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***Fusarium proliferatum* and fumonisin B1 co-occur with *Fusarium* species causing Fusarium Head Blight in durum wheat in Italy**

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Summary

Fusarium Head Blight caused by phytopathogenic *Fusarium* spp. with *Fusarium graminearum* as main causal agent is a major disease of durum wheat (*Triticum durum* Desf.). Mycotoxins in wheat are dominated by trichothecenes B. Fumonisin B1 has only occasionally been reported from wheat; their occurrence was attributed to *Fusarium proliferatum* and *Fusarium verticillioides*.

We investigated kernels of durum wheat grown in Italy in 2008 - 2010 for colonization with *Fusarium* spp. and for the content of *Fusarium* mycotoxins. Fungal biomass was determined using species-specific qPCR and mycotoxins were quantified by HPLC-MS/MS. *Fusarium graminearum* and *Fusarium culmorum* were dominating *Fusarium* species, followed by *Fusarium poae*, *Fusarium tricinctum* and *Fusarium proliferatum*. No *Fusarium verticillioides* DNA was found. Toxicologically relevant levels of deoxynivalenol and nivalenol but no trichothecenes A were detected. Enniatins, fumonisin B1 and beauvericin were present in grain in all three years. Based on these results and on the evaluation of previous published reports, we hypothesize that low levels of fumonisins commonly occur in wheat grains produced in warm climate; they may remain undetected as long as mycotoxin monitoring programs for wheat do not include fumonisins. The only relevant source of fumonisins in wheat grain appears to be *Fusarium proliferatum*.

Introduction

Fusarium head blight (FHB) is a worldwide-occurring cereal disease complex caused by several species of the fungal genus *Fusarium* that often cause indistinguishable symptoms. The disease leads to the reduction in grain yield, which can reach up to 70% (PARRY et al., 1995). FHB also affects quality parameters such as the size of kernels, protein content, baking quality of flour and mycotoxin content, making them unsuitable and unsafe for human and animal consumption. The prevailing species in FHB in Italy are *F. graminearum* Schwabe, *F. culmorum* (W.G. Smith) Sacc. and recently *F. poae* (Peck) Wollenweb. (SHAH et al., 2005; PANCALDI et al., 2010). INFANTINO et al. (2012) reported that *F. poae* was the most prevalent species found in organic bread and durum wheat in Italy.

F. graminearum Schwabe [*Gibberella zeae* (Schwein) Petch] is the predominant FHB pathogen worldwide (XU and NICHOLSON, 2009), producing a range of mycotoxins including type B trichothecenes nivalenol (NIV) and deoxynivalenol (DON). These mycotoxins disrupt cell functions by inhibiting protein synthesis via binding to the ribosome. For this reason, maximum levels of DON as major trichothecene contaminating FHB-affected kernels are regulated in food and feed by European directives EC n. 1881/2006, 126/2007. *F. graminearum* populations comprise chemotypes that synthesize either DON or NIV and their acetylated derivatives.

F. poae is a pathogen of increasingly importance as a cause of FHB in countries such as Argentina, Canada, Finland, Belgium,

Germany, Switzerland, Hungary, Slovakia, Italy, Ireland and the United Kingdom (AUDENAERT et al., 2009; VOGELGSANG et al., 2008; STENGLEIN, 2009; PANCALDI et al., 2010). The prevalence of *F. poae* in wheat reported in the last years is surprising because the species was believed to be less aggressive than other FHB pathogens (AUDENAERT et al., 2009; XU and NICHOLSON, 2009). Symptoms induced by *F. poae* are different from those caused by *F. graminearum*, characterized by small necrotic lesions on the wheat glume (VOGELGSANG et al., 2008).

PANCALDI and colleagues (2010) emphasized the importance of *F. poae* in the disease, showing that the isolation frequency of the species in North Italy increased from 9% to 23% from 1998 to 2007. Moreover, in the years where the isolation frequency of *F. poae* was high, *F. graminearum* was less often isolated; these observations are consistent with the research by PARRY et al. (1995).

Fumonisin B1 is a mycotoxin occurring in maize; fumonisins levels in corn are particularly high in Italy due to infection with thermophilic *F. verticillioides*. In wheat fumonisins have only occasionally been reported. In wheat plants artificially inoculated with *F. proliferatum* kernel black point symptoms were observed and fumonisin B1 was detected (DESJARDINS, 2007), indicating the potential of the fungus as natural contaminant in wheat fields. SHEPHARD et al. (2005) challenged the reports on fumonisins in wheat due to the occurrence of false positive signals when immunoaffinity clean-up, derivatization and fluorescence detection was used in fumonisin detection. More recent work, however, identified fumonisins in wheat unequivocally (e.g. PALACIOS et al., 2011; BUSMAN et al., 2012).

