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TITOLO TESI

Fusarium head blight: characterization of secondary *Fusarium* species, evaluation of their pathogenetic role and ability to produce mycotoxins

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Abstract

Fusarium head blight (FHB) is a worldwide cereal disease caused by a complex of *Fusarium* species resulting in high yield losses, reduction in quality and mycotoxin contamination of grain. In Europe, the principal species responsible for FHB are *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium poae*. However, a shift in *Fusarium* head blight community has been observed worldwide. For this reason, the present work aimed to analyze the evolution of Italian FHB community focusing the attention on species considered "secondary" in the past years such as members of *Fusarium tricinctum* species complex (FTSC) and *F. proliferatum*. The first goal of the study was to analyze the fungal community associated with Italian durum wheat. The first year of investigation, the study was conducted on a total number of 30 durum wheat samples harvested in three climatically different Italian cultivation areas (Emilia Romagna, Umbria and Sardinia), while, during the second year, 70 durum wheat samples harvested across Italian peninsula were analyzed. With a combination of two different isolation methods (deep-freezing blotter and isolation on potato dextrose agar), the fungal community was determinate, with a focus on FHB complex composition. In addition, the detection of fungal secondary metabolites in the grains was carried out. In both investigated years, the genus *Alternaria* was the main component of fungal community associated to durum wheat followed by *Fusarium*, even if regional differences in species composition were detected. Focusing on the FHB complex, the first year of investigation *F. poae* was the main species detected in particular in Northern and Central cultivation areas. Because of the highest *Fusarium* incidence, durum wheat harvested in the Northern cultivation area showed the highest presence of *Fusarium* secondary metabolites. The second year of investigation, at national level *F. avenaceum* and *F. proliferatum* were the main species detected on Italian durum kernels. A variable mycotoxins contamination was observed in the analyzed samples. Despite the higher *F. proliferatum* incidence, in both year a low level of fumonisins contamination was detected. The data obtained in the two-years investigations confirm that the durum wheat cultivated in Northern and Central Italy may be subject to a higher FHB infection risk and *Fusarium* mycotoxins accumulation.

Considering, the increased incidence of *F. avenaceum* and other members of FTSC in Italian FHB community and the risk connected with the production of secondary metabolites with effects still less known on animal and human health, the second aim of the present work was

to investigate genetic diversity among the FTSC and estimate the mycotoxin risk related to these species. A multilocus DNA sequence dataset comprising portions of three phylogenetically informative genes (*TEF1*, *RPB1* and *RPB2*) was constructed for 117 isolates from Italy and 6 from Iran to evaluate FTSC species diversity and their evolutionary relationships. Phylogenetic analyses revealed that *F. avenaceum* (FTSC 4) was the most common species in Italy, followed by an unnamed *Fusarium* sp., *F. tricinctum* and *F. acuminatum*. In addition to these four phylospecies, five other *F. tricinctum* clade species were sampled. These included strains of four newly discovered species (*Fusarium* spp. FTSC 11, 13, 14, 15) and *F. iranicum* (FTSC 6). Moreover, 59 FTSC isolates comprising 10 phylospecies were tested for their ability to produce mycotoxins *in vitro*. Of the 59 isolates tested for mycotoxin production on rice cultures, 54 and 55 strains, respectively, were able to produce quantitative levels of enniatins and moniliformin. Isolates were also assayed for their ability to produce the following five bioactive secondary metabolites: chlamydosporol, acuminatopyrone, longiborneol, fungerin and butenolide.

The two-years investigation as well as other several studies highlighted an increasing incidence of *F. proliferatum* in the FHB community. Despite the common detection of this species on wheat, the level of fumonisins, the most dangerous metabolites biosynthesized by *F. proliferatum*, appears lower compared to maize culture. For this reason, a preliminary study was conducted to evaluate the ability of a selected *F. proliferatum* isolate to produce fumonisins on wheat in open field and under natural climatic conditions. The three analogues (FB1, FB2 and FB3) were quantified by HPLC-FLD analysis on three grain fractions: kernels, chaff and rachis. Mycological analysis allowed to detect *F. proliferatum* in kernels, rachis and chaff. In addition, the typical black point symptom was observed on kernels while the chaff and the rachis appear browned. Fumonisins were detected in all the three investigated fractions without significant differences. FB1 was the main analogue detected. Even if the sum of FB1+FB2 was lower than the legal limit established by UE for maize, the risk connected with these mycotoxins should be not underestimated considering the global climate change as well as the possible co-presence of other toxic metabolites with effects on human and animal health still unknown.

General introduction

Fusarium head blight (FHB) is a global cereal disease caused by a complex of *Fusarium* species resulting in high yield losses and reduction in quality due to mycotoxin contamination of grain. FHB is caused by a dynamic "complex" of species each of which characterized by a specific mycotoxigenic profile (Bottalico and Perrone, 2002; Köhl et al., 2007; Osborne and Stein, 2007; Parry et al., 1995). The FHB community composition could be influenced by many factors first of all climatic condition (at micro and macro-scale level), agricultural practices, cultivated varieties (Beccari et al., 2019; Ferrigo et al., 2016; Scala et al., 2016; Xu, 2003). The main causal agents of this disease are considered the members of *Fusarium graminearum* species complex (type B trichothecenes producers), belonging to the *Fusarium sambucinum* species complex including both type A (e.g.: *F. poae*) and B trichothecenes producers (Ferrigo et al., 2016; Foroud and Eudes, 2009; O'Donnell et al., 2013). *F. graminearum*, traditionally prevalent in warm and wet area of Southern Europe and North and South America, has been recently detected also in cooler region of North Europe, while, an increased incidence of low aggressive species such as *F. poae* has been recorded in many wheat and barley cultivation area worldwide (Fredlund et al., 2013; Pancaldi et al., 2010; Pereira et al., 2020; Starkey et al., 2007; Stenglein, 2009; Tittlemier et al., 2013; Ward et al., 2008). However, other species considered secondaries such as the members of *Fusarium tricinctum* species complex (FTSC), the fumonisins producer *F. proliferatum* (member of *Gibberella Fujikuroi* species complex-GFSC) or species belonging to *Fusarium incarnatum-equiseti* species complex (FIESC), have become increasingly important due to several factors including changes in climatic conditions (Ferrigo et al., 2016). Among *Fusarium* secondary metabolites, type A (T-2 and HT-2 toxin) and B (DON and derivates, NIV) trichothecenes are the most monitored for their toxicity. In particular, DON is considered the most common cereals mycotoxin and it is cause of adverse effect on human and animal health such as chronic effect (growth reduction and anorexia) and acute effect (vomiting) (EFSA, 2005). For this reason, a legal limit for this compound on wheat grains as well as in others cereal grains has been established by EU (Commission Regulation (EC), 2006). T-2 and HT-2 toxins, the most common type A trichothecenes, are characterized by an high toxicity that can cause severe immunological and hematological

problems interfering the eukaryotic protein synthesis process (Cope, 2018). In this case, the EU settled a maximum recommendation level on cereal grains (Commission Recomandation (EC), 2013). In addition, in the last years, secondary metabolites such as depsipeptides (enniatins-ENNS and beauvericin-BEA) and moniliformin (MON) draw with an increasing interest the attention of scientific community (EFSA, 2018, 2014). Despite being reported as pathogens less aggressive than the member of FGSC, the "secondary" species associated to FHB community could cause a change in the secondary metabolites accumulated in the grains (Bakker et al., 2018; Valverde-Bogantes et al., 2019).

In addition, to estimate the mycotoxin risk related to the different *Fusarium* species a correct identification is fundamental. In the past, the taxonomic identification was based on morphological characters such as asexual structures (conidia shape and size). However, the absence of defined morphological characters and their variation depending on cultural media or environmental conditions made this method unstable and misleading (Geiser et al., 2004). This approach can cause an underestimation of species diversity as confirmed by *Genealogical concordance phylogenetic species recognition* (GCPSR) analysis, that in the last years, proves the presence of genetic differences among species sharing several morphological features (O'Donnell et al., 2018; Summerell, 2019; Summerell and Leslie, 2011).

In this context, the aim of the present study was, first of all, to monitor the evolution of Italian FHB community considering the climatic change scenario and the shift in FHB complex. Moreover, the mycotoxins contamination of the analyzed samples was investigated. In addition, considering the increased detection of FTSC member in FHB community, a phylogenetic study was conducted on Italian FTSC isolates to understand genetic diversity and estimate the mycotoxin risk related to these species.

In addition, a preliminary study was conducted to test the ability of a *F. proliferatum* isolate to produce fumonisins on wheat. Even if only few studies reported a higher fumonisins contamination on this substrate, in a global warming scenario, it is possible to hypothesize an increased incidence of fumonisins producers also on winter crops.

CHAPTER 1:

Regional differences in **Fusarium** *complex composition associated to* **Fusarium** *Head Blight in Italian durum wheat*

1.1 Durum wheat: Italian production and related sanitary problems

In 2019, with a global production of 38.1 million tons, durum wheat [*Triticum turgidum* L. *subsp. durum* (Desf.) Husn.] was one of the most important cultivated cereal species. The European Union (EU) is the highest producer worldwide followed by Canada, Turkey and United States (Xynias et al., 2020). Durum wheat production is concentrated on the Mediterranean area where durum wheat derivates such as flour, pasta, semolina and cous-cous are the basis of the alimentary diet. Among the EU, Italy is considered the leader in durum wheat production and, in particular, the South-Eastern regions are the most important cultivation area. In particular, 67% of durum wheat production comes from the Southern regions (Fagnano et al., 2012). However, in the last years, the durum wheat cultivation gradually expanded to the Central and Northern Italian regions. Over the season 2019-2020 the four regions with the higher durum wheat production were: Sicily [South Italy (827,951 t of harvested production)], Apulia [South Italy (708,190 t)], The Marche (Central Italy) and Emilia Romagna (North Italy) with a production of 370,169 and 341,945 t, respectively (Istat, 2020). Moreover, in 2017, with a production of 3.6 million t, Italy was the main producer and pasta exporter worldwide, for this reason, the high quality of raw material is one of the most important purposes of the Italian agri-food system (Visconti and Pascale, 2010). Even if the "expansion" of durum wheat cultivation area to northern regions caused an increased yield production, on the other hand, the specific climatic conditions could negatively affect the quality and the healthiness of the grains (Scala et al., 2016). Durum wheat is adapted to climate area with a prevalence of mild winter and dry summer, while Central and Northern Italian regions are characterized by high humidity that could promote fungal development and, consequently, reduce grain quality (Infantino et al., 2012; Quaranta et al., 2010; Shah et al., 2005).

Among the principal cereal diseases, *Fusarium* head blight (FHB) is one of the most destructive worldwide. About 17 *Fusarium* species have been traditionally associated to FHB (Parry et al., 1995). Even if several studies, both *in vivo* and *in vitro*, have been conducted to better understand the interspecific interactions among *Fusarium* species and the mycotoxins production response, the results have appeared to be not homogeneous. Xu et al. (2008) observed the reduction of biomass production in presence of more than one *Fusarium* species but, at the same time, an increasing of mycotoxins production. On the contrary, Siou and co-workers proved a decreasing of mycotoxins production in a co-

inoculation experiment. In detail, the authors highlighted how the pathogenicity of the single isolate can play a key role more than species specificity (Siou et al., 2015).

The dynamism of FHB complex across a season triggered not only a quantitative but also a qualitative modification of cereal mycotoxins contamination.

In this contest, a continuous and extensive monitoring of cereal crops in a specific cultivation area is essential to follow the FHB complex evolution as well as to predict the phytosanitary condition of the grains. For this reason, a two-years investigation in different durum wheat cultivation areas of Italy was conducted.

During the first-year investigation (2015), a total number of 30 durum wheat grain samples was collected around three climatically different Italian durum wheat cultivation areas (Emilia-Romagna, Umbria and Sardinia; Northern, Central and Southern Italy, respectively); during the second year (2018), in order to explore more in depth, the composition of Italian FHB community, the durum wheat grain samples (n=70) were collected around all different regions of the peninsula. The aims of this investigation were: analyzed the mycobiota composition using two different methods (Potato dextrose agar, PDA and deep-freezing blotter, DFB); identified the *Fusarium* species among the FHB complex; evaluated the presence of a wide range of fungal secondary metabolites using liquid chromatography tandem mass spectrometry (LC-MS/MS).

1.2 Materials and Methods

1.2.1 Durum wheat sampling and fungal isolation

The present study was conducted on durum wheat grain cereals cultivated in two different cultivation years: 2014-2015 and 2017-2018. During the first year, the investigation was carried out on a total number of 30 durum wheat samples collected from three Italian regions (10 samples per region) representative of three different Italian durum wheat cultivation areas (Emilia-Romagna-Northern Italy; Umbria-Central Italy; Sardinia-Southern Italy) (see TableS1 and Figure 1). In order to increase the representativeness of the investigation and to obtain a more detailed picture of Italian FHB community, during the season 2017-2018 a total number of 70 durum wheat samples was harvested along different regions of the peninsula. In detail, durum wheat kernels collected across Lombardy (n=4), Emilia Romagna (n=8), Veneto (n=4) (Northern Italy); Abruzzo (n=4), The Marche (n=5), Tuscany (n=5), Umbria (n=9), Latium (n=7) (Central Italy); Apulia (n=9), Campania (n=2), Molise (n=1), Sardinia (n=6), Sicily (n=6) (Southern Italy and Island) were analyzed (see TableS2 and Figure 2).

In both years, the sampling was conducted in order to cover the principal cultivation areas characterized by different climatic conditions.

After the harvest, the samples (about 500 g each) were divided into two representative sub-samples of about 250 g each: one used for mycological analysis and another for fungal secondary metabolites analysis by LC-MS/MS.

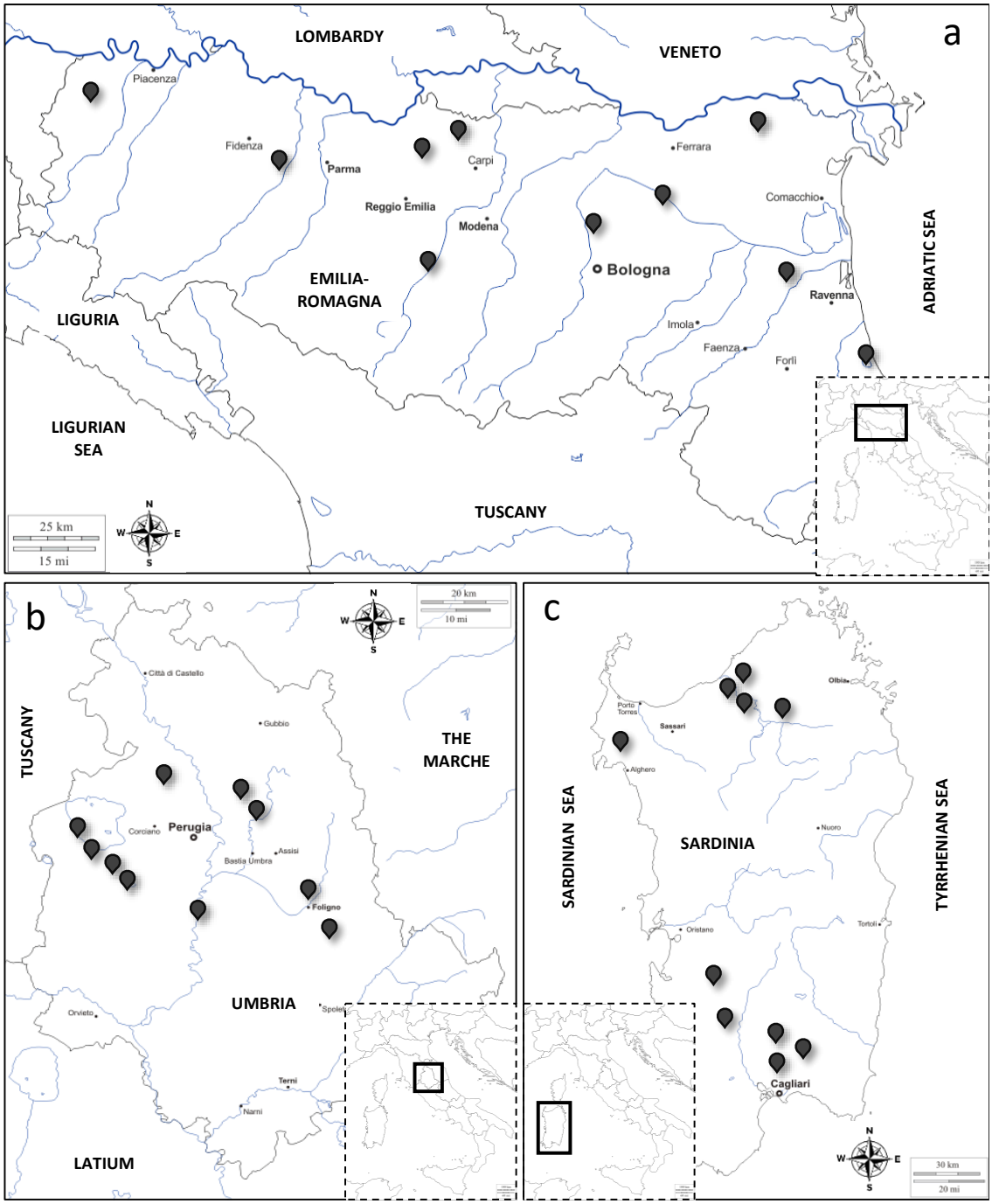

Figure 1: Location of 30 fields across three representative Italian regions where durum wheat samples were collected in 2015. In detail: map of Emilia Romagna region (Northern Italy) (a); map of Umbria region (Central Italy) (b); map of Sardinia region (Southern Italy) (c). The grey tags showed the sampling location.

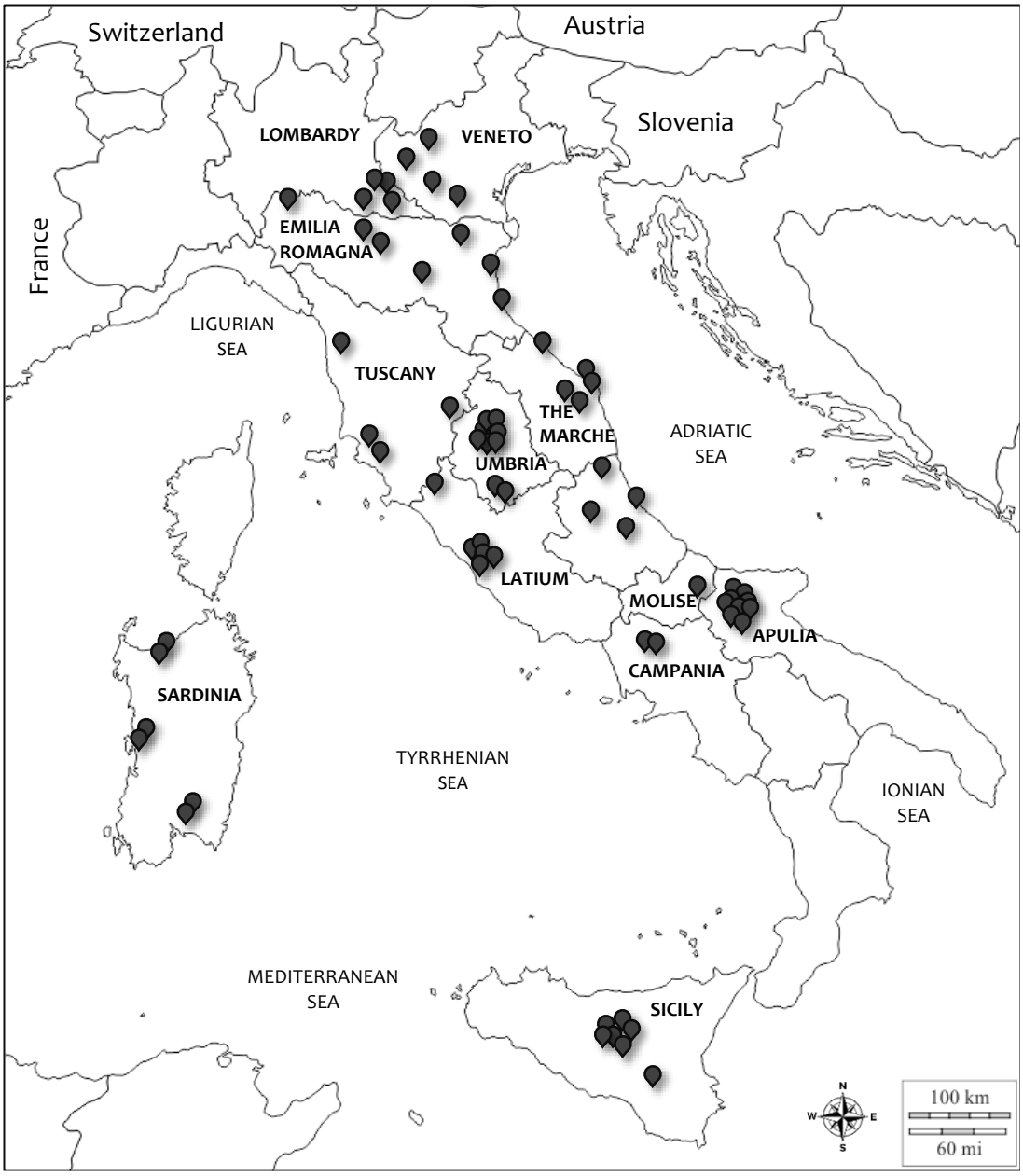

Figure 2: Location of 70 fields across Italian peninsula where durum wheat samples were collected in 2018. In detail Emilia Romagna, Lombardy and Veneto (Northern Italy); Tuscany, Umbria, The Marche, Abruzzo and Latium (Central Italy); Molise, Campania, Apulia, Sicily and Sardinia (Southern Italy). The grey tags showed the sampling location.

1.2.2 Determination of mycobiota composition

In both years of investigation, to detected potential differences on fungal isolation method, two methodologies were applied: isolation on Potato Dextrose Agar amended with antibiotics (MPDA) and deep-freezing blotter method (DFB).

Fungal isolation on MPDA was conducted as described by Beccari et al. (2018).

In brief, a sub-sample of 50 g of kernels per each wheat sample was randomly selected for plating. The kernels were superficially sterilized in a water-ethanol (95%)-sodium hypochlorite (7%) solution (82:10:8% vol.) for 2 min and then rinsed in sterile water, twice, for 1 min. After the sterilization process, 10 kernels were placed onto PDA (PDA, Biolife Italiana, Milan Italy) added with streptomycin sulphate (0.16 g L^{-1}, Sigma Aldrich) into a Petri dish (10 cm diameter). For each wheat sample, 10 Petri dishes were set up for a total of 100 kernels analyzed. The Petri dishes were incubated at 22 °C in the dark and after 5 days, each single kernel was subject to a combination of visual and stereomicroscope observations in order to obtain genera identification focusing on *Fusarium* genus (Leslie and Summerell, 2006).

Isolation of the fungal community infecting durum wheat grains by the DFB method was realized following the protocol described by Limonard (1966) with slight modifications. In brief, kernels were randomly collected and placed onto three sterilized layers of filter paper (90 mm diameter, grade 1) (Whatman, GE Healthcare, Amersham Place, UK) added with 7 mL of sterile deionized water into 10 petri dishes (100 mm diameter) containing 10 kernels each for a total number of 100 seeds analyzed for each sample. In order to promote the germination, the dishes were placed for 24 h at 24 °C under near ultraviolet light (NUV) and then transferred at -20 °C for 24 h to inhibit the germination process. Finally, the dishes were incubated at 24 °C under NUV light. After 7 days of incubation, a combination of visual and stereomicroscope (SZX9, Olympus, Tokyo, Japan) observations were carried out on each single kernel to assess fungal development. For the first year of investigation, the number of colonies (n) belonging to different fungal genera and developed from durum wheat kernels collected in each of the three investigated regions was reported as the average (±SE) of the 10 samples. For the second year of investigation, the number of colonies (n) belonging to different fungal genera was reported as the average (±SE) of all samples collected in the same region.

All the isolates identified as *Fusarium* spp. were transferred on new PDA plates and incubated for seven days at 22°C in the dark. The obtained *Fusarium* colonies were selected and organized on the basis of different morphotype based on phenotyping and morphological features. One colony for each representative morphotype was subjected to the obtainment of single spore culture and then further analyzed (Leslie and Summerell, 2006). The first year of investigation, all the *Fusarium* representative colonies obtained with both methods, MPDA and DFB, were subjected to molecular analysis, while, the second year, all the

Fusarium representative colonies obtained on DFB were identified based on morphological features.

1.2.3 DNA extraction and molecular identification of *Fusarium i*solates

After 1 week of incubation at 22 °C in the dark, the monosporic colonies representative for each morphotype were subject to DNA extraction using the method described by Prodi et al. (2011). In brief, the frozen mycelium was ground and 1500 µL of extraction buffer [CTAB: 20 mM of ethylenediaminetetraacetic acid, 100 mM of Tris–HCl pH 8.0, 1.4 M of NaCl, 20 g L^{-1} of EDTA (hexadecyltrimethylammonium bromide), 10 g L^{-1} of Na_2SO_3 and 20 g L^{-1} of PVP-40 (polyvinylpyrrolidone)], were added to each sample and it was incubated at 65 °C for 15 minutes.

The samples were centrifuged at 12000 rpm for 10 minutes (Centrifuge Microfuge 22R, Beckman) at room temperature and then 700 µL of the supernatant were added to an equal volume (700 µL) of a solution Isoamyl alcohol: chloroform (1:24). After a gently mixed, the samples were centrifuged at 12000 rpm for 10 min (room temperature) and 550 µL of supernatant liquid was added to a 1:24 (v/v) isoamyl alcohol: chloroform suspension in equal measure. The samples were further centrifuged (12000 rpm for 10 min). A total of 450 µL of the supernatant liquid was collected and added to 225 µL of 5 M NaCl and 450 µL of isopropanol. Samples were incubated for 60 minutes at -20°C and centrifuged (12000 rpm for 20 minutes at 4°C). Isopropanol was discharged and the DNA pellet was double washed in 70% (v/v) ethanol (500 µL). The pellet was dried applying vacuum and then resuspended in 50 µL nuclease- free UV sterilized water. The extracted DNA was stored at -20 until the use.

Each *Fusarium* colony was identified on the basis of the partial sequences of *translation elongation factor 1α* (*TEF1α*) obtained with the primers *EF1-EF2* (5′ ATGGGTAAGGAGGACAAGAC-3′; 5′-GGAAGTACCAGTGATCATG-3′) (O'Donnell et al., 1998). Each amplification reaction contained: 24.80 µL of nuclease-free sterilized water, 10 µL of reaction buffer 5X (Promega), 2 µL each of forward and reverse primer 10 mM (Sigma Aldrich), 1 µL of dNTPs mix 10mM (Promega), 0.20 µL of *Taq* DNA polymerase enzyme 5 U μL^{-1} (Promega), 10 µL of the DNA of the test sample for a final volume of 50 µL. All the amplifications were performed by using the thermocycler T3 (Biometra, Germany). Reactions were carried out at the following thermal conditions: 96

°C- 5 min, 94 °C- 45 s, 53 °C-30 s, 72°C-60s (30 cycles), 72 -5min. Expected PCR product about 700 bp.

PCR fragments were visualized on TBE 1X agarose gel (1%). Five µL of 100-1000 bp DNA ladder (Promega-1.0 µg/µL) were added to the electrophoretic run to obtain the size of the amplified fragment (bp). The applied tension for the electrophoretic run was about 120-130 volts for 40 minutes. After electrophoretic run, the gel was immersed in an ethidium bromide (EtBr) solution 0.4 µg/µL for 15 minutes. Separated DNA fragments were observed on an UV transilluminator (Spectroline, Model TVL-312°).

Purification and sequencing of PCR fragments was carried out by an external service (Genewize Genomics Europe, Takeley, United Kingdom). The obtained sequences were edited using Chromatogram Explorer Lite v40.0 (Heracle Biosoft srl 2011) and analyzed by comparing with those deposited on NCBI (Basic Local Alignment Search Tool (Blast) and Fusarium MLST (http://www.westerdijkinstitute.nl/fusarium/) (O'Donnell et al., 2012, 2010). The species identification was based on > 99.4% similarity between query and reference sequences (O'Donnell et al., 2015). The presence of each *Fusarium* species involved in the FHB complex was comprehended calculating the total number of isolates belong to the morphotype of which the identified isolate was representative. The first year of investigation, the number of isolates (n) belonging to the different *Fusarium* species developed from durum wheat kernels collected in each of the three investigated regions was reported as the average (±SE) of the 10 samples for both methods of isolation used; during the second year of investigation, the number of isolates (n) belonging to different *Fusarium* species was reported as the average (±SE) of the samples for each investigated region.

1.2.4 Mycotoxins analysis by LC-MS/MS

Detection and quantification of secondary metabolites were carried out at the Department of Agrobiotechnology (IFA- Tulln), University of Natural Resources and Applied Life Sciences, Vienna (BOKU). Sub-sampled durum wheat kernels were finely ground by a blender (IMETEC) and 5 g of each milled sample were extracted using 20 mL extraction solvent (acetonitrile-water-acetic acid, 79:20:1, v/v/v). Raw extracts were diluted 1+1 using acetonitrile-water-acetic acid 20:79:1 (v/v/v) and 5 µL were subsequently injected. The instrumental method used in this study is an extension of the version described in detail by Malachova and co-authors (Malachová et al., 2014). Briefly, a QTrap 5500 MS/MS system (Sciex, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source

was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4 × 3 mm i.d. (both Phenomenex, Torrance, CA, USA). Quantification was performed using external calibration based on serial dilution of a multi-analyte stock solution. Results were corrected using apparent recoveries that were determined for wheat by spiking experiments. The accuracy of the method is verified on a continuous basis by participation in a proficiency testing scheme organized by BIPEA (Gennevilliers, France) with a current success rate (i.e., a z-score between −2 and 2) of > 94% of the > 1100 results submitted and for 92 of the 95 results submitted for wheat, respectively. A total of 46 and 60 secondary metabolites was investigated during the first e second year respectively

1.2.5 Statistical analysis

Count data about the mycobiota composition were analyzed by using a generalized linear model (GLM) with binomial error and logit link. Species, isolation method and region were used as the explanatory factors. Back-transformed counts with delta standard errors were derived and reported in figures and tables. Data about the abundance of secondary metabolite concentrations (in µg/kg) were analyzed by using a gaussian linear model with regions and species as the explanatory factors and allowing the standard deviation to assume a different value for each region and species (heteroscedastic model, fitted with generalized least square (GLS) fitting. Both for GLM and GLS fits, pairwise comparisons were performed by using a general hypothesis testing procedure with multiplicity correction, as outlined in Bretz et al. (2011). All analyses were performed by using the R statistical environment, together with the packages ‘nlme’, ‘emmeans’ (Lenth, 2016) and ‘multcomp’ (Hothorn et al., 2008).

1.3 Results

1.3.1 Mycobiota composition of durum wheat grains

First year of investigation

After 5 days (MPDA) and 7 days (DFB) of incubation, the colonies developed from the durum wheat kernels were subject to visual observation. The attention was focused on *Alternaria*, *Fusarium*, *Epicoccum*, *Aspergillus* and *Penicillium* genera while the fungal colonies not belonging to any of the above-mentioned genera were included in the class "other". The average number (n) of fungal colonies per durum wheat samples belonging to the different fungal genera is reported in Table S3 and in Figure 3.

On both methods (MPDA and DFB), the most frequent genus within the Italian fungal community was *Alternaria* ($p = 1 \times 10^{-4}$). Moreover, the number of *Alternaria* colonies was significantly higher on DFB than those observed on MPDA in Umbria and Sardinia ($p = 1 \times 10^{-4}$), while in Emilia Romagna region no differences between the two methodologies were observed ($p = 0.41$). In the samples harvested in the latter region, the presence of *Alternaria* was significantly lower than that detected on MPDA and DFB in Umbria and Sardinia ($p \leq 1 \times 10^{-4}$).

The second most common genus on Italian durum wheat and in both methodologies used was *Fusarium.* In Umbria and Emilia Romagna regions the number of *Fusarium* colonies isolated on PDA was significantly higher than that isolated on DFB ($p = 1 \times 10^{-4}$). On the contrary, in Sardinia, *Fusarium* colonies developed on DFB were higher than those grown on MPDA ($p = 1 \times 10^{-4}$). On both MPDA and DFB, the gradient observed for *Fusarium* colonies incidence was Emilia Romagna > Umbria > Sardinia ($p = 1 \times 10^{-4}$). However, no significant differences were observed on DFB between the last two regions ($p = 0.24$). In addition to *Alternaria* and *Fusarium* genera, other fungal genera such as *Epicoccum*, *Aspergillus* and *Penicillium* were detected. The first one was isolated only from kernels placed in MPDA and not by DFB method. The incidence of *Epicoccum* was significantly higher in Emilia Romagna and Umbria than in Sardinia ($p \leq 1 \times 10^{-4}$). As for *Epicoccum,* also the genera *Aspergillus* and *Penicillium* were isolated only on MPDA except for Emilia Romagna samples.

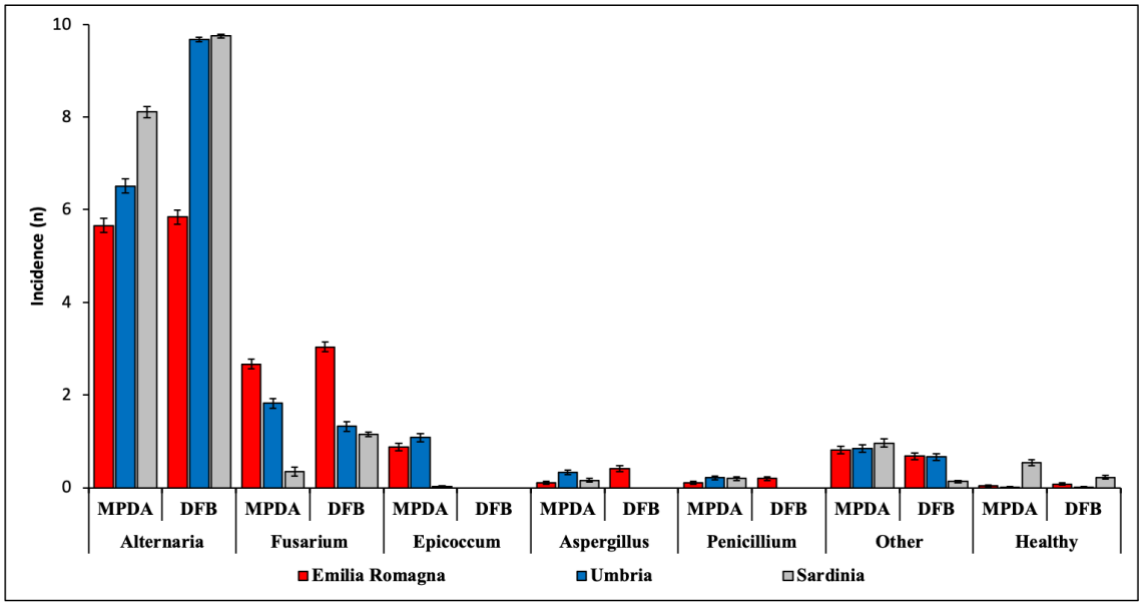

Figure 3: Average number of fungal colonies (n) per durum wheat sample belonging to different fungal genera as visually and microscopically assessed after their development from durum wheat kernels collected in three different Italian regions (Emilia Romagna, Umbria, Sardinia) with two different isolation methods (modified potato dextrose agar, MPDA; deep-freezing blotter, DFB). Columns represent the average (±standard error, SE) number of colonies belonging to different fungal genera developed from 10 analyzed durum wheat samples per each region and with each method.

Second year of investigation

After 5 days of incubation (MPDA) and 7 days of incubation (DFB), durum wheat kernels were subject to visual observation. Fungal colonies were ascribed to one of the following genera: *Alternaria*, *Fusarium*, *Microdochium*, *Cladosporium*, *Epicoccum*, *Aspergillus*, *Penicillium* and *Rhizopus* based on morphological features included color of the colony, mycelium aspect as well as microscopical observation of conidia. Colonies which did not belong to the aforementioned genera were classified as "other" (Figure 4).

Significant differences were detected among the two methodologies: the average number of fungal colonies developed on MPDA on each sample was statistically higher than those observed on DFB (100.8 and 84.5, respectively; $p < 1 \times 10^{-4}$). In details, MPDA method allowed to isolate a higher number of colonies belonging *Alternaria* ($p < 1 \times 10^{-4}$), *Fusarium* ($p < 1 \times 10^{-4}$), *Microdochium* ($p = 3 \times 10^{-4}$), *Epicoccum* ($p = 0.01$), and *Penicillium* ($p = 0.01$), compared with DFB; on the contrary, no differences related to the method were observed for *Aspergillus* ($p = 0.55$) and *Cladosporium* ($p = 0.06$) genera. On DFB method the number of fungal colonies isolated in Sicily was significantly lower compared with those developed from kernels collected in Emilia Romagna, Umbria, Tuscany and Veneto regions ($p \leq 0.03$); on the contrary, on MPDA method the only difference in term of fungal colonies

observed was detected between Apulia and Umbria regions (average number of fungal colonies 86.4 and 113.9 respectively; $p = 2 \times 10^{-3}$).

As expected, DFB methodology allowed to detect a higher number of healthy seeds compared with MPDA ($p < 1 \times 10^{-4}$). However, following the first method, the only difference observed in terms of number of healthy seeds was between Apulia and Umbria regions, while, on MPDA, the average number of seeds not contaminated in these two regions was different from that one observed in Sardinia, Latium, The Marche, Tuscany, Umbria and Abruzzo regions ($p \leq 0.02$).

Focusing on the fungal genera, on both methods, *Alternaria* and *Fusarium* were the two most detected genera associated to Italian durum wheat kernels. In details, the average number of *Alternaria* colonies was significantly higher than *Fusarium* ones on MPDA ($p < 1 \times 10^{-4}$) and DFB ($p < 1 \times 10^{-4}$). On both methods, the number of colonies belonging to these two genera was statistically different from those belonging to other genera observed ($p < 1 \times 10^{-4}$). On PDA, *Cladosporium*, *Microdochium* and *Epicoccum* showed a similar incidence (6.2, 4.8 and 3.9, $p \geq 0.56$) while on DFB, no differences were observed between *Cladosporium* and the average number of colonies classified as "Others" ($p = 0.87$).

Focusing on the single genera observed with DFB, the lowest *Alternaria* incidence was observed in Emilia Romagna even if it was significantly different only from Umbria, Latium, Apulia and Sardinia regions ($p \leq 0.02$). On the contrary, with this method, in Emilia Romagna was observed the higher *Fusarium* incidence that was significantly different from that observed in the Southern region such as Apulia, Campania and Sicily ($p \leq 2 \times 10^{-3}$). No differences were observed between Emilia Romagna and the other exanimated regions ($p \geq 0.05$). In addition, with DFB, two fungal genera not observed on MPDA were detected: *Gliocladium* and *Acremonium* (average number of colonies equal to 0.5 and 2.3, respectively). The first one was detected only in Tuscany (2.8) and Umbria (2.4), while *Acremonium* was present especially in The Marche (10.8) region. No significant differences were observed among regions for what concern the genera: *Microdochium* ($p \geq 0.30$), *Epicoccum* ($p \geq 0.41$), *Penicillium* ($p \geq 0.14$), *Rhizopus* ($p \geq 0.30$).

On MPDA, no significant differences were observed among regions in terms of *Alternaria* infection ($p \geq 0.25$). The highest average number of *Alternaria* colonies was observed in Campania (92) while the lower in Lombardy (54). With this method, the higher *Fusarium* incidence was reported in Abruzzo (28) and Umbria (22.5). However, Abruzzo and Umbria showed a significant difference exclusively compared to Sicily, Apulia, Campania, The Marche, Lombardy and Emilia Romagna regions ($p \leq 0.03$ and $p \leq 0.01$, respectively).

Finally, the genus *Microdochium*, also considered a Head Blight causal agent, was recorded without significant differences ($p \geq 0.13$) in samples collected across Central (Umbria, Tuscany, Abruzzo, Latium, The Marche) and Northern regions (Emilia Romagna, Veneto, Lombardy) while it was absent in Molise, Apulia and Sicily (Southern regions). Detailed information regarding the fungal genera isolated with the two different methods in each investigated region are reported in Figure 5 (MPDA) and Figure 6 (DFB) and in Table S5 and S6.

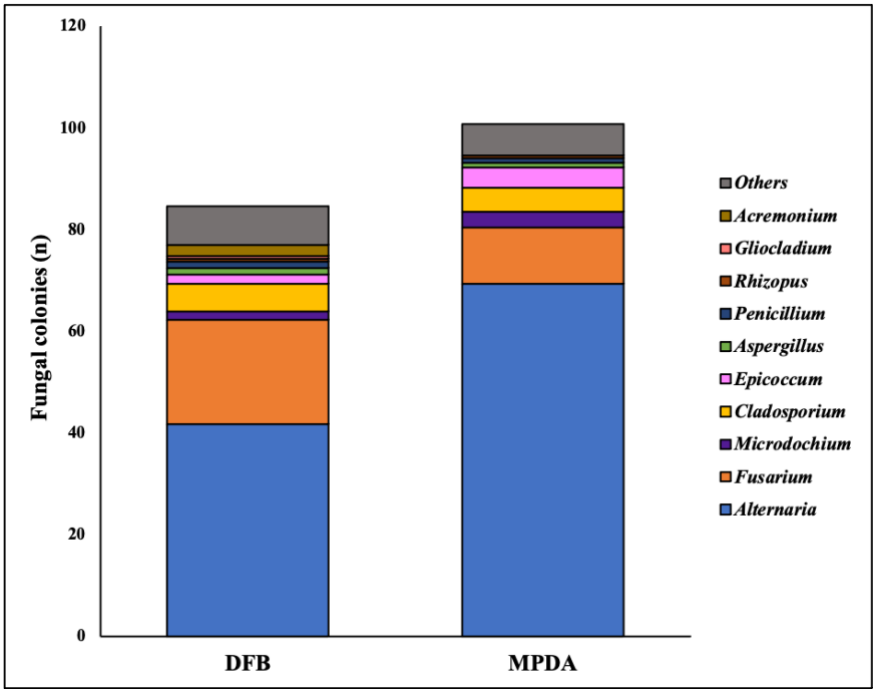

Figure 4: Global average number of fungal colonies (n) belonging to different fungal genera as visually and microscopically assessed after their development on thirteen Italian durum wheat samples.

