that documented weak associations or no association between FHB symptoms on kernels and DON contents in kernels infected by various *Fusarium* species (Birzele et al. 2002; Boyacioglu et al. 1992; Doohan et al. 1999; Edwards et al. 2001; Lemmens et al. 1997; Liu et al. 1997a; Martin and Johnston 1982).

FHB was not rated in our field experiment because *F. langsethiae* and *F. poae* do not produce visible FHB symptoms on heads (Imathiu et al. 2009; Imathiu et al. 2013a; Infantino et al. 2015; Liu et al. 1997a). On the other hand, Divon et al. (2012) reported symptoms after

injection or spray inoculation of oats, and Stenglein et al. (2014) reported visible symptoms in inoculation trials with *F. poae*. In some other cases, researchers have not detected a correlation between FHB symptoms on heads and toxin content (Edwards et al. 2001; Liu et al. 1997b).

In the present work, the incidence of infected kernels was correlated with toxin content for *F. sporotrichioides* and *F. poae* but not for *F. langsethiae*. A lack of correlation between incidence of kernel infection and DON content was also reported for *F. culmorum* (Liu et al. 1997a) and *F. graminearum* (Browne 2007). For *F. sporotrichioides* and *F. langsethiae*, Nazari et al. (2014) documented a correlation between infected florets and mycotoxin content at harvest.

The content of T-2 + HT-2 in kernels was greater with *F. sporotrichioides* than with *F. langsethiae* in the current study. As previously reported by Torp and Nirenberg (2004), *F. sporotrichioides* grows faster than *F. langsethiae* both in vitro and *in planta*, with the former species infecting more florets and kernels and developing more fungal biomass. The relationship between toxin data collected in vitro and *in planta* is unclear, with some authors reporting a significant correlation (Vogelgsang et al. 2008), variable results (Jestoi 2008), or lack of correlation between the two kinds of data (Nazari et al. 2014). The sum of T-2 and HT-2 concentration in the kernels was 1.7-times higher for plants inoculated with *F. sporotrichioides* than for those inoculated with *F. langsethiae*. This agrees with previous findings. Kokkonen et al. (2010), for example, found that *F. sporotrichioides* produced a greater amount and variety of toxins than *F. langsethiae*. Similar results were obtained when ripe durum kernels were inoculated with the two species, i.e., *F. sporotrichioides* produced 4-times more T-2 and 11-times more HT-2 in kernels than *F. langsethiae* (Nazari et al. 2014). An higher production of T-2 + HT-2 toxins in *F. sporotrichioides* inoculated kernels, if compared to *F. langsethiae* inoculated ones, was also reported by Yli-Mattila et al. (2008, 2009) in wheat and barley.

Most *Fusarium* species that cause FHB infect wheat at the flowering stage (Parry et al. 1995). The current results with *F. sporotrichioides* and *F. poae* were consistent with this in that the incidence of infected kernels and the amounts of DNA and mycotoxins were highest when wheat plants were inoculated at full flowering (BBCH 65). This was not, however, the case for *F. langsethiae*, i.e., incidence of infected kernels and amounts of DNA and mycotoxins were not affected by growth stage at inoculation. Several surveys revealed that *F. langsethiae* is present in fields between cereal (oat) flowering and harvest (Fredlund et al 2010; Parikka et al. 2007; Yli-Mattila et al. 2008). Imathiu et al. (2013a) hypothesized that *F. langsethiae* infects cereal heads before flowering, which differentiates this species from the other *Fusarium* causing head blight. Lukanowski and Lenc (2009) also stated that anthesis was not be the best time for infection by *F. langsethiae*.

Fungal inoculation in the current study did not affect wheat production. Vargo and Baumer (1986) reported that wheat plants inoculated with *F. sporotrichioides* filled and ripened normally, with no or minimal yield reduction. *F. langsethiae* has been widely reported to cause symptomless infection. *F. poae* is considered a weak pathogen and is commonly isolated from cereal glumes (Doohan et al. 1998; Polley and Turner 1995). *F. poae* did not reduce yield in a study on wheat in controlled condition by Doohan et al. (1999), but did reduce yield in some oat genotypes tested in a filed study by Kiecana et al. (2012).

Over the past few decades, evidence has been accumulating that trichothecenes act as non-host-selective toxins involved in the aggressiveness of *Fusarium*-related diseases (Arseniuk et al. 1993; Beremand et al. 1991; Mesterházy et al. 1999). In addition, some FHB-resistant plant genotypes have been shown to detoxify DON, primarily by glycosylation (reviewed in Boutigny et al. 2008). These data suggest that the ability of *Fusarium* species to cause disease is linked to trichothecene accumulation in the host, and that reduced aggressiveness may be linked to either reduced toxin production by the pathogen or removal/degradation of the toxin by the host.

The results from the present study increase our understanding of how infection, colonization, and mycotoxin production by *F. sporotrichioides*, *F. langsethiae*, and *F. poae* differ among host growth stage at inoculation and among different years. These results will be useful for developing FHB prediction models (Rossi et al. 2007), for timing of fungicide applications (Rossi et al. 2007), and for predicting the types and amounts of toxins that accumulate in kernels (Blaney and Dodman 2002; Hooker et al. 2002; Mateo et al. 2002; Rossi et al. 2001). Finally, given the potentially synergistic effects of multiple trichothecenes in the same agro-food products and the high toxicity of NIV and T-2/HT-2 toxins (Gutleb et al. 2002), models are needed to predict the risks related to a multi-toxin contamination of wheat kernels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animal The research did not involve human participant or animals.

Informed consent N/A.

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