The aim of this work was to investigate the occurrence of *Fusarium* species and their mycotoxins in durum wheat grain in Italy.

Materials and methods

Experimental design

A three years field trial was established in the experimental station of the University of Bologna (Cadriano, 44°33'4.15"N; 11°24'39.02"E), using the durum wheat cultivar Simeto, which is susceptible to FHB. The plot was used for artificial inoculation with *F. graminearum* and *F. culmorum* for several years before the sampling. The field was divided in 4 plots (each plot was 3×3 m²) for 2008 till 2010, which served as replicates.

The first evaluation of FHB symptoms – typical bleaching in the spike – was made three weeks after anthesis on a representative sample of 100 spikes/plot.

Disease incidence was determined by counting the number of infected spikes divided by the total number (n=100) of evaluated spikes.

$$\text{Incidence (DI)} = \frac{\text{number of infected spikes}}{\text{total number of evaluated spikes}} \times 100$$

Disease severity of every spike was determined by using an evaluation method modified by PURAHONG et al. (2011) that categorizes the bleached spikelet area per spike in percent in eight

severity classes (0 - 2 - 5 - 10 - 25 - 50 - 75 - 90%). Disease severity per plot (DS) was seen as the sum of infected spikes times the disease class divided by the total number of evaluated spikes in percent (including non-diseased spikes).

$$\text{Severity (DS)} = \frac{\sum_0^{90} \text{number of infected spikes} \times \text{disease class}}{\text{total number of evaluated spikes}} \times 100$$

Kernels of every plot were harvested at maturity with a combined harvester.

Mycological analysis and species identification

Mycological analyses were performed on 25 kernels per plot in 2008 and 2009 and 50 kernels per plot in 2010. Petri dishes, containing PDA (Potato Dextrose Agar, Difco, USA) were set up with 10 kernels each, previously washed in water for 5 minutes, sterilized in 2% sodium hypochlorite for 5 minutes and rinsed in sterile water. Agar media with kernels were incubated at 25°C for five days in dark. All *Fusarium*-like colonies were analyzed for their micro- and macroscopical features by optical microscopy (Labor lux 12, Leitz Wetzlar, Germany). Species identification was performed using the synoptic keys by TOUSSOUN and NELSON (1976) and LESLIE and SUMMERELL (2006). To confirm the belonging of a fungus to the species morphologically identified, DNA extraction from mycelium was performed using a CTAB (exadecyl-trimethyl-ammonium bromide) method (PRODI et al., 2011).

PCR specific for *F. graminearum* and *F. poae* was carried out using primer pairs Fg16 F/R (NICHOLSON et al., 1998) and Fp82 F/R (PARRY and NICHOLSON, 1996), respectively. Amplification was done in a T3 thermocycler (Biometra, Göttingen, Germany) under conditions described in the literature cited above.

DNA extraction from wheat flour and real-time PCR analysis

The kernels from each plot (2 to 5 kg) were well mixed, 500 g aliquots were removed and ground. DNA extraction from 1 g of wheat flour was carried out following the upscaled protocol by BRANDFASS and KARLOVSKY (2008). The DNA was dissolved in 200 µl of buffer TE (10 mM Tris HCl, 1 mM EDTA, adjusted to pH 8.0) and diluted 50 times, 1 µl of the dilutions was used as template for real-time PCR.

Real-time PCR based on SYBR Green I chemistry was used to evaluate the level of kernel infection with *Fusarium* spp. PCR specific for *F. culmorum*, *F. graminearum*, *F. proliferatum*, *F. poae*, *F. verticillioideis* and *F. tricinctum* (Tab. 1).

Serial dilutions of pure fungal DNA as standards were used, whereas the lowest standards were used as limits of quantification, which was 28 ng/g for all *Fusarium* species. Amplification was done under conditions based on the protocols described by the authors of the primers (Tab. 3) optimized for thermocycler CFX 384 (Biorad, Hercules, CVA, USA) with 384-well plates. DNA standards were quantified by comparative densitometry using known amounts of

lambda phage DNA (Biorad Multi-Analyst, Hercules CA, USA). Samples containing DNA below LOQ but with specific melting curve were allocated a value of ½ LOQ. Samples with unspecific melting curves were allocated a value of zero.