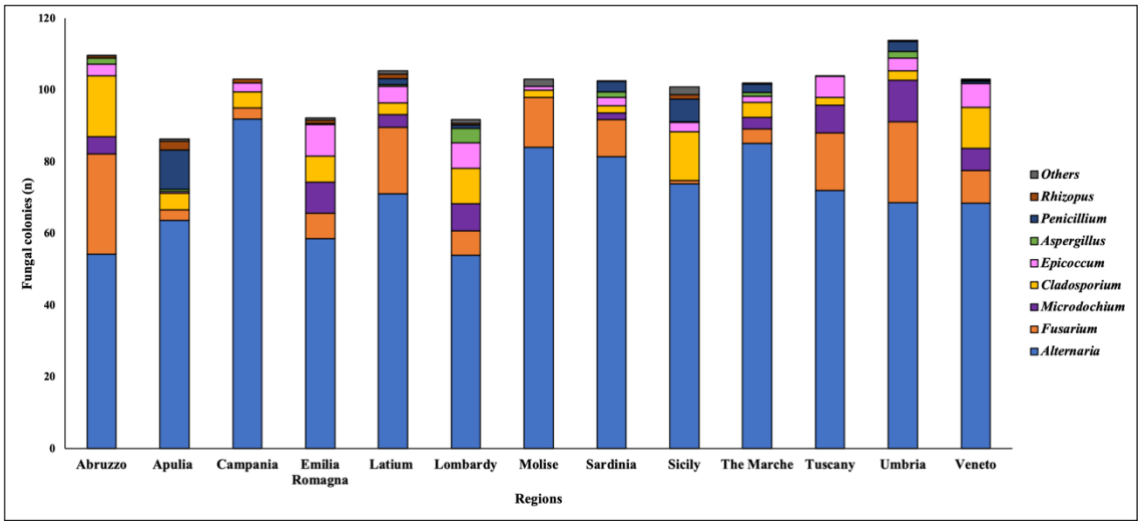

Figure 5: Average number of fungal colonies (n) per durum wheat samples belonging to different fungal genera as visually and microscopically assessed after their development from durum wheat kernels collected in 13 different Italian regions (Abruzzo, Apulia, Campania, Emilia Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria and Veneto) on modified potato dextrose agar (MPDA).

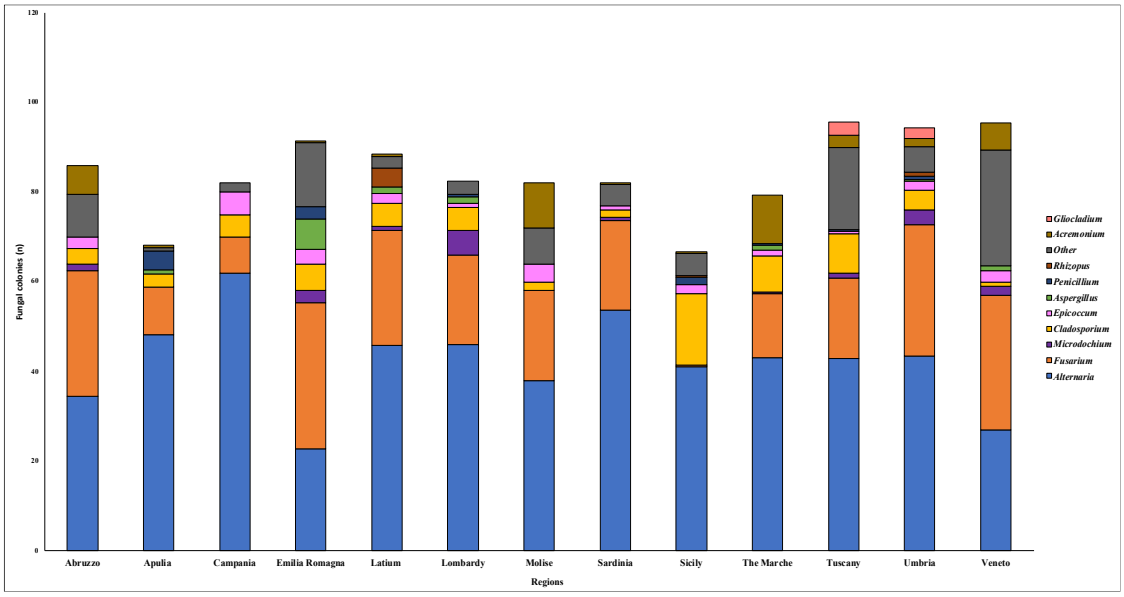

Figure 6: Average number of fungal colonies (n) per durum wheat samples belonging to different fungal genera as visually and microscopically assessed after their development from durum wheat kernels collected in 13 different Italian regions with deep-freezing blotter method (DFB).

1.3.2 Composition of *Fusarium* community of Italian durum wheat grains

First year of investigation

In the first year of investigation, the *Fusarium* community associated to durum wheat Italian samples harvested in 2015 was composed by 13 different species (*F. poae, F. avenaceum, F. graminearum, Fusarium culmorum, Fusarium sporotrichioides, Fusarium langsethiae, Fusarium tricinctum, Fusarium equiseti, Fusarium acuminatum, F. proliferatum, Fusarium verticillioides, Fusarium sambucinum, Fusarium crockwellense)*. However, differences between the three cultivation areas were detected. In detail, the average number of *Fusarium* strains isolated from the durum wheat samples followed the statistically significant gradient: Emilia Romagna > Umbria > Sardinia. This trend was observed in both PDA (11.8, 4.7, 0.74, respectively) ($p \leq 0.002$) and DFB method (15.2, 6.10, 1.96, respectively) ($p \leq 0.01$). No significant differences between the two methods in any of the investigated areas were detected ($p \geq 0.1$) even if the number of *Fusarium* colonies obtained with DFB was higher than that recovered on PDA.

A total of 11 different species were isolated on PDA and with DFB method from durum wheat seeds harvested in Emilia Romagna region. On PDA, *F. poae* was the most frequent species (6.1) ($p \leq 0.001$), followed by *F. graminearum* (3.0) ($p \leq 7 \times 10^{-4}$). In addition, *F. proliferatum, F. culmorum, F. avenaceum, F. equiseti, F. sambucinum, F. tricinctum, F. verticillioides* and *F. acuminatum* were isolated on that media. All these species showed a significantly lower presence than *F. poae* and *F. graminearum* ($p \leq 7 \times 10^{-4}$). With DFB, the species that showed a significantly highest incidence were *F. proliferatum* (5.98) and *F. poae* (4.33); a similar trend was observed on DFB method, where these species were followed by *F. graminearum* that was significantly lower than *F. proliferatum* ($p \leq 7 \times 10^{-4}$) but not than *F. poae* ($p = 0.079$). In addition, other six species (*F. avenaceum, F. equiseti, F. verticillioides, F. tricinctum, F. acuminatum* and *F. crockwellense*) were detected even if the colony number was significantly lower than the already mentioned species ($p \leq 2 \times 10^{-4}$). Differences in recovering species between PDA and DFB were found only for *F. culmorum* and *F. proliferatum*. In detail, *F. culmorum* was detected more on PDA (0.56) than on DFB (n.d.) ($p = 0.03$) while *F. proliferatum* showed a significantly higher presence on DFB (5.98) than on PDA (0.84) ($p = 1 \times 10^{-4}$).

In Umbria region, the *Fusarium* community was composed by 11 different species. On PDA, as well as in Emilia Romagna, *F. poae* showed a significantly higher presence (2.53) ($p \leq 0.001$) followed by *F. proliferatum* (0.67) and *F. graminearum* (0.66). These two species

were followed by *F. culmorum* and *F. avenaceum* that were not significantly different from the two main species ($p \geq 0.06$). Other 5 species (*F. langsethiae, F. sporotrichioides, F. tricinctum, F. acuminatum and F. verticillioides*) ($p \geq 0.29$) were detected with an incidence significantly lower than *F. poae* and *F. proliferatum*. Also, in this region, *F. proliferatum* was the species most detected with DFB (2.82) even if it did not show a significantly difference from *F. poae* (1.55) ($p = 6 \times 10^{-3}$). These species were followed by *F. graminearum*, significantly lower than *F. proliferatum* only ($p = 1 \times 10^{-3}$), *F. avenaceum, F. tricinctum, F. equiseti* and *F. acuminatum* that, in turn, showed a similar presence. In addition, only *F. proliferatum* showed a significantly higher incidence with DFB (2.82) than on PDA (0.67) ($p = 4 \times 10^{-4}$).

In Sardinia, only 7 species were detected with both PDA and DFB. In details, with the first method no differences were observed among 5 species (*F. poae, F. culmorum, F. graminearum, F. proliferatum, F. avenaceum*) ($p \geq 0.24$). With DFB, *F. proliferatum* was the most detected species (0.92) followed by *F. sporotrichioides* (0.47) ($p = 0.27$). In addition, colonies of *F. culmorum, F. poae, F. avenaceum, F. equiseti, F. verticillioides* were detected in significantly lower amount compared to *F. proliferatum* ($p \leq 0.02$). *F. proliferatum* and *F. sporotrichioides* showed a significantly higher presence with DFB than on PDA ($p = 0.005$ and 0.04, respectively).

Focusing on the single species: *F. poae* followed the significant gradient Emilia Romagna > Umbria > Sardinia (both for PDA and DFB); *F. graminearum* followed the significant gradient Emilia Romagna > Umbria > Sardinia (absent) (both for PDA and DFB); *F. avenaceum* followed the significant gradient Emilia Romagna ≥ Umbria ≥ Sardinia (only for PDA); *F. proliferatum* followed the significant gradient Emilia Romagna ≥ Umbria > Sardinia (both for PDA and DFB).

The average number (n) of *Fusarium* isolates ascribable to the different species is shown in Figure 7 and detailed in Table S4.

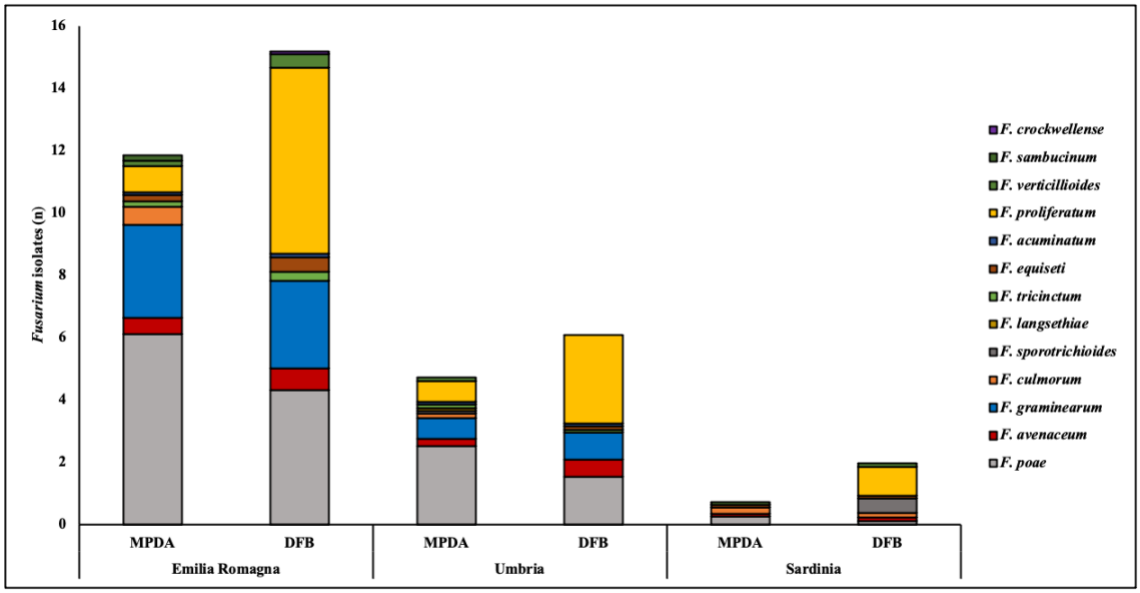

Figure 7: Average number of isolates (n) per durum wheat sample belonging to the different *Fusarium* species isolated from durum wheat kernels collected in three different Italian regions (Emilia-Romagna, Umbria, Sardinia) with two different isolation methods (modified potato dextrose agar, MPDA; deep-freezing blotter, DFB).

Second year of investigation

The *Fusarium* community associated to Italian durum wheat samples harvested in 2018 was composed by 21 different species (*F. graminearum, F. culmorum, F. poae, F. proliferatum, F. tricinctum,* members of FIESC, *F. sporotrichioides, F. sambucinum, Fusarium pseudograminearum, Fusarium brachigibbosum, Fusarium oxysporum, Fusarium nelsonii, Fusarium anthophilum, Fusarium lateritium, Fusarim globosum, F. langsethiae, Fusarium subglutinans, F. verticillioides, Fusarium semitectium, Fusarium clamydosporum*). As already mentioned, the representative isolates developed on MPDA were molecularly identified, while the isolates obtained with DFB were morphologically identified. The isolates obtained with DFB who were not recognized as any of the already mentioned species were classified as *Fusarium* sp.

Differences in the *Fusarium* detection were observed between the two methods (Figure 8). In general, the average number of *Fusarium* isolates obtained for each sample with DFB method (17.9) was higher than the ones observed on MPDA (10.9) ($p = 1 \times 10^{-4}$). In addition, differences in recovering *Fusarium* species between the two isolation methods in terms of the number of *Fusarium* isolates were recorded only for *F. tricinctum* and *F. proliferatum*. In detail, the first one showed a significant higher presence ($p = 3 \times 10^{-3}$) on MPDA (0.40)

compared with DFB (0.07), on the contrary, *F. proliferatum* was detected more on DFB (4.90) than on MPDA (1.10) ($p < 1 \times 10^{-4}$).

Even if with a low incidence, some species were isolates only on MPDA such as *F. pseudograminearum, F. brachigibbosum, F. oxysporum, F. nelsonii, F. antophilum,* while other species (*F. lateritium, F. globosum, F. langsethiae, F. subglutinans F. verticillioides, F. semitectum, F. clamydosporum*) were detected only with DFB. In addition, considering that *F. acuminatum* and *F. avenaceum* showed a similar phenotypical trait of the colony and similar conidia morphology, they can be difficult to distinguish based only on morphological characters. For this reason, the isolates obtained with DFB and identified as one of the two species were classified as "*F. acuminatum-F. avenaceum*".

Totally, the highest number of *Fusarium* colonies was isolated from regions of Central Italy: Abruzzo (26.7), Latium (24), Umbria (21). However, they were significantly different only compared to Sicily (0.66), Campania (4.2), Apulia (6.33) and The Marche (9.3) regions ($p \leq 0.04$).

With DFB, at national level, the species who showed the highest incidence was *F. proliferatum* followed by *F. globosum*, *F. acuminatum-F. avenaceum*, *F. verticillioides*; no significant differences were observed among the latter three species. In detail, *F. proliferatum* was detected in all investigated regions. It was particularly present in Umbria region (10.5), even if a significantly difference was observed only with Sicily, Sardinia, Campania, Apulia, Tuscany and The Marche region ($p \leq 0.03$). Interesting, *F. globosum* isolated exclusively with DFB, was particularly present in Abruzzo (6.25) and significant differences were observed with Sicily, Lombardy and Apulia regions ($p \leq 0.04$). In addition, this species was totally absent in Sicily and Campania regions.

The isolates identified based on morphological features as *F. avenaceum-F. acuminatum* followed the gradient: Latium (6.86) > 8 region (The Marche, Campania, Apulia, Veneto, Abruzzo, Tuscany and Emilia Romagna and Umbria) ($p < 1 \times 10^{-3}$). These two species were not detected in kernels collected across Sicily region.

F. verticilliodes, absent in Sicily, Campania and Sardinia, was isolated from the samples harvested in 8 regions (Emilia Romagna, Lombardy, Veneto, Latium, Umbria, Apulia, Molise and Abruzzo) without any significant difference ($p \geq 0.51$).

With DFB method, no significant differences were observed among the 13 investigated regions in terms of *F. sporotrichioides* ($p \geq 0.83$), *F. sambucinum* ($p \geq 0.55$), *F. tricinctum, F. langsethiae* and *F. semitectum* ($p \geq 0.93$) incidence.

On MPDA, *F. avenaceum* (average number of colonies for each sample equal to 3.4) was the most common species associated to Italian durum wheat samples followed by *F. graminearum* (1.69), *F. poae* (1.18), *F. proliferatum* (1.10) and *F. culmorum* (0.85). No significant differences were observed in the distribution of these 4 species in the surveyed territory ($p \geq 0.93$).

Absent in Sicily and Campania, *F. avenaceum* was significantly present in Latium. In detail, the incidence of *F. avenaceum* in this region was significantly higher than that observed in all the other regions ($p \leq 2 \times 10^{-3}$) with the exception of Sardinia, Abruzzo and Molise ($p \geq 0.96$).

F. graminearum was detected in higher amount in Umbria (5.2) however significant differences were observed only between this region and Apulia, Sardinia, Campania, Lombardy and Emilia Romagna ($p \leq 7 \times 10^{-3}$). It was completely absent in Sicily and The Marche regions.

F. poae was detected in all the investigated regions except for Molise, Sardinia and Campania. This species followed the significant gradient ($p \leq 0.02$) Latium = The Marche ($p = 1.0$) > Sicily = Apulia = Lombardy ($p \geq 0.99$). In addition, even if with a lower incidence, in Tuscany, Abruzzo, Emilia Romagna, Umbria and Veneto regions, the presence of this species was not significantly different from what observed in Latium and The Marche ($p \geq 0.16$).

On PDA, the higher incidence of *F. proliferatum* was observed in Abruzzo region (4.75).

In detail, the incidence of *F. proliferatum* in this region was not significantly different from that detected in Veneto (4.5), Umbria (1.33), Campania (1.0) and Emilia Romagna (0.75) ($p \geq 0.24$). *F. proliferatum* did not contaminated any of the investigated samples collected across The Marche, Sicily and Molise.

Finally, with the exception of Veneto, Sicily and Umbria, *F. culmorum* was detected in all the investigated regions without any significant differences ($p \geq 0.42$).

As well as for genera distribution, also the composition of *Fusarium* complex showed differences depending on the cultivation area. All the investigated regions showed the presence of, at least, 3 *Fusarium* species (Sicily and Campania 3 and 4 species, respectively), while the highest variability in the *Fusarium* complex composition was observed in Umbria, Emilia Romagna (17 species each), Abruzzo and Tuscany (16 species each).

The average number (n) of *Fusarium* isolates ascribable to the different species for each investigated region is shown in Figure 9 and Figure 10 and detailed in Table S6.

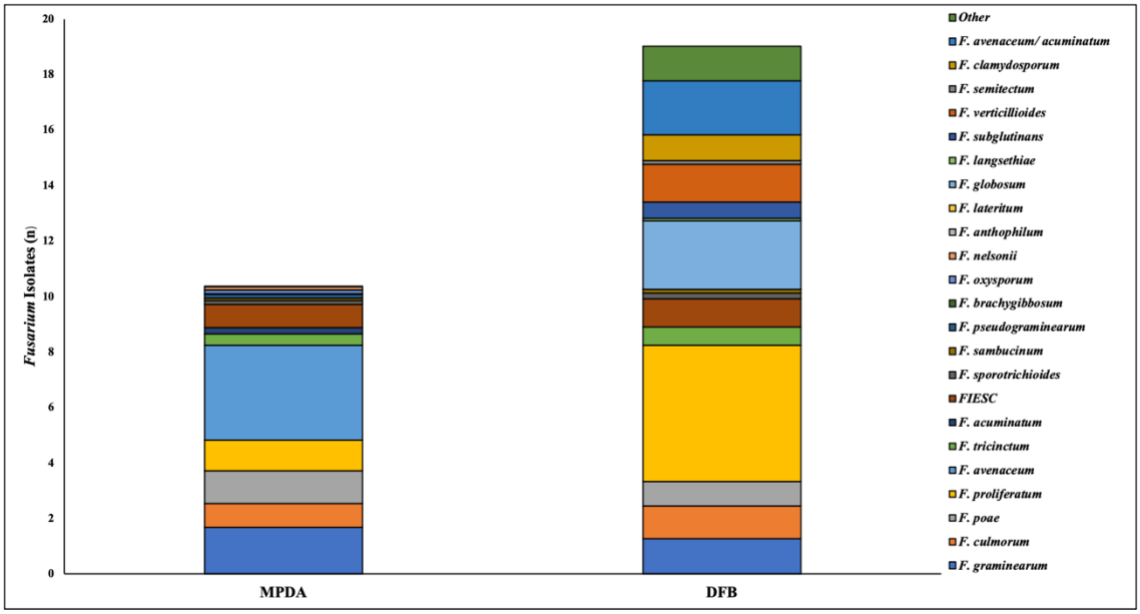

Figure 8: Average number of isolates (n) belonging to the different Fusarium species isolated from Italian durum wheat kernels collected across thirteen different Italian regions (Abruzzo, Apulia, Campania, Emilia Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto) with two different isolation methods (modified potato dextrose agar: MPDA; deep-freezing blotter DFB).

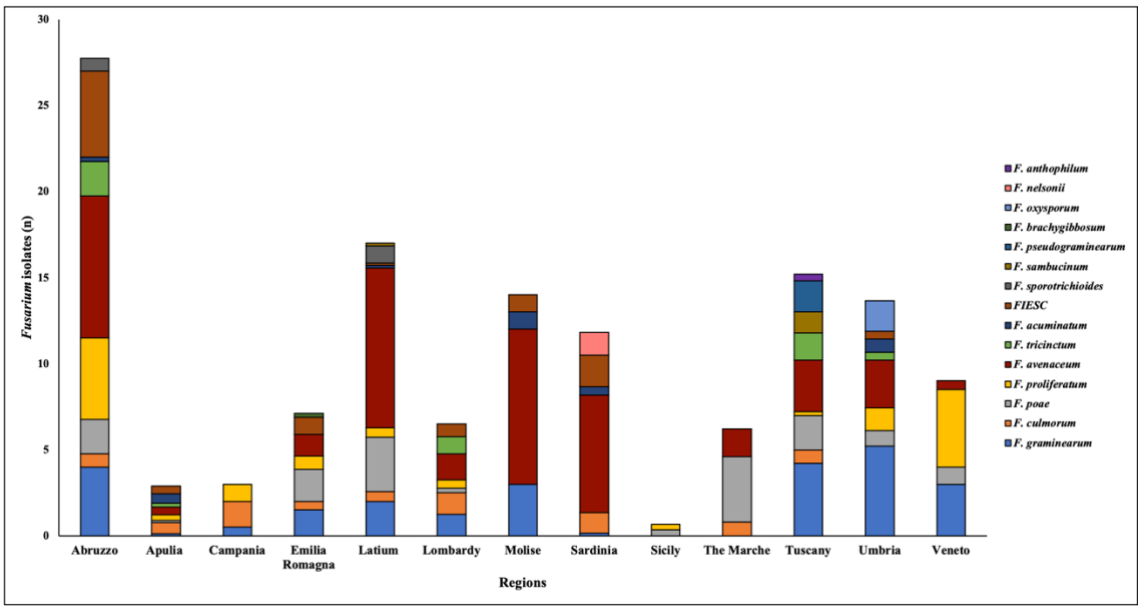

Figure 9: Average number of isolates (n) belonging to the different Fusarium species isolated from Italian durum wheat kernels collected across thirteen different Italian regions (Abruzzo, Apulia, Campania, Emilia Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto) with modified potato dextrose agar (MPDA).

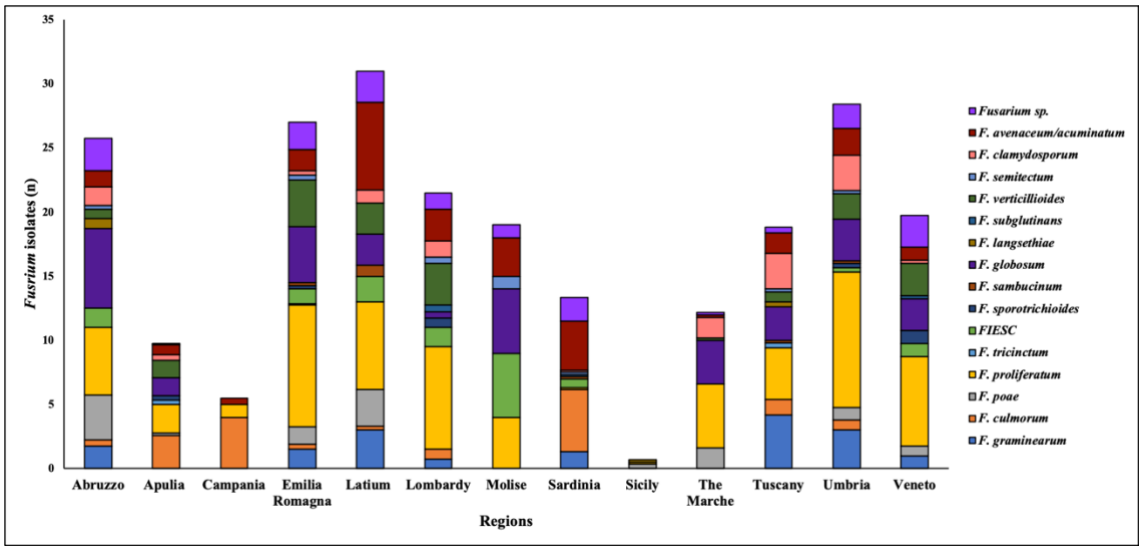

Figure 10: Average number of isolates (n) belonging to the different Fusarium species isolated from Italian durum wheat kernels collected across thirteen different Italian regions (Abruzzo, Apulia, Campania, Emilia Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto) with deep-freezing blotter (DFB).

1.3.3 Mycotoxins contamination in Italian durum wheat grain

First year of investigation

The fungal secondary metabolites (µg/kg) as quantified by LC/MS-MS in the durum wheat grains collected in the three Italian regions are summarized by category in Table 1 and detailed within each group in Table 2 (trichothecenes), Table 3 (depsipeptides), Table 4 (zearalenone, fumonisins and other *Fusarium* secondary metabolites), Table 5 (*Alternaria* secondary metabolites).

The samples collected in Emilia Romagna were particularly contaminated by *Fusarium* mycotoxins: in details, total trichothecenes followed the gradient Emilia Romagna > Umbria > Sardinia ($p \leq 0.05$) while depsipeptides followed the gradient Emilia Romagna > Umbria = Sardinia. Zearalenone and fumonisins were found in low percentage only in samples collected in Emilia Romagna. Conversely, in Umbria region was detected the great amount of *Alternaria* secondary metabolites if compared with the other analyzed regions; in detail, they followed the significant gradient: Umbria > Emilia Romagna > Sardinia.

Focusing on type B trichothecenes, DON was particularly present in Emilia Romagna grains while no differences in DON concentration were found between Umbria and Sardinia samples. The same gradient was observed for culmorin and 15-hydroxyculmorin. Among type A trichothecenes (NIV, HT-2 toxin, HT-2 glucoside and T-2 toxin), they were particularly present in Umbria and Emilia Romagna; in details, the followed gradient was Emilia Romagna = Umbria > Sardinia ($p \leq 0.05$).

In this study, depsipeptides such as ENs (enniatin A, ENA; enniatin A1, ENA1; enniatin B, ENB; enniatin B1, ENB1; enniatin B2, ENB2; enniatin B3, ENB3) and BEA were also investigated. They were particularly present in Emilia Romagna samples followed by Umbria and Sardinia ones. The EN analogues, detected in durum wheat grains from Emilia Romagna and Umbria, followed the gradient ENB1 > ENB > ENA1 > ENB2 > ENA > ENB3, while in samples harvested in Sardinia, the observed gradient was ENB1 > ENB > ENB2 > ENB3. Even if present in lower amount (compared with ENs) in all investigated region, BEA accumulation followed the significant gradient ($p \leq 0.03$) Emilia Romagna > Umbria > Sardinia.

Other *Fusarium* secondary metabolites were also investigated in the present study. In detail, some of them were present only in Emilia Romagna (antibiotic Y, chlamidospordiol, fusarin C, fusarinolic acid, sambucinol), others (aminodimethyloctadecanol, aurofusarin, butenolide, chalmydosporol, epiequisetin and equisetin) only in Emilia Romagna and

Umbria samples. Finally, MON was detected in all samples: in particular, this compound was significantly higher in Emilia Romagna samples compared to Umbria and Sardinia ($p \leq 0.02$, Emilia Romagna > Umbria = Sardinia).

With respect to *Alternaria* secondary metabolites such as tenuazonic acid, tentoxin, pyrenophorol and macrosporin, they were notably higher in the Umbrian grains, while some others (altersetin, alternariol, infectopyrone and alternariol methyl ether) were higher in the Emilia Romagna samples. However, no significant differences were recorded between Umbria and Emilia Romagna for the content of these single compounds. Conversely, significant differences between these two regions and Sardinia were detected for tenuazonic acid, alternariol methyl ether and infectopyrone (Umbria = Emilia Romagna > Sardinia, $p \leq 0.04$).

Table 1: Secondary metabolites divided for classes detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions.

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Trichothecenes	Mean (µg/ kg)	1326.00*** (±541.00)	104 (±28.1)	39.1 (±15.7)
	Mean Positive Samples (µg/ kg)	1326** (±541)	104 (±28.1)	65.1 (±20.1)
	Positive sample	10	10	6
	Incidence positive samples	100	100	60
Zearalenone	Mean (µg/ kg)	5.46 (±2.76)	nd§ -	nd -
	Mean Positive Samples (µg/ kg)	10.9 (±4.42)	nd -	nd -
	Positive sample	5	nd	nd
	Incidence positive samples	50	nd	nd
Fumonisins	Mean (µg/ kg)	6.65 (±5.31)	nd -	nd -
	Mean Positive Samples (µg/ kg)	33.3 (±19.6)	nd -	nd -
	Positive sample	2	nd	nd
	Incidence positive samples	20	nd	nd
Depsipeptides	Mean (µg/ kg)	323 (±115)	56.4 (±36.2)	0.06 (±0.05)
	Mean Positive Samples (µg/ kg)	323 (±115)	56.4 (±36.2)	0.23 (±0.15)
	Positive sample	10	10	3
	Incidence positive samples	100	100	30
Other *Fusarium* secondary metabolites	Mean (µg/ kg)	730 (±217)	95.1 (±43.6)	1.10 (±0.44)
	Mean Positive Samples (µg/ kg)	730 (±217)	106 (±47.3)	1.82 (±0.57)
	Positive sample	10	9	6
	Incidence positive samples	100	90	60
Alternaria secondary metabolites	Mean (µg/ kg)	366 (±35.8)	701 (±155)	205 (±19.4)
	Mean Positive Samples (µg/ kg)	366 (±35.8)	701 (±155)	205 (±19.4)
	Positive sample	10	10	10
	Incidence positive samples	100	100	100

**The value represents the average (± standard error) of positive samples of each region; ***the value represents the average (± standard error) of ten replicates for each region; §nd: not detected.

Table 2:Trichothecenes detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions (Emilia-Romagna, Umbria, Sardinia).

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Deoxynivalenol	Mean (µg/ kg)	499*** (±210)	15.5 (±10.3)	7.85 (±4.22)
	Mean Positive Samples (µg/ kg)	499** (±210)	51.9 (±25.7)	26.2 (±15.1)
	Positive sample	10	3	3
	Incidence positive samples	100	30	30
	Max Value (µg/ kg)	1740	103	34.6
Nivalenol	Mean (µg/ kg)	63.6 (±14.7)	35.1 (±14.9)	3.47 (±2.38)
	Mean Positive Samples (µg/ kg)	63.6 (±14.7)	35.1 (±14.9)	17.3 (±12.2)
	Positive sample	10	10	2
	Incidence positive samples	100	100	20
	Max Value (µg/ kg)	158	161	21.2
DeacetylNeosolaniol	Mean (µg/ kg)	12.4 (±6.65)	5.18 (±2.67)	nd§ -
	Mean Positive Samples (µg/ kg)	41.4 (±23.9)	17.3 (±9.98)	nd -
	Positive sample	3	3	nd
	Incidence positive samples	30	30	nd
	Max Value (µg/ kg)	51.2	20.4	nd
Diacetoxyscirpenol	Mean (µg/ kg)	1.06 (±0.97)	0.07 (±0.07)	nd -
	Mean Positive Samples (µg/ kg)	3.53 (±2.03)	0.70 (±0.07)	nd -
	Positive sample	3	1	nd
	Incidence positive samples	30	10	nd
	Max Value (µg/ kg)	9.80	0.70	nd
HT-2 glucoside	Mean (µg/ kg)	14.7 (±7.43)	10.7 (±3.62)	0.66 (±0.66)
	Mean Positive Samples (µg/ kg)	29.5 (±13.2)	6.30 (±2.20)	6.60 (±0.66)
	Positive sample	5	8	1
	Incidence positive samples	50	80	10
	Max Value (µg/ kg)	69.3	32.5	6.6
HT-2 toxin	Mean (µg/ kg)	17.9 (±8.52)	10.7 (±3.62)	0.42 (±0.28)
	Mean Positive Samples (µg/ kg)	29.9 (±12.2)	13.4 (±4.76)	2.10 (±1.48)
	Positive sample	6	8	2
	Incidence positive samples	60	80	20
	Max Value (µg/ kg)	78.8	32.5	2.10
T-2 toxin	Mean (µg/ kg)	3.74 (±1.98)	3.14 (±1.39)	0.20 (±0.20)
	Mean Positive Samples (µg/ kg)	9.35 (±4.67)	5.23 (±2.13)	2.00 (-)
	Positive sample	4	6	1
	Incidence positive samples	40	60	10
	Max Value (µg/ kg)	18.6	12.1	2.00
T-2 tetraol	Mean (µg/ kg)	3.94 (±2.47)	1.26 (±0.66)	nd -
	Mean Positive Samples (µg/ kg)	13.1 (±7.58)	4.20 (±2.42)	nd -
	Positive sample	3	3	nd
	Incidence positive samples	30	30	nd
	Max Value (µg/ kg)	23.5	5.40	nd
Monoacetoxyscirpenol	Mean (µg/ kg)	7.66 (±3.63)	2.00 (±1.10)	nd -
	Mean Positive Samples (µg/ kg)	9.57 (±3.38)	5.00 (±2.50)	nd -
	Positive sample	8	4	nd
	Incidence positive samples	80	40	nd
	Max Value (µg/ kg)	39.5	11.1	nd *

**the value represents the average (± standard error) of positive samples of each region; ***the value represents the average (± standard error) of ten replicates for each region; §nd: not detected.

Table 3: *Fusarium* depsipeptides secondary metabolites detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions (Emilia-Romagna, Umbria, Sardinia).

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Enniatin A	Mean (µg/ kg)	4.24*** (±3.00)	0.70 (±0.26)	nd[§] -
	Mean Positive Samples (µg/ kg)	5.30** (±1.87)	1.16 (±0.47)	nd -
	Positive sample	8	6	nd
	Incidence positive samples	80	60	nd
	Max Value (µg/ kg)	30.8	2.2	nd
Enniatin A1	Mean (µg/ kg)	46.2 (±28.6)	6.16 (±2.61)	nd -
	Mean Positive Samples (µg/ kg)	66.0 (±24.9)	12.3 (±5.50)	nd -
	Positive sample	7	5	nd
	Incidence positive samples	70	50	nd
	Max Value (µg/ kg)	296	23.9	nd
Enniatin B	Mean (µg/ kg)	123 (±32.5)	24.2 (±18.8)	0.02 (±0.02)
	Mean Positive Samples (µg/ kg)	137 (±45.8)	40.4 (±16.4)	0.20 (±0.20)
	Positive sample	9	6	1
	Incidence positive samples	124	24.3	0.03
	Max Value (µg/ kg)	(±32.5)	(±18.8)	(±0.02)
Enniatin B1	Mean (µg/ kg)	133 (±52.6)	21.9 (±13.9)	0.03 (±0.03)
	Mean Positive Samples (µg/ kg)	148 (±49.5)	43.8 (±19.6)	0.30 (±0.30)
	Positive sample	9	5	1
	Incidence positive samples	90	50	10
	Max Value (µg/ kg)	564	144	0.30
Enniatin B2	Mean (µg/ kg)	7.44 (±1.99)	1.40 (±1.10)	0.01 (±0.01)
	Mean Positive Samples (µg/ kg)	7.44 (±1.99)	2.80 (±1.25)	0.10 (±0.10)
	Positive sample	10	5	1
	Incidence positive samples	7.45	1.41	0.02
	Max Value (µg/ kg)	(±1.99)	(±1.10)	(±0.01)
Enniatin B3	Mean (µg/ kg)	0.07 (±0.02)	0.01 (±0.01)	nd -
	Mean Positive Samples (µg/ kg)	0.11 (±0.04)	0.10 (±0.10)	nd -
	Positive sample	6	1	nd
	Incidence positive samples	0.07	0.01	nd
	Max Value (µg/ kg)	(±0.02)	(±0.01)	-
Beauvericin	Mean (µg/ kg)	7.15 (±0.89)	1.95 (±0.88)	0.01 (±0.01)
	Mean Positive Samples (µg/ kg)	7.15 (±0.89)	1.95 (±0.88)	0.10 (±0.10)
	Positive sample	10	10	1
	Incidence positive samples	100	100	10
	Max Value (µg/ kg)	13.0	9.60	0.10

[**]the value represents the average (± standard error) of positive samples of each region; [***]the value represents the average (± standard error) of ten replicates for each region; [§]nd: not detected.

Table 4: Zearalenone, fumonisins and other *Fusarium* secondary metabolites detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions (Emilia-Romagna, Umbria, Sardinia).

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Culmorin	Mean (µg/ kg)	314*** (±141)	16.7 (±7.08)	12.2 (±5.42)
	Mean Positive Samples (µg/ kg)	314** (±142)	23.8 (±9.01)	30.5 (±15.2)
	Positive sample	10	7	4
	Incidence positive samples	100	70	40
	Max Value (µg/ kg)	1222	74.8	44.6
15-Hydroxyculmorin	Mean (µg/ kg)	203 (±83.8)	9.49 (±5.94)	14.2 (±5.99)
	Mean Positive Samples (µg/ kg)	203 (±83.8)	23.7 (±11.8)	28.5 (±12.7)
	Positive sample	10	4	5
	Incidence positive samples	100	40	50
	Max Value (µg/ kg)	698	59.7	50.7
15-Hydroxyculmoron	Mean (µg/ kg)	52.2 (±75.4)	nd§ -	nd -
	Mean Positive Samples (µg/ kg)	86.9 (±35.4)	nd -	nd -
	Positive sample	6	nd	nd
	Incidence positive samples	60	nd	nd
	Max Value (µg/ kg)	203	nd	nd
5-Hydroxyculmorin	Mean (µg/ kg)	133 (±64.0)	nd -	nd -
	Mean Positive Samples (µg/ kg)	266 (±119)	nd -	nd -
	Positive sample	5	nd	nd
	Incidence positive samples	50	nd	nd
	Max Value (µg/ kg)	564	nd	nd
Zearalenone-sulfate	Mean (µg/ kg)	5.07 (±2.59)	nd -	nd -
	Mean Positive Samples (µg/ kg)	15.5 (±3.91)	nd -	nd -
	Positive sample	4	nd	nd
	Incidence positive samples	40	nd	nd
	Max Value (µg/ kg)	23.9	nd	nd
Zearalenone	Mean (µg/ kg)	0.39 (±0.20)	nd -	nd -
	Mean Positive Samples (µg/ kg)	0.98 (±0.31)	nd -	nd -
	Positive sample	4	nd	nd
	Incidence positive samples	40	nd	nd
	Max Value (µg/ kg)	1.8	nd	nd
Fumonin B1	Mean (µg/ kg)	6.05 (±4.73)	nd -	nd -
	Mean Positive Samples (µg/ kg)	30.3 (±16.5)	nd -	nd -
	Positive sample	2	nd	nd
	Incidence positive samples	20	nd	nd
	Max Value (µg/ kg)	46.8	nd	nd
Fumonisin B2	Mean (µg/ kg)	0.60 (±0.60)	nd -	nd -
	Mean Positive Samples (µg/ kg)	6.04 -	nd -	nd -
	Positive sample	1	nd	nd
	Incidence positive samples	10	nd	nd
	Max Value (µg/ kg)	6.04	nd	nd

**the value represents the average (± standard error) of positive samples of each region; ***the value represents the average (± standard error) of ten replicates for each region; §nd: not detected.

Zearalenone, fumonisins and other *Fusarium* secondary metabolites detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions (Emilia-Romagna, Umbria, Sardinia) (CONTINUED).

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Aminodimethyloctadecanol	Mean (µg/ kg)	112 (±28.4)	460 (±142)	44.3 (±12.9)
	Mean Positive Samples (µg/ kg)	112 (±28.4)	460 (±142)	44.3 (±12.9)
	Positive sample	10	10	10
	Incidence positive samples	100	100	100
	Max Value (µg/ kg)	321	1450	155
Antibiotic Y	Mean (µg/ kg)	26.7 (±14.6)	nd -	nd -
	Mean Positive Samples (µg/ kg)	66.8 (±33.4)	nd -	nd -
	Positive sample	4	nd	nd
	Incidence positive samples	40	nd	nd
	Max Value (µg/ kg)	143	nd	nd
Moniliformin	Mean (µg/ kg)	171 (±58.2)	24.2 (±17.4)	0.16 (±0.14)
	Mean Positive Samples (µg/ kg)	171 (±58.2)	48.4 (±21.6)	0.80 (±0.56)
	Positive sample	10	5	2
	Incidence positive samples	100	50	20
	Max Value (µg/ kg)	610	177	1.50
Apicidin	Mean (µg/ kg)	38.3 (±25.7)	14.5 (±7.72)	0.28 (±0.17)
	Mean Positive Samples (µg/ kg)	47.9 (±16.9)	24.2 (±9.90)	0.93 (±0.53)
	Positive sample	8	6	3
	Incidence positive samples	80	60	30
	Max Value (µg/ kg)	249	75.8	1.70
Aurofusarin	Mean (µg/ kg)	398 (±139)	31.2 (±17.6)	nd -
	Mean Positive Samples (µg/ kg)	398 (±139)	62.5 (±27.9)	nd -
	Positive sample	10	5	nd
	Incidence positive samples	100	50	nd
	Max Value (µg/ kg)	1400	144	nd
Chlamydospordiol	Mean (µg/ kg)	1.37 (±0.91)	nd -	nd -
	Mean Positive Samples (µg/ kg)	6.85 (±4.84)	nd -	nd -
	Positive sample	2	nd	nd
	Incidence positive samples	20	nd	nd
	Max Value (µg/ kg)	7.00	nd	nd
Chlamydosporol	Mean (µg/ kg)	6.72 (±3.35)	0.39 (±0.39)	nd -
	Mean Positive Samples (µg/ kg)	16.8 (±8.4)	3.90 (±3.90)	nd -
	Positive sample	4	1	nd
	Incidence positive samples	40	10	nd
	Max Value (µg/ kg)	30.9	3.90	nd

[**]the value represents the average (± standard error) of positive samples of each region; [***]the value represents the average (± standard error) of ten replicates for each region; [§]nd: not detected.

Table 5: *Alternaria* secondary metabolites detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in three different Italian regions (Emilia-Romagna, Umbria, Sardinia).

Secondary metabolites		Emilia Romagna	Umbria	Sardinia
Tenuazonic acid	Mean (µg/ kg)	112*** (±28.4)	460 (±142)	44.3 (±12.9)
	Mean Positive Samples (µg/ kg)	112** (±28.4)	460 (±142)	44.3 (±12.9)
	Positive sample	10	10	10
	Incidence positive samples	100	100	100
	Max Value (µg/ kg)	321	1450	155
Alternariol	Mean (µg/ kg)	0.75 (±0.44)	0.56 (±0.26)	nd§ -
	Mean Positive Samples (µg/ kg)	1.50 (±0.67)	1.12 (±0.50)	nd -
	Positive sample	5	5	nd
	Incidence positive samples	50	50	nd
	Max Value (µg/ kg)	4.50	2.40	nd
Alternariol methyl ether	Mean (µg/ kg)	0.55 (±0.25)	0.74 (±0.43)	0.02 (±0.02)
	Mean Positive Samples (µg/ kg)	0.61 (±0.20)	0.74 (±0.43)	0.20 (±0.20)
	Positive sample	9	10	1
	Incidence positive samples	90	100	10
	Max Value (µg/ kg)	2.80	4.50	0.20
Altersetin	Mean (µg/ kg)	36.6 (±11.9)	12.4 (±5.66)	nd -
	Mean Positive Samples (µg/ kg)	36.6 (±11.9)	12.4 (±5.66)	nd -
	Positive sample	10	10	nd
	Incidence positive samples	100	100	nd
	Max Value (µg/ kg)	113	46.7	nd
Tentoxin	Mean (µg/ kg)	0.33 (±0.04)	0.83 (±0.35)	0.28 (±0.13)
	Mean Positive Samples (µg/ kg)	0.33 (±0.04)	0.83 (±0.35)	0.46 (±0.19)
	Positive sample	10	10	6
	Incidence positive samples	100	100	60
	Max Value (µg/ kg)	0.60	3.90	1.40
Infectopyrone	Mean (µg/ kg)	214 (±15.2)	217 (±25.9)	159 (±9.38)
	Mean Positive Samples (µg/ kg)	214 (±15.2)	217 (±25.9)	159 (±9.38)
	Positive sample	10	10	10
	Incidence positive samples	100	100	100
	Max Value (µg/ kg)	296	329	220
Pyrenophorol	Mean (µg/ kg)	0.80 (±0.80)	5.89 (±3.17)	nd -
	Mean Positive Samples (µg/ kg)	8.00 (±8.00)	11.8 (±5.27)	nd -
	Positive sample	1	5	nd
	Incidence positive samples	10	50	nd
	Max Value (µg/ kg)	8.00	32.3	nd

**the value represents the average (± standard error) of positive samples of each region; ***the value represents the average (± standard error) of ten replicates for each region; §nd: not detected.

Second year of investigation

The fungal secondary metabolites (µg/kg) as quantified by LC/MS-MS in the durum wheat grains collected in the 13 investigated Italian regions are detailed in Table 6 (trichothecenes in samples harvested in Northern and Central Italy), Table 7 (trichothecenes in samples harvested in Southern Italy and island), Table 8 (depsipeptides in samples harvested in Northern and Central Italy), Table 9 (depsipeptides in samples harvested in Southern Italy and island), Table 10 (zearalenone, fumonisin B1 and other *Fusarium* secondary metabolites in samples harvested in Northern and Central Italy), Table 11 (zearalenone, fumonisin B1 and other *Fusarium* secondary metabolites in Southern Italy and island), Table 12 (*Alternaria* metabolites detected in samples harvested in Northern and Central Italy), Table 13 (*Alternaria* metabolites detected in samples harvested in Southern Italy and island).

Globally the highest concentration of *Fusarium* secondary metabolites was recorded in kernels harvested in Abruzzo region where the average amount of these compounds was equal to 7831.1 µg/kg, while, as expected, durum wheat samples from Sicily region resulted the less contaminated by *Fusarium* mycotoxins (average 66 µg/kg). *Alternaria* metabolites were detected in all investigated regions ranging from the main value of 454 µg/kg detected in Sicily to 2792 µg/kg observed in samples collected in Tuscany. Low levels of *Aspergillus* and *Penicillium* mycotoxins were recorded in all the investigated samples.

Focusing on the individual *Fusarium* mycotoxins classes, the lower trichothecenes contamination was observed in durum wheat samples harvested in Southern region: Sicily, Campania, and Sardinia (average of 62.9, 112.9 and 150.6 µg/kg, respectively), while compounds of this chemical family were particularly present in Abruzzo (1388.4 µg/kg) and Tuscany (1376.4 µg/kg) regions. Among trichothecenes, DON was present in 67 up to 70 analyzed durum wheat samples. Even if without any significant differences among regions, this compound was present at higher level in Abruzzo, Tuscany, Veneto, and Umbria where 4 durum wheat samples (one for each region) largely exceeded the maximum level for DON contamination (1750 µg/kg) established by the EU. In details, LC-MS/MS analysis showed a DON concentration of 3730 µg/kg, 3720 µg/kg, 3370 µg/kg and 2600 µg/kg in the samples 27 (Umbria), 26 (Tuscany), 52 (Abruzzo) and 5 (Veneto), respectively. On the contrary, the lowest DON concentration was observed in Apulia, Campania and Sicily (all South Italy regions). Among Type B trichothecenes, nivalenol showed the highest incidence in samples harvested in Latium region (average 238.4 µg/kg) followed by Abruzzo, Tuscany and Umbria even if without significant difference ($p \geq 0.99$). Among type A trichothecenes, T-2 toxin was detected in 7 regions up to 13 (Abruzzo, Apulia, Latium, Lombardy, The

Marche, Tuscany and Umbria) without any significant difference. Instead, HT-2 toxin was detected in all investigated regions. In particular, durum wheat samples collected across Apulia showed an average contamination level (31.9 µg/kg) significantly different from what observed in Campania, Emilia Romagna, Latium, Sardinia and Sicily regions ($p \leq 0.03$). Among all the analyzed samples, the 40 (Umbria) and the 51 (Abruzzo) exceeded the maximum level for the sum of T-2+ HT-2 toxin set by the EU Recommendation (2013/165/EU) with a level of these two toxins equal to 131.1 and 131.2, respectively. Low levels of neosolaniol were reported in 3 regions up to 13 (Apulia, Latium and Umbria). Focusing on depsipeptides compounds, they were mainly detected in the Abruzzo region where the highest average contamination (1453.5 µg/kg) was recorded. In details, depsipeptides followed the gradient: Abruzzo=Umbria=Emilia-Romagna=Apulia >The Marche> Sicily. The ENNs analogues were accumulated in all regions following the gradient: ENB1> ENB> ENA1> ENB2> ENA> ENB2 with the exception of samples harvested in Campania, Emilia Romagna, Umbria and Veneto regions where the average concentration of ENB was higher than ENB1. ENB3 was found only in traces in the 77% of the analyzed samples.

Considering BEA contamination, it was detected in low amount without significant differences in all investigated regions with the exception of Campania.

Fumonisin B1 was found in 5 analyzed regions up to 13 (Abruzzo, Apulia, Emilia Romagna, The Marche and Umbria). However, the concentration of this mycotoxin did not exceed the average value of 27.70 µg/kg observed in samples collected across Abruzzo. Zearalenone was detected in low amount in 7 regions up to 13 (Abruzzo, Emilia Romagna, Latium, Lombardy, Tuscany Umbria, Veneto).

LC-MS/MS analysis allowed the detection also of other *Fusarium* secondary metabolites. Several compounds (culmorin, 15-hydroxyculmorin, moniliformin) were particularly present in durum wheat grains collected in Central Italy. In details, culmorin and 15-hydroxyculmorin were mainly detected in samples harvested in Tuscany (mean of 1195.07 and 638.47 µg/kg, respectively) and Abruzzo (1081.15 µg/kg and 815.40 µg/kg, respectively). Finally, the higher moniliformin content was detected in Abruzzo, Latium, Tuscany and Umbria samples with mean values of 827.20 µg/kg, 473.48 µg/kg, 268.04 µg/kg and 348.80 µg/kg, respectively.

With regard to *Alternaria* secondary metabolites, tenuazonic acid was detected in all investigated regions. In details, its concentration was significantly higher in samples collected across Umbria region compared to durum wheat harvested in Sicily, Campania,

Emilia Romagna, The Marche and Veneto regions ($p \leq 0.02$). Alternariol and alternariolmethylether were detected in low amount. Finally, infectopyron was detected in all analyzed sample.

Table 6: Trichothecenes detected by liquid chromatography tandem mass spectrometry on durum wheat grain samples harvested in North (Lombardy, Veneto, Emilia Romagna) and Central Italian regions (Abruzzo, The Marche, Umbria, Latium).

		Lombardy	Veneto	Emilia Romagna	Tuscany	Abruzzo	The Marche	Umbria	Latium
Deoxynivalenol	Mean (µg/ kg)	308.00* (±134.00)**	1190.00 (±564.00)	363.00 (±201.00)	1124.00 (±685.00)	1030.00 (±787.00)	140.00 (±73.80)	883.00 (±366.00)	469.00 (±199.00)
	Mean Positive Samples (µg/ kg)	308.00*** (±134.00)	1190.00 (±564.00)	363.00 (±201.00)	1123.00 (±685.00)	1030.00 (±787.00)	140.30 (±73.80)	883.00 (±366.00)	469.00 (±199.00)
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	668.00	2600.00	1720.00	3720.00	3370.00	422.00	3730.00	1440.00
DON-3-glucoside	Mean (µg/ kg)	11.60 (±8.09)	57.40 (±25.30)	10.20 (±2.37)	33.50 (±19.80)	43.00 (±36.50)	5.93 (±1.99)	30.50 (±6.27)	11.70 (±4.82)
	Mean Positive Samples (µg/ kg)	23.10 (±11.10)	57.40 (±25.30)	10.20 (±2.37)	41.80 (±23.20)	86.00 (±65.60)	7.41 (±1.71)	30.50 (±6.27)	16.30 (±5.46)
	Positive sample	2	4	8	4	2	4	9	5
	Incidence positive samples	50.00	100.00	100.00	80.00	50.00	80.00	100.00	71.40
	Max Value (µg/ kg)	34.30	103.00	23.60	105.00	152.00	11.90	65.20	31.40
Nivalenol	Mean (µg/ kg)	25.00 (±2.70)	31.60 (±9.75)	23.50 (±6.18)	164.00 (±73.00)	192.00 (±81.10)	32.90 (±17.30)	123.75 (±44.96)	283.00 (±117.00)
	Mean Positive Samples (µg/ kg)	25.00 (±2.70)	31.60 (±9.75)	26.80 (±5.99)	205.00 (±77.30)	192.00 (±81.10)	41.00 (±19.60)	139.00 (±48.00)	283.00 (±117.00)
	Positive sample	4	4	7	4	4	4	8	7
	Incidence positive samples	100.00	100.00	87.50	80.00	100.00	80.00	88.90	100.00
	Max Value (µg/ kg)	31.80	57.00	51.20	415.00	406.00	98.00	412.00	775.00
Nivalenol Glucoside	Mean (µg/ kg)	nd -	nd -	nd -	21.50 (±8.49)	28.70 (±13.40)	nd -	14.80 (±5.84)	33.80 (±16.50)
	Mean Positive Samples (µg/ kg)	nd -	nd -	nd -	26.90 (±8.47)	38.30 (±13.20)	nd -	26.60 (±6.64)	59.10 (±21.40)
	Positive sample	nd	nd	nd	4	3	nd	5	4
	Incidence positive samples	nd	nd	nd	80.00	75.00	nd	55.60	57.14
	Max Value (µg/ kg)	nd	nd	nd	50.20	58.90	nd	43.40	97.72
T-2 toxin	Mean (µg/ kg)	2.70 (±0.98)	nd -	nd -	2.05 (±1.26)	5.11 (±3.51)	3.60 (±1.96)	1.48 (±1.34)	0.34 (±0.22)
	Mean Positive Samples (µg/ kg)	3.60 0.54	nd -	nd -	5.11 0.35	6.81 4.34	4.51 2.25	6.66 5.46	1.20 0.00
	Positive sample	3	nd	nd	2	3	4	2	2
	Incidence positive samples	75.00	nd	nd	40.00	75.00	80.00	22.20	28.60
	Max Value (µg/ kg)	4.57	nd	nd	5.46	15.30	11.10	12.10	1.20
HT-2 toxin	Mean (µg/ kg)	29.00 (±12.70)	8.37 (±4.69)	4.53 (±2.26)	17.70 (±9.20)	43.90 (±24.80)	28.70 (±9.96)	16.90 (±12.90)	2.89 (±2.17)
	Mean Positive Samples (µg/ kg)	29.00 (±12.70)	11.20 (±5.34)	12.10 (±1.48)	17.70 (±9.20)	43.90 (±24.80)	28.70 (±9.96)	30.50 (±22.30)	10.10 (±5.10)
	Positive sample	4	3	3	5	4	5	5	2
	Incidence positive samples	100.00	75.00	37.50	100.00	100.00	100.00	55.60	28.60
	Max Value (µg/ kg)	66.10	21.00	13.70	51.00	116.00	60.80	119.00	15.20
Neosolaniol	Mean (µg/ kg)	nd -	nd -	nd -	nd -	nd -	nd -	0.16 (±1.45)	0.21 (±1.45)
	Mean Positive Samples (µg/ kg)	nd -	nd -	nd -	nd -	nd -	nd -	1.45 -	1.45 -
	Positive sample	nd	nd	nd	nd	nd	nd	1	1
	Incidence positive samples	nd	nd	nd	nd	nd	nd	11.10	14.30
	Max Value (µg/ kg)	nd	nd	nd	nd	nd	nd	1.45	1.45
T2-Tetraol	Mean (µg/ kg)	15.20 (±8.85)	nd -	nd -	nd -	25.60 25.60	nd -	4.89 (±4.89)	nd -
	Mean Positive Samples (µg/ kg)	30.39 (±2.83)	nd -	nd -	nd -	102.00 -	nd -	44.00 -	nd -
	Positive sample	2	nd	nd	nd	1	nd	1	nd
	Incidence positive samples	50.00	nd	nd	nd	25.00	nd	11.11	nd
	Max Value (µg/ kg)	33.20	nd	nd	nd	102.00	nd	44.00	nd
Monoacetoxyscirpenol	Mean (µg/ kg)	nd -	1.25 (±0.72)	nd -	0.50 (±0.50)	1.58 (±1.58)	nd -	2.00 (±1.14)	4.13 (±1.78)
	Mean Positive Samples (µg/ kg)	nd -	2.50 0.00	nd -	2.50 -	6.34 -	nd -	4.50 (±2.00)	7.22 (±1.90)
	Positive sample	nd	2	nd	1	1	nd	4	4
	Incidence positive samples	nd	50.00	nd	20.00	25.00	nd	44.40	57.10
	Max Value (µg/ kg)	nd	2.50	nd	2.50	6.34	nd	10.50	11.80
Diacetoxyscirpenol	Mean (µg/ kg)	nd -	nd -	nd -	nd -	0.40 (±0.40)	nd -	0.32 (±0.32)	0.63 (±0.30)
	Mean Positive Samples (µg/ kg)	nd -	nd -	nd -	nd -	1.59 -	nd -	2.91 -	1.46 (±0.13)
	Positive sample	nd	nd	nd	nd	1	nd	1	3
	Incidence positive samples	nd	nd	nd	nd	25.00	nd	11.10	42.90
	Max Value (µg/ kg)	nd	nd	nd	nd	1.59	nd	2.91	1.71
Deacetylneosolaniol	Mean (µg/ kg)	5.84 (±3.58)	0.54 (±0.54)	nd -	5.07 (±3.16)	6.46 (±4.19)	0.43 (±0.43)	3.96 (±3.45)	nd -
	Mean Positive Samples (µg/ kg)	7.79 (±4.25)	2.15 -	nd -	12.70 (±1.89)	12.90 (±4.68)	2.15 -	17.81 (±13.51)	nd -
	Positive sample	3	1	nd	2	2	1	2	nd
	Incidence positive samples	75.00	25.00	nd	40.00	50.00	20.00	22.20	nd
	Max Value (µg/ kg)	16.10	2.15	nd	14.50	17.60	2.15	31.30	nd

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 7: Trichothecenes detected by liquid chromatography on durum wheat samples from South Italy (Molise, Apulia, Campania) and Island (Sardinia, Sicily).

		Molise	Apulia	Campania	Sardinia	Sicily
Deoxynivalenol	Mean (µg/ kg)	64.50* -	88.50 (±25.70)**	55.50 (±55.50)	142.00 (±76.70)	23.90 (±21.10)
	Mean Positive Samples (µg/ kg)	64.50 -	88.50*** (±25.70)	111.00 -	170.00 (±87.20)	47.80 (±40.60)
	Positive sample	1	9	1	5	3
	Incidence positive samples	100.00	100.00	50.00	83.30	50.00
	Max Value (µg/ kg)	64.50	284.00	111.00	514.00	129.00
DON-3-glucoside	Mean (µg/ kg)	3.61 -	5.12 (±1.58)	4.79 (±4.79)	4.60 (±3.79)	5.10 (±3.93)
	Mean Positive Samples (µg/ kg)	3.61 -	7.68 (±1.44)	9.58 -	13.80 (±9.42)	15.30 (±8.68)
	Positive sample	1	6	1	2	2
	Incidence positive samples	100.00	66.67	50.00	33.30	33.30
	Max Value (µg/ kg)	3.61	13.90	9.58	23.20	24.00
Nivalenol	Mean (µg/ kg)	19.60 -	4.42 (±2.24)	47.80 (±30.90)	3.30 (±3.30)	28.60 (±28.60)
	Mean Positive Samples (µg/ kg)	19.60 -	13.30 (±1.28)	47.80 (±30.90)	19.80 -	171.00 -
	Positive sample	1	3	2	1	1
	Incidence positive samples	100.00	33.30	100.00	16.70	16.70
	Max Value (µg/ kg)	19.60	15.80	78.70	19.70	171.50
Nivalenol Glucoside	Mean (µg/ kg)	nd -	nd -	nd -	nd -	3.63 (±3.63)
	Mean Positive Samples (µg/ kg)	nd -	nd -	nd -	nd -	21.77 -
	Positive sample	nd	nd	nd	nd	1
	Incidence positive samples	nd	nd	nd	nd	16.70
	Max Value (µg/ kg)	nd	nd	nd	nd	21.80
T-2 toxin	Mean (µg/ kg)	nd -	7.72 (±3.36)	nd -	nd -	nd -
	Mean Positive Samples (µg/ kg)	nd -	9.93 3.96	nd -	nd -	nd -
	Positive sample	nd	7	nd	nd	nd
	Incidence positive samples	nd	77.80	nd	nd	nd
	Max Value (µg/ kg)	nd	32.40	nd	nd	nd
HT-2 toxin	Mean (µg/ kg)	41.80 -	32.00 (±6.76)	4.74 (±4.74)	0.96 (±0.96)	0.42 (±0.42)
	Mean Positive Samples (µg/ kg)	41.80 -	36.00 (±6.19)	9.49 -	5.74 -	2.50 -
	Positive sample	1	8	1	1	1
	Incidence positive samples	100.00	88.90	50.00	16.70	16.70
	Max Value (µg/ kg)	41.80	59.30	9.49	5.74	2.50
Neosolaniol	Mean (µg/ kg)	nd -	0.67 (±4.58)	nd -	nd -	nd -
	Mean Positive Samples (µg/ kg)	nd -	3.01 (±1.56)	nd -	nd -	nd -
	Positive sample	nd	2	nd	nd	nd
	Incidence positive samples	nd	22.20	nd	nd	nd
	Max Value (µg/ kg)	nd	4.58	nd	nd	nd
T2-Tetraol	Mean (µg/ kg)	21.90 -	23.00 (±7.82)	nd -	nd -	nd -
	Mean Positive Samples (µg/ kg)	21.90 -	41.40 (±5.46)	nd -	nd -	nd -
	Positive sample	1	5	nd	nd	nd
	Incidence positive samples	100.00	55.60	nd	nd	nd
	Max Value (µg/ kg)	21.90	52.80	nd	nd	nd
Monoacetoxyscirpenol	Mean (µg/ kg)	nd -	0.71 (±0.71)	nd -	nd -	0.83 (±0.53)
	Mean Positive Samples (µg/ kg)	nd -	6.41 -	nd -	nd -	2.50 0.00
	Positive sample	nd	1	nd	nd	2
	Incidence positive samples	nd	11.10	nd	nd	33.30
	Max Value (µg/ kg)	nd	6.41	nd	nd	2.50
Diacetoxyscirpenol	Mean (µg/ kg)	nd -	0.40 (±0.40)	nd -	nd -	0.15 (±0.15)
	Mean Positive Samples (µg/ kg)	nd -	3.58 -	nd -	nd -	0.89 -
	Positive sample	nd	1	nd	nd	1
	Incidence positive samples	nd	11.10	nd	nd	16.70
	Max Value (µg/ kg)	nd	3.58	nd	nd	0.89
Deacetylneosolaniol	Mean (µg/ kg)	14.80 -	9.58 (±2.50)	nd -	nd -	nd -
	Mean Positive Samples (µg/ kg)	14.80 -	12.30 (±2.25)	nd -	nd -	nd -
	Positive sample	1	7	nd	nd	nd
	Incidence positive samples	100.00	77.80	nd	nd	nd
	Max Value (µg/ kg)	14.80	20.00	nd	nd	nd

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 8: Depsipeptides detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in North (Lombardy, Veneto, Emilia Romagna) and Central Italian regions (Abruzzo, The Marche, Umbria, Latium).

		Lombardy	Veneto	Emilia Romagna	Tuscany	Abruzzo	The Marche	Umbria	Latium
Enniatin A	Mean (µg/ kg)	4.23* (±3.57)**	0.68 (±0.51)	0.83 (±0.29)	5.21 (±4.12)	8.66*** (±3.60)	0.37 (±0.23)	2.10 (±0.69)	1.08 (±0.49)
	Mean Positive Samples (µg/ kg)	4.23*** (±3.57)	0.90 (±0.65)	0.95 (±0.31)	5.21 (±4.12)	8.66 (±3.60)	0.37 (±0.23)	2.10 (±0.69)	1.51 (±0.59)
	Positive sample	4	3	7	5	4	5	9	5
	Incidence positive samples	100.00	75.00	87.50	100.00	100.00	100.00	100.00	71.43
	Max Value (µg/ kg)	14.91	2.19	2.35	21.6	18.6	1.24	6.78	3.19
Enniatin A1	Mean (µg/ kg)	105.00 (±81.80)	26.5 (±20.00)	27.2 (±7.18)	148.00 (±113.00)	230.5 (±111.00)	8.59 (±5.04)	54.3 (±12.10)	48.5 (±24.30)
	Mean Positive Samples (µg/ kg)	105.00 (±81.80)	26.5 (±20.00)	27.2 (±7.18)	148.00 (±113.00)	230.5 (±111.00)	8.59 (±5.04)	54.3 (±12.10)	48.5 (±24.30)
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	349.00	85.60	63.50	597.00	550.00	27.50	122.00	161.00
Enniatin B	Mean (µg/ kg)	242.00 (±101.00)	164.00 (±106.00)	207.00 (±68.8)	288.00 (±102.00)	471.00 (±138.00)	13.00 (±5.97)	291.00 (±63.80)	280.00 (±124.00)
	Mean Positive Samples (µg/ kg)	242.00 (±101.00)	164.00 (±106.00)	207.00 (±68.8)	288.00 (±102.00)	471.00 (±138.00)	13.00 (±5.97)	291.00 (±63.80)	280.00 (±124.00)
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	446.00	474.00	512.00	593.00	783.00	33.00	716.00	770.00
Enniatin B1	Mean (µg/ kg)	317.00 (±181.00)	149.00 (±109.0)	159.00 (±50.80)	413.00 (±242.00)	694.00 (±325.00)	20.10 (±9.89)	260.00 (±60.40)	291.00 (±149.00)
	Mean Positive Samples (µg/ kg)	317.00 (±181.00)	149.00 (±109.00)	159.00 (±50.80)	413.00 (±242.00)	694.00 (±325.00)	20.10 (±9.89)	260.00 (±60.40)	291.00 (±149.00)
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	822.00	472.00	372.00	1350.00	1620.00	51.90	666.00	982.00
Enniatin B2	Mean (µg/ kg)	15.80 (±8.17)	9.04 (±6.11)	12.70 (±4.45)	16.02 (±6.16)	39.80 (±20.40)	0.56 (±0.31)	20.20 (±7.01)	25.83 (±14.00)
	Mean Positive Samples (µg/ kg)	15.80 (±8.17)	9.04 (±6.11)	14.50 (±4.69)	16.00 (±6.16)	39.80 (±20.40)	0.93 (±0.39)	20.20 (±7.01)	25.80 (±14.00)
	Positive sample	4	4	7	5	4	3	9	7
	Incidence positive samples	100.00	100.00	87.50	100.00	100.00	60.00	100.00	100.00
	Max Value (µg/ kg)	37.30	27.03	33.90	33.60	98.70	1.62	73.50	93.70
Enniatin B3	Mean (µg/ kg)	0.11 (±0.05)	0.07 (±0.05)	0.11 (±0.04)	0.14 (±0.06)	0.38 (±0.20)	0.001 0.001	0.17 (±0.07)	0.23 (±0.13)
	Mean Positive Samples (µg/ kg)	0.11 (±0.05)	0.07 (±0.05)	0.12 (±0.04)	0.14 (±0.06)	0.38 (±0.20)	0.001 0.001	0.17 (±0.07)	0.26 (±0.15)
	Positive sample	4	4	7	5	4	2	9	6
	Incidence positive samples	100.00	100.00	87.50	100.00	100.00	40.00	100.00	85.70
	Max Value (µg/ kg)	0.23	0.23	0.28	0.28	0.94	0.01	0.72	0.88
Beauvericin	Mean (µg/ kg)	1.70 (±1.13)	1.00 (±0.69)	0.60 (±0.06)	1.37 (±0.39)	8.93 (±6.56)	1.83 (±0.86)	1.52 (±0.61)	1.20 (±0.68)
	Mean Positive Samples (µg/ kg)	1.70 (±1.13)	1.00 (±0.69)	0.60 (±0.06)	1.37 (±0.39)	8.93 (±6.56)	1.83 (±0.86)	1.52 (±0.61)	1.68 (±0.88)
	Positive sample	4	4	8	5	4	5	9	5
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	71.40
	Max Value (µg/ kg)	5.07	3.04	0.90	2.62	28.50	5.05	6.06	4.80

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 9: Depsipeptides detected by liquid chromatography on durum wheat samples from South Italy (Molise, Apulia, Campania) and Island (Sardinia, Sicily).

		Molise	Apulia	Campania	Sardinia	Sicily
Enniatin A	**Mean (µg/ kg)**	1.43* -	2.53 (±1.06)**	0.16 (±0.04)	4.28 (±4.03)	nd -
	Mean Positive Samples (µg/ kg)	1.43 -	2.53*** (±1.06)	0.16 (±0.04)	4.28 (±4.03)	nd -
	Positive sample	1	9	2	6	nd
	Incidence positive samples	100.00	100.00	100.00	100.00	nd
	Max Value (µg/ kg)	1.43	7.52	0.21	24.4	nd
Enniatin A1	**Mean (µg/ kg)**	38.00 -	63.2 (±25.30)	4.51 (±1.17)	130.00 (±118.00)	0.04 (±0.04)
	Mean Positive Samples (µg/ kg)	38.00 -	63.2 (±25.30)	4.51 (±1.17)	130.00 (±118.00)	0.26 -
	Positive sample	1	9	2	6	1
	Incidence positive samples	100.00	100.00	100.00	100.00	16.67
	Max Value (µg/ kg)	38.00	192.00	5.68	721.00	0.26
Enniatin B	**Mean (µg/ kg)**	67.4 -	92.00 (±30.80)	29.10 (±18.50)	240.00 (±115.00)	0.17 0.07
	Mean Positive Samples (µg/ kg)	67.4 -	92.00 (±30.80)	29.10 (±18.50)	240.00 (±115.00)	0.26 (±0.06)
	Positive sample	1	9	2	6	4
	Incidence positive samples	100.00	100.00	100.00	100.00	66.67
	Max Value (µg/ kg)	67.40	235.00	47.60	747.00	0.44
Enniatin B1	**Mean (µg/ kg)**	103.00 -	154.00 (±55.90)	24.00 (±13.60)	348.00 (±264.00)	0.16 (±0.09)
	Mean Positive Samples (µg/ kg)	103.00 -	154.00 (±55.90)	24.00 (±13.60)	348.00 (±264.00)	0.32 (±0.13)
	Positive sample	1	9	2	6	3
	Incidence positive samples	100.00	100.00	100.00	100.00	50.00
	Max Value (µg/ kg)	103.00	391.00	37.60	1660.00	0.58
Enniatin B2	**Mean (µg/ kg)**	3.17 -	3.86 (±1.33)	1.31 (±0.69)	15.80 (±9.65)	nd -
	Mean Positive Samples (µg/ kg)	3.17 -	4.34 (±1.40)	1.31 (±0.69)	19.00 (±11.20)	nd -
	Positive sample	1	8	2	5	nd
	Incidence positive samples	100.00	88.90	100.00	83.30	nd
	Max Value (µg/ kg)	3.17	10.70	2.00	62.10	nd
Enniatin B3	**Mean (µg/ kg)**	0.01 -	0.03 (±0.01)	0.00 (±0.00)	0.12 (±0.08)	nd -
	Mean Positive Samples (µg/ kg)	0.01 -	0.05 (±0.02)	0.01 -	0.15 (±0.09)	nd -
	Positive sample	1	6	1	5	nd
	Incidence positive samples	100.00	66.70	50.00	83.30	nd
	Max Value (µg/ kg)	0.01	0.11	0.01	0.51	nd
Beauvericin	**Mean (µg/ kg)**	0.10 -	1.26 (±1.23)	nd -	0.64 (±0.64)	2.89 (±2.89)
	Mean Positive Samples (µg/ kg)	0.10 -	5.67 (±5.43)	nd -	3.81 -	17.40 -
	Positive sample	1	2	nd	1	1
	Incidence positive samples	100.00	22.20	nd	16.70	16.70
	Max Value (µg/ kg)	0.10	11.10	nd	3.81	17.40

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 10: Zearalenone, fumonisin B1 and other *Fusarium* secondary metabolites detected by liquid chromatography tandem mass spectrometry on durum wheat grain samples harvested in North (Lombardy, Veneto, Emilia Romagna) and Central Italian regions (Abruzzo, The Marche, Umbria, Latium).

		Lombardy	Veneto	Emilia Romagna	Tuscany	Abruzzo	The Marche	Umbria	Latium
Zearalenone	Mean (µg/ kg)	0.76*	25.80	0.66	0.65	0.16	nd	3.25	0.57
		0.54**	19.50	0.62	0.57	0.16	-	2.27	0.33
	Mean Positive Samples (µg/ kg)	1.52***	25.80	2.64	1.63	0.63	nd	4.88	1.33
		0.77	19.50	2.33	1.31	-	-	3.28	0.52
	Positive sample	2	4	2	2	1	nd	6	3
	Incidence positive samples	50.00	100.00	25.00	40.00	25.00	nd	66.70	42.90
	Max Value (µg/ kg)	2.29	84.20	4.97	2.93	0.63	nd	21.02	2.00
Fumonisin B1	Mean (µg/ kg)	nd	nd	4.29	nd	27.70	14.30	12.90	nd
		-	-	4.29	-	11.60	14.30	7.19	-
	Mean Positive Samples (µg/ kg)	nd	nd	34.30	nd	36.90	71.70	38.60	nd
		-	-	-	-	9.87	-	11.10	-
	Positive sample	nd	nd	1	nd	3	1	3	nd
	Incidence positive samples	nd	nd	12.50	nd	75.00	20.00	33.30	nd
	Max Value (µg/ kg)	nd	nd	34.30	nd	55.70	71.70	59.30	nd
Culmurin	Mean (µg/ kg)	210.00	912.00	198.00	1190.00	1080.00	184.00	817.00	554.00
		95.70	518.00	89.10	848.00	773.00	115.00	294.00	218.00
	Mean Positive Samples (µg/ kg)	210.00	912.00	198.00	1190.00	1080.00	115.00	817.00	554.00
		95.70	518.00	89.10	848.00	773.00	115.00	294.00	218.00
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	474.00	2260.00	768.00	4530.00	3370.00	642.00	3040.00	1390.00
15-Hydroxyculmorin	Mean (µg/ kg)	195.00	571.00	204.00	638.00	815.00	40.60	492.00	447.00
		73.00	261.00	107.00	332.00	445.00	16.70	227.00	104.00
	Mean Positive Samples (µg/ kg)	195.00	571.00	234.00	638.00	815.00	40.60	492.00	447.00
		72.60	261.00	118.00	332.00	445.00	16.70	227.00	104.00
	Positive sample	4	4	7	5	4	5	9	7
	Incidence positive samples	100.00	100.00	87.50	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	374.00	1210.00	926.00	1940.00	2120.00	100.00	2240.00	917.00
Fungerin	Mean (µg/ kg)	0.60	0.33	nd	1.56	0.44	nd	nd	0.25
		0.60	0.33	-	1.56	0.25	-	-	0.16
	Mean Positive Samples (µg/ kg)	2.41	1.31	nd	7.80	0.87	nd	nd	0.89
		-	-	-	-	0.08	-	-	0.05
	Positive sample	1	1	nd	1	2	nd	nd	2
	Incidence positive samples	25.00	25.00	nd	20.00	50.00	nd	nd	28.60
	Max Value (µg/ kg)	2.41	1.31	nd	7.80	0.95	nd	nd	0.94
Butenolid	Mean (µg/ kg)	nd	nd	nd	19.40	19.00	nd	15.30	16.40
		-	-	-	19.40	19.00	-	7.90	10.70
	Mean Positive Samples (µg/ kg)	nd	nd	nd	97.20	76.10	nd	46.00	57.40
		-	-	-	-	-	-	6.64	6.84
	Positive sample	nd	nd	nd	1	1	nd	3	2
	Incidence positive samples	nd	nd	nd	20.00	25.00	nd	33.30	28.60
	Max Value (µg/ kg)	nd	nd	nd	97.20	76.10	nd	58.60	64.20
Apicidin	Mean (µg/ kg)	8.67	4.93	1.03	14.70	27.60	5.20	5.87	nd
		3.05	2.35	0.58	13.80	10.00	1.74	4.85	-
	Mean Positive Samples (µg/ kg)	8.67	6.57	2.75	36.90	27.60	6.50	17.60	nd
		3.05	2.37	0.88	33.00	10.00	1.49	13.40	-
	Positive sample	4	3	3	2	4	4	3	nd
	Incidence positive samples	100.00	75.00	37.50	40.00	100.00	80.00	33.30	nd
	Max Value (µg/ kg)	16.20	11.10	4.49	69.90	55.10	9.05	44.30	nd
Apicidin D2	Mean (µg/ kg)	1.35	0.52	nd	4.01	5.74	nd	0.90	nd
		0.81	0.31	-	4.01	2.44	-	0.90	-
	Mean Positive Samples (µg/ kg)	2.71	1.04	nd	20.10	7.65	nd	8.13	nd
		0.49	0.18	-	-	2.14	-	-	-
	Positive sample	2	2	nd	1	3	nd	1	nd
	Incidence positive samples	50.00	50.00	nd	20.00	75.00	nd	11.10	nd
	Max Value (µg/ kg)	3.20	1.22	nd	20.10	11.90	nd	8.13	nd
Equisetin	Mean (µg/ kg)	0.25	5.46	3.56	2.98	20.10	nd	0.11	5.58
		0.25	2.22	1.98	1.45	19.10	-	0.11	4.11
	Mean Positive Samples (µg/ kg)	1.00	7.28	7.13	4.96	20.10	nd	1.00	19.50
		-	1.79	3.14	1.46	19.10	-	-	9.03
	Positive sample	1	3	4	3	4	nd	1	2
	Incidence positive samples	25.00	75.00	50.00	60.00	100.00	nd	11.11	28.60
	Max Value (µg/ kg)	1.00	10.10	15.80	7.54	77.50	nd	1.00	28.60

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Zearalenone, Fumonisin B1 and other Fusarium secondary metabolites detected by liquid chromatography tandem mass spectrometry in durum wheat grain samples harvested in North (Lombardy, Veneto, Emilia Romagna) and Central Italian regions (Abruzzo, The Marche, Umbria, Latium) (CONTINUED).

		Lombardy	Veneto	Emilia Romagna	Tuscany	Abruzzo	The Marche	Umbria	Latium
Siccanol	Mean (µg/ kg)	nd	nd	65.90	nd	353.00*	40.30	76.90	128.00
		-	-	43.20	-	152.00**	27.50	41.30	51.40
	Mean Positive Samples (µg/ kg)	nd	nd	264.00	nd	353.00***	101.00	231.00	179.00
		-	-	13.00	-	152.00	38.50	51.90	57.10
	Positive sample	nd	nd	2	nd	4	2	3	5
	Incidence positive samples	nd	nd	25.00	nd	100.00	40.00	33.30	71.40
	Max Value (µg/ kg)	nd	nd	277.00	nd	803.00	139.00	290.00	393.00
Fusaric acid	Mean (µg/ kg)	nd	93.30	nd	6.56	118.00	11.70	78.70	14.60
		-	93.30	-	6.56	33.03	11.70	32.30	9.44
	Mean Positive Samples (µg/ kg)	nd	373.00	nd	32.80	118.00	58.60	177.00	51.10
		-	-	-	-	33.00	-	21.60	2.08
	Positive sample	nd	1	nd	1	4	1	4	2
	Incidence positive samples	nd	25.00	nd	20.00	100.00	20.00	44.40	28.60
	Max Value (µg/ kg)	nd	373.00	nd	32.80	166.00	58.60	232.00	53.20
Aurofusarin	Mean (µg/ kg)	174.00	439.00	148.00	224.00	1730.00	10.00	470.00	161.00
		75.20	123.00	63.60	88.10	821.00	6.12	176.00	91.60
	Mean Positive Samples (µg/ kg)	174.00	439.00	197.10	224.00	1730.00	25.00	470.00	282.30
		75.00	123.00	74.90	88.10	821.00	0.00	176.00	134.00
	Positive sample	4	4	6	5	4	2	9	4
	Incidence positive samples	100.00	100.00	75.00	100.00	100.00	40.00	100.00	57.10
	Max Value (µg/ kg)	352.00	649.00	407.00	451.00	3330.00	25.00	1670.00	606.00
W493	Mean (µg/ kg)	6.82	6.17	5.14	8.11	8.91	5.98	7.51	5.37
		2.55	4.69	1.61	1.76	1.75	1.26	2.00	1.81
	Mean Positive Samples (µg/ kg)	9.10	6.17	5.14	8.11	8.91	5.98	7.51	5.37
		1.63	4.69	1.61	1.76	1.75	1.26	2.00	1.81
	Positive sample	3	4	8	5	4	5	9	7
	Incidence positive samples	75.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	11.10	20.30	13.70	12.00	13.46	8.82	16.80	14.80
Moniliformin	Mean (µg/ kg)	159.00	152.00	257.00	268.00	827.00	19.50	349.00	473.00
		75.20	91.50	82.20	81.70	442.00	15.80	85.30	219.00
	Mean Positive Samples (µg/ kg)	159.00	152.00	257.00	268.00	827.00	24.40	349.00	473.00
		75.20	91.50	82.20	81.70	442.00	19.40	85.30	219.00
	Positive sample	4	4	8	5	4	4	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	80.00	100.00	100.00
	Max Value (µg/ kg)	370.00	418.00	620.00	477.00	2120.00	82.50	943.00	1400.00
Aminodimethyloctadecanol	Mean (µg/ kg)	14.90	52.80	81.00	157.00	1080.00	nd	266.00	238.00
		14.90	30.60	41.50	68.00	701.00	-	134.00	155.00
	Mean Positive Samples (µg/ kg)	59.70	106.00	217.00	196.00	1080.00	nd	399.00	554.00
		-	7.47	36.80	71.90	701.00	-	180.00	283.00
	Positive sample	1	2	3	4	4	nd	6	3
	Incidence positive samples	25.00	50.00	37.50	80.00	100.00	nd	66.70	42.90
	Max Value (µg/ kg)	59.70	113.00	287.00	406.00	3070.00	nd	1230.00	1120.00
Antibiotic Y	Mean (µg/ kg)	nd	nd	10.30	nd	79.30	nd	21.20	17.50
		-	-	8.46	-	30.90	-	15.20	9.73
	Mean Positive Samples (µg/ kg)	nd	nd	41.40	nd	106.00	nd	63.60	40.90
		-	-	26.90	-	22.80	-	37.80	13.60
	Positive sample	nd	nd	2	nd	3	nd	3	3
	Incidence positive samples	nd	nd	25.00	nd	75.00	nd	33.30	42.90
	Max Value (µg/ kg)	nd	nd	68.20	nd	131.00	nd	138.00	59.60
Chlamydospordiol	Mean (µg/ kg)	1.11	1.41	2.17	11.30	20.70	0.37	9.19	9.45
		0.68	1.41	0.85	5.18	9.58	0.37	2.82	4.39
	Mean Positive Samples (µg/ kg)	2.21	5.66	4.35	11.30	20.70	1.83	9.19	16.50
		0.59	-	0.49	5.18	9.58	-	2.82	5.34
	Positive sample	2	1	4	5	4	1	9	4
	Incidence positive samples	50.00	25.00	50.00	100.00	100.00	20.00	100.00	57.10
	Max Value (µg/ kg)	2.80	5.66	5.46	28.10	47.00	1.83	23.20	30.80
Chlamydosporol	Mean (µg/ kg)	2.78	7.73	11.80	47.00	80.10	nd	42.80	25.60
		2.78	7.73	4.93	20.40	37.80	-	12.10	15.30
	Mean Positive Samples (µg/ kg)	11.12	30.90	23.60	47.00	107.00	nd	55.00	59.70
		-	-	4.49	20.40	37.80	-	11.80	25.00
	Positive sample	1	1	4	5	3	nd	7	3
	Incidence positive samples	25.00	25.00	50.00	100.00	75.00	nd	77.80	42.90
	Max Value (µg/ kg)	11.10	30.90	32.50	105.00	182.00	nd	104.00	104.80
Chrisogin	Mean (µg/ kg)	43.60	36.10	34.40	36.40	86.60	5.79	39.30	67.20
		20.50	18.80	10.50	14.20	29.90	1.45	8.64	29.50
	Mean Positive Samples (µg/ kg)	43.56	36.10	34.40	36.40	86.60	5.79	39.30	67.20
		20.55	18.80	10.50	14.20	29.90	1.45	8.64	29.50
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	104.49	89.40	80.60	91.00	159.20	11.40	97.70	201.00

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 11: Zearalenone, fumonisin B1 and other *Fusarium* secondary metabolites detected by liquid chromatography tandem mass spectrometry on durum wheat samples from South Italy (Molise, Apulia, Campania) and Island (Sardinia, Sicily).