Mycotoxin analysis

Wheat meal was processed, extracted and defatted as described by ADEJUMO et al. (2007). HPLC-MS/MS for the content of DON and NIV was carried out as described below. Non-defatted acetonitrile extracts were kept at -20°C as counter samples. PCR analysis of DNA extracted from the flour carried out four to six years later indicated that kernels could also be contaminated with fumonisins and depsipeptides. Therefore, the counter-samples were re-analyzed for the presence of fumonisin B1, enniatins and beauvericin; because stability of these toxins in acetonitrile extracts over extended periods of time is unknown, the purpose of the analysis was to obtain a lower boundary for their concentration in kernels. Samples for the analysis of beauvericin and enniatins were processed by separating acetonitrile-water supernatants from flour by centrifugation, followed by drying supernatants in vacuum and redissolving the residues in methanol/water (1:1) without defatting. Samples for the analysis of fumonisin B1 were dried in vacuum, re-extracted with methanol/water (3:1) and cleaned up by SPE on strong anion exchanger columns (SAX, Agilent, Waldbronn, Germany) according to SHEPHARD et al. (1990).

Standards for the quantification of DON and NIV were prepared by spiking uncontaminated wheat flour with pure mycotoxins and processing the spiked samples in the same way as unknown samples (matrix-matched standards). Chromatographic separation on a reverse-phase column, ionization and quantification in multiple reaction monitoring mode of a triple quadrupole detector (trichothecenes) and an ion trap detector (the other mycotoxins) was carried out on a system described by RATZINGER et al. (2009), using published mass transitions (RASMUSSEN et al., 2012; ADEJUMO et al., 2007). The limit of quantification (LOQ) was 10 µg/kg for DON, 30 µg/kg for NIV, 10 µg/kg for HT-2 and T-2, 5 µg/kg for DAS, 50 µg/kg for NEO, 3 µg/kg for fumonisin B1, and 14 µg/kg for beauvericin and enniatins. Limits of detection (LOD) values were 3 µg/kg for DON, 10 µg/kg for NIV, 5 µg/kg for HT-2 and T-2, 3 µg/kg for DAS, 20 µg/kg for NEO, 1 µg/kg for fumonisin B1, and 5 µg/kg for beauvericin and enniatins.

Results

Fungal colonization and mycotoxin content of kernels

In the years 2008 and 2010 the infection pressure was rather high while in 2009 only a limited incidence of FHB was observed (Fig. 1). Disease severity was highest in 2010 and very low in 2009; in spite of the relatively high incidence of FHB in 2008, on the average only 5% of the spikes were bleached (Fig. 1).

Colonization of wheat kernels with *F. graminearum* as the major causal agent of FHB was investigated by placing 100 to 200 surface-sterilized kernels from each year's harvest on agar media and counting kernels from which *F. graminearum* isolates were obtained. The isolation frequencies (Tab. 2) matched the ranking of disease severity among years.

The biomass of *Fusarium* spp. in wheat kernels was estimated by species-specific real-time PCR analysis of DNA extracted from representative samples of kernels. The analysis was carried out for the major pathogen of FHB *F. graminearum* and for the following species: *F. culmorum*, *F. poae*, *F. proliferatum*, *F. verticillioideis* and *F. tricinctum* (Fig. 2). *F. graminearum* and *F. culmorum* were found in high amounts in all three years (except for *F. culmorum* in 2009). *F. poae*, *F. proliferatum* and *F. tricinctum* occurred in 10 to 100 times lower amounts; no *F. verticillioideis* was found in any sample.