		Molise	Apulia	Campania	Sardinia	Sicily
Zearalenone	**Mean (µg/ kg)**	nd	nd	nd	nd	nd
		-	-	-	-	-
	Mean Positive Samples (µg/ kg)	nd	nd	nd	nd	nd
		-	-	-	-	-
	Positive sample	nd	nd	nd	nd	nd
	Incidence positive samples	nd	nd	nd	nd	nd
	Max Value (µg/ kg)	nd	nd	nd	nd	nd
Fumonisin B1	**Mean (µg/ kg)**	nd	4.47*	nd	nd	nd
		-	4.47**	-	-	-
	Mean Positive Samples (µg/ kg)	nd	40.20***	nd	nd	nd
		-	-	-	-	-
	Positive sample	nd	1	nd	nd	nd
	Incidence positive samples	nd	11.10	nd	nd	nd
	Max Value (µg/ kg)	nd	40.20	nd	nd	nd
Culmurin	**Mean (µg/ kg)**	70.80	133.00	176.00	148.00	18.20
		-	31.20	66.30	87.00	18.20
	Mean Positive Samples (µg/ kg)	70.80	133.00	176.00	178.00	109.00
		-	31.20	66.30	100.00	-
	Positive sample	1	9	2	5	1
	Incidence positive samples	100.00	100.00	100.00	83.30	16.70
	Max Value (µg/ kg)	70.80	373.00	242.00	577.00	109.00
15-Hydroxyculmorin	**Mean (µg/ kg)**	84.20	122.00	221.00	303.00	42.00
		-	62.90	150.00	163.00	42.00
	Mean Positive Samples (µg/ kg)	84.20	122.00	221.00	364.00	251.00
		-	62.90	150.00	186.00	-
	Positive sample	1	9	2	5	1
	Incidence positive samples	100.00	100.00	100.00	83.00	16.70
	Max Value (µg/ kg)	84.20	611.00	371.00	1080.00	251.00
Fungerin	**Mean (µg/ kg)**	nd	1.23	nd	1.44	nd
		-	1.01	-	1.16	-
	Mean Positive Samples (µg/ kg)	nd	5.54	nd	4.33	nd
		-	3.57	-	2.76	-
	Positive sample	nd	2	nd	2	nd
	Incidence positive samples	nd	22.20	nd	33.30	nd
	Max Value (µg/ kg)	nd	9.11	nd	7.09	nd
Butenolid	**Mean (µg/ kg)**	nd	nd	14.50	nd	nd
		-	-	14.50	-	-
	Mean Positive Samples (µg/ kg)	nd	nd	29.00	nd	nd
		-	-	-	-	-
	Positive sample	nd	nd	1	nd	nd
	Incidence positive samples	nd	nd	50.00	nd	nd
	Max Value (µg/ kg)	nd	nd	29.00	nd	nd
Apicidin	**Mean (µg/ kg)**	9.69	27.60	nd	nd	nd
		-	6.96	-	-	-
	Mean Positive Samples (µg/ kg)	9.69	31.10	nd	nd	nd
		-	6.85	-	-	-
	Positive sample	1	8	nd	nd	nd
	Incidence positive samples	100.00	88.90	nd	nd	nd
	Max Value (µg/ kg)	9.69	55.30	nd	nd	nd
Apicidin D2	**Mean (µg/ kg)**	1.50	5.24	nd	nd	nd
		-	1.91	-	-	-
	Mean Positive Samples (µg/ kg)	1.50	7.87	nd	nd	nd
		-	2.16	-	-	-
	Positive sample	1	6	nd	nd	nd
	Incidence positive samples	100.00	66.70	nd	nd	nd
	Max Value (µg/ kg)	1.50	17.70	nd	nd	nd
Equisetin	**Mean (µg/ kg)**	1.00	3.06	nd	1.52	0.17
		-	1.85	-	1.52	0.17
	Mean Positive Samples (µg/ kg)	1.00	9.19	nd	9.15	1.00
		-	3.60	-	-	-
	Positive sample	1	3	nd	1	1
	Incidence positive samples	100.00	33.30	nd	16.70	16.70
	Max Value (µg/ kg)	1.00	16.30	nd	9.15	1.00

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Zearalenone, fumonisin B1 and other *Fusarium* secondary metabolites detected by liquid chromatography tandem mass spectrometry on durum wheat samples from South Italy (Molise, Apulia, Campania) and Island (Sardinia, Sicily) (CONTINUED).

		Molise	Apulia	Campania	Sardinia	Sicily
Siccanol	**Mean (µg/ kg)**	nd	70.30	nd	18.80	nd
		-	46.60	-	18.80	-
	Mean Positive Samples (µg/ kg)	nd	316.00	nd	113.00	nd
		-	20.20	-	-	-
	Positive sample	nd	2	nd	1	nd
	Incidence positive samples	nd	22.20	nd	16.70	nd
	Max Value (µg/ kg)	nd	336.00	nd	113.00	nd
Fusaric acid	**Mean (µg/ kg)**	nd	21.50	nd	17.10	nd
		-	21.50	-	17.10	-
	Mean Positive Samples (µg/ kg)	nd	193.00	nd	103.00	nd
		-	-	-	-	-
	Positive sample	nd	1	nd	1	nd
	Incidence positive samples	nd	11.10	nd	16.70	nd
	Max Value (µg/ kg)	nd	193.00	nd	103.00	nd
Aurofusarin	**Mean (µg/ kg)**	25.00	37.00	nd	223.00	nd
		-	20.70	-	155.00	-
	Mean Positive Samples (µg/ kg)	25.00	83.20	nd	446.00	nd
		-	36.00	-	265.00	-
	Positive sample	1	4	nd	3	nd
	Incidence positive samples	100.00	44.40	nd	50.00	nd
	Max Value (µg/ kg)	25.00	173.00	nd	936.00	nd
W493	**Mean (µg/ kg)**	1.65	1.02	1.62	nd	1.88
		-	0.74	1.62	-	1.53
	Mean Positive Samples (µg/ kg)	1.65	3.07	3.24	nd	5.63
		-	1.87	-	-	3.74
	Positive sample	1	3	1	nd	2
	Incidence positive samples	100.00	33.30	50.00	nd	33.30
	Max Value (µg/ kg)	1.65	6.81	3.24	nd	9.37
Moniliformin	**Mean (µg/ kg)**	40.90	78.80	7.28	277.00	0.42
		-	42.20	4.78	119.00	0.42
	Mean Positive Samples (µg/ kg)	40.90	101.00	7.28	332.00	2.50
		-	51.70	4.78	130.00	-
	Positive sample	1	7	2	5	1
	Incidence positive samples	100.00	77.80	100.00	83.00	16.70
	Max Value (µg/ kg)	40.90	332.00	12.00	752.60	2.50
Aminodimethyloctadecanol	**Mean (µg/ kg)**	nd	20.30	nd	438.00	nd
		-	20.30	-	173.00	-
	Mean Positive Samples (µg/ kg)	nd	183.00	nd	657.00	nd
		-	-	-	164.00	-
	Positive sample	nd	1	nd	4	nd
	Incidence positive samples	nd	11.10	nd	66.70	nd
	Max Value (µg/ kg)	nd	183.00	nd	1070.00	nd
Antibiotic Y	**Mean (µg/ kg)**	nd	5.90	nd	46.61	nd
		-	4.39	-	30.76	-
	Mean Positive Samples (µg/ kg)	nd	26.50	nd	93.21	nd
		-	12.00	-	50.60	-
	Positive sample	nd	2	nd	3	nd
	Incidence positive samples	nd	22.20	nd	50.00	nd
	Max Value (µg/ kg)	nd	38.60	nd	194.24	nd
Chlamydospordiol	**Mean (µg/ kg)**	0.15	4.21	nd	12.60	nd
		-	2.57	-	9.35	-
	Mean Positive Samples (µg/ kg)	0.15	7.57	nd	18.90	nd
		-	4.18	-	13.40	-
	Positive sample	1	5	nd	4	nd
	Incidence positive samples	100.00	55.60	nd	66.70	nd
	Max Value (µg/ kg)	0.15	21.40	nd	58.30	nd
Chlamydosporol	**Mean (µg/ kg)**	nd	24.20	nd	50.70	nd
		-	18.10	-	42.00	-
	Mean Positive Samples (µg/ kg)	nd	109.00	nd	152.00	nd
		-	51.00	-	105.00	-
	Positive sample	nd	2	nd	2	nd
	Incidence positive samples	nd	22.20	nd	33.30	nd
	Max Value (µg/ kg)	nd	160.00	nd	257.00	nd
Chrisogin	**Mean (µg/ kg)**	18.70	20.90	6.51	33.80	1.15
		-	5.25	3.11	13.40	1.15
	Mean Positive Samples (µg/ kg)	18.70	20.90	6.51	33.80	6.87
		-	5.25	3.11	13.40	-
	Positive sample	1	9	2	6	1
	Incidence positive samples	100.00	100.00	100.00	100.00	16.67
	Max Value (µg/ kg)	18.70	57.20	9.62	80.10	6.87

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 12: *Alternaria* secondary metabolites detected by liquid chromatography tandem mass spectrometry on durum wheat grain samples harvested in North (Lombardy, Veneto, Emilia Romagna) and Central Italian regions (Abruzzo, The Marche, Umbria, Latium).

		Lombardy	Veneto	Emilia Romagna	Tuscany	Abruzzo	The Marche	Umbria	Latium
Tenuazonic acid	Mean (µg/ kg)	226.00* (±129.00)**	58.80 (±19.00)	na (±41.70)	243.00 (±78.30)	288.00 (±83.70)	76.60 (±56.90)	426.31 (±86.10)	658.00 (±363.00)
	Mean Positive Samples (µg/ kg)	301.00*** (±147.00)	58.80 (±19.00)	117.80 (±45.00)	243.00 (±78.30)	288.00 (±83.70)	128.00 (±86.70)	426.31 (±86.10)	658.00 (±363.00)
	Positive sample	3	4	7	5	4	3	9	7
	Incidence positive samples	75.00	100.00	87.50	100.00	100.00	60.00	100.00	100.00
	Max Value (µg/ kg)	589.00	99.90	321.00	475.00	438.00	301.00	913.00	2770.00
Alternariol	Mean (µg/ kg)	1.11 (±0.82)	2.80 (±2.18)	5.57 (±3.23)	4.32 (±1.30)	9.90 (±8.44)	0.94 (±0.72)	20.45 (±14.60)	6.38 (±3.65)
	Mean Positive Samples (µg/ kg)	2.22 (±1.26)	5.61 (±3.57)	8.91 (±4.66)	4.32 (±1.30)	9.90 (±8.44)	2.36 (±1.37)	20.45 (±14.60)	14.88 (±5.48)
	Positive sample	2	2	5	5	4	2	9	3
	Incidence positive samples	50.00	50.00	62.50	100.00	100.00	40.00	100.00	42.80
	Max Value (µg/ kg)	3.48	9.18	25.19	8.88	35.21	3.73	135.09	25.12
Alternariolmethylether	Mean (µg/ kg)	0.33 (±0.19)	0.38 (±0.23)	1.53 (±0.81)	2.35 (±0.83)	0.58 (±0.27)	0.16 (±0.11)	12.45 (±8.86)	3.02 (±1.85)
	Mean Positive Samples (µg/ kg)	0.66 (±0.02)	0.51 (±0.26)	2.04 (±1.00)	2.35 (±0.83)	0.77 (±0.26)	0.39 (±0.14)	14.01 (±9.89)	7.04 (±3.15)
	Positive sample	2	3	6	5	3	2	8	3
	Incidence positive samples	50.00	75.00	75.00	100.00	75.00	40.00	88.90	42.90
	Max Value (µg/ kg)	0.68	1.04	6.64	5.23	1.07	0.53	81.80	13.20
Macrosporin	Mean (µg/ kg)	1.30 (±0.49)	1.13 (±0.49)	8.88 (±4.44)	38.40 (±20.40)	3.47 (±2.05)	10.10 (±4.81)	13.30 (±2.97)	16.10 (±7.32)
	Mean Positive Samples (µg/ kg)	1.30 (±0.49)	1.50 (±0.45)	8.88 (±4.44)	38.40 (±20.40)	3.47 (±2.05)	10.10 (±4.81)	13.30 (±2.97)	16.10 (±7.32)
	Positive sample	4	3	8	5	4	5	9	7
	Incidence positive samples	100.00	75.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	2.76	2.31	37.30	115.00	9.51	27.50	28.60	56.50
Infectopyron	Mean (µg/ kg)	679.00 (±181.00)	926.00 (±174.00)	958.00 (±291.00)	1074.00 (±230.00)	693.00 (±101.00)	529.00 (±84.40)	641.00 (±68.90)	815.00 (±93.10)
	Mean Positive Samples (µg/ kg)	679.00 (±181.00)	926.00 (±174.00)	958.00 (±291.00)	1074.00 (±230.00)	693.29 (±100.53)	529.00 (±84.40)	641.00 (±68.90)	815.00 (±93.10)
	Positive sample	4	4	8	5	4	5	9	7
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	1120.00	1270.00	2730.00	1850.00	961.00	692.00	906.00	1120.00
Altersolanol	Mean (µg/ kg)	nd -	nd -	283.00 (±175.00)	1400.00 (±1030.00)	299.00 (±251.00)	446.00 (±286.00)	763.00 (±241.00)	277.00 (±89.50)
	Mean Positive Samples (µg/ kg)	nd -	nd -	567.00 (±298.00)	1740.00 (±1250.00)	399.14 (±325.55)	743.00 (±402.00)	858.00 (±251.00)	323.00 (±90.70)
	Positive sample	nd	nd	4	4	3	3	8	6
	Incidence positive samples	nd	nd	50.00	80.00	75.00	60.00	88.90	85.70
	Max Value (µg/ kg)	nd	nd	1420.00	5480.00	1050.00	1540.00	2200.00	748.00
Altertoxin-I	Mean (µg/ kg)	nd -	0.60 (±0.60)	2.06 (±1.18)	5.21 (±0.95)	3.80 (±2.28)	nd -	6.91 (±1.41)	0.92 (±0.92)
	Mean Positive Samples (µg/ kg)	nd -	2.40 -	5.49 (±1.90)	5.21 (±0.95)	5.07 (±2.67)	nd -	6.91 (±1.41)	6.42 -
	Positive sample	nd	1	3	5	3	nd	9	1
	Incidence positive samples	nd	25.00	37.50	100.00	75.00	nd	100.00	14.30
	Max Value (µg/ kg)	nd	2.40	8.94	8.36	10.40	nd	15.60	6.42
Tentoxin	Mean (µg/ kg)	1.72 (±0.81)	2.15 (±0.63)	3.14 (±0.79)	4.07 (±0.64)	6.70 (±2.47)	1.43 (±0.93)	6.32 (±2.15)	5.47 (±1.57)
	Mean Positive Samples (µg/ kg)	2.29 (±0.80)	2.15 (±0.63)	3.14 (±0.79)	4.07 (±0.64)	6.70 (±2.47)	1.79 (±1.11)	6.32 (±2.15)	5.47 (±1.57)
	Positive sample	3	4	8	5	4	4	9	7
	Incidence positive samples	75.00	100.00	100.00	100.00	100.00	80.00	100.00	100.00
	Max Value (µg/ kg)	3.88	4.02	7.83	5.52	13.80	5.11	21.30	14.20

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

Table 13: *Alternaria* secondary metabolites detected by liquid chromatography tandem mass spectrometry on durum wheat samples from South Italy (Molise, Apulia, Campania) and Island (Sardinia, Sicily).

		Molise	Apulia	Campania	Sardinia	Sicily
Tenuazonic acid	**Mean (µg/ kg)**	235.00* -	285.00 (±156.00)**	70.20 (±20.70)	371.00 (±229.00)	85.50 (±20.80)
	Mean Positive Samples (µg/ kg)	235.00*** -	367.00 (±191.00)	70.20 (±20.70)	446.00 (±265.00)	85.50 (±20.80)
	Positive sample	1	7	2	5	6
	Incidence positive samples	100.00	77.78	100.00	83.00	100.00
	Max Value (µg/ kg)	235.00	1410.00	90.93	1460.00	154.00
Alternariol	**Mean (µg/ kg)**	0.84 -	2.14 (±1.88)	0.43 (±0.43)	0.66 (±0.50)	0.11 (±0.11)
	Mean Positive Samples (µg/ kg)	0.84 -	9.61 (±7.42)	0.86 -	1.98 (±1.10)	0.68 -
	Positive sample	1	2	1	2	1
	Incidence positive samples	100.00	22.20	50.00	33.30	16.70
	Max Value (µg/ kg)	0.84	17.04	0.86	3.08	0.68
Alternariolmethylether	**Mean (µg/ kg)**	nd -	0.35 (±0.17)	0.46 (±0.46)	0.31 (±0.31)	0.08 (±0.05)
	Mean Positive Samples (µg/ kg)	nd -	0.78 (±0.26)	0.92 -	1.84 -	0.25 0.00
	Positive sample	nd	4	1	1	2
	Incidence positive samples	nd	44.40	50.00	16.70	33.30
	Max Value (µg/ kg)	nd	1.48	0.92	1.80	0.25
Macrosporin	**Mean (µg/ kg)**	2.03 -	2.02 (±0.62)	1.29 (±0.62)	10.12 (±5.17)	1.23 (±0.62)
	Mean Positive Samples (µg/ kg)	2.03 -	2.02 (±0.62)	1.29 (±0.62)	10.10 (±5.17)	1.84 (±0.76)
	Positive sample	1	9	2	6	4
	Incidence positive samples	100.00	100.00	100.00	100.00	66.70
	Max Value (µg/ kg)	2.03	4.94	1.91	35.50	3.88
Infectopyron	**Mean (µg/ kg)**	1510.00 -	1070.00 (±216.00)	1200.00 (±70.00)	1250.00 (±211.00)	320.00 (±115.00)
	Mean Positive Samples (µg/ kg)	1510.00 -	1070.00 (±216.00)	1200.00 (±70.00)	1250.00 (±211.00)	320.00 (±115.00)
	Positive sample	1	9	2	6	6
	Incidence positive samples	100.00	100.00	100.00	100.00	100.00
	Max Value (µg/ kg)	1510.00	2300.00	1270.00	1930.00	731.00
Altersolanol	**Mean (µg/ kg)**	68.90 -	12.00 (±12.00)	nd -	468.00 (±362.00)	44.70 (±44.70)
	Mean Positive Samples (µg/ kg)	68.90 -	108.29 -	nd -	937.00 (±660.00)	268.00 -
	Positive sample	1	1	nd	3	1
	Incidence positive samples	100.00	11.10	nd	50.00	16.70
	Max Value (µg/ kg)	68.80	108.00	nd	2250.00	268.00
Altertoxin-I	**Mean (µg/ kg)**	nd -	0.53 (±0.35)	nd -	0.80 (±0.51)	nd -
	Mean Positive Samples (µg/ kg)	nd -	2.40 0.00	nd -	2.40 0.00	nd -
	Positive sample	nd	2	nd	2	nd
	Incidence positive samples	nd	22.20	nd	33.30	nd
	Max Value (µg/ kg)	nd	2.40	nd	2.40	nd
Tentoxin	**Mean (µg/ kg)**	3.32 -	1.09 (±0.25)	3.06 (±0.17)	3.64 (±1.54)	1.13 (±0.80)
	Mean Positive Samples (µg/ kg)	3.32 -	1.22 (±0.23)	3.06 (±0.17)	3.64 (±1.54)	2.27 (±1.38)
	Positive sample	1	8	2	6	3
	Incidence positive samples	100.00	88.90	100.00	100.00	50.00
	Max Value (µg/ kg)	3.32	2.71	3.23	10.20	5.02

*The value represents the total average for each region; **the value between brackets represents the standard error; ***the value represents the average (± standard error) of positive samples of each region the value represents the average of the replicates for each region; - standard error to calculated nd: not detected.

1.4 Discussion and conclusions

Durum wheat is one of the most important cereals worldwide, in particular, it is one of the main crops in the Mediterranean area (Fagnano et al., 2012). However, the long biological cycle and the adaptability to a large range of different climatic conditions made this crop particularly susceptible to biotic and abiotic stress that can compromise yield and quality (Xu, 2003). Considering the biotic stress, fungal diseases are very common, and their complexity is increased by the co-presence of more than one fungal genus. In addition, several species belonging to the same genera and with a different metabolic profile, can co-exist. Among the fungal diseases, FHB caused by species belonging to *Fusarium* genus is one of the most dangerous for quantity and qualities losses (Ferrigo et al., 2016; Osborne and Stein, 2007; Parry et al., 1995). The composition of FHB complex is influenced by many factors including climatic conditions, agronomic practices, cultivar resistance, timing of infection, use of fungicides (Beccari et al., 2020; Decleer et al., 2018; Ferrigo et al., 2016; Giraud et al., 2011; Scala et al., 2016; Tini et al., 2020). However, among them, the climatic conditions, at both macro and micro-scale level, play a key role in the dynamic change of fungal complex associated to durum wheat grains (Scala et al., 2016). The present survey analyzed the Italian fungal community on durum wheat and the related mycotoxins content in two different cultivation years: 2014/2015 and 2017/2018. The first-year survey was conducted on 30 samples of durum wheat grains in 3 different climatic Italian areas (corresponding to Emilia Romagna, Umbria and Sardinia regions); the survey carried out in the second year aimed to analyze a higher number of samples (n=70) collected across all the Italian peninsulas. In addition, the investigation was conducted following two different isolation method (MPDA and DFB) in order to have an image as realistic as possible of the mycoflora diversity associated to Italian durum wheat kernels.

In both years, two mycotoxigenic genera, *Alternaria* and *Fusarium*, were the principal ones associated to Italian durum wheat kernels.

The ubiquitarian genus *Alternaria* has been associated to cereal kernels not only as saprotrophic fungus but also as pathogen able to cause “black point disease” resulting in a reduction of technological and sanitary grain quality (Masiello et al., 2020; Patriarca et al., 2007). The symptom consisted in a germ discoloration that limits the use of this raw material for the production of pasta and other cereal derivates. In both investigated years and with both methods adopted, *Alternaria* spp. was present particularly in Southern regions of the country compared with Northern and Central ones. This trend could be due to the climatic

conditions and to the cosmopolitan characters of this fungal genus; in fact, even if *Alternaria* is frequently associated to high moisture and warm temperatures, its incidence can be high also in a dry environmental (Patriarca et al., 2007; Tralamazza et al., 2018). These data are in accord with several other studies conducted in different countries, including Italy, that recorded a high frequency of *Alternaria* genus on cereal kernels (Beccari et al., 2018b, 2018a, 2017, 2016; Masiello et al., 2020; Patriarca et al., 2007; Pereira et al., 2020; Pinto and Patriarca, 2017; Ramires et al., 2018; Somma et al., 2019; Tralamazza et al., 2018).

Fusarium spp. was the second fungal genus associated to durum wheat kernels cultivated in Italy in both surveyed years and with both methods. The contamination of durum wheat by *Fusarium* species has been reported worldwide as well as in several Italian regions (Alkadri et al., 2013; Beccari et al., 2018a; Cerón-Bustamante et al., 2018; Covarelli et al., 2015; Cowger et al., 2020; Ferrigo et al., 2016; Garmendia et al., 2018; Gräfenhan et al., 2013; Infantino et al., 2012; Ioos et al., 2004; Nazari et al., 2019; Nielsen et al., 2011; Pancaldi et al., 2010; Pereira et al., 2020; Shah et al., 2005; Tittlemier et al., 2013; Vanheule et al., 2014). As already mentioned, the incidence of *Fusarium* genus is depending on many factors first of all climatic conditions at regional level (macroscale) especially during the anthesis of wheat that it is considered the stage of maximum susceptibility of wheat *Fusarium* infection (Beccari et al., 2019; Parry et al., 1995). However, also other factors such as crop rotation, fungicide application and cultivated variates can play a key role in the disease evolution (Ferrigo et al., 2016; Scala et al., 2016). During both years of investigation, a higher *Fusarium* infection in North and Central Italy compared with South Italy area was highlighted. This trend is in agreement to that observed in previous studies that showed an increased incidence of *Fusarium* species moving from Southern to Northern Italy as well as the high contribution in FHB development of climatic condition at microscale level (Infantino et al., 2012; Pancaldi et al., 2010; Scala et al., 2016; Shah et al., 2005).

During the second year of investigation and using MPDA method, the presence of nontoxigenic genus *Microdochium* among fungal community was highlighted. In details, *M. nivale* and *M. majus* are common in cooler area even if, their incidence can differ during years as well as within season (Xu et al., 2008). However, considering the slower growth rate compared to FHB agents, these species can be underestimated in case of mixed infection (Nicholson et al., 1996). Even if not able biosynthesize mycotoxins, the presence of *Microdochium* spp. on durum wheat should be not underestimate considering its ability to affect the grain gluten quality producing gluten-degrading proteases and its association to black point symptoms (Aamot et al., 2020).

Climatic and agronomic factors can affect not only the balance of *Fusarium* species with the fungal species belonged to the other fungal genera, but they can influence also the dynamics of the single species within the *Fusarium* community. During 2015, in the three investigated areas (Emilia Romagna, Umbria and Sardinia), *F. poae* and *F. proliferatum* were the main species associated to durum wheat grains. The prevalence of *F. poae* in FHB complex was highlighted also in previous studies that demonstrated how this species can assume a predominant role in the *Fusarium* community when climatic conditions during anthesis are not particularly favorable to the main FHB causal agents such as *F. graminearum* (Beccari et al., 2018b, 2017; Covarelli et al., 2015; Infantino et al., 2012; Valverde-Bogantes et al., 2019; Vogelgsang et al., 2019; Xu and Nicholson, 2009). In details, this species seems to be favor by high temperature during anthesis and relatively drier conditions (Beccari et al., 2018a; Xu, 2003).

In both surveyed years, *F. proliferatum* was the principal species detected with DFB method. This species has been associated to cereals as both saprotroph and pathogen and it is able to cause kernel black point symptoms (Busman et al., 2012). *F. proliferatum* is traditionally considered one of the most important species associated to maize grains but, at the same time, it has been detected in a wide range of other hosts including durum wheat (Amato et al., 2015; Palacios et al., 2015; Proctor et al., 2010). During the second year of investigation, through the "DFB method-morphological identification" approach, it was possible detected also *F. globosum*, a fumonisins producer species, member of the GFSC as well as *F. proliferatum*. This species, first reported in Europe (Sardinia-Italy) as causal agents of dark necroses on tomato plants, has been indicated as wheat and maize pathogen in Japan and in South Africa respectively (Aoki and Nirenberg, 1999; Sydenham et al., 1997). Phylogenetically closely related to *F. proliferatum*, *F. globosum* can be morphologically distinguished by the production of globose microconidia (Aoki and Nirenberg, 1999; Kvas et al., 2009). However, considering the genetic similarity of the species belonging to GFSC, further investigation and detailed molecular analysis will be needed in order to confirm its correct taxonomic collocation.

In the second year of investigation, in addition to *F. proliferatum*, *F. avenaceum* was one of the principal species associated with FHB community in Italy even if some regional differences were observed. This cosmopolitan species, member of FTSC (*Fusarium tricinctum* species complex) with *F. tricinctum* and *F. acuminatum*, has been associated especially to low temperature and high humidity levels during anthesis (Beccari et al., 2017; Brennan et al., 2003). For this reason, in the past, *F. avenaceum* has been reported as main

causal agents of FHB in North America and North Europe (Gräfenhan et al., 2013; Ioos et al., 2004; Kosiak et al., 2003; Nielsen et al., 2011; Niessen et al., 2012; Tittlemier et al., 2013; Uhlig et al., 2007; Yli-Mattila et al., 2018, 2006, 2004). However, in the last years, the members of FTSC increased their presence also in temperate area suggesting high adaptability to a wide range of host plant species and climatic conditions (Alkadri et al., 2013; Beccari et al., 2017, 2016; Cerón-Bustamante et al., 2018; Cowger et al., 2020; Pereira et al., 2020). This hypothesis is supported by studies that proved its high genetic variability (Kulik et al., 2011). In details, the genome sequencing highlights the presence of a wide range of protein families involved in transcription factors, redox reactions and signal transduction how could favor its response to environmental signals and, consequently, its adaptability to several ecological niches (Lysøe et al., 2014). Regarding Italian cereal crops, *F. avenaceum* was the main species associated to malting barley harvested in Umbria region in 2012 and 2013 while, in 2014, the 32.5% of FHB community associated to the same cereal species was composed by FTSC members (Beccari et al., 2018b, 2017, 2016). Finally, this species was also the most frequent associated to durum wheat harvested in 2009 and 2013 in Umbria region (Beccari et al., 2018a; Covarelli et al., 2015). In both investigated year *F. proliferatum* growth was favor by DFB method with respect to PDA medium. This trend could be explained by the absence of surface disinfection in DFB method that could promote *F. proliferatum* development. On the contrary, the higher detection *F. tricinctum* on PDA could be due to the slow growth rate of this species that could be disadvantaged by the absence of disinfection with DFB. These data showed the importance to apply both methods in order to do not underestimate or overestimate the fungal biodiversity associated with the kernels.

The multi-mycotoxins analysis allowed to detect, in both years, a high number of secondary metabolites contaminated Italian durum wheat kernels. Some of them have been studied in depth, others are still under investigation for their toxicity for human and animal health. Globally, durum wheat samples collected across South Italy showed lower mycotoxins content. Even if *F. graminearum* was not the principal species in both investigated years, DON contamination was observed in particular on durum wheat harvested across Italy during 2018. If in 2015 durum wheat harvested in Emilia Romagna was the most contaminated by this mycotoxin, in 2018, DON was detected in high amount also in samples collected in Central Italy. Generally, type A trichothecenes such as T-2 and HT-2 toxins were detected in low amount in both years with the exception of two samples, in 2018, exceeding the limit of 100 µg/kg established by the EU recommendation (EFSA, 2017).

These data are in according to the mycological analysis that showed a low incidence of type A trichothecenes producers such as *F. sporotrichioides* and *F. langsethiae*. The latter species, usually associated to cold climate of North and Central Europe, has been reported in Italy in 2007 and it is constantly under monitoring considering the high toxicity of aforementioned compounds (Imathiu et al., 2013; Infantino et al., 2007; Lattanzio et al., 2013). Surprisingly, despite the significant *F. proliferatum* presence in both years of investigation, low fumonisin levels were detected, confirming what previously observed in Italy and other wheat-cultivation areas such as Syria, United States, Spain, Canada, Tunisia and Argentina (Alkadri et al., 2013; Amato et al., 2015; Beccari et al., 2017; Busman et al., 2012; Castellá et al., 1999; Palacios et al., 2011; Roscoe et al., 2008; Serrano et al., 2012). The low level of these compounds could be explained hypothesizing that wheat matrix is not particular suitable as maize for fumonisin biosynthesis (Busman et al., 2012; Shephard et al., 2005). However, in some seasons and in presence of particular climatic conditions, also wheat could be a favorable substrate for fumonisins production as observed in Brazil and Argentina (Cendoya et al., 2014; Mendes et al., 2015). This evidence highlights the possible human exposure through wheat products to the risk connected with these compounds. For this reason, more detailed studies could be interesting to better understand the interaction between *F. proliferatum* and wheat with particular emphasis on fumonisins production on this substrate (See Chapter 3).

Additionally, in both investigated years, a higher contamination of depsipeptides and moniliformin was observed across Italian durum wheat cultivation areas. In the last years, these secondary metabolites produced by several *Fusarium* species (e.g.: members of FTSC and *F. poae*), draw the attention of scientific community resulting in two scientific opinions on the risks to human and animal health related to their presence in feed and food (Liuzzi et al., 2017; Orlando et al., 2019). In both cases, given the overall lack of toxicity data, no conclusions have been drawn for what concerns chronic exposure and for this reason, no legislative maximum levels have been established (EFSA, 2018, 2014; Fraeyman et al., 2017). However, considering the increasing of their detection and the possible chronic toxic effect as well as the co-occurrence and possible synergisms with other secondary metabolites, the risk connected with ENNs and MON should not be underestimated.

In addition to *Fusarium* mycotoxins, species belonging to *Alternaria* genus are able to accumulate in the kernels genotoxic and mutagenic secondary metabolites such as AOH and AME (Ostry, 2008; Ramires et al., 2018; Somma et al., 2019). Despite the proved toxic effect of these secondary metabolites and the increased attention of scientific community,

no limits in food and feed matrices have been established worldwide. In 2011, the European Union published an opinion regarding the evaluation of risks for animal and human health related to *Alternaria* mycotoxins, concluding that available data about toxicity and occurrence were not enough to set limits for *Alternaria* toxins in feed and food (EFSA, 2011). However, in 2018, the Bavarian Health and Food Safety authority limited the content of TA in infant food at 500 ppb (Tralamazza et al., 2018).

Alternaria metabolites were also detected in Italian durum wheat samples in particular on kernels harvested in Central Italy. This evidence highlights the risk connected with the simultaneous presence of several mycotoxigenic fungi and, consequently, a wide range of secondary metabolites. Few studies focused the attention on the competition among toxigenic fungi. In details, they hypothesized that, when climatic conditions are favorable for fungal development, both genera can compete for the nutrient and the infection site, co-habits the substrate and produce mycotoxins causing a combinate effect still poor investigated (Müller and Korn, 2013; Streit et al., 2013; Vanheule et al., 2014).

The present study confirms a "dynamism" within the FHB community already observed in Italy and consequently, a "shift" also in mycotoxins associated to wheat grains. As already mentioned, the change in *Fusarium* complex could be due to different causes: first of all, the climatic conditions that can influence the total and the relative abundance of a single *Fusarium* species involved in FHB (De Wolf et al., 2003). In fact, as well as for *F. poae*, also FTSC members could be advantage by temperature and moisture conditions during anthesis that are not particularly favorable to "main" FHB species such as *F. graminearum*. Agronomic factors cultivar varieties can play an important role in FHB incidence and FHB complex composition as well as mycotoxins contamination (Ferrigo et al., 2016). Several studies observed how crop rotations with no-cereals cultures resulted in a reduction of FHB incidence and relative mycotoxins contamination (Schaafsma et al., 2005). Other studies observed the effect of farming system on the mycotoxins level in cereals. The majority of these studies focused on DON, T-2 and HT-2 concentration in the organic and conventional farming systems. In details many of them reported no differences between farming systems or a lower DON or HT-2 and T-2 contamination in wheat cultivated in the organic one (Bernhoft et al., 2012, 2010; Edwards, 2009; Góral et al., 2019; Karlsson et al., 2017; Váňová et al., 2008). This trend could be explained considering that organic farming system often practice rotations with non-cereals crop or that other factors such as weather condition, temperatures, year, location may be more important than farming system in fungal development and mycotoxins production (Brodal et al., 2016; Champeil et al., 2004).

Contrasting results are reported for what concerns agronomic practices such as tillage system (Champeil et al., 2004). Sipiläand co-workers (2012) observed how increasing the decomposers fungi soil component, no-till practices management could favor the competition and the antagonism among soilborne community and, consequently, reduce the impact of pathogen such as *Fusarium* spp. (Sipilä et al., 2012). On the contrary, other studies suggested an increase of *Fusarium* incidence in the case of minimally prepared soil (Blandino et al., 2012; Dill-Macky and Jones, 2000; Steinkellner and Langer, 2004; Vogelgsang et al., 2019). Among cultural practices also the high agricultural intensity and in particular the number of pesticide applications and amount of nitrogen fertilizer applied can influence the FHB community promoting secondary species development such as *F. tricinctum* (Karlsson et al., 2017). In addition, fungicide treatments and the time of application can influence the fungal community as well as the mycotoxins content (Decleer et al., 2018; Giraud et al., 2011; Tini et al., 2020). In details, Decleer and co-workers (2018) studied the impact of fungicide application on depsipeptides production by *Fusarium* sp. and concluded, that fungicide can apply a selective pressure on *Fusarium* community promoting *F. poae* and *F. avenaceum* development and consequently have no impact on ENs and BEA contamination.

In conclusion, the present work, according to other previous studies, confirms the risk connected with the gradual expansion of durum wheat cultivation along the peninsula. In addition, this survey highlights as the climatic conditions of South Italy are suitable to obtain a durum wheat raw material of high quality for the food industries and the final consumers.

Collaborations

This work was carried out with the scientific support of G. Beccari (University of Perugia), V. Balmas (University of Sassari), A. Infantino (Council for Agricultural Research and Agricultural Economy Analysis-CREA, Rome) (data collection), A. Onofri (University of Perugia) (statistical analysis). Mycotoxins detection was carried out by M. Sulyok at Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Konrad Lorenz Strasse, 20, A-3430 Tulln.

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Supplementary Material

Table S1:

Durum wheat samples analyzed in the present study and collected in three different regions (Emilia Romagna, Umbra, Sardinia). Sample ID, province and location of sampling, variety are reported.

Sample ID	Region	Province	Location of Sampling	Variety
1	Emilia-Romagna	Parma	Noceto	PR22D78
2	Emilia-Romagna	Bologna	Molinella	Achille
3	Emilia-Romagna	Ravenna	Cervia	Tyrex
4	Emilia-Romagna	Ferrara	Jolanda di Savoia	Obelix
5	Emilia-Romagna	Bologna	Argelato	Monastir
6	Emilia-Romagna	Ravenna	Mezzano	Marakas
7	Emilia-Romagna	Modena	Sassuolo	Odisseo
8	Emilia-Romagna	Reggio Emilia	Novellara	Orobel
9	Emilia-Romagna	Reggio Emilia	Reggiolo	Odisseo
10	Emilia-Romagna	Piacenza	Sarmato	Orobel
1	Umbria	Perugia	Casalina	Odisseo
2	Umbria	Perugia	Ramazzano	Iride
3	Umbria	Perugia	Ramazzano	Prospero
4	Umbria	Perugia	Castiglione del Lago	Colorado
5	Umbria	Perugia	Foligno	Avispa
6	Umbria	Perugia	Spello	PR22D40
7	Umbria	Perugia	Colle Umberto	Dylan
8	Umbria	Perugia	Bagnaia	Dylan
9	Umbria	Perugia	Panicale	Claudio
10	Umbria	Perugia	Panicarola	Dylan
1	Sardinia	Sassari	Valledoria	Karalis
2	Sardinia	Sassari	Valledoria	Iride
3	Sardinia	Cagliari	Sestu	Karalis
4	Sardinia	Cagliari	San Gavino	Karalis
5	Sardinia	Sassari	Santa Maria Coghinas	Karalis
6	Sardinia	Cagliari	Ussana	Saragolla
7	Sardinia	Sassari	Bachileddu	Saragolla
8	Sardinia	Cagliari	Samassi	Rusticano
9	Sardinia	Sassari	Laerru	Karalis
10	Sardinia	Cagliari	Ussana	Karalis

Table S2:

Durum wheat samples analyzed in the present study and collected in 2018 across thirteen different Italian regions (Lombardy, Veneto, Emilia Romagna, The Marche, Tuscany, Umbria, Latium, Abruzzo, Molise, Campania, Apulia, Sicily, Sardinia). Province and location of sampling, variety and sample ID are reported.

Sample ID	Region	Province	Location of Sampling	Variety
1	Lombardy	Mantua	Mantova	PR22D66
2	Lombardy	Mantua	Ostiglia	Pigreco
3	Lombardy	Mantua	Ostiglia	Oliver
4	Lombardy	Mantua	Mantova	Levante
5	Veneto	Verona	Cerea	Obelix
6	Veneto	Padua	Magliadino San Fidenzio	Mimmo
7	Veneto	Vicenza	Noventa Vicentina	Odisseo
8	Veneto	Rovigo	Canaro	Levante
9	Emilia Romagna	Rimini	Rimini	Ettore
10	Emilia Romagna	Ferrara	Argenta	Miradoux
11	Emilia Romagna	Bologna	Budrio	Zetae
12	Emilia Romagna	Parma	Sissa	Monastir
13	Emilia Romagna	Ravenna	Ravenna	Nazareno
14	Emilia Romagna	Reggio Emilia	Viano	Levante
15	Emilia Romagna	Piacenza	Piacenza	Athoris
16	Emilia Romagna	Modena	Carpi	Tito Flavio
17	The Marche	Ancona	Filottrano	Achille
18	The Marche	Pesaro e Urbino	Monte Porzio	San Carlo
19	The Marche	Macerata	Montefano	Achille
20	The Marche	Macerata	San Severino	San Carlo
21	The Marche	Ancona	Corinaldo	Odisseo
22	Tuscany	Livorno	Campiglia Marittima	Ariosto
23	Tuscany	Grosseto	Buriano	Massimo Meridio
24	Tuscany	Grosseto	Scansano	Casanova
25	Tuscany	Arezzo	Castiglion Fiorentino	Nazareno
26	Tuscany	Arezzo	Riccio	Claudio
27	Umbria	Perugia	Porto	Achille
28	Umbria	Perugia	Pozzuolo	Claudio
29	Umbria	Perugia	Castiglion del Lago	Marco Aurelio
30	Umbria	Terni	Montecastrilli	Antalis
31	Umbria	Terni	Avigliano Umbro	Don Matteo
32	Umbria	Perugia	Spello	Miradoux
33	Umbria	Perugia	Marsciano	P22D84
34	Umbria	Perugia	Deruta	Odisseo
35	Umbria	Perugia	Perugia	Ramirez
36	Latium	Rome	Roma	Ettore
37	Latium	Rome	Roma	San Carlo
38	Latium	Viterbo	Montalto di Castro	Marco Aurelio
39	Latium	Rome	Centro Grande	Tirex
40	Latium	Rome	Fiumicino	Colombo
41	Latium	Rome	Montelibretti	Claudio
42	Latium	Rome	Montelibretti	Ariosto
43	Abruzzo	Pescara	Capagatti	Marco Aurelio
44	Abruzzo	Chiet	Ortona	Marco Aurelio
45	Abruzzo	L'Aquila	Sulmona	Marco Aurelio
46	Abruzzo	Teramo	Sant'Omero	Marco Aurelio
47	Molise	Campobasso	Campobasso	Marco Aurelio
48	Campania	Benevento	Benevento	Aureo
49	Campania	Benevento	Benevento	Svevo
50	Apulia	Foggia	Foggia	Svevo
51	Apulia	Foggia	Sannicandro Garganico	Antalis
52	Apulia	Foggia	San Severo	Antalis
53	Apulia	Foggia	Apricena	Antalis
54	Apulia	Foggia	Foggia	Antalis
55	Apulia	Foggia	San Marco in Lamis	Antalis
56	Apulia	Foggia	Motta Montecorvino	Antalis
57	Apulia	Foggia	Troia	Iride
58	Apulia	Foggia	Lucera	Saragolla
59	Sardinia	Sassari	Pozzo S. Nicola	Karalis
60	Sardinia	Sassari	Saccheddu	Ramirez
61	Sardinia	Oristano	Riola	Ramirez
62	Sardinia	Oristano	Simala	Santo Graal
63	Sardinia	Cagliari	Nuraminis	Kanakis
64	Sardinia	Cagliari	Quartuccio	Karalis
65	Sicily	Enna	Aidone	Marco Aurelio
66	Sicily	Enna	Regalbuto	Saragolla
67	Sicily	Enna	Assoro	Simeto
68	Sicily	Caltanissetta	Caltanissetta	Marco Aurelio
69	Sicily	Caltanissetta	Butera	Simeto
70	Sicily	Caltanissetta	Mazzarino	Core

Table S3:

Colonies belonging to the different fungal genera as visually and microscopically assessed after their development from durum wheat kernels collected in three different Italian regions (Emilia-Romagna, Umbria, Sardinia) with two different isolation method.

Region	Method	Fungal genera					
		Alternaria	***Fusarium***	***Epicoccum***	***Aspergillus***	***Penicillium***	**Other**
Emilia-Romagna	MPDA[†]	5.66 (±0.15)[*]	2.67 (±0.10)	0.88 (±0.08)	0.11 (±0.03)	0.11 (±0.03)	0.81 (±0.08)
	DFB[‡]	5.84 (±0.15)	3.04 (±0.10)	nd[§]	0.42 (±0.06)	0.20 (±0.04)	0.68 (±0.07)
Umbria	MPDA	6.51 (±0.15)	1.82 (±0.10)	0.66 (±0.26)	0.33 (±0.05)	0.21 (±0.04)	0.85 (±0.08)
	DFB	9.68 (±0.05)	1.32 (±0.10)	nd	nd	nd	0.66 (± 0.07)
Sardinia	MPDA	8.11 (±0.12)	0.35 (±0.10)	0.03 (±0.01)	0.16 (±0.04)	0.20 (±0.04)	0.96 (±0.09)
	DFB	9.75 (±0.04)	1.15 (±0.05)	nd	nd	nd	0.13 (±0.03)

[†]modified potato dextrose agar; [‡]Deep-freezing blotter; [*]the value represents the average (± standard error) of 10 replicates; [§]nd: not detected.

Table S4:

Fusarium species as identified by partial *translation elongation factor 1α* sequencing after their isolation with two different methods from durum wheat kernels collected in three different Italian regions (Emilia-Romagna, Umbria, Sardinia).

Region	Method	*Fusarium* species						
		F. poae	***F. avenaceum***	***F. graminearum***	***F. culmorum***	***F. sporotrichioides***	***F. langsethiae***	***F. tricinctum***
Emilia-Romagna	MPDA[†]	6.11 (±0.80)[**]	0.51 (±0.23)	3.01 (±0.56)	0.56 (±0.24)	nd	nd	0.18 (±0.13)
	DFB[‡]	4.33 (±0.65)	0.67 (±0.25)	2.82 (±0.52)	nd	nd	nd	0.28 (±0.16)
Umbria	MPDA	2.53 (±0.52)	0.23 (±0.15)	0.66 (±0.26)	0.13 (±0.11)	0.10 (±0.07)	0.10 (±0.07)	0.10 (±0.08)
	DFB	1.55 (±0.40)	0.53 (±0.23)	0.87 (±0.30)	nd	nd	nd	0.10 (±0.09)
Sardinia	MPDA	0.26 (±0.16)	0.10 (±0.07)	nd[§]	0.18 (±0.13)	nd	nd	nd
	DFB	0.13 (±0.12)	0.10 (±0.09)	nd	0.14 (±0.13)	0.47 (±0.23)	nd	nd

Region	Method	*Fusarium* species					
		F. equiseti	***F. acuminatum***	***F. proliferatum***	***F. verticillioides***	***F. sambucinum***	***F. crockwellense***
Emilia-Romagna	MPDA[†]	0.20 (±0.13)	0.10 (±0.07)	0.84 (±0.29)	0.15 (±0.12)	0.20 (±0.14)	nd
	DFB[‡]	0.48 (±0.21)	0.10 (±0.07)	5.98 (±0.76)	0.42 (±0.20)	nd	0.10 (±0.07)
Umbria	MPDA	nd	0.10 (±0.07)	0.67 (±0.26)	0.10 (±0.08)	nd	nd
	DFB	0.10 (±0.07)	0.10 (±0.07)	2.82 (±0.55)	nd	nd	nd
Sardinia	MPDA	0.10 (±0.08)	nd	nd	0.10 (±0.07)	nd	nd
	DFB	0.10 (±0.09)	nd	0.92 (±0.33)	0.10 (±0.09)	nd	nd

[†]modified potato dextrose agar; [‡]deep-freezing blotter; [**]the value represents the average (± standard error) of 10 replicates; [§]nd: not detected

Table S5:

Colonies belonging to the different fungal genera as visually and microscopically assessed after their development from durum wheat kernels collected in the investigated Italian regions (Abruzzo, Apulia, Campania, Emilia-Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto) with two different isolation method (MPDA, modified potato dextrose agar; DFB deep-freezing blotter).

Region	Method	Fungal Genera										
		Alternaria	*Fusarium*	*Microdochium*	*Cladosporium*	*Epicoccum*	*Aspergillus*	*Penicillium*	*Rhizopus*	*Acremonium*	*Gliocladium*	Others
Abruzzo	MPDA	54.25*(±7.70)**	28.00 (±5.53)	4.75 (±2.28)	17.00 (±4.31)	3.25 (±1.88)	1.75 (±1.38)	nd	0.50 (±0.74)	nd	nd	0.25 (±0.52)
	DFB	34.50 (±6.14)	28.00 (±5.53)	1.50 (±1.28)	3.50 (±1.95)	2.50 (±1.65)	nd	nd	nd	6.5 (±2.50)	nd	9.50 (±3.22)
Apulia	MPDA	63.67 (±5.56)	2.89 (±1.18)	nd***	4.67 (±1.50)	0.44 (±0.46)	0.67 (±0.57)	11.00 (±2.31)	2.44 (±1.09)	nd	nd	0.67 (±0.57)
	DFB	48.22 (±4.84)	10.67 (±2.27)	nd	2.89 (±1.18)	nd	0.89 (±0.66)	4.22 (±1.43)	nd	0.67 (±0.53)	nd	2.67 (±1.14)
Campania	MPDA	92.00 (±14.20)	3.00 (±2.56)	nd	4.50 (±3.13)	2.50 (±2.34)	nd	nd	1.00 (±1.48)	nd	nd	nd
	DFB	62.00 (±11.60)	8.00 (±4.18)	nd	5.00 (±3.30)	5.00 (±3.30)	nd	nd	nd	nd	nd	2.00 (±2.09)
Emilia Romagna	MPDA	58.50 (±5.65)	7.13 (±1.97)	8.63 ±(2.17)	7.38 (±2.01)	8.75 (±2.19)	0.38 (±0.45)	nd	0.88 (±0.70)	nd	nd	0.63 (±0.58)
	DFB	22.75 (±3.52)	32.50 (±4.21)	2.75 (±1.23)	6.00 (±1.81)	3.25 (±1.33)	6.75 (±1.92)	2.75 (±1.23)	nd	0.50 (±0.49)	nd	14.25 (±2.79)
Latium	MPDA	71.14 (±6.66)	18.43 (±3.39)	3.57 (±1.49)	3.29 (±1.43)	4.57 (±1.69)	0.57 (±0.60)	1,71 (±1.03)	1.14 (±1.48)	nd	nd	1.00 (±0.79)
	DFB	45.71 (±5.34)	25.71 (±4.01)	0.86 (±0.73)	5.14 (±1.79)	2.29 (±1.19)	1.43 (±0.94)	nd	4.29 (±1.64)	0.57 (±0.56)	nd	2.57(±1.27)
Lombardy	MPDA	54.00 (±7.68)	6.75 (±2.71)	7.50 (±2.86)	10.00 (±3.30)	7.00 (±2.76)	4.00 (±2.09)	1.00 (±1.04)	0.50 (±0.74)	nd	nd	1.00 (±1.04)
	DFB	46.00 (±7.09)	20.00 (±4.67)	5.50 (±2.45)	5.00 (±2.34)	1.00 (±1.04)	1.50 (±1.28)	0.50 (±0.74)	nd	nd	nd	3.00 (±1.81)
Molise	MPDA	84.00 (±19.2)	14.00 (±7.82)	nd	2.00 (±2.96)	1.00 (±2.09)	nd	nd	nd	nd	nd	2.00 (±2.96)
	DFB	38.00 (±0.13)	30.00 (±11.4)	nd	2.00 (±2.96)	4.00 (±4.18)	nd	nd	nd	10 (±6.2)	nd	8.00 (±5.91)
Sardinia	MPDA	81.50 (±7.70)	10.33 (±2.74)	1.83 (±1.16)	2.00 (±1.21)	2.33 (±1.30)	1.50 (±1.04)	3.00 (±1.48)	0.17 (±0.35)	nd	nd	nd
	DFB	53.67 (±6.25)	20.00 (±3.82)	0.67 (±0.70)	1.67 (±1.10)	1.00 (±0.85)	nd	nd	nd	0.33 (±0.46)	nd	4.67 (±1.84)
Sicily	MPDA	73.83 (±7.33)	1.00 (±0.85)	nd	13.50 (±3.13)	2.67 (±1.39)	0.17 (±0.35)	6.33 (±2.15)	1.17 (±0.92)	nd	nd	2.17 ±(1.26)
	DFB	41.00 (±5.46)	0.33 (±0.49)	nd	16.00 (±3.41)	2.00 (±1.21)	nd	1.67 (±1.10)	0.33 (±0.49)	0.33 (±0.46)	nd	5.00 (±1.91)
The Marche	MPDA	85.20 (±8.63)	4.00 (±1.87)	3.20 (±1.67)	4.20 (±1.92)	1.60 (±1.18)	1.20 (±1.02)	2.20 (±1.39)	0.40 (±0.59)	nd	nd	nd
	DFB	43.60 (±6.17)	14.40 (±3.55)	0.40 (±0.59)	8.00 (±2.64)	1.20 (±1.02)	1.20 (±1.02)	0.40 (±0.59)	nd	10.8 (±2.88)	nd	nd
Tuscany	MPDA	72.00 (±7.39)	16.00 (±3.74)	7.80 (±2.61)	2.20 (±1.39)	5.80 (±2.25)	nd	nd	nd	nd	nd	0.20 (±0.42)
	DFB	42.80 (±6.11)	18.00 (±3.96)	1.20 (±1.02)	8.80 (±2.77)	0.40 (±0.59)	nd	nd	nd	2.80 (±1.46)	2.80 (±1.46)	18.40 (±4.01)
Umbria	MPDA	68.67 (±5.77)	22.56 (±3.31)	11.56 (±2.37)	2.67 (±1.14)	3.44 (±1.29)	1.89 (±0.96)	2.78 (±1.16)	0.11 (±0.32)	nd	nd	0.22 (±0.33)
	DFB	43.33 (±4.59)	29.33 (±3.77)	3.33 (±1.27)	4.44 (±1.47)	2.00 (±0.99)	0.44 (±0.46)	0.67 (±0.57)	0.89 (±0.66)	1.78 (±0.87)	2.44 (±1.02)	5.78 (±1.67)
Veneto	MPDA	68.50 (±8.65)	9.00 (±3.13)	6.25 (±2.61)	11.50 (±3.54)	6.50 (±2.66)	nd	0.75 (±0.91)	0.25 (±0.52)	nd	nd	0.25 (0.52)
	DFB	27.00 (±5.43)	30.00 (±5.72)	2.00 (±1.48)	1.00 (±1.04)	2.50 (±1.65)	1.00 (±1.04)	nd	nd	6.00 (±2.40)	nd	26.00 (±5.33)

**the value represents the average number of each fungal genera detected for each region **the value between brackets represents the standard error; nd: not detected.

Table S6:

Fusarium species as identified with two different methods from durum wheat kernels collected across Italian regions (Abruzzo, Apulia, Campania, Emilia-Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto).

Region	Method	Fusarium species											
		FG	FC	FP	FPr	FAv	FT	Fac	FIESC	FS	FSa	FPs	FB
Abruzzo	MPDA	4.0 (± 1.09)	0.75 (± 0.47)	2.0 (± 0.77)	4.75 (± 1.09)	8.25 (± 1.56)	2.0 (± 0.77)	0.25 (± 0.27)	5.0 (± 1.21)	0.75 (± 0.47)	nd	nd	nd
	DFB	1.75 (± 0.71)	0.50 (± 0.38)	3.5 (± 1.01)	5.25 (± 1.24)	nd	nd	nd	1.5 (± 0.66)	nd	nd	nd	nd
Apulia	MPDA	0.11 (± 0.12)	0.68 (± 0.30)	0.11 (± 0.12)	0.33 (± 0.21)	0.44 (± 0.24)	0.22 (± 0.17)	0.56 (± 0.27)	0.44 (± 0.24)	nd	nd	nd	nd
	DFB	nd	2.56 (± 0.58)	0.22 (± 0.17)	2.22 (± 0.54)	nd	0.33 (± 0.21)	nd	nd	0.33 (± 0.21)	nd	nd	nd
Campania	MPDA	0.50 (± 0.54)	1.5 (± 0.94)	nd	1.00(± 0.77)	nd	nd	nd	nd	nd	nd	nd	nd
	DFB	nd	4.0 (± 1.53)	nd	1.00(± 0.77)	nd	nd	nd	nd	nd	nd	nd	nd
Emilia Romagna	MPDA	1.50 (± 0.47)	0.50 (± 0.27)	1.87 (± 0.52)	0.75 (± 033)	1.25 (± 0.43)	nd	nd	1.00 (± 0.38)	nd	nd	nd	0.25 (± 0.19)
	DFB	1.50 (± 0.47)	0.37 (± 0.23)	1.37 (± 0.45)	9.5 (± 1.18)	nd	0.12 (±0.13)	nd	1.12 (± 0.40)	0.25 (± 0.19)	0.25 (± 0.19)	nd	nd
Latium	MPDA	2.0 (± 0.58)	0.57 (± 0.31)	3.14 (± 0.73)	0.57 (± 0.31)	9.29 (± 1.25)	nd	0.14 (± 0.15)	0.14 (± 0.15)	1.0 (± 0.41)	0.14 (± 0.15)	nd	nd
	DFB	3.0 (± 0.71)	0.29 (± 0.22)	2.86 (± 0.70)	6.86 (± 1.07)	nd	nd	nd	2.0 (± 0.58)	nd	0.86 (± 0.38)	nd	nd
Lombardy	MPDA	1.25 (± 0.61)	1.25 (± 0.61)	0.25 (± 0.27)	0.50 (± 0.38)	1.5 (± 0.66)	1.0 (± 0.54)	nd	0.75 (± 0.47)	nd	nd	nd	nd
	DFB	0.75 (± 0.47)	0.75 (± 0.47)	nd	8.0 (± 1.53)	nd	nd	nd	1.5 (± 0.66)	0.75 (± 0.47)	nd	nd	nd
Molise	MPDA	nd	3.0 (±1.89)	nd	nd	3.0 (±3.25)	nd	1.0 (± 1.08)	1.0 (± 1.08)	nd	nd	nd	nd
	DFB	nd	nd	nd	4.00 (± 2.17)	nd	nd	nd	5.0 (±2.42)	nd	nd	nd	nd
Sardinia	MPDA	0.17(± 0.18)	1.17(± 0.48)	nd	nd	6.83 (± 1.16)	nd	0.50 (± 0.31)	1.83 (± 0.60)	nd	nd	nd	nd
	DFB	1.33(± 0.51)	4.8 (± 0.98)	nd	0.167(± 0.18)	nd	nd	nd	0.67 (± 0.36)	nd	0.167(± 0.18)	nd	nd
Sicily	MPDA	nd	nd	0.33 (± 0.25)	0.33 (± 0.21)	nd	nd	nd	nd	nd	nd	nd	nd
	DFB	nd	nd	0.33 (± 0.25)	0.167(± 0.18)	nd	nd	nd	nd	nd	nd	nd	nd
The Marche	MPDA	nd	0.80 (± 0.43)	3.80 (± 0.95)	nd	1.60 (± 0.61)	nd	nd	nd	nd	nd	nd	nd
	DFB	nd	nd	1.60 (± 0.61)	5.00 (± 1.08)	nd	nd	nd	nd	nd	nd	nd	nd
Tuscany	MPDA	4.2 (± 0.99)	0.80 (± 0.43)	2.0 (± 0.69)	0.2 (± 0.22)	3.0 (± 0.84)	1.6 (± 0.61)	nd	nd	nd	1.2 (± 0.53)	1.8 (± 0.65)	nd
	DFB	4.2 (± 0.99)	1.2 (± 0.53)	nd	4.00 (± 0.97)	nd	0.40 (± 0.30)	nd	nd	nd	0.20 (± 0.22)	nd	nd
Umbria	MPDA	5.22 (± 0.83)	nd	0.89 (± 0.34)	1.33 (± 0.42)	2.78 (± 0.60)	0.44 (± 0.24)	0.78 (± 0.32)	0.44 (± 0.24)	nd	nd	nd	nd
	DFB	3 (± 0.62)	0.78 (± 0.32)	1.00 (± 0.36)	10.56 (± 1.17)	nd	nd	nd	0.33 (± 0.21)	0.33 (± 0.21)	0.22 (± 0.17)	nd	nd
Veneto	MPDA	3.0 (± 0.94)	nd	1.0 (± 0.54)	4.5 (± 1.15)	0.5 (± 0.38)	nd	nd	nd	nd	nd	nd	nd
	DFB	1 (± 0.54)	nd	0.75 (± 0.47)	7.0 (± 1.43)	nd	nd	nd	1.0 (± 0.540)	1.0 (± 0.540)	nd	nd	nd

FG: F. *graminearum*, FC: *F. culmorum*, FP: *F. poae*, FPr: *F. proliferatum*, FAv: *F. avenaceum*, FT: *F. tricinctum*, Fac: *F. acuminatum*, FIESC (*Fusarium incarnatum equiseti* species complex), FS: *F. sporotrichioides*, FSa: *F. sambucinum*, FPs: *F. pseudogaminearum*, FB: *F. brachygibbosum*,

*the value represents the average number of each *Fusarium* species detected for each region; **the value between brackets represents the standard error; nd: not detected; na: not available

Table S6- continued

Fusarium species as identified with two different methods from durum wheat kernels collected across Italian regions (Abruzzo, Apulia, Campania, Emilia-Romagna, Latium, Lombardy, Molise, Sardinia, Sicily, The Marche, Tuscany, Umbria, Veneto).

Region	Method	*Fusarium* species												
		FO	FN	FAn	FL	FGl	FLa	FSu	FV	FSe	FCl	FA/Fac	*Fusarium* sp.	*Fusarium* sp.
Abruzzo	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	6.25 (± 1.36)	0.75 (± 0.47)	nd	0.75 (± 0.47)	0.25 (± 0.27)	1.5 (± 0.66)	1.25 (± 0.61)	2.50 (± 0.86)	2.50 (± 0.86)
Apulia	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	1.44 (± 0.43)	nd	nd	1.22 (± 0.42)	nd	0.44 (± 0.24)	0.78 (± 0.32)	0.11 (± 0.12)	0.11 (± 0.12)
Campania	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.50 (± 0.54)	nd	nd
Emilia Romagna	MPDA	0.25 (± 0.19)	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	4.37 (± 0.80)	nd	nd	3.62 (± 0.73)	0.37 (± 0.23)	0.37 (± 0.23)	1.62 (± 0.49)	2.12 (± 0.56)	2.12 (± 0.56)
Latium	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	2.43 (± 0.64)	nd	nd	2.43 (± 0.64)	nd	1.0 (± 0.41)	6.86 (± 1.07)	2.43 (± 0.64)	2.43 (± 0.64)
Lombardy	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	0.50 (± 0.38)	nd	0.50 (± 0.38)	3.25 (± 0.98)	0.50 (± 0.38)	1.25 (± 0.61)	2.50 (± 0.86)	1.25 (± 0.61)	1.25 (± 0.61)
Molise	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	5.0 (± 2.43)	nd	nd	1.0 (± 1.08)	nd	nd	3.0 (±1.89)	1.0 (± 1.08)	1.0 (± 1.08)
Sardinia	MPDA	nd	1.33 (± 0.51)	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	nd	0.17 (± 0.18)	nd	nd	0.17 (± 0.18)	0.17 (± 0.18)	3.83 (± 0.87)	1.83 (± 0.60)	1.83 (± 0.60)
Sicily	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	0.17 (± 0.18)	nd	nd	nd	nd	nd	nd	nd	nd
The Marche	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	0.20 (± 0.22)	3.40 (± 0.89)	nd	nd	0.20 (± 0.22)	nd	1.60 (± 0.61)	0.20 (± 0.22)	0.20 (± 0.22)	0.20 (± 0.22)
Tuscany	MPDA	nd	nd	0.40 (± 0.31)	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	2.60 (± 0.78)	0.40 (± 0.31)	nd	0.80 (± 0.43)	0.20 (± 0.22)	2.80 (± 0.81)	1.60 (± 0.61)	0.40 (± 0.31)	0.40 (± 0.31)
Umbria	MPDA	1.78 (± 0.48)	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	3.22 (± 0.65)	nd	nd	2.00 (± 0.51)	0.22 (± 0.17)	2.77 (± 0.51)	2.11 (± 0.52)	1.89 (± 0.50)	1.89 (± 0.50)
Veneto	MPDA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	na	na	na
	DFB	nd	nd	nd	nd	2.50 (± 0.86)	nd	2.50 (± 0.86)	2.50 (± 0.86)	nd	0.25 (± 0.27)	1.0 (± 0.54)	2.50 (± 0.86)	2.50 (± 0.86)

FO: *F. oxysporum*, FN: *F. nelsonii*, FAn: F. *anthophilum*, FL: *F. langsethiae*, FGl: *F. globosum*, FLa: *F. lateritium*, FSu: *F. subglutinans*, FV: *F. verticillioides*, FSe: *F. semitectum*, FCl: *F. clamydosporum*, FA/Fac: *F.avenaceum/F.acuminatum*,

**the value represents the average number of each *Fusarium* species detected for each region; **the value between brackets represents the standard error; nd: not detected; na: not available

CHAPTER 2:

Phylogeny of **Fusarium***: the case study of* **Fusarium tricinctum** *species complex*

2.1 *Fusarium* recognition and phylogeny: state of the art

2.1.1 History of *Fusarium* recognition and taxonomy

Fusarium is a widespread genus, composed by many morphologically and phylogenetically different species characterized by very different lifestyle. This genus includes several toxigenic species (about 300) that could live as saprotroph or endophytes or live as pathogen on other fungi, plants and animals (Aoki et al., 2014).

During the past years, many taxonomic changes have been made to classify the different species correctly. Historically, the taxonomic identification was based on morphological characters such as asexual structures (conidia shape and size). However, the absence of defined morphological characters and their variation depending on cultural media or environmental conditions made this method unstable and misleading (Geiser et al., 2004). Moreover, this approach could be exhaustive for a classification at ordinal, familial or genus level but could be unreliable at species level (Raja et al., 2017). For this reason, molecular methods were developed to understand species diversity and phylogenetic relationships among the different species. The concept of *Fusarium* was introduced for the first time in 1809 by Link who admitted as first distinctive character the falciform-shaped conidia (Summerell, 2019). However, the bases of the modern *Fusarium* taxonomy were provided by Wollenweber and Reinking in 1935. They organized the genus in 16 non-monophyletic sections containing 65 species, 55 varieties and 22 forms. In addition, the authors described a detailed method for the morphological identification and the collection of phenotypic features. The guideline for the *Fusarium* identification adopted by these two authors resulted excessively specific and difficult to apply for many pathologists considering that they required a microscopical observation of the conidia. For this reason and in order to facilitate the morphological recognition, an extreme simplification of that system was made by Snyder and Hansen in the 1940s. They reduced drastically the number of *Fusarium* species counting only 9. Considering the unsuitability of the later system, in 1983, Booth described 44 species on the basis of Wollenweber and Reinking guideline. On this path, Gerlach and Nirenberg (1982) count 90 morphological distinct species while Nelson and his collaborators described 12 section including 30 “well-documented” and 16 “insufficiently documented” species. To combine morphological, biological and phylogenetic features Leslie and Summerell published in 2006 “The *Fusarium* Laboratory Manual” recognizing 70 species and giving, in addition, information regarding the taxonomy, the pathogenicity and the range of plant hosts

(Leslie and Summerell, 2006; O'Donnell et al., 2015). Historically, the division in section of the genus *Fusarium* is based on morphological and physiological similarities, however as demonstrated by Kristensen and co-workers (2005), species that share morphological features could be members of different phylogenetic clades. The same authors observing a clear connection between the phylogenetic relationship and the potential ability to biosynthesize a specific class of secondary metabolites highlighting the needed to revise the old classification considering also this genetic aspect. On the basis of a more detailed phylogenetic analysis, the artificial classification proposed in the past was replaced by monophyletic species complex (O'Donnell et al., 2013). For this reason, during the last years, new species have been described also with the molecular tools support. Even if the use of molecular analysis makes easier the comprehension of genealogy and to differentiate between species, there is still a pronounced discussion on the correct identification method. The high morphological and genetic variability makes hard the differentiation between species, even at molecular level. Despite it is estimated that *Fusarium* has more than three hundred distinct phylogenetic species, only half of them have been described (Kulik, 2008).

Another discussed aspect of *Fusarium* taxonomy is the definition of its teleomorph form. Several teleomorph genera were linked with *Fusarium*. Among them *Gibberella* Sacc. 1877, *Cyanonectria* Samuels & P. Chaverri, *Albonectria* Rossman & Samuels, *Geejayessia* Schroers, Grafenhan & Seifert, and *Haematonectria* (Aoki et al., 2014). However, considering that the sexual form has been observed only for the 20% of *Fusarium* species and that the dual-nomenclature could create issue regarding the taxonomic definition, the use of both definitions has been abolished in the International Botanical Congress in 2011, with the changes of the International Code of Nomenclature for algae, fungi, and plants (ICNafp) (Leslie and Summerell, 2006). For this reason, the dual nomenclature for anamorph and teleomorph name of *Fusarium* species had to be unified by the 1st January 2013 (Aoki et al., 2014; Summerell, 2019).

In conclusion, following the current classification, the genus *Fusarium* belongs to the kingdom of *Fungi*, Division *Ascomicota*, class *Sordariomycetes*, order *Hypocreales* and family of *Nectriaceae* (Aoki et al., 2014; Leslie and Summerell, 2006)

2.1.2 Molecular identification of *Fusarium* species

As previously mentioned, morphological features can be indicative for species identification, however, they can cause an underestimation of species diversity (Summerell and Leslie, 2011). For example, morphological species recognition (MSR) was able to recognize only 6 species out of 16 among the *Fusarium graminearum* species complex (FGSC), highlighting the needed of more sensitive diagnostic tools (Aoki et al., 2012).

Several genes were tested for their ability to distinguish species within *Fusarium* genus, among them: *Internal transcribed space* (*ITS*), intergenic spacer (*IGS*), β-*tubulin* (*β-tub*), calmodulin, histones, *Translation elongation factor 1a* (*TEF1a)* and two genes encoding the largest and second largest subunits of RNA polymerase (*RPB1* and *RPB2*, respectively) (Summerell, 2019; Summerell and Leslie, 2011).

β-tub was one of the first protein-encoding gene used in *Fusarium* phylogenesis. It showed to be a better reliable marker compared with other genes such as 28rDNA and *ITS* 2 (O'Donnell et al., 1998; O'Donnell and Cigelnik, 1997). However, the detection of divergent paralogs in some species complex (i. e.: *F. solani* and *F. chlamidosporum*), demonstrated its uselessness in phylogenetic studies (O'Donnell et al., 2015). Later, *ITS* has been used to infer phylogenetic relationships among several fungal genera included *Fusarium.* However, the presence of non-orthologous copies could cause an incorrect identification as observed in the *Gibberella fujikuroi* species complex (GFSC) (O'Donnell et al., 1998). Moreover, its use is limited by the frequent lack of informativeness at species level and the possibility to align only fungal sequences obtained from species belonging to same complex or its closely related ones (O'Donnell et al., 2015).

Successively, researchers focused their attention on gene markers characterized by a rich intron-portions useful to infer phylogenetic relationship among species such as *TEF1a*. Present in single-copies in *Fusarium, TEF1a* is involved in the protein translation process (Geiser et al., 2004; Kristensen et al., 2005; O'Donnell et al., 2012). Because of its highly informativeness at specie level, this gene has demonstrated to be a suitable genetic marker for *Fusarium* single-locus identification also due to: the absence of non-orthologous copies and the availability of universal primers able to work across the entire genus (Geiser et al., 2004). However, the high-informative and variable introns, which characterized about one-third of the amplicons obtained with the designed primers (about

700 bp), allowed the alignment of that sequences only in the species complex or in the closely related ones but it is not useful for phylogenetic study across the entire genus (O'Donnell et al., 2015, 2013). For this reason, to infer phylogenetic relationship within all *Fusarium* genus, *RPB1* and *RPB2* were selected for their informative capacity. The latter gene demonstrated to be easier to align across *Fusarium*, compared with *TEF1a*, considering the lack of intron portions (O'Donnell et al., 2013, 2012). In addition to the already mentioned phylogenetic markers, several studies focused the attention on other genes in order to establish evolutionary relationships between *Fusarium* species. Among them: *CYP51* gene, encoding for sterol *14 α- demethylase* and aminoadipate reductase gene (*lys2*) (Fernández-Ortuño et al., 2010, 2011; Watanabe et al., 2011).

CYP51 gene has been proved to be useful in the discrimination of monophyletic taxa well linked with the mycotoxigenic profiles. This gene showed a resolving phylogenetic power higher than other common-used markers such as *β-tub* or *ITS* sequences (Fernández-Ortuño et al., 2010). *Lys2* gene, as observed for species belonging to *Byssochlamys* genus, demonstrated to be a good phylogenetic marker also in *Fusarium* showing the highest nucleotide substitution rated if compared to 5.8S rDNA, *ITS* 11, 28rDNA and *β-tub* (Watanabe et al., 2011).

However, sometimes, a single locus analysis resulted not enough to give a unique identification especially for species complex poor represented in the online database such the FTSC (Harrow et al., 2010). In this contest, *Genealogical concordance phylogenetic species recognition* (GCPSR), based on multiple gene genealogies, demonstrated its utility for a unique identification (O'Donnell et al., 2018; Summerell, 2019). This analysis allowed the identification of about 300 separated phylogenetic species within *Fusarium* genus (at least 69 of clinical importance), 20 species complex and 9 monophyletic lineages (O'Donnell et al., 2015). These species were detected on the bases of phylogenetic analyses of fusaria collected in the ARS Culture Collection (NRRL), the CBS-KNAW Biodiversity Centre (CBS) and the *Fusarium* Research Center (FRC). The term "species complex" was introduced to move away from the subgeneric and polyphyletic concept of section. It is defined as "a grouping of species with shared morphological characteristics and phylogenetic markers, and some level of cryptic speciation". Even if the concept of "complex" is not formally recognized by the International Code of Nomenclature (ICN), this term allowed to group species strongly linked by a phylogenetic point of view and how share features (i.e. phenotypic characteristics and metabolic profile) (Summerell, 2019).

Despite the great numbers of *Fusarium* species detected worldwide, only few of them have been described and resulted distinguishable from other species on the basis of morphological features and many of them are still unnamed (Aoki et al., 2014; O'Donnell et al., 2010). Several online sequences databases were developed to help researchers in diagnosis and diagnostic of fungal species. Among them: GenBank at the National Center for Biotechnology Information, GenBank NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) *Fusarium* MLST (https://fusarium.mycobank.org/) and *Fusarium* ID (isolate.fusariumdb.org/blast.php).

However, not all of them are able to give a correct identification. In fact, the absence of quality control on sequences deposited on GenBank and the taxonomy of many sequences not update and out of date could result in a misidentification (O'Donnell et al., 2010). For this reason, it is recommended the use of online databases such as *Fusarium* MLST and *Fusarium* ID where well-characterized sequences are deposited.

2.1.3 Main *Fusarium* species complex

Currently, within *Fusarium* genus, about 23 species complexes have been described, among them (Summerell, 2019) (Figure 1) :

***Fusarium sambucinum* species complex (FSAMSC)**

FSAMSC comprises the principal causal agents of *Fusarium* head blight in small grain cereals worldwide (*F. graminearum, Fusarium poae, Fusarium culmorum, Fusarium cerealis, Fusarium pseudograminearum, Fusarium sporotrichioides*) (Pancaldi et al., 2010; Ward et al., 2008). Nested within the FSAMSC is the *Fusarium graminearum* species complex (FGSC) which includes at least 16 phylogenetically distinct species (e.g. *F. graminearum* and *Fusarium asiaticum*) that could differed in biogeography, biology, morphological features and secondary metabolites profile (Aoki et al., 2012; O'Donnell et al., 2013; Summerell, 2019). Member of FSAMSC are able to produce a wide range of secondary metabolites included both type of trichothecenes (A and B), zearalenone, beauvercin and several other mycotoxins (O'Donnell et al., 2018; Ward et al., 2008).

***Fusarium fujikuroi* species complex (FFSC)**

This complex comprises about 50 phylogenetically distinct species that are known for economically important plant disease such as bakanae disease of rice (*Oryza sativa* L.) (*F. fujiikuroi*), Pitch canker disease of pine (*Fusarium circinatum*), pink ear rot in maize (*Fusarium verticillioides, Fusarium proliferatum, Fusarium subglutinans*) (Aoki et al., 2014; O'Donnell et al., 1998). The species belonging this complex are able to produce several secondary metabolites such as fumonisins, beauvericin and moniliformin (Amato et al., 2015; Covarelli et al., 2012; O'Donnell et al., 2018; Summerell and Leslie, 2011). *F. proliferatum* will be described in more detail in Chapter 3.

***Fusarium oxysporum* species complex (FOSC)**

The members of this complex are known to be both endophyte and saprotroph (Harrow et al., 2010). FOSC species are causal agents of some of the most economically important plant diseases (Leslie and Summerell, 2006). They are able to cause vascular wilts, crown and root rot and damping-off in many horticultural and ornamental plants such as tomato, tobacco, cotton, melon and tulips (Aoki et al., 2014; Leslie and Summerell, 2006). The great phylogenetic variability among this complex is proved by the description of about 70 *formae specialis* (f. sp.) (most of them paraphyletic or polypholetic), concept based on the infected plant host from which the isolate was detected (e.g.: *F. oxysporum* f. sp. *cubense* specific banana pathogen) (Laurence et al., 2014). In addition, members of this complex are reported also as human pathogens (Summerell, 2019).

***Fusarium incarnatum-equiseti* species complex (FIESC)**

Among this complex, GCPSR allowed identifying about 33 phylogenetically distinct and cryptic species organized in 2 different major clades (*Equiseti* and *Incarnatum*). The majority of them have not been described (Villani et al., 2019). These species could co-occur as saprotroph or opportunistic plant pathogen in several cereal cultures (O'Donnell et al., 2018; Villani et al., 2016). The members of FIESC are able to produce trichothecenes (both type A and B), beauvericin, equisetin, butanolide and zearalenone (Desjardins, 2006; O'Donnell et al., 2018).

***Fusarium solani* species complex (FSSC)**

FSSC comprises about 60 phylogenetically distinct species organized in three clades (Nalim et al., 2011; O'Donnell et al., 2018). The members of this complex are reported

not only as causal agents of foot and root rot in several hosts (e.g.: soybean sudden death syndrome-SDS) but also as human pathogens (Aoki et al., 2014; O'Donnell et al., 2008).

Fusarium tricinctum species complex **(FTSC)** will be described in detail in the following paragraph (2.1.4).

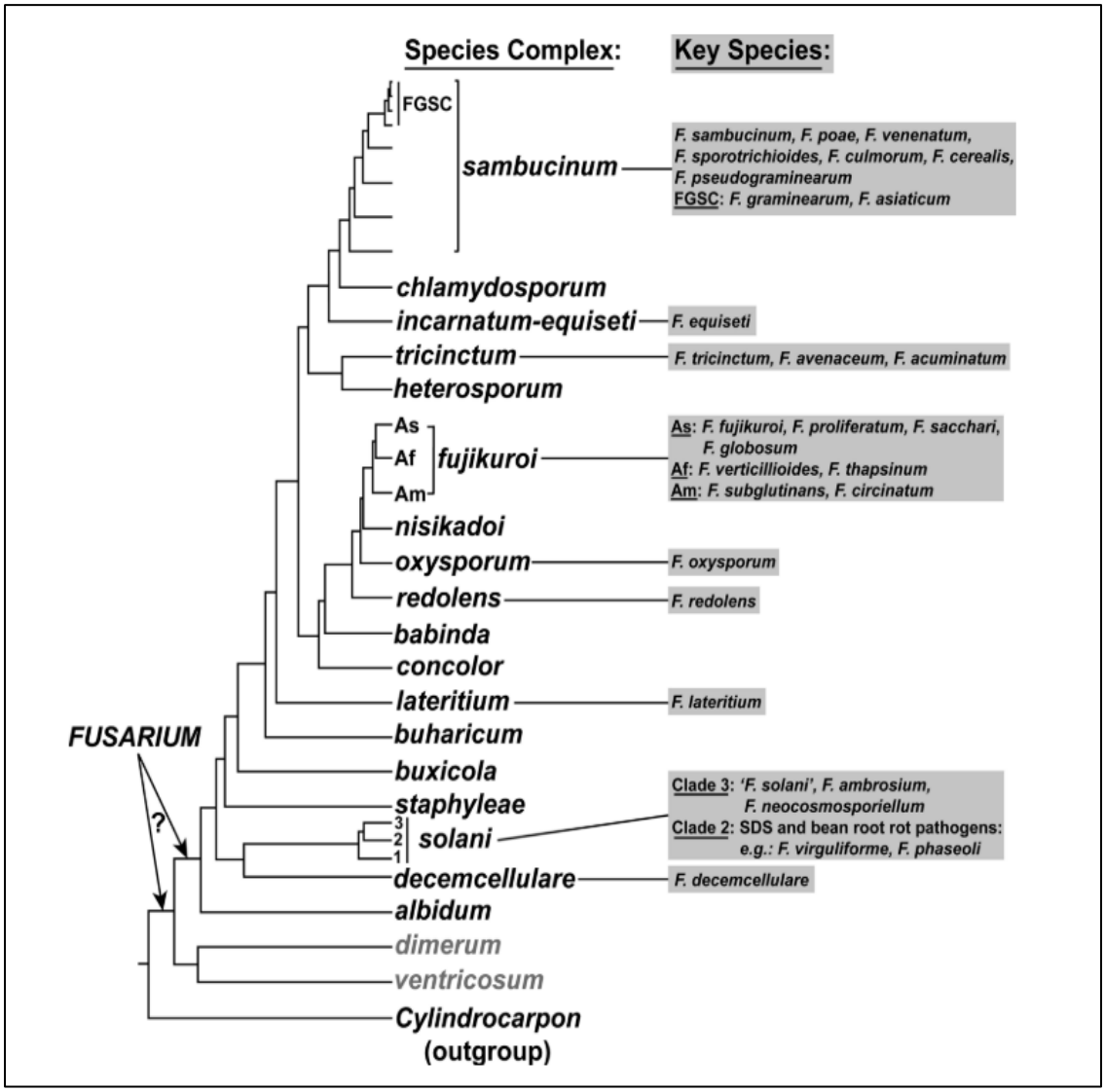


Figure 1: Phylogenetic relationships among *Fusarium* species as described by O'Donnell et al.2013 (Aoki et al., 2014)

2.1.4 *Fusarium tricinctum* species complex

The *Fusarium tricinctum* species complex, according to O'Donnell and co-workers (2018), is mainly constituted by *F. avenaceum* (FTSC 4), *F. tricinctum* (FTSC 3) and *F. acuminatum* (FTSC 2). In addition to well-known species, an informal ad hoc nomenclature using Arabic numerals (e.g., FTSC 1-to-11) was proposed to distinguish the named and unnamed species within this complex (O'Donnell et al., 2018). As well as for other *Fusarium* species complex, also the members of FTSC share genetic and biological features.