Tab. 1: Primers used for species-specific quantification of *Fusarium* DNA

Fungal species	Primer name	Reference
<i>F. culmorum</i>	OPT18F/OPT18 R	SCHILLING et al., 1996
<i>F. graminearum</i>	Fg16 F/R	NICHOLSON et al., 1998
<i>F. poae</i>	Fp82 F/R	PARRY and NICHOLSON, 1996
<i>F. tricinctum</i>	Tri1/Tri2	KULIK, 2008
<i>F. proliferatum</i>	Fp3-F/Fp4-R	JURADO et al., 2006
<i>F. verticillioideis</i>	VER 1/2	MULE et al., 2004

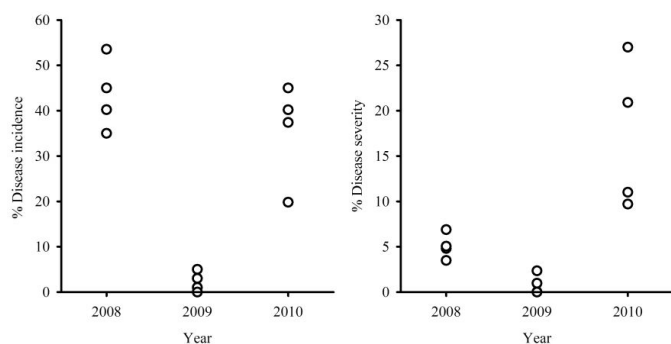


Fig. 1: Incidence and severity of *Fusarium* head blight on durum wheat cultivar Simeto grown in a field near Bologna, Italy

Tab. 2: Isolation frequency of *F. graminearum* from naturally infected wheat kernels

	2008	2009	2010
<i>F. graminearum</i>	18 (n=100)	2 (n=100)	51 (n=200)

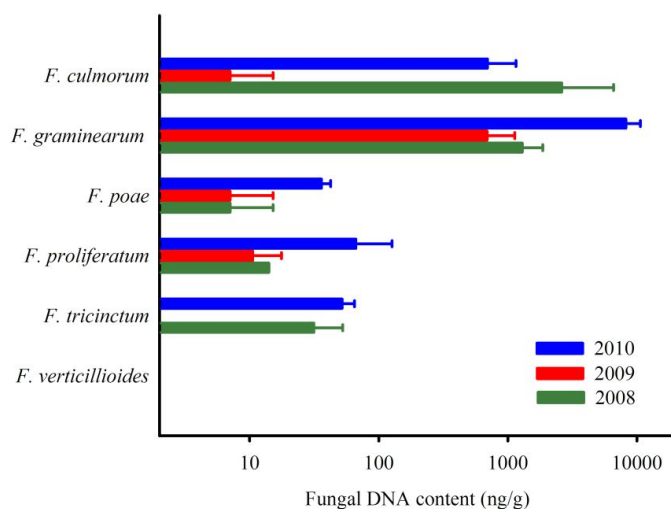


Fig. 2: *Fusarium* DNA in naturally infected durum wheat kernels

Mycotoxins DON and NIV were found in all three years in amounts raising toxicological concerns (Fig. 3); it has to be pointed out, however, that a strong inoculum of trichothecene B producers is likely to be established at the location due to long-year inoculation experiments (see also Discussion). Because PCR analysis revealed the presence of fumonisin and beauvericin producer *F. proliferatum* and enniatin producer *F. tricinctum*, the counter samples kept at -20°C were used to determine lower boundaries for concentrations of these toxins in kernels. Fumonisin B1 was found in all samples; four daughter ions generated from the molecular ion possessed the same m/z ratios and occurred in similar relative abundances as daughter ions of authentic fumonisin B1 standard (Fig. 4). Together with the retention time and m/z value of the molecular ion, these results proved convincingly that fumonisin B1 was present in durum wheat kernels. The presence of beauvericin and enniatins A1, B and B1 was supported by their retention times, the molecular ions and three daughter ions for each analyte.