All the species of the FTSC are characterized by the absence or the low frequency of sexual stage and, on PDA medium, they show a slow grown, a floccose to cottony aerial mycelia with a white and pink pigmentation (Balmas et al., 2010, Leslie *et al.*, 2007). Hereafter, the components of FTSC are described.

Fusarium avenaceum (Fries) Saccardo

F. avenaceum is the main members of FTSC. Classified by Nelson et al. in the section *Roseum*, this species can be easily confused with the other members of the complex on the basis of morphological characters. However, the recent phylogenetic analysis place *F. avenaceum* within the FTSC (O'Donnell et al., 2018, 2013). The known sexual stage (*Gibberella avenacea*) of this species is uncommon (Yli-Mattila et al., 2002). The colony morphology of *F. avenaceum* is characterized by an high variability that reflect also the high genetic variability of its genomic DNA (Benyon et al., 2000). Morphologically, this species presents long and slender macroconidia (3-5 septate), straight to slightly curved. The apical cell appears long and tapering to a point while the basal cell is usually notched. They are produced in abundance in large pale orange-colored sporodochia. Ovoid microconidia are produced rarely only by some isolates. No chlamydospores production has been observed. On PDA medium, the colony morphology could be highly variable (Leslie and Summerell, 2006). Usually, *F. avenaceum* produces abundant mycelium ranging from a white to yellow to greyish rose color. *F. avenaceum* has been classified as both saprotrophic and aggressive pathogen associated not only to FHB on wheat and barley (See Chapter 1) but also to damping-off, root and crown rots of alfalfa (*Medicago sativa* L.), lisianthus (*Eustoma grandiflorum*), stalk rots and fruit rots of a wide range cereals legume, and vegetable crops (Bottalico and Perrone, 2002; Desjardins et al., 2000; Kollmorgen, 1974; Leslie and Summerell, 2006; Nalim et al., 2009; Salter et al., 1994).

This species has been reported as causal agents of wet core rot in apples where the diseased fruit exhibited light-brown wet rot, developing from the core to cortex (Sørensen et al., 2009).

High genetic variability among *F. avenaceum* isolates was observed worldwide.

Finnish *F. avenaceum* isolates, no discernible based on morphological features, were phylogenetically divided into two groups (I, II), separated clearly on the basis of *β-tub* gene and identified using specific primers (Yli-Mattila et al., 2006, 2002).

Analyzing several *F. avenaceum* isolates from different countries, Kulik and co-workers (2011) observed a high level of genetic variation without, however, any relationship with geographic or host origin indicated that these two characteristics do not play a main role in its evolution (Kulik et al., 2011). In addition, this variability was observed also in *F. avenaceum* population isolated from cereals harvested in the north-west and central region of Russia. In fact, phylogenetic analysis based on *partial phosphate permease* gene (*PHO*) showed a high intraspecific diversity (seven intraspecific groups) confirmed also by the different length of *PHO* amplicons (Stakheev et al., 2016). Finally, Ceròn-Bustamante and co-workers (2018), on the basis of both single and combined phylogeny, highlighted the presence of, at least, 4 distinct evolutionary lineages among Mexican *F. avenaceum* isolates, suggesting the presence of a novel species diversity among the complex (Cerón-Bustamante et al., 2018).

***Fusarium tricinctum* (Corda) Saccardo**

F. tricinctum, despite has been assigned by Nelson et al. (1983) to the section *Sporotrichiella*, shares with the member of FTSC the same morphology and the same metabolite profile. It is distinguished from the other species of the complex FTSC for the production of different kinds of microconidia (lemon-shape, pyriform, napiform, oval, ellipsoid, reniform 0-1 septate). However, *F. tricinctum* may be confused with other members of the section *Sporotrichiella* as *F. poae*, *F. sporotrichioides* and *F. chlamydosporum* which are characterized by an abundant microconidia and chlamydospores production that prove a convergent evolution of *F. tricinctum* with members of *Sporotrichiella* section (Leslie and Summerell, 2006; Stakheev et al., 2016; Turner et al., 1998). Moreover, *F. tricinctum* is the only member of this section which does not have the ability to produce trichothecenes considering the lack of the *TRI5* gene involved in trichothecenes biosynthesis (Harrow et al., 2010; Niessen and Vogel, 1998; Turner et al., 1998). On PDA cultures, *F. tricinctum* grows slowly and forms abundant

mycelium, initially of white colour, which becomes pink, red or purple with the age, forming red pigments in the agar. The macroconidia have slender and falcate shape, reaching almost a lunate form with an apical cell curved and tapering and a foot cell developed in a foot shape. The number of septa is often three, but occasionally four or five septate. Abundant microconidia (7-18 x 3.5-8 µm) are present in the aerial mycelium. They can present different shapes (napiform, oval, pyriform and sometimes citriform), usually without septa or occasionally one septum. Unlike macroconidia and microconidia, chlamydospores are formed only by some isolates and, for this reason, they are not a reliable diagnostic feature. They have a globose aspect with a smooth wall that becomes brown with age. On PDA substrate, *F. tricinctum* could be confused with *F. poae*, *F. chlamydosporum* and *F. sporotrichioides*, but the microconidial aspect and the exclusive production of monophialiadic conidiogenous cell, typical of *F. tricinctum*, allow to distinguish between the different species. In addition, *F. poae* monophialides have an urn shape easily discernible from those of *F. tricinctum* (Harrow et al., 2010; Leslie and Summerell, 2006; Zemánková and Lebeda, 2018). On SNA cultures, the growth rate is of 30-50 mm in four days and the aerial mycelium has a white or pink pale aspect, with dark vinaceous to blood pigment. *F. tricinctum* occurs as a saprotroph or weak pathogen in temperate area of the world; however, under certain conditions, it could be the main causal agents of plant diseases (Alkadri et al., 2013; Beccari et al., 2018a; Bottalico and Perrone, 2002; Kulik, 2008; Zemánková and Lebeda, 2018). Some of them are well known (i.e.: FHB- see Chapter 1), while others are less common and studied.

In Turkey, this species has been reported as causal agents of crown rot on wheat (*Triticum* spp.) (Shikur Gebremariam et al., 2018). Recent studies highlight the pathogenicity of this *Fusarium* species on pumpkin (*Cucurbita moschata* Duchesne), garlic (*Allium sativum* L.), carrot (*Daucus carota* L.). In Korea, *F. tricinctum* has been studied as the causal agent of severe post-harvest fruit rot in pumpkins (Aktaruzzaman et al., 2018). In France, this species has been indicated as cause of rots and wilt in carrots for seeds production. Diseased plants become dried prematurely compromising seed development (Le Moullec-Rieu et al., 2019). In Serbia, during 2016, infected garlic occurred in storage and warehouses. Garlic bulbs and cloves were softened, spongy, or sunken and covered with white, light pink or reddish mycelium. The disease was proven to be caused by *F. tricinctum* (Ignjatov et al., 2017). Recently, in China, *F. tricinctum* has been described as causal agents of several plant disease on rice (*Oryza sativa* L.), lily (*Lilium sp*. L.), lotus (*Nelumbo nucifera* Gaertn.), and alfalfa (*Medicago sativa* L.). On rice, this species has

been reported as agents of seedling blight (Li et al., 2019). Symptoms that ranged from wilting and small lesions (black spots) up to internal browning were observed on lotus roots, during the post-harvest (Li et al., 2016). Lily presented symptoms of wilting, stem and root rot, brown spots, rot and spalling on bulbs, plus a progressive yellowing and defoliation of the leaves from the base (Li et al., 2013). Alfalfa showed chlorotic, stunted and wilted leaves and root rot symptoms (brownish or reddish water-soaked lesions and white mycelium) (Cong et al., 2016). In Italy, *F. tricinctum* has been described as the causal agent of the pink root on onion (*Allium cepa* L.). Typical symptoms of this disease include pink discoloration and necrosis on rotten roots, reddening of the outer layers of the bulb and stunting plants (Carrieri et al., 2013).

***Fusarium acuminatum* Ellis & Everhart.**

Found in temperate region, *F. acuminatum* is reported not only as a soil saprotroph fungus but also as a maize pathogen in Germany, Nigeria and Iran, as causal agents of crown rot in Europe, USA and Australia and as weak pathogen in FHB small grain cereals (Adejumo et al., 2007; Akinsanmi et al., 2004; Bec et al., 2015; Beccari et al., 2019; Marín et al., 2012; Williams et al., 2002). In addition, this species has been reported as pathogen on several legume species and pumpkin (Leslie and Summerell, 2006; Okello and Mathew, 2019). *F. acuminatum,* included in the polyphyletic section *Gibbosum* as well as *F. equiseti*, is characterized by a toxigenic profile and genetic features that made it more closely related to *F. avenaceum* and *F. tricinctum*. For this reason, *F. acuminatum* has been included in the FTSC as FTSC 2 (O'Donnell et al., 2018). Morphologically, this species rarely produces microconidia. For this reason, this feature cannot be used as an identification character. Macroconidia, similar to those produced by *F. avenaceum*, formed in pale orange sporodochia, are characterized by 3 to 5 septa, a dorsoventral moderate curvature and a distinct foot shape. The production of chlamydospores appears slow. On PDA, as all members of FTSC, *F. acuminatum* presents a slow growth rate, a white to pink abundant mycelium (Harrow et al., 2010; Leslie and Summerell, 2006).

Few phylogenetic studies have been conducted on this species. Marin and co-workers (2012) highlight the homogeneity of Spanish *F. acuminatum* population without any relation with geographic or host origin suggesting, according to Harrows and co-workers, that its low spread could be due to the biogeographic limitation and the low genetic variability (Harrow et al., 2010).

This specie has been reported as an ENNs, MON, BEA, T-2 and HT-2, diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MON), neosolaniol (NEO) producer (Bottalico and Perrone, 2002; Marín et al., 2012). Its ability to biosynthesize type B trichothecenes such as DON is still unclear and discussed. Although at low level, Marin and co-workers (2012) observe DON production in 7 out of 12 analyzed strains. However, further studies will be necessary to confirm these data.

***Fusarium flocciferum* Corda**

In the old classification, *F. flocciferum* had been classified as member of section *Gibbosum* as well as *F. equiseti* although the different metabolic profile (Kristensen et al., 2005). Instead, accurate studies suggested similarity in genetic and secondary metabolites production between *F. flocciferum* and members of FTSC. On the basis of these data, *F. flocciferum* was included in the previous complex (O'Donnell et al., 2018; Summerell, 2019). From a morphological point of view, this species is characterized by the presence of long macroconidia 3-7 septate.

This species, common in temperate regions, has been found associated to FHB and wheat crown and root rot in several countries (Dehghanpour-Farashah et al., 2020; Shikur Gebremariam et al., 2018). In details, Dehghanpour-Farashah and co-workers (2020) observed a high level of aggressiveness on seedling of this species. Recently, *F. flocciferum* has been reported as causal agent of root rot in pea (*Pisum sativum* L.) and fava bean (*Vicia faba* L.) (Šišić et al., 2020).

F. torolosum was included by Wollenweber & Reinking (1935) in the section *Discolor* as well as, *F. graminearum* and *F. culmorum* although it is a non-trichothecene producer species. Instead, *F. torolosum* appears to be closely related to *Fusarium tricinctum* and *Fusarium acuminatum (O'Donnell et al., 2013)*. In fact, the differentiation between these species based on morphology and molecular markers has been shown to be difficult (Harrow et al., 2010; Kristensen et al., 2005; Yli-Mattila et al., 2002). From a morphological point of view, this species could be confused with *F. culmorum* and *F. sambucinum* due to the similarity of colony morphology and macroconidia shape (Leslie and Summerell, 2006).

Even if not included by O'Donnell et al (2018) in the FTSC, *Fusarium reticulatum* appears closely related to the latter complex sharing several morphological and cultures

characters. It has been reported as pathogen on U.S, Canadian and Brazilian cereals and on *Medicago* seeds (Bec et al., 2015; Lamprecht, 1986; Pereira et al., 2020; Strausbaugh et al., 2005). This species is characterized by short macroconidia, curved and with a less defined apical cell while microconidia and chlamydospores are rarely formed (Bec et al., 2015; Gräfenhan et al., 2013).

In addition, following Yli-Mattila and co-workers research, *F. anguioides* (old section Artrosporiella) could be considered a real species despite the several morphological and genetic similarity with *F. avenaceum* (Yli-Mattila et al., 2018, 2002).

Gerlach and Nirenberg described *F. arthorosporiodes* as new species. However, inadequate morphological features and molecular marker have been identified in order to distinguish this species from the closely related species as *F. avenaceum*. Morphological differences such as the absence of orange color of sporodochia and pyriform conidia of *F. arthrosporioides* are not enough to describe a new monophyletic species (Yli-Mattila et al., 2002). Moreover, the hypothesis of a novel species have been not supported by several phylogenetic studies based on partial DNA sequences of *acl1* and *TEF1a*, *β-tub* and enzyme enniatin synthetase (*Esyn*1) genes confirming that *F. arthorosporioides* could form a distinct lineage in the clade but that the latter and *F. avenaceum* belonged to a single phylogenetic species (Gräfenhan et al., 2013; Kristensen et al., 2005; Niessen et al., 2012; Stakheev et al., 2016; Yli-Mattila et al., 2018, 2002).

The introduction of GCPSR allowed the identification of potentially novel species and the detection of novelty within the already known species complex. Unnamed and not well described species (FTSC 1, FTSC 5 and FTSC 11) were included in the complex by O'Donnell et al 2013, 2018.
Within Iranian fungicolous isolates, Torbati and co-workers described two novel species closely related with the member of FTSC: *Fusarium iranicum* and *Fusarium gamsii.* The first one differs from *F. flocciferum* for the production of less septate and strongly curved macroconidia; the second one, morphologically similar to *F. torolosum*, is characterized by the presence of slender conidia with long apical and basal cells (Torbati et al., 2019).

2.1.5 FTSC: phylogeny history

As already mentioned, a classification based mainly on morphological features could be influenced by many factors like the culture medium, the incubation parameters, unique strains characteristics causing an incorrect identification of the isolates. In addition, the phylogenetic identification could be complicated for those species complex poor represented in online database such as FTSC (Geiser et al., 2004).

Considering the high genetic similarity among the species complex, the phylogenetic analysis becomes an essential tool in order to obtain a unique isolates identification. Several genes were tested to defined species boundaries among the FTSC.

In 1998, Turner and co-worker (1998) proved that *ITS* gene was not sufficient to adequately distinguish between *F. avenaceum* and *F. tricinctum*.

In 2002, Yli-Mattila and co-workers carried out a phylogenetical study based on *ITS, IGS, mtSSU* and *β-tub* sequences with the aim to distinguish *F. avenaceum, F. arthrosporioides, F. anguioides, F. tricinctum, F. graminum* and *F. acuminatum* strains. According to Turner et al (1998), also in this study, *ITS* was inadequate to separate *F. tricinctum* from the other species while a higher resolutive power was observed in phylogenesis based on *IGS* and *β-tub*. Moreover, *mtSSU* was not able to separate *F. tricinctum* from *F. avenaceum* strains. In details, they concluded that phylogenetic relationship within species can be better resolved by *IGS* region, characterized by a greater variability, while *mtSSU*, *ITS* and *β-tub* could be more useful at species level (Yli-Mattila et al., 2004, 2002). Another gene successfully used in phylogenetic analysis among members of *Fusarium* genus is the *ATP-citrase lyase 1* gene (*ACL)* involved in the formation of cytosolic acetyl-CoA and, subsequentially, in the fatty acids and sterols biosynthesis. The presence of a single, large intron (300-400 bp) in the larger *acl1* subunit, allow to identified species boundaries (Gräfenhan et al., 2011; Niessen et al., 2012).

As already demonstrated for *F. graminearum, F. pseudograminearum, F. culmorum* and *F. cerealis, partial phosphate permease* gene (*PHO*) is characterized by a high resolutive power for both inter and intra and inter specific analysis. On the basis of these previous studies, Stakheev and co-workers (2016) carried out a phylogenetic study on FTSC members based on *PHO* gene that demonstrated to be more phylogenetic informative than *TEF1a* and *β-tub* (high number of variable and parsimony informative sites).

The high genetic similarity is also testified by the difficult to design specific primers for each species of the complex. The availability of primers specific is made more difficult not only by the high interspecific homology among *Fusarium* species but also by the continuous changing in phylogenetic structure of the closely related species and the evolution in molecular analysis. Several genes were analyzed in order to design specific PCR system for both diagnostic and identification on closely related species. Among them: *ITS, Esyn1, IGS* rDNA, *acl1, TEF1a.*

Species-specific primers for *F. avenaceum* were designed by Schilling and co-workers (1996) based on *ITS* sequences. However, their specificity was later denied considering their inability to differentiate *F. avenaceum* from *F. tricinctum* (Cullen et al., 2005; Turner et al., 1998).

Kulik and co-worker (2007) focused their attention on *Esyn1* gene encoding for the multifunctional enzyme enniatin synthetase to obtain species-specific primers for the detection of species able to produce ENNS. However, cross-reaction was observed between *F. avenaceum* and *F. tricinctum* (Kulik et al., 2007). Later, primers based on single nucleotide-polymorphism of *IGS* rDNA were designed to distinguish between *F. tricinctum* and the species closely related. In this case too, false positive amplicons were obtained from two isolates identified as *F. acuminatum* and *Fusarium nurragi* (Kulik, 2008). Initially, this last was defined as subspecies of *F. avenaceum*, on the basis of morphological characteristics such as macroconidia shape. Later, considering the genetic differences in both nuclear and mitochondrial genomes, *F. nurragi* was reintroduced as species (Benyon et al., 2000). In 2011, Stakheev and co-workers designed specific qPCR primers based on *TEF1a* sequences aiming to detect species with a similar metabolic profile such as *Fusarium avenaceum/Fusarium tricinctum* (Stakheev et al., 2011).

Based on interspecific divergence among *CYP51C* gene in *Fusarium* genus, specific PCR primers for a simultaneous identification of *F. avenaceum* and *F. tricinctum* were designed. However, the primers were tested on few isolates and other species of the complex such as *F. acuminatum* or *F. flocciferum* were not included in the analysis for the primer specificity (Fernández-Ortuño et al., 2011).

In 2012, Niessen and co-workers, as an alternative to classic PCR, applied the Loop-mediated isothermal amplification (LAMP technology) for the specific detection of *F. tricinctum* using primers designed on *acl1* gene. However, cross reaction with 8 out of 13 *F. acuminatum* isolates and 1 out of 2 *F. reticulatum* strains was observed. In addition,

only 21 of the 23 *F. tricinctum* isolates included in the analysis were detected (Niessen et al., 2012).

On the contrary, no cross-reactivity was observed in qPCR assay developed by Stakheev and co-workers (2016) who, on the basis of *PHO* gene, designed a set of four primer pairs and probes for the detection of *F. avenaceum*-*F. arthrosporioides*, *F. tricinctum*, *F. acuminatum* and *F. torulosum.*

2.1.6 Mycotoxins production within *Fusarium tricinctum* species complex

The members of FTSC have been reported as "emerging mycotoxins" producers, where with the term "emerging" we referred to the mycotoxins that are not routinely monitored and are not subject to any regulation. Among them, enniatins (ENNS), beauvericin (BEA) and moniliformin (MON) are the most detected worldwide. Moreover, even if these secondary metabolites, in particular ENNS, are frequently detected in food, feed, and grains, to date, no legal maximum levels have been set (Gruber-Dorninger et al., 2017).

BEA and ENNS are cyclic hexadepsipeptide showing a similar chemical structure. They are synthesized by *Esyn1* gene which consists of a polypeptide chain with a molecular mass of 347 kDa (Liuzzi et al., 2017). Twenty-nine enniatin analogues have been identified; seven of those (A, A1, B, B1, B2, B3 and B4) have been found in cereals, and four of them (A, A1, B and B1) are also frequently detected in foods and feeds (EFSA, 2014; Covarelli *et al.*, 2015).

Most of the bioactivities are related to the ionophoric properties of ENNs. In details, the oxygen atoms of the carbonyl groups and the tertiary amino nitrogen of the amide bonds have free electron pairs that can act as nucleophiles and form weak chemical (ion-dipole) interactions with cations. This is noteworthy because, due to this chemical conformation, BEA and ENNs can exploit their toxic effects, acting as ionophores and promoting the transport of cations through the membranes. This leads to an abnormal increase of cations in the cells, compared to their physiological level, affecting its ionic homeostasis and so bringing to an increase of the calcium cations, which activate the calcium dependent endonucleases causing subsequent DNA fragmentation, a typical step of apoptosis (Gruber-Dorninger et al., 2017).

The ENNS and BEA toxicity is not yet fully understood however several *in vitro* studies were conducted. Ivanova *et al.* (2006) demonstrated that their cytotoxicity is comparable to DON while Svingen et al. (2016) observed a significant citoxicity of ENNB and BEA compared to aflatoxin B1 in a quadroprobe assay. Prosperini *et al.* (2013) proved that the

presence of BEA and ENNs in human colon adenocarcinoma can increase the Reactive Oxygen Species (ROS) production and cause cell death, induced by a mitochondrial dependent apoptotic process as result of the reduction of mitochondrial membrane potential (Ivanova et al., 2006; Prosperini et al., 2013). The toxic effect on the reproductive system has been observed (Fraeyman et al., 2017). In addition, due to the iohophoric proprieties of ENNS, the synergistic interaction with other mycotoxins should be not underestimated (Gautier et al., 2020). Moreover, considering the lack of *in vivo* studies, the EFSA Panel on Contaminants in the Food Chain (CONTAM) concluded that: "due to the lack of studies, acute exposure to BEA and ENNs does not indicate concern for human health, but there might be a concern respect the chronic exposure but no firm conclusion could be drawn" (Gruber-Dorninger et al., 2017). For this reason, no limits in food and feed have been established.

Even if these mycotoxins have to be studied further for what concerns human and mammal toxicity, many other properties are well documented (Uhlig et al., 2007). Over the phytotoxic activity demonstrated against wheat growth and promotion of root rot (Burmeister, H. R. & Plattner, 1987), BEA and ENNS exhibit a wide range of biological activities such as insecticidal activity against blowflies and tiger mosquito (Grove and Pople, 1980), antibiotic activity against different fungi (among which also some species of *Fusarium* and *Botrytis cinerea*) and bacteria (a drug, called Fusafungine, particularly active against some species of *Streptococcus*, was marketed against oral infections even if now is not anymore). Moreover, BEA and ENNs have strong inhibitor activity of acyl-CoA cholesterol acyltransferase (ACAT) that can be useful for the treatment and prevention of atherosclerosis and hypercholesterolemia, and their activity as ionophores is studied for its anticancer properties (Sy-Cordero et al., 2012).

About 30 *Fusarium* species, belonging to 8 different *Fusarium* species complex (included FFSC and FTSC), have been reported as ENNS producers (Gautier et al., 2020). However, several studies reported *F. avenaceum* and *F. tricinctum* as main ENNS producers although the high variability of the toxigenic potential linked with the single isolate (Beccari et al., 2018a; Covarelli et al., 2015b; Jestoi, 2008; Stepień and Waśkiewicz, 2013; Uhlig et al., 2006). As well as for all secondary metabolites, the biosynthesis of ENNs is regulated, at genetic level, by several mechanisms in response to external factors such as temperature, water activity, *pH* and both nutrient composition and availability. Kokkonen ad co-workers (2010), in an *in-vitro* trial, observed that the ENNs biosynthesis by *F. avenaceum* was independent from water activity and

temperatures concluding that this species is able to produce mycotoxins in many environmental conditions. This ability could be linked with the cosmopolitan character of these species that is able to contaminate a wide variety of host plants in different pedo-climatic conditions (Gautier et al., 2020; Kokkonen et al., 2010).

In addition, the high mycotoxins potential of *F. avenaceum* in response to environmental signals is confirmed by the genomic data how have showed an over-representation of genes involved in stress-related response (Lysøe et al., 2014). To date, the role of ENNS in pathogenesis on cereal has not been proved. However, Hermann et co-workers (1996) observed the involvement of ENNS in the virulence of *F. avenaceum* on potato (Herrmann et al., 1996).

Several studies have been conducted worldwide to monitor the level of emerging mycotoxins in food and feed. In particular, the researchers focused their attention on cereals and cereal derivates. For what concern the North European countries (Denmark, Norway and Finland) the observed ENNS gradient was often the same B1>B>A1>A (Gautier et al., 2020). Svingen and co-workers detected ENNB and ENNB1 in the 100% and the 91% of the Danish grain samples, respectively (Svingen et al., 2016); great amount of ENNB was found in Norwegian and Finnish grain samples were the highest concentration was of 5800 ppb and 18,300 kg respectively (Jestoi, 2008; Jestoi et al., 2004; Uhlig et al., 2006). The same trend was observed by Orlando and et al. (2019) who analysed a huge number of small grains cereals samples harvested in France from 2012-2014. In details, *F. tricinctum* was identified as main ENNS producers in barley and durum wheat (Orlando et al., 2019). ENNS were detected also on all Swedish wheat samples collected in 2009 and 2011. In addition, a strong correlation between *F. avenaceum* + *F. tricinctum* DNA and these mycotoxins was observed in the same study (Lindblad et al., 2013). According to North Europe data, also the survey conducted on Canadian durum wheat reported ENNB as the major depsipeptide followed by ENNB1 (Tittlemier et al., 2013). On the contrary, in the Mediterranean countries ENNS of group A appears to be most prevalent compared to ENNS of group B. Covarelli and co-workers (2015a) observed a variability in ENNS gradient depending on cultures and year of cultivation. In 2009 the gradient was ENNA>ENNA1>ENNB>ENNB1 and ENNA>ENNB>ENNA1>ENNB1 in durum and soft wheat respectively, while, in 2010, the trend was the same for both cultures (ENNB>ENNA>ENNB1>ENNA). On Spanish cereal samples, ENNA1 was the most detected (max value 814.42 ppm on rice sample) followed by ENNB, ENNB1 (Meca et al., 2010). The same trend was observed in raw

cereals collected in Morocco: ENNA1 contaminated the 39% (maximum value 445 ppm on corn) (Zinedine et al., 2011). In the same country, for the first time in 2011, emerging mycotoxins were detected also in rice. However, in this study, the most common was ENNB, present in the 30% of analysed samples, while the highest detected value was for ENNA (448.7 ppm) (Sifou et al., 2011).

For what concern the Italian situation, recent studies suggested an increase in the ENNS contamination on wheat and barley. In details, depsipeptides were detected in the 100% of durum wheat samples harvested in North and Central Italy (Emilia Romagna and Umbria region) and the 30% of samples from South Italy (Sardinia region) with average values of 323, 56.4 and 0.23 ppb, respectively. In Emilia Romagna and Umbria regions, the gradient observed was ENNB1>ENNB>ENNA1>ENNB2>ENNA>ENNB3 (see Chapter 1). Relatively high occurrence of ENNS (51% of positive samples- ENNB the main analogous) and *F. avenaceum* isolates was recovered in malting barley harvested in 2013 (Beccari et al., 2016). In 2014, ENNS were detected in all barley samples with ENNB principal analogue (max value 73 ppb). Also in this case, an high incidence of species able to produce these secondary metabolites was detected; in details, *F. avenaceum*, *F. tricinctum* and *F. acuminatum* constituted for the 32.5 % the FHB community (Beccari et al., 2018).

Considering that ENNS and BEA are produced by the same *Fusarium* species and by the same metabolic pathway a high co-occurrence is expected (Jestoi, 2008).

However, BEA concentration is usually lower than those of ENNS with a maximum concentration in the ppb range (EFSA, 2014; Jestoi et al., 2004; Meca et al., 2010; Svingen et al., 2016; Uhlig et al., 2006). Among the European and Mediterranean cereal samples, the higher BEA concentration was reported on maize and oat; in details, 327 ppb in Sweden oats samples (Fredlund et al., 2013) and 73.9 ppb on Mediterranean maize (Serrano et al., 2013). Several studies confirmed a low level of BEA contamination also in Italian small grains samples (durum and soft wheat, barley) (Beccari et al., 2020, 2018b, 2016; Covarelli et al., 2015b; Juan et al., 2013). In all the cited studies, the average concentration of BEA was lower than 10 ppb except for malting barley harvested in 2013 were an average value of 54 ppb was observed (Beccari et al., 2016).

Moniliformin is a sodium or potassium 1-hydroxycyclobut-1-ene-3,4 dion salt, isolated for the first time in 1972 from a *F. moniliforme* (now *F. verticilliodes*) strain (Fraeyman et al., 2017). This metabolite has been proved to cause the inhibition of thiamine pyrophosphate depending enzymes, such as pyruvate dehydrogenase. Moreover, this

molecule permits the entrance of pyruvate into the Krebs's cycle and therefore, if inhibited, a decrease in mitochondrial respiration is recorded. In addition, it inhibits gluconeogenesis, aldose reductase and glutathione peroxidase and reductase. Chronic exposure resulted in intestinal problems, immunosuppression and myocardial lesion, necrosis and death (Gruber-Dorninger et al., 2017; Jestoi, 2008; Jestoi et al., 2004). In addition, moniliformin intoxication has been put in relation with Keshan disease, due to the similarities of the lesions on animals during toxicity tests and heart lesions on people, affected by this disease (Uhlig et al., 2007). Natural occurrence of MON has been reported worldwide, however, data are collected mostly in northern Europe (Gräfenhan et al., 2013). In details, Uhlig and co-worker (2004) observed a high prevalence of moniliformin especially in Norwegian wheat (maximum value 950 ppb) harvested in 2000 and 2001. In the same years, Jestoi and co-workers (2004) collected Finnish grain samples to investigate the presence of emerging mycotoxins including MON. The highest levels detected were 750 and 810 ppb in barley (2002) and soft wheat (2001), respectively. Different amounts of MON were detected also on Swedish oat and wheat with incidence and average values depending on cultivation year and cultures (Fredlund et al., 2013; Lindblad et al., 2013). In addition, a great correlation between *F. avenaceum* on Swedish wheat ad MON levels was observed by Lindblad and co-workers (2013) corroborating the data reported by Uhlig et al. (2004) on Norwegian cereals and Jestoi et al. (2004) on Finnish wheat. Surprisingly, a lack of relationship between the presence of *F. avenaceum* and the MON produced was observed on Canadian oats and rye where the measured concentration of this mycotoxin was similar to those reported in the European country. In Italy, MON was detected only in 5 up to 43 and 12 up to 52 barley samples harvested in 2013 and 2014, respectively (average value of 317 and 5 ppb) (Beccari et al., 2018b, 2016). A significantly higher amount of MON was also detected on North Italian durum wheat samples (max value 610 ppb) compared with those collected in Central and South Italy (See Chapter 1).

2.2 Species diversity and mycotoxin production by members of the *Fusarium tricinctum* species complex

2.2.1 Introduction

Fusarium head blight (FHB) is a global cereal disease caused by a complex of *Fusarium* species resulting in high yield losses and reduction in quality mainly due to mycotoxin contamination of grain (McMullen et al., 2012). Geographic distribution of the etiological agents appears to be related to climatic conditions such as temperature and humidity (Bakker et al., 2018; Vaughan et al., 2016). Although the principal species responsible for FHB in Europe are *F. graminearum*, *F. culmorum* and *F. poae* (Bottalico and Perrone, 2002), members of the *F. tricinctum* species complex (FTSC) have become increasingly important contributors to FHB (Beccari et al., 2018a, 2016). These include several formally named species with Latin binomials (*F. acuminatum*, *F. tricinctum*, *F. avenaceum*, *F. iranicum*, *F. flocciferum*, and *F. torulosum*) (Leslie and Summerell, 2006; Torbati et al., 2019) and several unnamed phylospecies. An informal ad hoc nomenclature using Arabic numerals (e.g. FTSC 1-to-11) was proposed to distinguish the named and unnamed species within this complex (O'Donnell et al., 2018; Stakheev et al., 2016).

F. avenaceum has been reported from the cooler regions of Northern Europe, Canada and Central Europe (Gräfenhan et al., 2013; Karlsson et al., 2017; Kosiak et al., 2003; Logrieco et al., 2002; Uhlig et al., 2007; Yli-Mattila et al., 2004). This species and *F. tricinctum* are saprotroph and plant pathogens of a variety of hosts including barley and wheat (Leslie and Summerell, 2006; Uhlig et al., 2007). However, an increase in their incidence has also been reported recently in warmer regions throughout the world (Beccari et al., 2016, 2017, 2018a,b; Cerón-Bustamante et al., 2018; Cowger et al., 2020; Harrow et al., 2010). *F. acuminatum* has also been recovered from cereals in Canada, Italy and Spain, but at a lower frequency compared to *F. avenaceum* and *F. tricinctum* (Beccari et al., 2018a,b; Gräfenhan et al., 2013; Marín et al., 2012).

Members of the FTSC complex produce several "emerging" mycotoxins (Jestoi, 2008), including moniliformin (MON) and enniatins (ENNs) that pose a threat to food safety and human health (Covarelli et al., 2015; Gautier et al., 2020; Jestoi, 2008; Kokkonen et al., 2010; Logrieco et al., 2002; Uhlig et al., 2007). Of the 29 ENN analogues characterized to date, A, A1, B and B1 are the most commonly reported due to their widespread occurrence (Liuzzi et al., 2017; Sy-Cordero et al., 2012). ENNs are able to increase oxidative stress, induce cell apoptosis and cause mitochondrial dysfunction in

mammals, while a main target of MON is cardiac muscle where its toxicity has been shown to vary among different cell lines (Gautier et al., 2020; Gruber-Dorninger et al., 2017; Jestoi, 2008; Uhlig et al., 2004; Wu et al., 2018). In addition, FTSC taxa have been reported to produce other toxic secondary metabolites including aurofusarin, chlamydosporol, 2-Amino-14,16-dimethyloctadecan-3-ol, and antibiotic Y (Beccari et al., 2018a,b; Munkvold, 2017; Uhlig et al., 2005).

Because of the morphological similarity, identification of FTSC isolates based exclusively on morphological data poses a daunting challenge even to skilled *Fusarium* taxonomists (Stakheev et al., 2016). For this reason, DNA sequence data from several marker loci have been used to resolve phylogenetic relationship within the FTSC, including *ACL1*, β-tubulin, *ITS* rDNA, *PHO*, *RPB1*, *RPB2*, *TEF1*, but with varying success (Bakker et al., 2018; Gräfenhan et al., 2013; Harrow et al., 2010; Niessen et al., 2012; O'Donnell et al., 2018; Yli-Mattila et al., 2018, 2002). Genealogical concordance phylogenetic species recognition (GCPSR); (Taylor et al., 2000) has become the gold standard for identifying exclusive species level lineages within *Fusarium* that may differ in pathogenicity and production of toxic secondary metabolites (O'Donnell et al., 2018). Although several multilocus molecular phylogenetic studies have been conducted to assess FTSC genetic diversity in Northern Europe (Gräfenhan et al., 2013; Stakheev et al., 2016; Yli-Mattila et al., 2018), similar studies have not been conducted in Italy. Given the increased detection of FTSC species in FHB pathogen surveys within Italy over the past 10 years, the risk posed by toxin contaminated cereals remains to be determined. Therefore, the objectives of this study were to: a) assess the phylogenetic diversity and evolutionary relationships of Italian FTSC isolates recovered from symptomatic wheat and barley via analyses of multilocus DNA sequence data (*TEF1*, *RPB1* and *RPB2*); and b) determine their ability to produce mycotoxins on rice.

2.2.2 Materials and Methods

2.2.2.1 Fungal isolates

A total of 123 single-spore isolates belonging to the FTSC were collected and analyzed phylogenetically in this study together with sequences of 17 reference isolates (Table 1). Isolates marked with "F" (N = 68) are part of a collection of the Department of Agricultural and Food Science (University of Bologna - *Alma Mater Studiorum*). Of the 50 "P" strains, 43 were isolated from wheat and barley harvested in Italy during the 2017/2018 growing season. Six strains from Iran (five "R" and one "F") were included

in this study to determine whether they represent FTSC species present in both countries. Isolates were deposited in the culture collection of the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy. P-strains were isolated from 21 durum wheat (*Triticum durum* Desf.) samples from seven different climatic regions in Italy (Fig. 2; Table 1). Pathogen surveys were conducted in Northern (Lombardy, Veneto and Emilia Romagna), Central (Abruzzo and Molise) and Southern Italy (Campania and Apulia). P strains were isolated as described by Beccari et al. (2016). In brief, a 50 g sub-sample of kernels from each wheat sample was randomly selected for plating. Kernels were surface sterilized in a water-ethanol (95%)-sodium hypochlorite (7%) solution (82:10:8% vol.) for 2 min and then rinsed twice in sterile water for 1 min with each exchange. After the kernels were blotted dry using sterile paper towels, they were placed on potato dextrose agar (PDA, Biolife Italiana, Milan, Italy) supplemented with streptomycin sulphate (0.16 g/L, Sigma Aldrich) in Petri dishes. The 100 kernels analyzed from each wheat sample were divided evenly among 10 Petri dishes. Petri dishes were incubated at 22 °C in the dark and after 5 days examined for fungal growth using a stereomicroscope. All isolates identified as *Fusarium* were transferred to new PDA plates, incubated at 22 °C in the dark for 7 days and then single-spored. The set isolates identified morphologically as members of the FTSC, using criteria described by Leslie and Summerell (2006), were subjected to further molecular analysis.

Figure 11: Geographic distribution of the P strain origin across Italy peninsula.

2.2.2.2 Genomic DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from mycelium of the 123 single-spored isolates after 7 days growth on PDA using a CTAB (cetyltrimethylammonium bromide) protocol (Gardes and Bruns, 1993) with slight modifications. Portions of the following three phylogenetically informative genes were PCR amplified and sequenced to identify the 123-strain set: translation elongation factor 1-*α* (*TEF1*), DNA- directed RNA polymerase I largest (*RPB1*) and second largest (*RPB2*) subunits. Amplicons were obtained using the following primer pairs: EF-1 (5′ ATGGGTAAGGAGGACAAGAC-3′) × EF-2 (5′-GGAAGTACCAGTGATCATG-3′) for *TEF1* (O'Donnell et al., 1998)*;* 5f2 (5′-GGGGWGAYCAGAAGAAGGC-3′) × 11ar (5-′ GCRTGGATCTTRTCRTCSACC-3′)

for *RPB2* (Liu et al., 1999; Reeb et al., 2004); and Amp3f (5'-GAYTACATCTTCAAYCGTCAGCC-3') × Amp3r (5'-GTTCTTGGAHGACACACCRGCG-3') for *RPB1*. *RPB1* PCR primers were designed in this study aligning *RPB1* sequences of 16 FTSC isolates and two closely related species, *F. graminum* NRRL 20692 and *F. heterosporum* NRRL 20693 from the *F. heterosporum* species complex (FHSC). In addition, all isolates were screened for their potential ability to produce ENNs by PCR amplification of a portion of the *Esyn1* gene, which is a nonribosomal peptide synthetase involved in ENNs biosynthesis (Kulik et al., 2007).

Each 25 μL PCR reaction contained 50 mM $MgSO_4$, 2 mM of each deoxynucleoside triphosphate, 0.6 mM of each primer pair, 1 U of *Taq* High Fidelity polymerase (Invitrogen) and 50 ng of genomic DNA. Reactions were carried out using the following PCR cycling conditions: EF-1/EF-2 primer pairs, 96 °C 2 min, 94 °C 30 s, 56 °C 30 s, 68 °C 60 s (35 cycles), 68 °C 60 s, followed by a 4 °C soak; for the 5f2/11ar and Amp3f/Amp3r primer pairs, 96 °C 2 min, 94 °C 30 s, 56 °C 30 s, 68 °C 60 (40 cycles), 68 °C 60 s, followed by a 4 °C soak; and for the esya1/esya2 primer pair, 96 °C 2min, 96 °C 30 s, 58 °C 30 s, 68 °C 60 s (35 cycles), 68 °C 60 s, followed by a 4 °C soak. Amplicons and a 1-10 kb ladder (Invitrogen) were separated by electrophoresis in a 1.5% agarose gel that was run at 80 V for 1 h, stained with ethidium bromide and then visualized over a UV transilluminator. Once amplicons were purified with MultiScreen-PCR_{96} filter plates (Millipore), they were sequenced using a Big Dye Terminator Sequencing kit ver. 3.1 (Applied Biosystems) and then analyzed with an ABI 3730 DNA Analyzer (Applied Biosystems). ABI chromatograms were edited using Sequencher (ver. 5.0, Gene Codes, Ann Arbor, MI), exported as fasta files that were aligned using MUSCLE (Edgar, 2004). Sequences were used to conduct BLASTn queries of *Fusarium MLST* (https://fusarium.mycobank.org/) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to obtain preliminary identifications of the 123 FTSC isolates (O'Donnell et al., 2015).

2.2.2.3 Phylogenetic analyses

Aligned sequences of the 123 FTSC isolates collected in this study were combined with those of sequences from 17 FTSC reference strains (Table 1) (O'Donnell et al., 2018; Torbati et al., 2019) and then analyzed via maximum likelihood bootstrapping (ML-BS)

using IQ-TREE 1.6.12 (Nguyen et al., 2015) (http://www.iqtree.org/). Once the best-fit model of molecular evolution was determined for *TEF1* (TIMe+G4), *RPB1* (TNe+G4) and *RPB2* (TNe+I+G4), using ModelFinder (Kalyaanamoorthy et al., 2017) based on the Bayesian information criterion (BIC) scores (Chernomor et al., 2016), a combined partition ML-BS analysis was conducted with IQ-TREE ver. 1.6.12. Statistical support for the branches was evaluated by conducting a ML-BS bootstrap analysis of 5000 replicates. Sequences of two outgroup species from the FHSC, *F. graminum* NRRL 20692 and *F. heterosporum* NRRL 20693 were chosen for rooting the trees, and sequences of *F. nurragi* NRRL 36452, the closest sister lineage to the FTSC (Geiser et al., 2020), were also included in the analyses.

2.2.2.4 Production of mycotoxins and other biologically active secondary metabolites in vitro

Based on results of the phylogenetic analyses, 59 isolates that included representatives 10 FTSC phylospecies were selected to evaluate their ability to synthesize mycotoxins and several bioactive secondary metabolites in vitro on rice cultures. After strains were grown on V8 juice agar plates (20% V8 juice, 0.3% $CaCO_3$, 2% agar) for 7 days at 28 °C, two 5 mm diam plugs from each plate were placed in dram vials contained autoclaved rice (4.4 g + 1.8 mL of water). After 10-12 days of incubation in the dark at 25 °C, the rice cultures were extracted with 10 mL ethyl acetate for 30 min with shaking. One mL of each extract was transferred to a 1-dram vial and dried under nitrogen with heat. Once the dried extracts were resuspended in 100 µL ethyl acetate, they were analyzed with an Agilent 5873 GC-MS fitted with a HP-5MS column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness) and a 5973-mass spectrometer with an electron impact source. Samples were injected at 150 °C, the temperature was held for 1 min and then the column was heated at 30 °C/min to 280 °C and then held for 7.7 min. Individual peaks in chromatograms were examined and compounds were identified based on retention time, comparison of ion fragmentation patterns with a NIST library, and a library prepared with purified standards. Under these conditions, butenolide (BUT) was detected at 3.10 min, longiborneol (LONG) at 3.94 min, acuminatopyrone (ACU) at 5.23 min, chlamydosporol (CHL) at 5.43 min, and fungerin (FUN) at 5.85 min.

HPLC-MS analysis was performed using a Dionex Ultimate U3000 liquid chromatography system coupled to a QExactive high resolution mass spectrometer

equipped with an electrospray ionization (ESI) source (ThermoFisher Scientific). ENNs and AOD-ol were separated using a Phenomenex Kinetex 2 mm x 50 mm XB-C18 100A column (2.6 µm particle size, 100 Å pore size). Elution of the metabolites was accomplished in a binary gradient flow of mobile phase A [water / acetic acid (99.7: 0.3 v/v)] and mobile phase B [methanol / acetic acid (99.7: 0.3 v/v)], in which the injection volume was 10 µL. The gradient of 20-95% mobile phase B over 5 min was delivered at a flow rate of 0.6 mL/min. The HPLC flow was coupled to the mass spectrometer operated in positive mode utilizing the following parameters: 320 °C capillary temperature, 310 °C heater temperature, and spray voltage of 4.00 kV for positive ESI. For analysis of MON the HPLC utilized a gradient of mobile phase A [water / formic acid (99.1: 0.1 v/v)] and mobile phase B [methanol / formic acid (99.1: 0.1 v/v)]; injection volume: 10 µL, HPLC column: Waters XBridge 4.6 mm x 150 mm BEH-C18 column (5 µm particle size, 130 nm pore size). The gradient of 5-95% mobile phase B over 5 min was delivered at a flow rate of 0.8 mL/min. The HPLC flow was coupled to the mass spectrometer operated in negative mode utilizing the following parameters: 320 °C capillary temperature, 310 °C heater temperature, and spray voltage of -4.00 kV for negative ESI. For both positive and negative mode experiments the mass spectrometer was operated in full MS mode (m/z range 150/2000 and 70,000 resolution). Quantification and identification of each metabolite was performed by comparison to purified standards. Instrument operation and data processing were done using Xcalibur Data Acquisition and Interpretation Software (ThermoFisher Scientific). Limits of quantitation for ENNA, ENNA1, ENNB, ENNB1, AOD-ol and MON were 1 ng/µl.

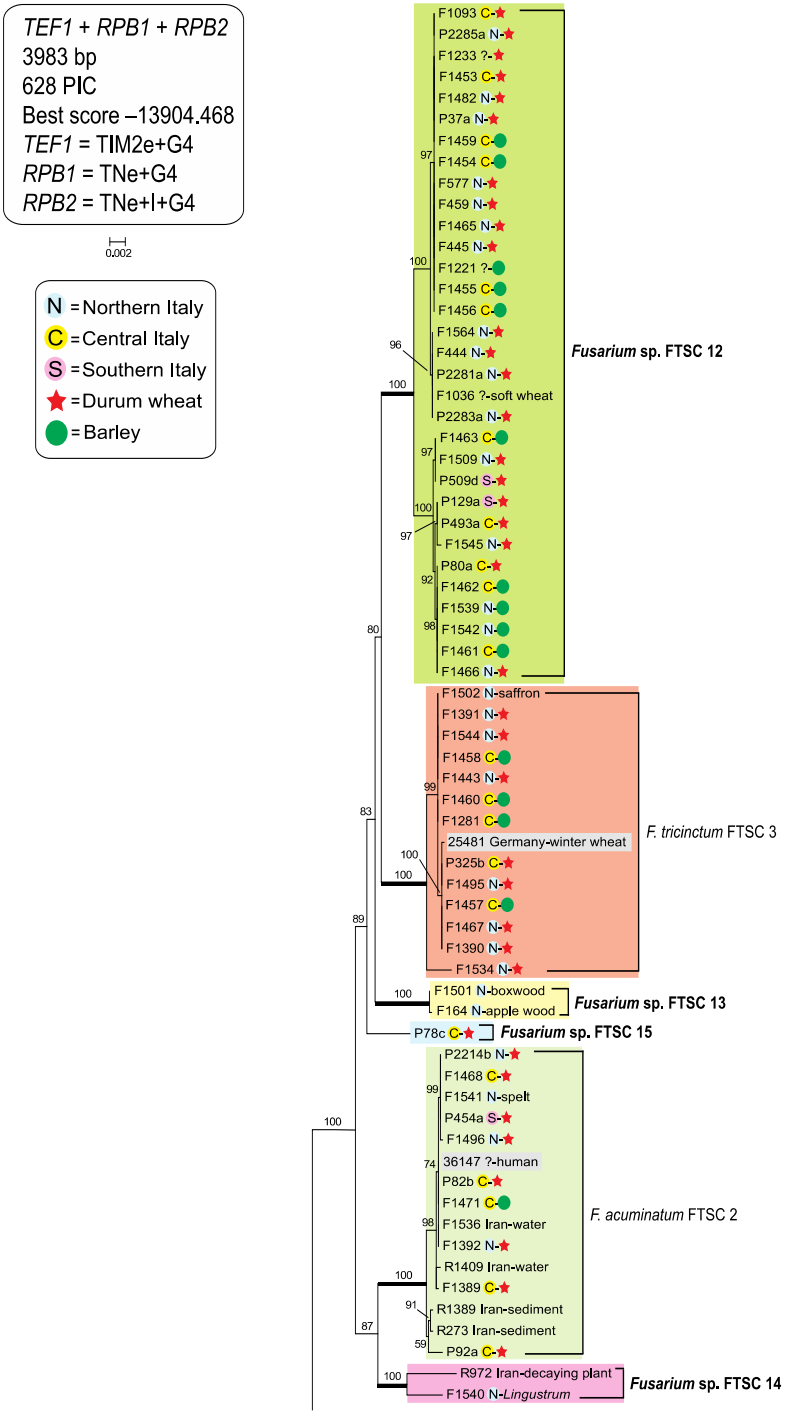
2.2.3 Results

2.2.3.1 Phylogenetic analyses

All the 123 analyzed strains resulted positive for the presence of *Esyn1* gene confirming their potential ability to biosynthesize these secondary metabolites.

Partial *TEF1* sequences of the 117 Italian and six Iranian isolates recovered in our pathogen surveys were used to conduct BLASTn queries of *Fusarium MLST* and NCBI GenBank to obtain preliminary species level identifications. In addition, maximum likelihood bootstrap (ML-BS) analyses of the three individual partitions, which contained sequences of 17 reference strains, identified the optimal model of molecular evolution as TIM2e+G4 for *TEF1* [Supplemental Fig. S1: 684 bp alignment, 138 parsimony

informative characters (PICs)] TNe+G4 for *RPB1* (Supplemental Fig. S2: 1606 bp alignment, 233 PIC) and TNe+I+G4 for *RPB2* (Supplemental Fig. S3: 1693 bp alignment, 256 PIC). These three models were used to conduct a partitioned ML-BS analysis of the combined 3-locus dataset (Fig. 3: 3983 bp alignment, 628 PIC). ML-BS analyses of the three individual and combined dataset resolved the 117 Italian and 6 Iranian isolates, respectively, as 9 and 4 FTSC species (Fig. 3, Table 2). Of the 10 *F. tricinctum* clade species represented by 2 or more stains, *TEF1* strongly supported 8/10 as reciprocally monophyletic (94-100% ML-BS), *RPB1* all 10 (86-100% ML-BS), *RPB2* 8/10 (75-100% ML-BS), and the combined dataset all 10 (99-100% ML-BS). Although *F. gamsii* (FTSC 1) and *F. avenaceum* (FTSC 4) in analyses of *TEF1* and *F. iranicum* (FTSC 6) and *Fusarium* sp. (FTSC 14) in the ML-BS *RPB2* phylogeny were not supported as genealogically exclusive, their monophyly was not contradicted. Four phylospecies accounted for 111/117 (94.9%) of the FTSC from Italy (Fig. 2), and these included in descending prevalence: *F. avenaceum* (FTSC 4, N = 56), *Fusarium* sp. (FTSC 12, N = 32), *F. tricinctum* (FTSC 3, N = 13), and *F. acuminatum* (FTSC 2, N = 10). Except for *F. tricinctum*, which was only found in Northern and Central Italy, isolates of the other three species from wheat (N = 78 total) were collected in Northern, Central and Southern Italy. The same four species were recovered from barley, but in low numbers ranging from 1-to-10, and of the 17 total, 2 and 15, respectively, were from Northern and Central Italy. Five other FTSC species were present in the Italian collection, and these included two isolates of *Fusarium* sp. (FTSC 13), one from *Malvus domestica* (apple wood) and *Buxus sempervirens* (boxwood), and singletons of the following 4 FTSC: *F. iranicum* (FTSC 6) and *Fusarium* spp. (FTSC 11, 14 and 15). The 6 environmental Iranian strains that were typed included *F. acuminatum* (FTSC 2) from water and sediment and one isolate of *F. gamsii* (FTSC 1) from foam and *Fusarium* sp. (FTSC 14) from decaying vegetation (Fig. 2, Table 1). Analyses of the three individual and combined dataset suggests that *Fusarium petersiae* from Dutch soil (Lombard, 2017) is a later synonym of *F. flocciferum* (Fig. 2, Supplemental Figs. 1-3).


TEF1 + RPB1 + RPB2
3983 bp
628 PIC
Best score –13904.468
TEF1 = TIM2e+G4
RPB1 = TNe+G4
RPB2 = TNe+I+G4
0.002
N = Northern Italy
C = Central Italy
S = Southern Italy
= Durum wheat
= Barley
F1093 C-
P2285a N-
F1233 ?-
F1453 C-
F1482 N-
P37a N-
F1459 C-
F1454 C-
F577 N-
F459 N-
F1465 N-
F445 N-
F1221 ?-
F1455 C-
F1456 C-
F1564 N-
F444 N-
P2281a N-
F1036 ?-soft wheat
P2283a N-
F1463 C-
F1509 N-
P509d S-
P129a S-
P493a C-
F1545 N-
P80a C-
F1462 C-
F1539 N-
F1542 N-
F1461 C-
F1466 N-
Fusarium sp. FTSC 12
F1502 N-saffron
F1391 N-
F1544 N-
F1458 C-
F1443 N-
F1460 C-
F1281 C-
25481 Germany-winter wheat
P325b C-
F1495 N-
F1457 C-
F1467 N-
F1390 N-
F1534 N-
F. tricinctum FTSC 3
F1501 N-boxwood
F164 N-apple wood
Fusarium sp. FTSC 13
P78c C-
Fusarium sp. FTSC 15
P2214b N-
F1468 C-
F1541 N-spelt
P454a S-
F1496 N-
36147 ?-human
P82b C-
F1471 C-
F1536 Iran-water
F1392 N-
R1409 Iran-water
F1389 C-
R1389 Iran-sediment
R273 Iran-sediment
P92a C-
F. acuminatum FTSC 2
R972 Iran-decaying plant
F1540 N-Lingustrum
Fusarium sp. FTSC 14

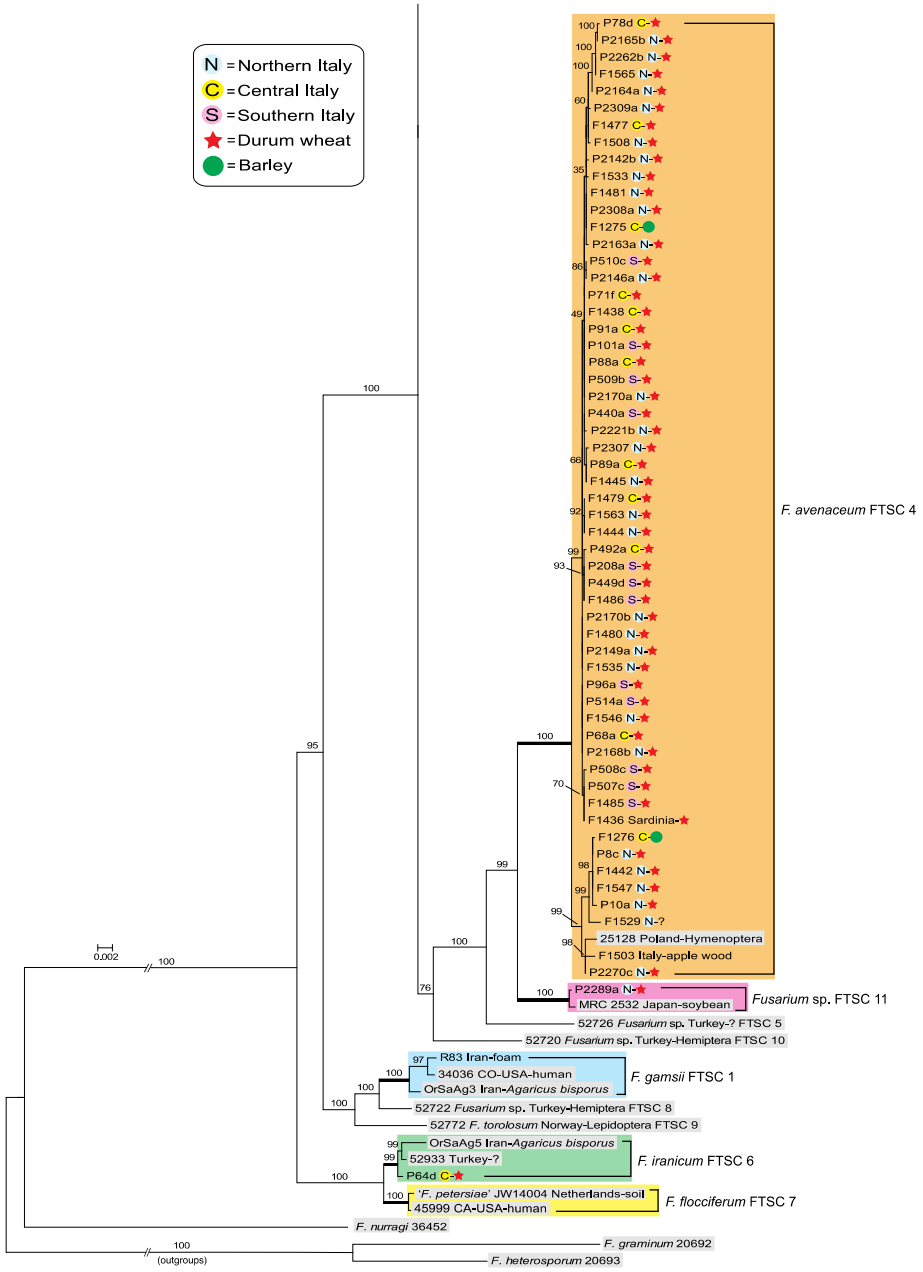


Figure 3: Maximum likelihood phylogeny based on a concatenated DNA alignment of *TEF1*, *RPB1* and *RPB2* sequences from 117 FTSC analysed in the present study in relation to FTSC reference sequences (highlight in grey). Maximum likelihood (ML) bootstrap values are indicated above nodes based on 5000 pseudoreplicates of the data.

2.2.3.2 Production of mycotoxins and other biologically active secondary metabolites in vitro

Based on phylogenetic analyses, 59 isolates comprising 10 FTSC phylospecies were selected to assess their ability to produce mycotoxins and other secondary metabolites *in-vitro* on rice (Table 3). Forty-three isolates were from wheat, 10 from barley, four *non-graminaceous* hosts, and one each from decaying vegetation and foam (Table 1). At least one isolate of all 10 species was able to produce quantitatively detectable levels of one or more enniatin; however, ENNs were not detected in three strains of *Fusarium* sp. (FTSC 12) from wheat and barley and *Fusarium* sp. (FTSC 14) from *Ligustrum*. Although ENNA was only produced by one isolate of three phylospecies, ENNA1 was detected in one or more isolate of 9 species (39/59 = 66.1%), and ENNB (54/59 = 91.5%) and ENNB1 (51/59 = 86.4%) in all 10 species. The highest total ENNs production by a strain of the four most common species recovered from Italian wheat and barley (Table 3) was 701.8 μg/kg in *F. avenaceum* F1503 (FTSC 4, all 23 produced), 594.1 μg/kg in *Fusarium* sp. F1509 (FTSC 12, 12/15 produced), 853.5 μg/kg in *F. acuminatum* F1389 (FTSC 2, all 9 produced), and 498.8 μg/kg in *F. tricinctum* F1460 (FTSC 3, all 5 produced).

Detectable levels of moniliformin (MON) were recorded in 55/59 isolates (Table 3), but not in three isolates of *Fusarium* sp. (FTSC 12) and the single isolate of *F. iranicum* (FTSC 6) tested. MON production by strains of the four most common species recovered from Italian wheat and barley ranged from 207.7-803.9 μg/kg in *F. avenaceum* (FTSC 4, all 23 produced), 12.4-322.2 μg/kg in *Fusarium* sp. (FTSC 12, 12/15 produced), 55.7-417.8 μg/kg in *F. acuminatum* (FTSC 2, all 9 produced), and 71.6-702.8 μg/kg in *F. tricinctum* (FTSC 3, all 5 produced). Similarly, detectable levels of 2-Amino-14,16-dimethyloctadecan-3-ol, (AOD-ol) were recorded in 52/59 isolates (Table 3). Capacity for AOD-ol production appears to be distributed broadly across FTSC (Kim et al., 2020).

A qualitative screen of the rice culture extracts revealed that some of the 59 FTSC isolates could produce several other secondary metabolites. These included chlamydosporol production by 26 isolates representing 6 species, acuminatopyrone by 20 isolates comprising four species, longiborneol by 13 isolates from three species, fungerin by 23 strains representing eight species, and butenolide by 8 isolates from five of the species (Table 3).

2.2.4 Discussion and Conclusions

The present research is the first to assess FTSC species diversity associated with FHB of wheat and barley in Italy using phylogenetic species recognition based on genealogical exclusivity (GCPSR sensu) (Taylor et al., 2000) and to experimentally test the ability of 59 strains representing 10 FTSC phylospecies to produce mycotoxins and other biologically active secondary metabolites *in vitro*. Consistent with the findings of a recent GCPSR-based study of toxigenic fusaria (O'Donnell et al., 2018), our pathogen survey of wheat and barley symptomatic for FHB revealed that nine FTSC species were represented among the 117 isolates from Italy, including five unnamed phylospecies new to science (i.e., *Fusarium* spp. FTSC 11 - 15). To aid in identifying the Italian collection, sequences of 17 isolates comprising 11 FTSC species were included as a reference (O'Donnell et al., 2018, 2012, 2009). Because nine of the 15 FTSC species included in this study lack Latin binomials, the named and unnamed species were distinguished informally using Arabic numbers (i.e., FTSC 1 - 15). An ad hoc nomenclature using Arabic numbers was also employed in prior studies of other species-rich complexes in *Fusarium* (Laraba et al., 2021; O'Donnell et al., 2012, 2009, 2008), given that GCPSR studies have consistently revealed morphological species recognition greatly underestimates species diversity within this genus. As reported here, published multilocus molecular phylogenetic analyses of FTSC pathogen collections have consistently encountered novel species diversity (Cerón-Bustamante et al., 2018; Gräfenhan et al., 2013; Harrow et al., 2010; Niessen et al., 2012; O'Donnell et al., 2018; Ponts et al., 2020; Torbati et al., 2019). Some of this species level diversity, however, has been misinterpreted as infraspecific variation within *F. avenaceum* (Kulik et al., 2011; Stakheev et al., 2016; Yli-Mattila et al., 2002), strongly suggesting the FTSC comprises well over 15 phylospecies. Recent taxonomic advances within the FTSC include formal descriptions of two species from *Agaricus bisporus* in Iran as *F. gamsii* (FTSC 1) and *F. iranicum* (FTSC 6) (Torbati et al., 2019); however, *F. petersiae* from Dutch soil (Lombard, 2017) is treated here as illegitimate because it appears to be a later synonym of *F. flocciferum* (FTSC 7) based on our phylogenetic analyses and those of Ponts et al. (2020). Future molecular systematic advances within the FTSC need to take advantage of the rich genomic resources that should be exploited to develop additional phylogenetic marker loci needed to infer robust GCPSR-based hypotheses of FTSC species diversity (Kim et al., 2020; Lysøe et al., 2014).

Our maximum likelihood bootstrap analyses of DNA sequence from portions of three phylogenetically informative genes (i.e., *TEF1*, *RPB1* and *RPB2*), and the combined 3-locus 140 isolate dataset, strongly supported the monophyly of the 10 FTSC phylospecies represented by two or more strains, employing the highly conservative criteria of genealogical concordance and nondiscordance under GCPSR (Dettman et al., 2003) (Table 2). These analyses revealed that 4/11 FTSC species accounted for 111/117 (94.9%) of the FHB-associated collection from Italian wheat and barley. *F. avenaceum* (FTSC 4) was the predominant species comprising close to half of the Italian collection (N 56/117 = 47.9%), followed by the newly discovered phylospecies *Fusarium* sp. FTSC 12 (N 32/117 = 27.4%), *F. tricinctum* FTSC 3 (N 13/117 = 11.1%), and *F. acuminatum* FTSC 2 (N 10/117 = 8.5%). As reported here, *F. avenaceum* (FTSC 4) was one of most common FTSC species recovered in prior pathogen surveys on Italian barley and durum wheat (Beccari et al., 2020, 2018a, 2018b).

While *F. avenaceum* has generally been reported from the cooler region of Northern Europe and Canada (Gräfenhan et al., 2013; Stakheev et al., 2016; Uhlig et al., 2007; Xu et al., 2008; Yli-Mattila et al., 2004), it was recently recovered in FHB pathogen surveys in Mexico (Cerón-Bustamante et al., 2018), Brazil (Moreira et al., 2020), North Carolina, U.S. (Cowger et al., 2020), and New Zealand (Harrow et al., 2010). Although members of *Fusarium graminearum* species complex (FGSC), especially *F. graminearum*, are the most important and aggressive FHB pathogens on small grain cereals in warm and temperate regions worldwide (Astolfi et al., 2011; Bottalico and Perrone, 2002; Garmendia et al., 2018; Ji et al., 2019; Reynoso et al., 2011; Ward et al., 2008) a recent putative "shift" in the *Fusarium* community composition was observed involving species thus far considered secondary invaders such as *F. poae* and members of the FTSC (Beccari et al., 2018b, 2016; Cerón-Bustamante et al., 2018). The increased presence of *F avenaceum* and other closely related species might be due to climate change and/or agricultural practices (Cowger et al., 2020; Gräfenhan et al., 2013; Karlsson et al., 2017; Vogelgsang et al., 2019). Our working hypothesis is that when climatic conditions are unfavorable for *F. graminearum*, secondary invaders such as *F. avenaceum* become more competitive (Beccari et al., 2017). In addition, infection timing during anthesis might help explain the increased presence of *F. avenaceum* (Beccari et al., 2019).

The novel unnamed taxon *Fusarium* sp. FTSC 12, which was supported in our analyses as sister to *F. tricinctum* FTSC 3, was the second most common *tricinctum* clade species recovered from Italian wheat and barley, comprising slightly more than one-quarter of

our Italian pathogen collection (N 32/117 = 27.4%). The available data suggests that this novel phylospecies may have been reported as *F. tricinctum* in prior phylogenetic studies of the FTSC (Niessen et al., 2012; Ponts et al., 2020). Only 8.5% (10/117) of the Italian isolates were identified as *F. acuminatum*, consistent with prior results that found it was present at low frequencies on wheat and malting barley in Italy (Beccari et al., 2018a, 2018b). This species was recently recovered from wheat in Spain, Canada and North Carolina in the U.S. where it was reported as a minor contaminant except in North Carolinian wheat fields (Cowger et al., 2020; Gräfenhan et al., 2013; Marín et al., 2012). Cowger et al. (2020), however, reported that it accounted for approximately half of the North Carolinian FTSC isolates (122/249 = 49%), suggesting that it can become a significant contaminant under favorable conditions. Future studies are also needed to determine whether endophytic fusaria in native plants near cultivated areas serve as a reservoir of pathogen diversity, given the report that 50% of the endophytes present in symptomless wild grasses in Minnesota were members of the FTSC (Lofgren et al., 2018).

The present research adds to a growing number of studies that have shown that members of the FTSC are able to produce significant levels of MON and ENN mycotoxins (Bottalico and Perrone, 2002; Kokkonen et al., 2010; O'Donnell et al., 2018; Orlando et al., 2019; Pereira et al., 2020; Schütt et al., 1998; Sørensen et al., 2009). These mycotoxins are defined as "emerging" because maximum levels have not been established by the European Union and elsewhere (EFSA, 2018, 2014) and because they are not monitored (Gruber-Dorninger et al., 2017). Reports that FTSC can produce trichothecenes, however, appear to be due to the misidentification of *F. armeniacum*, a species within the *F. sambucinum* species complex, as *F. acuminatum.* Moreover, while *F. avenaceum* has been reported to produce beauvericin (Logrieco et al., 2002; Morrison et al., 2002), this mycotoxin was not detected in the current and several other surveys of the FTSC (Cerón-Bustamante et al., 2018; Covarelli et al., 2015a; Jestoi et al., 2004; O'Donnell et al., 2018; Uhlig et al., 2007, 2006; Vogelgsang et al., 2008). However, because BEA and ENNs are produced by the same biosynthetic pathway, cultural conditions may not have been optimal for BEA production in the aforementioned studies (Sørensen and Giese, 2013). Moreover, because comparative genomic analyses have shown toxins and other bioactive secondary metabolites are frequently not produced even when the gene clusters that encode them are intact (Kim et al., 2020), future studies are warranted to assess whether FTSC species can produce BEA.

Extending the finding of prior studies that established *F. avenaceum* and *F. tricinctum* produce significant levels of MON and ENNs (Beccari et al., 2020; Fredlund et al., 2013; Orlando et al., 2019; Uhlig et al., 2006), our analyses revealed that all but one of the 10 FTSC phylospecies we analyzed produced significant levels of these toxins *in vitro* on rice. Therefore, it should be assumed that all FTSC possess the genetic potential to contaminate cereals with MON and ENNs until proven otherwise. Until recently, these secondary metabolites were thought to be concern primarily for Northern European countries (Ivanova et al., 2006); however, Santini et al. (2012) proved not only high levels of ENNs on grain-based foods within the Mediterranean, but also the resistance of these compunds to food processing. In addition to MON and ENNs, GC-MS analyses of rice culture extracts reported here revealed that several FTSC species possess the ability to produce *in vitro* lesser-known secondary metabolites such as the lactone chlamydosporol (Sørensen et al., 2009), heterocyclic ketone acuminatopyrone, sesquiterpene alcohol longiborneol, antifungal alkaloid fungerin, and lactone butanolide (Beccari et al., 2018a, 2018b). Although limited data is available concerning acute and chronic toxicity, chlamydosporol was reported to be toxic to human cells (Solfrizzo et al., 1994) and acuminatopyrone toxic to mouse cells, human fibroblasts and chick embryos (Solfrizzo et al., 1994; Solfrizzo and Visconti, 1996). Kim et al. (2020) clarified the gene cluster responsible for the production of AOD-ol and discovered that this cluster is widely distributed in the FTSC. Early studies showed AOD-ol to be cytotoxic in a variety of assays (Uhlig et al., 2008). More recently, it has been shown that AOD-ol induced a transient accumulation of vacuoles in the cells of the HepG2 human model liver cell line (Solhaug et al., 2020).

In conclusion, the present study significantly increases our knowledge of FTSC species diversity and mycotoxin potential associated with FHB-symptomatic wheat and barley in Italy. Because MON and ENNs toxin levels in cereals and other food and feed are currently not regulated by the European Food Safety Authority, the toxin data reported here should provide a robust framework for improving our understanding of the risk they pose to human health and food security (EFSA, 2018, 2014). Towards this end, in-depth toxicological studies are urgently needed to inform science-based regulatory decisions.

Collaborations

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Supplementary Material

Table S1: Histories of strains included in the present study

Strain #[a]	Geographic Origin[b]	Host	Cultivar[c]	Year isolated
F164	Emilia Romagna (Northern Italy)	Apple wood - *Malus domestica*	Unknown	Unknown
F444	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Duillio	2006
F445	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Levante	2006
F459	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Neodur	2006
F577	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Neodur	2006
F1036	Italy	Soft wheat- *Triticum aestivum*	Unknown	2009
F1093	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Unknown	2009
F1221	Italy	Barley - *Hordeum vulgare*	Unknown	2012
F1233	Italy	Barley - *Hordeum vulgare*	Unknown	2012
F1275	Umbria (Central Italy) - Perugia	Barley - *Hordeum vulgare*	Naturel	2014
F1276	Umbria (Central Italy) - Perugia	Barley - *Hordeum vulgare*	Naturel	2014
F1281	Umbria (Central Italy) - Perugia	Barley - *Hordeum vulgare*	Naturel	2014
F1389	Umbria (Central Italy) - Perugia	Durum wheat - *Triticum durum*	Iride	2015
F1390	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Odisseo	2015
F1391	Emilia Romagna (Northern Italy) - Piacenza	Durum wheat - *Triticum durum*	Orobel	2015
F1392	Emilia Romagna (Northern Italy) - Jolanda Di Savoia	Durum wheat - *Triticum durum*	Obelix	2015
F1436	Sardinia	Durum wheat - *Triticum durum*	Karalis	2016
F1438	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Colorado	2015
F1442	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Odisseo	2015
F1443	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Orobel	2016
F1444	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Orobel	2016
F1445	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Orobel	2016
F1453	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2016
F1454	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2016
F1455	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2016
F1456	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2016
F1457	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1458	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1459	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1460	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2013
F1461	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1462	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1463	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Quench	2014
F1465	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Odisseo	2016
F1466	Emilia Romagna (Northern Italy) - Modena	Durum wheat - *Triticum durum*	Odisseo	2016
F1467	Emilia Romagna (Northern Italy) - Modena	Durum wheat - *Triticum durum*	Odisseo	2016
F1468	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Unknown	2010
F1471	Umbria (Central Italy)	Barley - *Hordeum vulgare*	Unknown	2016
F1477	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Unknown	2014
F1479	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Unknown	2009
F1480	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Unknown	2009
F1481	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Unknown	Unknown
F1482	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Cesare	2017
F1485	Apulia (Southern Italy)	Durum wheat - *Triticum durum*	Unknown	Unknown
F1486	Apulia (Southern Italy)	Durum wheat - *Triticum durum*	Unknown	Unknown
F1495	Emilia Romagna (Northern Italy) - Ravenna	Durum wheat - *Triticum durum*	Tyrex	2017
F1496	Emilia Romagna (Northern Italy) - Ravenna	Durum wheat - *Triticum durum*	Tyrex	2017
F1501	Emilia Romagna (Northern Italy)	Boxwood - *Buxus sempervirens*	Unknown	2017
F1502	Emilia Romagna (Northern Italy)	Saffron - *Crocus sativus*	Unknown	2017
F1503	Italy	Apple wood - *Malus domestica*	Unknown	2017
F1508	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Rusticano	2018
F1509	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Saragolla	2018
F1529	Emilia Romagna (Northern Italy) - Piacenza	Unknown	Athoris	2018
F1533	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2018
F1534	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2018
F1535	Emilia Romagna (Northern Italy) - Parma	Durum wheat - *Triticum durum*	Monastir	2018
F1536	Iran	Water	NA	2018
F1539	Emilia Romagna (Northern Italy) - Bologna	Barley - *Hordeum vulgare*	Ketos	2018
F1540	Emilia Romagna (Northern Italy) - Bologna	*Ligustrum* sp.	Unknown	2018
F1541	Emilia Romagna (Northern Italy) - Bologna	Spelt -*Triticum monococcum*	Unknown	2018
F1542	Emilia Romagna (Northern Italy) - Bologna	Barley - *Hordeum vulgare*	Ketos	2018
F1544	Liguria (Northern Italy) - Albaro	Durum wheat - *Triticum durum*	Jubilar	2018
F1545	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Consort	2018
F1546	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Unknown	2018
F1547	Emilia Romagna (Northern Italy) - Bologna	Durum wheat - *Triticum durum*	Marco Aurelio	2018
F1563	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Oliver	2018
F1564	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Oliver	2018
F1565	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	PR22D66	2018

[a] F, P and R strains belong to a collection maintained at the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy; ARSEF, ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York, USA; BBA, Biologische Bundesanstalt für Land-und Forstwirtschaft, Institute für Mikrobiologie, Berlin, Germany; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; MRC, former South African Medical Research Council Collection, currently housed at the Agricultural Research Council, Pretoria, South Africa; NRRL, ARS Culture Collection, Peoria, Illinois, USA; UTHSC, University of Texas Health Sciences Center, San Antonio, TX, USA. Strains followed by an Asterix were used as a reference (See Fig. 2).
[b] See Fig. 1 for map showing regions where pathogen surveys were conducted.
c NA = not applicable.

Table 1: Histories of strains included in the present study (Continued).

Strain #[a]	Geographic Origin[b]	Host	Cultivar[c]	Year isolated
P8c	Veneto (Northern Italy) - Verona	Durum wheat - *Triticum durum*	Obelix	2019
P10a	Veneto (Northern Italy) - Verona	Durum wheat - *Triticum durum*	Obelix	2019
P37a	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Oliver	2019
P64d	Abruzzo (Central Italy) - L'Aquila	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P68a	Abruzzo (Central Italy) - L'Aquila	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P71f	Abruzzo (Central Italy) - L'Aquila	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P78c	Abruzzo (Central Italy) - Chieti	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P78d	Abruzzo (Central Italy) - Chieti	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P80a	Abruzzo (Central Italy) - Chieti	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P82b	Abruzzo (Central Italy) - Chieti	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P88a	Molise (Central Italy) - Campobasso	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P89a	Molise (Central Italy) - Campobasso	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P91a	Molise (Central Italy) - Campobasso	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P92a	Molise (Central Italy) - Campobasso	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P96a	Campania (Southern Italy) - Benevento	Durum wheat - *Triticum durum*	Aureo	2019
P101a	Campania (Southern Italy) - Benevento	Durum wheat - *Triticum durum*	Aureo	2019
P129a	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Anthalis	2019
P208a	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Anthalis	2019
P325b	Abruzzo (Central Italy) - Teramo	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P440a	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Antalis	2019
P449d	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Iride	2019
P454a	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Iride	2019
P492a	Abruzzo (Central Italy) - Teramo	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P493a	Abruzzo (Central Italy) - Teramo	Durum wheat - *Triticum durum*	Marco Aurelio	2019
P507c	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P508c	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P509b	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P509d	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P510c	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P514a	Apulia (Southern Italy) - Foggia	Durum wheat - *Triticum durum*	Saragolla	2019
P2142b	Emilia Romagna (Northern Italy) - Parma	Durum wheat - *Triticum durum*	Monastir	2019
P2146a	Emilia Romagna (Northern Italy) - Parma	Durum wheat - *Triticum durum*	Monastir	2019
P2149a	Emilia Romagna (Northern Italy) - Parma	Durum wheat - *Triticum durum*	Monastir	2019
P2163a	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2164a	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2165b	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2168b	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2170a	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2170b	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat - *Triticum durum*	Levante	2019
P2214b	Emilia Romagna (Northern Italy) - Piacenza	Durum wheat - *Triticum durum*	Athoris	2019
P2221b	Emilia Romagna (Northern Italy) - Carpi	Durum wheat - *Triticum durum*	Tito Flavio	2019
P2262b	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	PR22D66	2019
P2270	Lombardy (Northern Italy) - Ostiglia	Durum wheat - *Triticum durum*	Pigreco	2018
P2281a	Lombardy (Northern Italy) - Ostiglia	Durum wheat - *Triticum durum*	Pigreco	2019
P2283a	Lombardy (Northern Italy) - Ostiglia	Durum wheat - *Triticum durum*	Pigreco	2019
P2285a	Lombardy (Northern Italy) - Ostiglia	Durum wheat - *Triticum durum*	Pigreco	2019
P2289a	Lombardy (Northern Italy) - Ostiglia	Durum wheat - *Triticum durum*	Pigreco	2019
P2307	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Levante	2019
P2308a	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Levante	2019
P2309a	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Levante	2019
R83	Iran	Foam	NA	2018
R273	Iran	Sediment	NA	2018
R972	Iran	Submerged decaying stem or root	NA	2018
R1389	Iran	Sediment	NA	2018
R1409	Iran	Water	NA	2018
NRRL 34036 = UTHSC 01-1965*	CO, USA	Ethmoid aspirate	NA	Unknown
CBS 143609 = OrSaAg3*	Orumieh-Salmas, Iran	*Agaricus bisporus*	NA	2016
NRRL 36147 = CBS 109232*	Unkown	Human bronchial secretion	Unknown	Unknown
NRRL 25481 = CBS 393.93 = BBA 64485 (neotype)*	Germany	Winter wheat	Diplomat	1984
NRRL 25128 = ARSEF 1331*	Poland	*Hymenoptera ichneumonidae*	NA	Unknown
NRRL 52726 = ARSEF 8299*	Turkey	Unknown	Unknown	Unknown
NRRL 52933 = ARSEF 8648*	Turkey	Unknown	Unknown	Unknown
CBS 143611 = OrSaAg3*	Orumieh-Salmas, Iran	*Agaricus bisporus*	NA	2016
NRRL 45999 = UTHSC 06-3449 *	CA, USA	Human scalp	Unknown	Unknown
CBS 143231 = JW14004*	Netherlands	Soil	NA	2017
NRRL 52722 = ARSEF 6401*	Turkey	*Eurygaster* sp.	NA	1999
NRRL 52772 = ARSEF 5560*	Norway	*Galleria mellonella* larva	NA	Unknown
NRRL 52720 = ARSEF 6410*	Turkey	*Eurygaster* sp.	NA	1999
MRC 2532*	Japan	Soybean	Unknown	Unknown
NRRL 20692*	Ethiopia	*Cynodon dactylon*	Unknown	Unknown
NRRL 20693*	Netherlands	*Claviceps purpurea* on *Lolium perenne*	NA	Unknown
NRRL 36452 = CBS 831.85 = BBA 64346*	Australia	Soil	NA	Unknown

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[b] See Fig. 1 for map showing regions where pathogen surveys were conducted.
c NA = not applicable.

Table S2: Loci sequenced and monophyly support.

***Fusarium* species (FTSC #[a])**	***TEF1***	***RPB1***	***RPB2***	**Combined**
PIC/bp/% PIC[b]	138/684/20.2	233/1606/14.5	256/1693/15.1	628/3983/15.8
F. gamsii (FTSC 1)	52	100	100	100
F. acuminatum (FTSC 2)	99	100	99	100
F. tricinctum (FTSC 3)	99	100	87	100
F. avenaceum (FTSC 4)	69	99	100	100
Fusarium sp. (FTSC 5)	NA[c]	NA	NA	NA
F. iranicum (FTSC 6)	94	96	<[d]	99
F. flocciferum (FTSC 7)	100	86	97	100
Fusarium sp. (FTSC 8)	NA	NA	NA	NA
F. torulosum (FTSC 9)	NA	NA	NA	NA
Fusarium sp. (FTSC 10)	NA	NA	NA	NA
Fusarium sp. (FTSC 11)	100	100	88	100
Fusarium sp. (FTSC 12)	98	100	75	100
Fusarium sp. (FTSC 13)	98	100	100	100
Fusarium sp. (FTSC 14)	94	99	<	100
Fusarium sp. (FTSC 15)	NA	NA	NA	NA

[a] The FTSC # represents an informal ad nomenclature used to distinguish taxa because only seven of the 15 species included in this study have Latin binomials.[b] PIC/bp = parsimony informative characters per base pair.[c] NA = not applicable for five species represented by single strains where monophyly cannot be assessed. [d] < = monophyly neither supported nor contradicted by bootstrapping.

Table S3: Mycotoxins and other secondary metabolites produced *in-vitro* by 59 selected isolates.