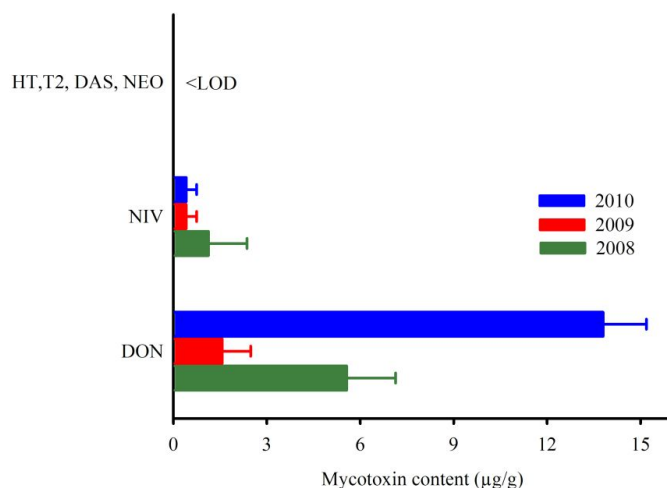


Fig. 3: Deoxynivalenol and nivalenol in naturally infected durum wheat kernels

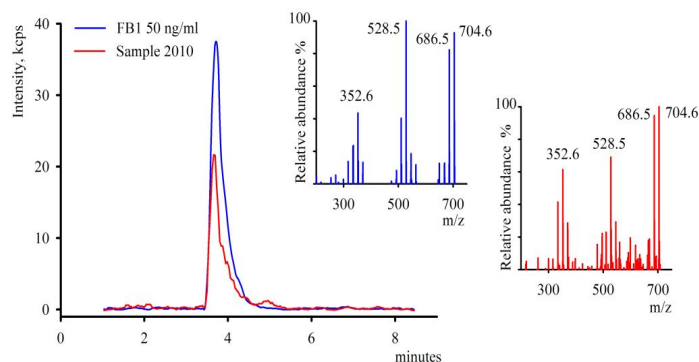


Fig. 4: Fumonisin B1 in naturally infected durum wheat kernels. Identity of fumonisin B1 was confirmed by comparing authentic standard (blue) with sample extract (red) regarding retention time, m/z of the mother ion and fragmentation spectra

Discussion

The original goal of our investigation of FHB of durum wheat in Italy was to obtain a deeper insight into the role of *F. poae*, which attracts increasing attention as a component of FHB complex and a source of highly toxic trichothecenes type A in general (STENGLEIN, 2009) and in Italy in particular (PANCALDI et al., 2010; INFANTINO et al., 2012). Because the area sampled for our analysis was used for inoculation experiments with *F. graminearum* and *F. culmorum* in the last years, we expected a strong infection pressure of these pathogens due to inoculum originating from crop residues. Real-time PCR showed that *F. graminearum* was the dominant *Fusarium* species in wheat kernels (Fig. 2). The presence of *F. culmorum* itself was not surprising. The DNA levels appeared rather high, concerning the climate in Emilia Romagna and the displacement of *F. culmorum* by *F. graminearum* in the last decade (YLI-MATTILA, 2010). We speculate that high levels of *F. culmorum* DNA can be accounted for by the inoculum built after years of inoculation experiments. Colonization of kernels with *F. poae* was negligible: we found *F. poae* in very few kernels by agar plating (data not shown) and the content of *F. poae* DNA in kernels was much lower than the content of *F. graminearum* DNA (Fig. 2). In line with these results, we have not found any of trichothecenes A typically produced by *F. poae* (Fig. 3). The presence of *F. poae* in durum wheat might fluctuate,

Tab. 3: Lower boundary for the content of fumonisin B1, beauvericin and enniatins

The content of fumonisins and depsipeptides was determined in samples that were stored for 4 to 6 years in acetonitrile-water at -20°C. Because stability of mycotoxins under these conditions is unknown, we regard the values as lower boundaries for mycotoxin content in freshly harvested kernels. Mean of four plots per year \pm standard deviation is shown except for enniatin B, for which all four values are listed. Limit of detection (LOQ) was 3 ng/g for fumonisin B1 and 14 ng/g for the other mycotoxins. "n.d.": signal not detectable; "<LOQ": positive signals too low to be quantified.

	2008	2009	2010
Beauvericin [ng/g]	<LOQ	<LOQ	48 \pm 19
Enniatin A [ng/g]	n.d.	n.d.	<LOQ
Enniatin A1 [ng/g]	62 \pm 9	88 \pm 14	46 \pm 30
Enniatin B [ng/g]	3200, 3100, 73, 1900	3000, 3500, 5200, 2400	1030, 5200, 1100, 490
Enniatin B1 [ng/g]	390 \pm 80	510 \pm 140	140 \pm 110
Fumonisin B1 [ng/g]	6.2 \pm 3.4	5.3 \pm 2.6	33 \pm 18

as shown by COVARELLI et al. (2015) who reported that over 20% of *Fusarium* isolates from durum wheat ears belonged to *F. poae* in 2009 but rarely any *F. poae* was found in the following year.

F. tricinctum DNA at moderate levels was found in wheat kernels in two years. Although the species is rarely monitored in FHB, according to published reports it dominated *Fusarium* microflora of wheat in Syria (ALKADRI et al., 2013) and was one of the dominant species in barley in (NIELSEN et al., 2014).

qPCR analysis revealed the presence of DNA of *F. proliferatum* in all years. In year 2009, colonization of wheat kernels with *F. proliferatum* was comparable to the colonization with *F. culmorum* (Fig. 2). *F. proliferatum* produces fumonisins, which are proven carcinogens in rodents and suspected carcinogens in humans. Contamination of wheat with fumonisins is therefore highly relevant for food safety. Fumonisin regularly occur in maize grown in warm climate; major fumonisin producers in maize are *F. verticillioides* and *F. proliferatum* (FANDOHAN et al., 2003) with *F. verticillioides* often reported to dominate (REYES-VELAZQUEZ et al., 2011). While trichothecene content in wheat has been documented in hundreds of publications, fumonisins were only occasionally detected in wheat (DESJARDINS et al., 2007; PALACIOS et al., 2011; BUSMAN et al., 2012). Founding DNA of *F. proliferatum* in the total DNA extracted from wheat kernels motivated us to extend the analysis of mycotoxin content to fumonisins and beauvericin, which are both produced by *F. proliferatum*. We also analyzed the content of enniatins, which are produced by many *Fusarium* spp. including *F. tricinctum* which we detected in wheat kernels in two years (Fig. 2). Fumonisin were detectable in wheat kernels in all three years, though the biomass of *F. proliferatum* was hundred times lower than the biomass of *F. graminearum*.

In analogy to maize, the origin of fumonisins in wheat is generally attributed to *F. proliferatum* and *F. verticillioides*; the results reported by INFANTINO et al. (2012) indicated that *F. verticillioides* could be the major source of fumonisins in wheat in Italy. Our durum wheat samples were consistently contaminated with fumonisins but we have not found any DNA of *F. verticillioides*. *F. proliferatum*, which was found in the kernels consistently in all three years, appeared to be the only source of fumonisin B1. *F. verticillioides* is ubiquitous in Italian maize fields; inoculum in form of airborne conidia likely reached wheat fields, too. We are confident that our qPCR analysis reliably distinguished between *F. proliferatum* and *F. verticillioides*. The primers targeted intergenic spacers of rRNA genes, which were shown to be taxonomically informative for the differentiation between the two species (VISENTIN et al., 2009), and they have been extensively tested before (NUTZ et al., 2011). Even if *F. verticillioides* can infect wheat seeds, as reported by INFANTINO et al. (2012), fumonisins in wheat do not seem to originate from *F. verticillioides* because DESJARDINS et al. (2007) and BUSMAN

et al. (2012) reported that *F. verticillioides* artificially inoculated into wheat did not produce any fumonisins, even though the isolates produced large amounts of fumonisins *in vitro* and in inoculated maize plants.

The DON and NIV levels in kernels reached toxicologically relevant levels in all three years. In 2008 and 2010 the amount of DON exceeded by a large margin the legal limit of 1,750 μ g/kg for unprocessed durum wheat established by European Regulations EC n. 1881/2006 and EC n. 1126/2007. The high levels of DON can likely be accounted for by inoculation experiments carried out in the field with *F. graminearum* in last years, which cause build-up of inoculum on crop residues. Which *Fusarium* species was the source of DON and NIV in our samples? We found a significant positive correlation between the concentration of NIV and the amount of DNA of *F. graminearum* in kernels. DON concentration and *F. graminearum* DNA were positively correlated, too, but the significance of the correlation was not statistically supported (data not shown). The amount of *F. culmorum* DNA in kernels has not correlated with NIV nor DON. We therefore assume that the dominant source of trichothecenes in kernels was *F. graminearum*, which is in line with the view that *F. graminearum* is the major causal agent of FHB in Europe (YLI-MATTILA, 2010). The amount of DON in grain exceeded the amount of NIV by a factor of 4 to 10, which is in line with the observation that *F. graminearum* chemotypes producing DON and its derivatives dominate over NIV producers in wheat in North Italy (PRODI et al., 2009; PRODI et al., 2011).

High competitiveness of *F. graminearum* was reported by XU and NICHOLSON (2009) in co-inoculations *in vitro* trials in controlled environmental conditions. Wheat kernels in our samples were consistently colonized with *F. proliferatum* though they contained high levels of *F. graminearum* DNA. Co-inoculation experiments with *F. proliferatum* have not been conducted in wheat so far. To elucidate the interaction of *F. proliferatum* and other minor members of the *Fusarium* complex on wheat kernels with *F. graminearum*, we intend to investigate individual ears and kernels for the presence of *Fusarium* species.

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