Isolate #[a]	Species (FTSC #[b])	CHL[c]	ACU[c]	LONG[c]	FUNG[c]	BUT[c]	ENNA[d]	ENNA1[d]	ENNB[d]	ENNB1[d]	MON[d]	AOD-ol[d]
R83	*F. gamsii* (FTSC 1)	-[e]	+[e]	-	-	-	< LOQ	4.9	8	1.1	16.1	< LOQ
F1389	*F. acuminatum* (FTSC 2)	+	+	-	-	-	1.1	187.5	318.5	346.4	63.1	5.9
F1392	*F. acuminatum* (FTSC 2)	+	+	-	-	-	< LOQ	80.6	236.4	211	55.7	186.3
F1468	*F. acuminatum* (FTSC 2)	-	-	-	-	-	< LOQ	2.8	6.4	6.9	72.6	2139.3
F1471	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	23.4	109.4	81.7	417.8	182.8
F1541	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	32.3	102.9	92.8	276.8	208.3
P82b	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	77.1	197.9	187.5	169	226.7
P92a	*F. acuminatum* (FTSC 2)	+	+	-	+	-	< LOQ	30.6	94.5	82.2	304.8	127.1
P454a	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	188.9	283.3	304	218.6	< LOQ
P2214b	*F. acuminatum* (FTSC 2)	+	+	-	-	+	< LOQ	41.9	119.3	116.1	366.4	337.5
F1281	*F. tricinctum* (FTSC 3)	-	-	+	-	-	< LOQ	63.8	110	122.2	702.8	269.6
F1458	*F. tricinctum* (FTSC 3)	-	-	-	+	-	< LOQ	22.1	91.6	72	345.8	104.1
F1460	*F. tricinctum* (FTSC 3)	+	-	-	+	-	2.6	110.6	186	199.6	162.6	193.2
F1502	*F. tricinctum* (FTSC 3)	-	-	-	+	-	< LOQ	15	88.2	60	550.4	302.7
P325b	*F. tricinctum* (FTSC 3)	-	-	-	+	+	< LOQ	9.8	25	24.6	71.6	47.6
F1275	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	< LOQ	16.2	1.6	286.5	259.8
F1436	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	57.6	296.1	227.2	348.9	< LOQ
F1444	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	4.5	28.8	136.2	420	75.3
F1479	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	2.1	80.5	17.6	462.8	629.1
F1480	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	< LOQ	13.8	1.7	287.6	227.7
F1481	*F. avenaceum* (FTSC 4)	+	+	+	-	+	< LOQ	< LOQ	41.6	8.4	303.5	233.3
F1486	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	< LOQ	48.5	8.4	331.4	< LOQ

F1503	*F. avenaceum* (FTSC 4)	+	+	-	+	-	2.5	117.5	306.9	274.9	469.4	86.8
F1533	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	9	130.2	50	426.3	218.0
F1565	*F. avenaceum* (FTSC 4)	-	-	-	-	+	< LOQ	< LOQ	73.5	9.9	611.4	577.5
P8C	*F. avenaceum* (FTSC 4)	+	-	-	-	-	< LOQ	< LOQ	12	1.8	365.8	221.8
P78d	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	10.7	117	45.2	772.5	244.2
P88a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	30.2	3.8	376.7	193.7
P89a	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	2.4	61.3	7.2	558.4	165.8
P91a	*F. avenaceum* (FTSC 4)	+	-	-	-	-	< LOQ	<LOQ	97.4	10.8	482.9	160.4
P101a	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	10.3	214.8	77.4	503.1	421.4
P208a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	3.2	< LOQ	319.6	290.0
P440a	*F. avenaceum* (FTSC 4)	+	+	+	-	+	< LOQ	< LOQ	1.3	< LOQ	445.6	335.3
P507c	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	< LOQ	33.1	5.6	362.9	154.3
P514a	*F. avenaceum* (FTSC 4)	+	-	+	-	-	< LOQ	6	160.4	48.9	207.7	320.6
P2149a	*F. avenaceum* (FTSC 4)	-	-	-	-	+	< LOQ	1.5	69.1	1.4	803.9	165.8
P2164a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	63.7	9.3	338.6	66.6
P2221b	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	6.9	< LOQ	283.8	313.5
P64d	*F. iranicum* (FTSC 6)	+	-	-	+	-	< LOQ	60.8	2.7	20	< LOQ	406.4
P2289a	*Fusarium* sp. (FTSC 11)	-	-	-	+	-	< LOQ	< LOQ	74.3	12.6	159.5	6.2
F444	*Fusarium* sp. (FTSC 12)	+	-	+	+	-	< LOQ	3.1	11.3	9.6	138	< LOQ
F445	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	< LOQ	1.5	1.5	12.4	238.2
F1036	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	15.2	59.2	43.5	171.5	239.0
F1093	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	78.4	12.4	136	176.5	194.8
F1233	*Fusarium* sp. (FTSC 12)	+	-	+	+	-	< LOQ	< LOQ	< LOQ	< LOQ	27.6	119.1
F1456	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	23.2	80.4	64	187.4	228.6
F1459	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	47.9	128.7	115.6	277.2	85.7
F1463	*Fusarium* sp. (FTSC 12)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	16.8

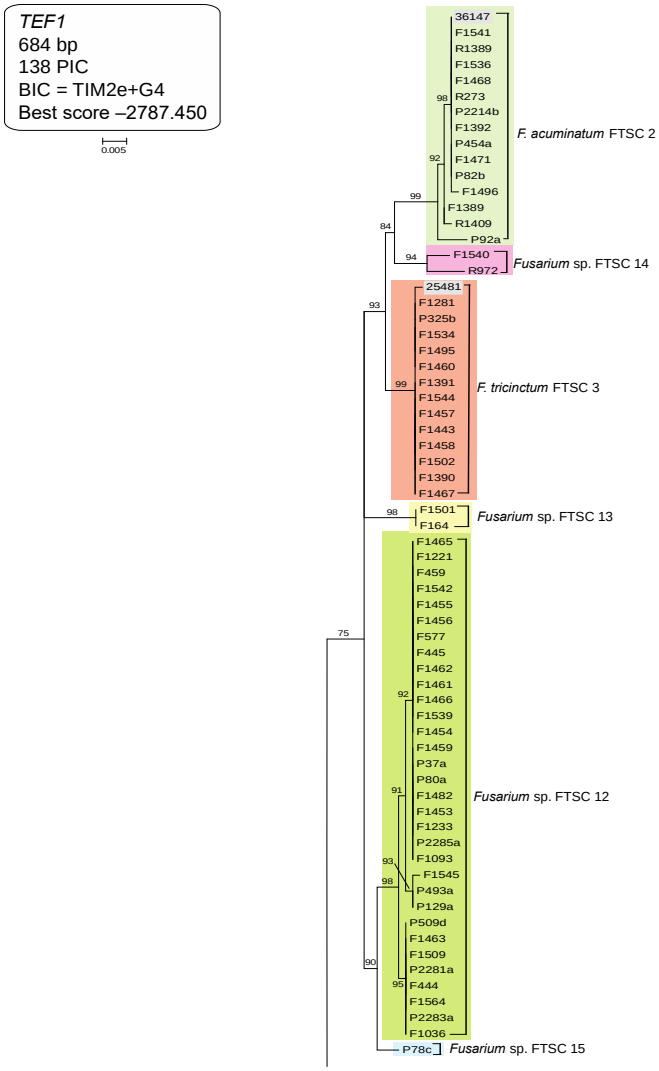
F1465	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	76.4
F1509	*Fusarium* sp. (FTSC 12)	-	-	-	+	-	< LOQ	136.2	220.5	237.4	256	< LOQ
F1539	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	123.4	192.4	207.1	89.1	310.4
P37a	*Fusarium* sp. (FTSC 12)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	14.7
P80a	*Fusarium* sp. (FTSC 12)	-	-	-	+	+	< LOQ	31.2	82.6	66.6	94.1	14.7
P129a	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	27.8	64.8	62.3	< LOQ	471.6
P2283a	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	48.7	142.2	125.4	322.2	6.4
F1501	*Fusarium* sp. (FTSC 13)	-	-	-	+	-	< LOQ	16.5	96.6	67.2	584.1	19.1
R972	*Fusarium* sp. (FTSC 14)	-	-	-	-	+	< LOQ	36.1	110.9	97.5	295.8	< LOQ
F1540	*Fusarium* sp. (FTSC 14)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	574.5	333.0
P78c	*Fusarium* sp. (FTSC 15)	+	-	-	+	-	< LOQ	13	64.8	46.5	483.3	35.3

[a]Isolate collection maintained at the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy.
[b]An informal ad hoc nomenclature employing Arabic numerals was used to distinguish taxa because six of the 10 species tested for mycotoxin production in vitro lack Latin binomials.
[c]Mycotoxins and other secondary metabolites analyzed by GC/MS: CHL = chlamydosporol; ACU = acuminatopyrone; LONG = longiborneol; FUNG = fungerin; BUT = butenolide.
[d]Mycotoxins and other secondary metabolites analyzed by LC-MS: ENNA = enniatin A; ENNA1 = enniatin A1; ENNB = enniatin B; ENNB1 = enniatin B1; MON = moniliformin; AODol = 2-Amino-14,16-dimethyloctadecan-3-ol. "< LOQ" indicates below method limit of quantitation (1 ng/µl)
[e] +/- = detected / not detected

.

Figure S1:

Maximum likelihood tree based on *TEF1* sequences (684 bp alignment) of 123 analyzed FTSC isolates collected across Italy and Iran in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TIM2e + G4 model of molecular evolution. Bootstrap value (% based on 5000 pseudoreplications) are shown on branches.



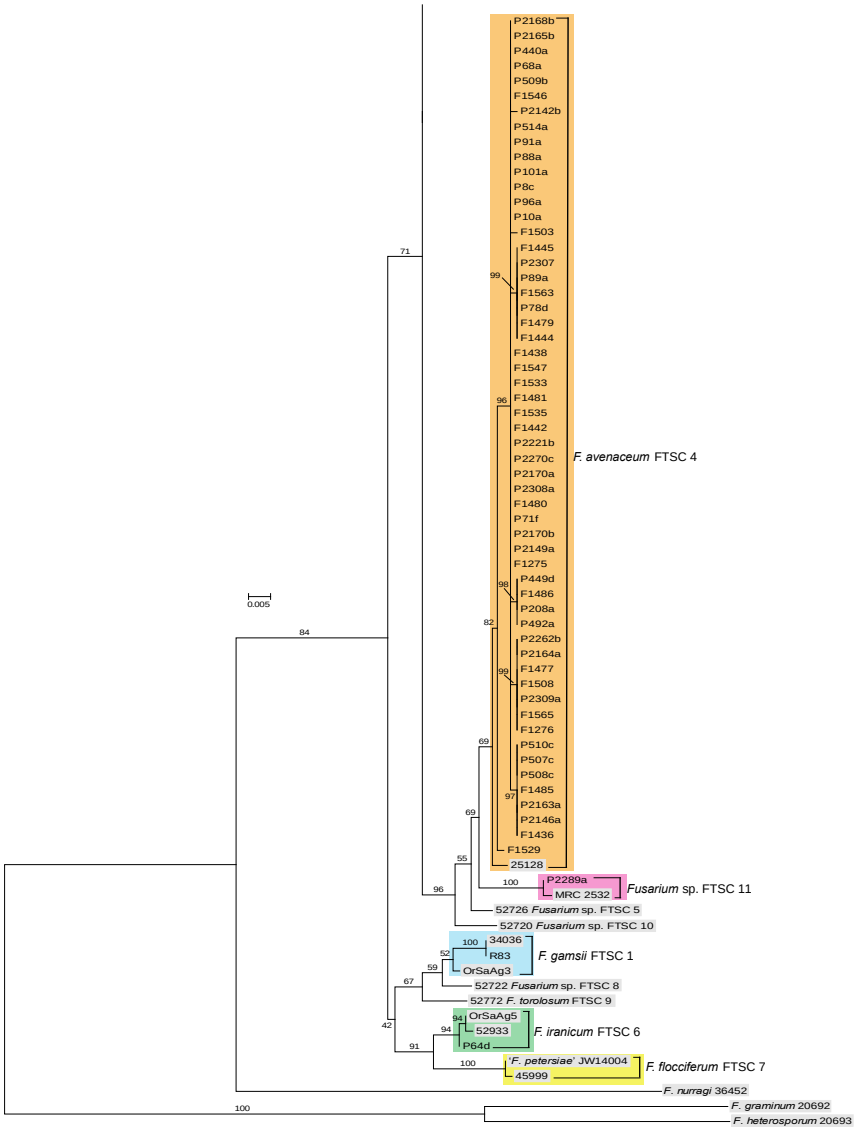
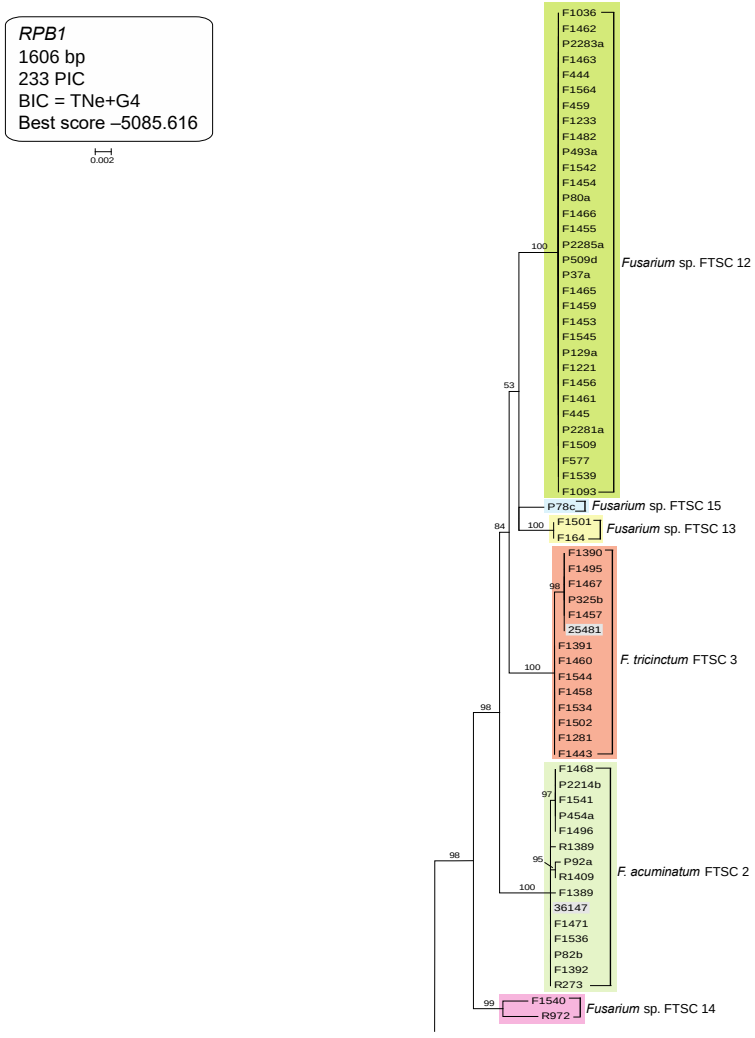


Figure S1 (Continued)

Figure S2:

Maximum likelihood tree based on *RPB1* sequences (1606 bp alignment) of 123 analyzed FTSC isolates collected across Italy (n= 117) and Iran (n= 6) in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TNe + G4 model of molecular evolution. Bootstrap value (% based on 5000 pseudoreplications) are shown on branches.



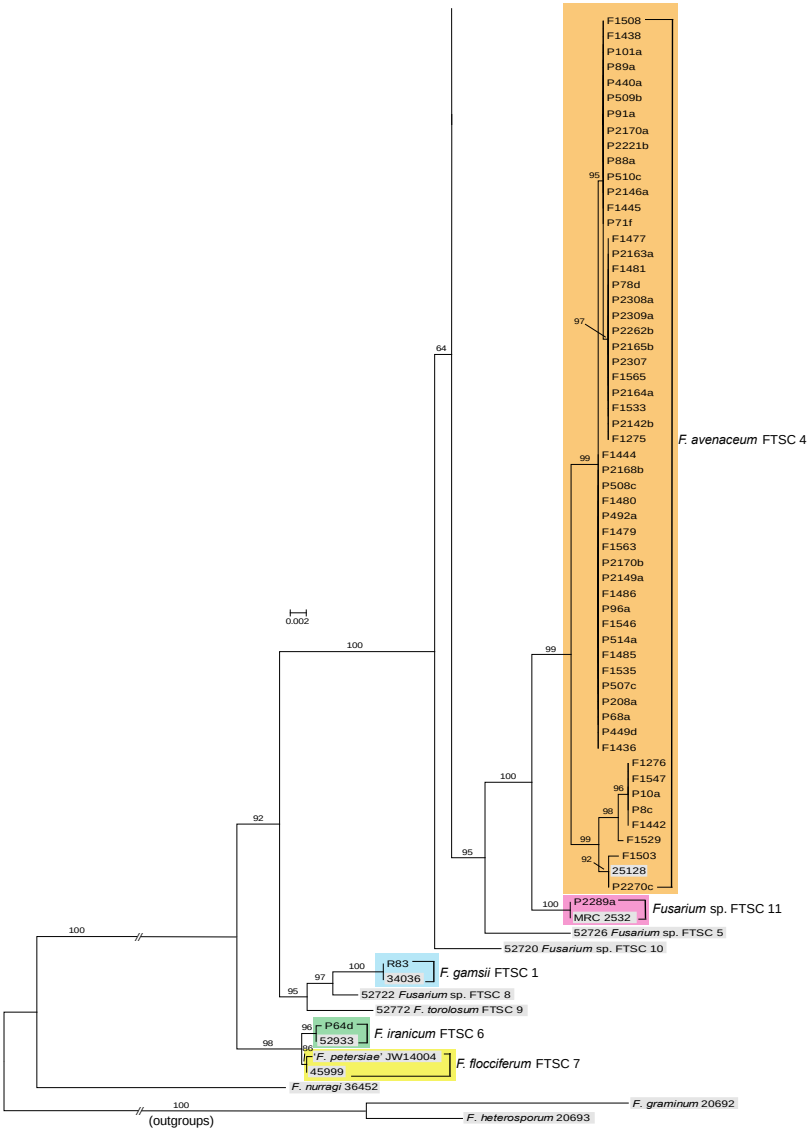
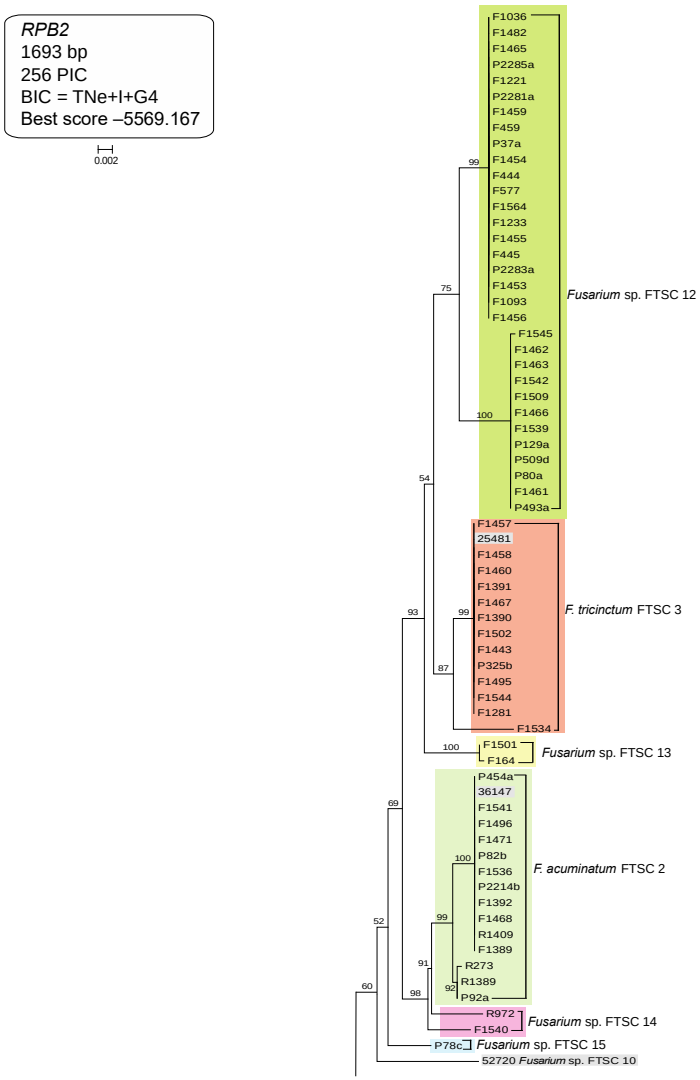


Figure S2 (Continued)

Figure S3: Maximum likelihood tree based on *RPB2* sequences (1693 bp alignment) of 123 analyzed FTSC isolates collected across Italy and Iran in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TNe + I + G4 model of molecular evolution. Bootstrap value (% based on 5000 pseudoreplications) are shown on branches



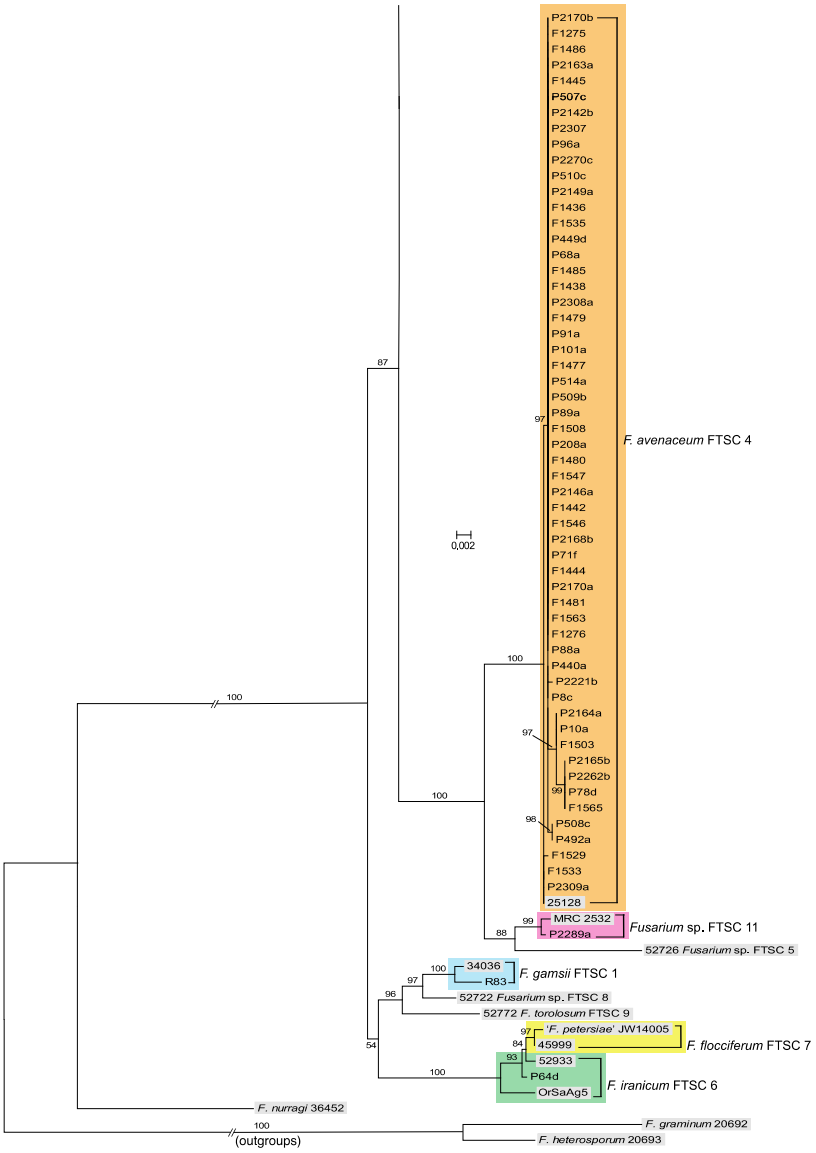


Figure S3 (Continued)

CHAPTER 3:

Preliminary study about the distribution of fumonisins, biosynthesized by F. proliferatum, *in three different fractions (kernels, rachis and chaff) of durum wheat head*

3.1 Introduction

Fusarium head blight (FHB) is a small-grain cereal disease complex worldwide spread. It is caused by at least 17 *Fusarium* species (Parry et al., 1995) and it is responsible for economic losses also related to reduction of quality parameters and mycotoxin accumulation (Becher *et al.,* 2013). Despite *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* are recognized as major FHB pathogens, other species could contribute to the development of the disease (Parry et al., 1995; Xu and Nicholson, 2009). In particular, the presence of members of *F. tricinctum* species complex (FTSC) and *F. proliferatum* is variable according to different climatic conditions. Indeed, recent works identified *F. proliferatum* as a pathogen associated with mycotoxin contamination also in wheat (Amato et al., 2015; Busman et al., 2012; Cendoya et al., 2018; Palacios et al., 2011). *F. proliferatum* is a polyphagous and widespread pathogenic species belonging to *Gibberella fujikuroi* species complex (GFSC) together with other 40 closely related species included the well-known *F. verticillioides* and *F. subglutinans* (Leslie and Summerell, 2006; O'Donnell et al., 2000). Its pathogenic capacity is proved on different horticulture crops, like onion, garlic, asparagus (Bargen et al., 2009; Dugan et al., 2003; Stankovic et al., 2007; Tonti et al., 2017) and on several cereals especially maize on which it is identified as one of the main responsible of ear rot (Desjardins et al., 2000; Logrieco et al., 2002). In addition, even if it has been reported also as a minor component of FHB, it shows the ability to cause "black point disease" on wheat kernels together with other fungal species (Desjardins et al., 2007). The "black point symptom" occurs after spikelet infections at early anthesis stage and it is characterized by a brown to black discoloration of the embryo ends of grains (Desjardins et al., 2007). The presence of black point can reduce the grain quality for industrial production (Mak et al., 2006). In detail, according to US standard, US No.1 grade wheat must have less than 2% black point kernels while US No. 2 wheat must have less than 4% black point kernels (Busman et al., 2012).

As well as the loss of grain quality, FHB also causes the mycotoxins accumulation on affected plant matrices. *F. proliferatum* can produce a wide variety of mycotoxins, that can cause both humans and animals food and feed contamination. Fumonisins, beauvericin, fusaproliferin, fusarin, and moniliformin are only some of the most important mycotoxins biosynthesized by *F. proliferatum* (Guo et al., 2016; Leslie and Summerell, 2006). Among them fumonisins are certainly the most common and toxic ones, especially considering their heat-stability (Ferrara et al., 2019; Marasas et al., 2004). In details, the biosynthetic pathway

for fumonisins production is regulated by the *FUM* gene cluster constituted by 17 genes including a gene encoding polyketide synthase (PKS), two genes encoding fatty acid synthases, nine genes encoding cytochrome P450 monooxygenases, dehydrogenases, transporter proteins, an aminotransferase, and a dioxygenase (Sun et al., 2019).

Among fumonisins analogues, fumonisin B1 (FB1) is the most frequent form produced by *F. proliferatum*, while fumonisin B2 (FB2), fumonisin B3 (FB3) and fumonisin B4 (FB4) are, in order, less prevalent (Cendoya et al., 2018; Voss et al., 2017). Due to toxicological effects in animals and humans, FB1 is classified by IARC (International Agency for Research on Cancer) as "2B" (possible carcinogen to humans) (Cendoya et al., 2017). To date, the Commission Regulation (EC) No 1126/2007 establishes the maximum fumonisin limits for human consumption in maize (4000 ppb) and maize-based foods, but not for wheat or wheat-based foods (Cendoya et al., 2018; Commission Regulation (EC), 2006). Unlike other well-known mycotoxins such as deoxynivalenol, the pathogenicity role of these harmful secondary metabolites is still not clear. However, considering that strains from field unable to produce fumonisins are rare, it is possible to suppose an ecological function of these compounds (Waalwijk et al., 2004). Several hypotheses have been advanced about their functionality. Since the production of secondary metabolites increases with oxidative stress, it could be possible that, besides providing ecological advantages, mycotoxins play a key role in maintaining the fungal oxidative state at non-harmful levels (Reverberi et al., 2010). Desjardins et al. (2002) demonstrated that fumonisins are not required for *F. verticillioides* to cause ear infection in maize. On the contrary, Williams and co-workers (2007) showed that *F. verticilliodes* fumonisin non-producing isolates were not able to cause leaf lesions and have less negative effect on root and stalk development. Glenn and co-worker (2008) suggested that fumonisins production in *F. verticillioides* is necessary for the development of foliar symptoms in some maize genotypes but that the incidence and the severity of the disease are depended on both maize genotype and amount of mycotoxins. Recently, Sun and co-workers (2019) observed that the inhibition of fumonisins biosynthesis resulted in a reduction of strains pathogenicity confirming their possible involvement in the virulence process.

In cereals, the production of fumonisins by *F. proliferatum* was recorded with high frequency in maize and maize-based products (Proctor et al., 2004; Zöllner and Mayer-Helm, 2006), but in the last ten years an increasing interest to evaluate the capacity to produce these compounds also by isolates obtained from durum and common wheat has been recorded (Amato et al., 2015; Cendoya et al., 2018; Guo et al., 2018; Palacios et al., 2011).

In addition, in a global warming scenario, low precipitation and high temperature could increase the spread of fumonisins producers species also in wheat (Braun and Wink, 2018; Dall'Asta and Battilani, 2016). The increased *F. proliferatum* frequency among FHB community has been observed on Italian durum wheat. In detail, in two investigations conducted, *F. proliferatum* was recorded on all investigated areas and regions confirming its high spread distribution and its adaptability to different agroclimatic conditions. Despite its high frequency, the level of fumonisins was found low in both years (see Chapter 1).

For these reasons, the present study aimed to test the ability of a *F. proliferatum* strain to produce fumonisins in field conditions after artificial inoculation of durum wheat head evaluating their distribution among three different fractions (kernels, chaff and rachis).

3.2 Materials and Methods

3.2.1 Fungal strain and inoculum preparation

The choice of *F. proliferatum* strain used for this study was made on the basis of previous studies. In brief, *F. proliferatum* strain F1383 isolated from durum wheat grains and stored in the Phytopathological Mycology Laboratory of the Agricultural Science Department of the Bologna (DISTAL), was selected in a previous field experiment conducted in the experimental station of University of Bologna (Cadriano) from November 2015 to July 2016. To test its pathogenicity on durum wheat, strain F1383 was inoculated at three different timings: 10% of extruded anthers (GS59) (Time 1), 30% of extruded anthers (GS61) (Time 2) and 70% of extruded anthers (Time 3). In addition, its ability to produce *in vitro* fumonisins was confirmed by a previous HPLC-FLD analysis. The disease incidence and severity evaluated 14 and 21 days post-inoculation, showed a high aggressiveness of F1383 at GS61 (Time 2). Moreover, an ELISA test of durum wheat grains obtained from the inoculated heads confirms the ability of this strain to produce fumonisins in open field.

The strain F1383 was previously cultured at 25°C on potato dextrose agar (PDA) in a 9-cm plate and then, a mycelial plug (5 mm of diameter) was transferred into 1 L flask containing 500 mL of V8 broth obtained diluting pure V8 juice (Campbell's, Camden, NJ, USA) previously sterilized by autoclaving for 15 min at 121°C.

Flask was incubated in an orbital incubator shaker (Thermo Scientific, USA) at 130 rpm with a temperature of 25°C. After 5 days of incubation, the conidia suspension was filtered and resuspended in sterile water. The inoculum concentration was adjusted at 1 x 10^6

conidia/mL by using a Thoma cell counting chamber. Fresh inoculum was used for the field inoculation.

3.2.2 Artificial inoculation of *F. proliferatum* on durum wheat head and sampling

The study was conducted in 2016-2017 season on the durum wheat variety "Saragolla". Plants were grown in 3 x 2 m field experimental plot arranged in randomized block design and located in the experimental station of University of Bologna (Cadriano 44°33'4.15" N; 11°24'39.02" E). The variety "Saragolla" was selected on the basis of the high susceptibility to FHB and its widespread cultivation in Emilia Romagna region. The previous crop was maize, and no fungicides were applied.

The experimental design included 3 replicates for the inoculated plots (FP) and 2 replicates for control. The variety was sown in late autumn. Each experimental plot was artificially inoculated at GS61 stage with 180 ml of spore suspension containing 1 x 10^6 conidia/mL of *F. proliferatum* F1383 strain.

At maturation stage, durum wheat heads were harvested and successively, kernels, rachis and chaff were hand-separated. The three fractions were subjected to visual examination.

Two sub-samples of harvested materials were arranged: one for the HPLC-FLD analysis (see next paragraph) and another one used for the mycological analysis. This last was conducted to detect the presence of *F. proliferatum* on the investigated fractions. In detail, kernels, rachis and chaff were surface disinfected with a 2% solution of NaClO for 3 minutes. After disinfection, the kernels, rachis and chaff were rinsed with sterile water and placed on Petri dishes containing Potato Dextrose Agar (PDA). After 6 days of incubation at 25 °C, plates were subject to visual and microscopic observation to assess the *F. proliferatum* development.

3.2.3 Fumonisins detection and quantification in different durum wheat head fractions by HPLC-FLD analysis

Chemicals and reagents

All reagents and solvents were of analytical grade. Fumonisin Mixture 50 µg/mL in acetonitrile, water, analytical standard, methanol, acetonitrile, water Lichrosolv HPLC Plus,

sodium phosphate monobasic monohydrate (NaH_2PO_4-H_2O), orthophosphoric acid were purchased from Sigma-Aldrich Co (St.Louis, MO, USA). Distilled water (RPE) for sample extraction was purchased from Carlo Erba Reagents (Milano, Italy). Fumonisin B3 (FB3) 50,3 μg/mL in acetonitrile-water was purchased from Romer Laboratories GMbH (Tulln, Austria). FumoniTest Developer A and B for HPLC, phosphate buffer solution (PBS), and immunoaffinity FumoniTest WB columns were purchased from Vicam (Milford, MA).

Fumonisins extraction and purification

Fumonisins were extracted according to the method suggested by Solfrizzo et al. (2001). In detail, samples were finely grounded in a blender (IMETEC) obtaining a sub-sample of 20 g for kernels, 10 g for chaff and rachis for each analyzed thesis (3 biological replicates for inoculated plots and 2 biological replicates for control plots). The grounded material was transferred in a centrifuge tube and added with 50 mL of a mixture of acetonitrile-methanol-water (25:25:50 v/v), vortex-stirred and placed in a pivoting stirrer at 210/min for 1 hour. An additional intermediate stirring was performed with vortex after 30 min and centrifuge (3000 rpm; 5 min) and then the supernatant was filtered on Buchner with Whatman No. 4 paper filter. The residual pellet was resuspended in 50 mL of extraction solvent and the previous operations were repeated leaving it agitated on a shaker for 30 minutes. Finally, the two filtrates were mixed. Ten mL of the filtrate were added into 40 mL of PBS (1:5), centrifuged (3000 rpm for 10 min.) and filtered as described above. A total of 10 mL of filtrate, equivalent to 0.4 g of sample, was purified by passing through an immunoaffinity column with a flow rate of 1-2 drops/s. Columns were washed with 10 mL of PBS and then the fumonisins were eluted in 1.5 mL of methanol. The solution was dried with nitrogen in thermoblock (40°C) and finally suspended in 200 μL of MeOH-H2O (50:50 v/v). An aliquot of 50 μL was derivatized by adding 225 μL of Developer A + B. Developer A was previously activated with 10 μL of Developer B. After 1 min. of derivatization, the sample was analyzed by HPLC.

FB1, FB2 and FB3 were identified comparing their retention times to standard solutions prepared by making appropriate working dilutions: FB1 (0.05-5.00 ug/mL), FB2, FB3 0.05-2.50 ug/mL for both analogues. For linearity, six-point calibration curves were prepared using the linear least squares regression procedure of peak area versus concentration. Three independent replicates were performed for each concentration.

HPLC–FLD analysis

HPLC–FLD analyses were performed on an Agilent 1260 Infinity system, equipped with a Series 1100 autosampler and fluorescence detector (FLD), Agilent Technologies, (Palo Alto, CA) operating at an excitation wavelength of 335 nm and an emission wavelength of 440 nm. The analytical column was a Kinetex C18 (150 mm × 4,6 mm, 5 μm particles) (Phenomenex, Torrance, CA, USA), preceded by a security guard C18 ultra cartridges. The elution was performed using 0.1 M methanol/ NaH_2PO_4-H_2O (77/23, v/v), brought to pH 3.3 with H_3PO_4. A total of 100 μL of samples was injected into the HPLC system at a flow rate of 0.8 mL/min at room temperature. Acquired data were analyzed with Chemstation for LC3D software (Agilent) (Tonti et al., 2017).

3.2.4 Statistical analysis

The fumonisins concentration for each analyzed fraction is reported as average value (±standard error, SE) of three independent replicates for the inoculated plots and two independent replicates for the control plots. The statistical analysis was conducted using Statgraphics 2.1 software. Data distribution was evaluated by Shapiro-Wilk test. Once tested the normal distribution of the data, the ANOVA (One-way analysis of variance) analysis was performed. The differences with a p-value < 0.05 were considered statistically significant.

3.3 Results and discussion

The present preliminary study aimed to evaluate the ability of *F. proliferatum* F1383 strain to produce fumonisins in durum wheat head and to evaluate the distribution of these compounds in three different durum wheat head fractions (kernels, rachis and chaff). Even if, several *in vitro* studies have been conducted on wheat-based media to test the ability of *F. proliferatum* to produce fumonisins (Cendoya et al., 2017, 2014a; Palacios et al., 2015), no-one of them tested the ability of this species to produce mycotoxins in open field and natural environment.

Mycological analysis allowed to isolate *F. proliferatum* in all three investigated fractions. The colonies were identified as *F. proliferatum* based on morphological features (Leslie and Summerell, 2006). White to pale-violet colonies with a floccose mycelium developed from the head tissue placed on PDA. In detail, macroconidia appear long, slender, usually 3-5 septate and thin-walled while microconidia were 0-septate, obovoid with a truncate base, ellipsoid or oval. Typical mono and poly-phialides were observed.

Kernels produced by artificially inoculated heads showed the typical black-point symptoms associated to *F. proliferatum*, while browning was also observed on chaff and rachis (Figure 1). No symptoms were observed in the three different fractions produced by non-inoculated heads.

HPLC–FLD analyses allowed to detect fumonisins (FB1, FB2 and FB3) in all investigated fractions (kernels, rachis and chaff) of artificially inoculated heads at GS61 (Figure 2). However, no significant differences were observed between the total fumonisins (FB1+FB2+FB3) content of the three investigated fractions (p = 0.53). The higher amount of total fumonisins (FB1+ FB2+ FB3) was detected in chaff (556.7 ± 125 μg/kg), while kernels and rachis showed a total amount of FB1+FB2+FB3 equal to 500 μg/kg (±125.0 μg/kg) and 333.3 μg/kg (± 109.7 μg/kg), respectively. According to several studies (Cendoya et al., 2014b; Chehri et al., 2010; Cirillo et al., 2003), FB1 was the main analogues that contaminated the three-fractions followed by FB2 and FB3. FB1 was detected mainly in the kernels (average 370 ± 115.3 μg/kg) followed by chaff and rachis that showed an average concentration of this analogue of 350 (± 115.3 μg/kg) and 260 (± 75.7 μg/kg) μg/kg, respectively. No significant differences were observed in the FB1 distribution among the investigated fractions (p = 0.74). FB2 was detected mainly in chaff (average 106.7 ± 33.8 μg/kg) followed by kernels (63.3 ± 6.7 μg/kg) and rachis (36.7± 20.3 μg/kg). The same trend was observed for FB3. In detail, for this analogue an average concentration of 100, 66.7 and

36.7 µg/kg was observed for chaff, kernels and rachis respectively. Considering the not-inoculated control, kernels were the only wheat fraction naturally contaminated with low levels of the three analogues (average amount of 15 µg/kg for FB1 and 25 µg/kg for both FB2 and FB3). On the contrary, in chaff and rachis of the non-inoculated plots, only FB1 analogue was detected (60, 30 and 15 µg/kg, in chaff, kernels and rachis, respectively).

Figure 1: Shrinking and black point symptoms observed on durum wheat kernels contaminated by *F. proliferatum* (a); dark-brown symptoms on chaff (b) and rachis (c).

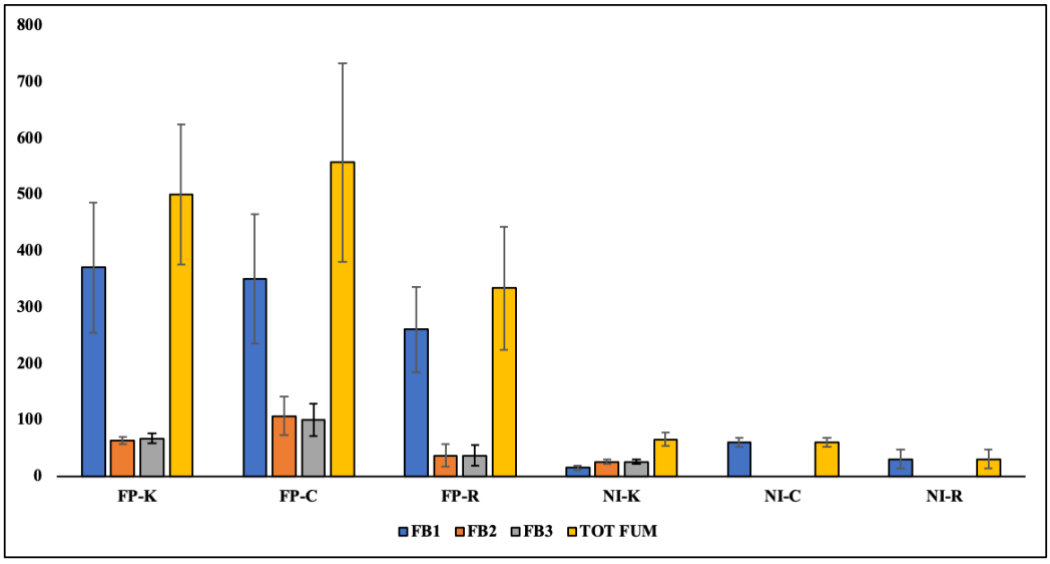


Figure 2: Average amount of fumonisins detected in the three investigated fractions (Kernels, chaff and rachis) in the two analyzed theses (inoculated and not inoculated). FP-K: inoculated kernels; FP-C: inoculated chaff; FP-R inoculated thesis rachis; NI-K: not-inoculated thesis kernels: NI-C: not-inoculated thesis chaff; NI-R: not-inoculated thesis rachis. FB1: Fumonisin B1, FB2: Fumonisin B2; FB3: Fumonisins B3; TOT FUM: sum of FB1, FB2 and FB3.

F. proliferatum has been often associated with wheat, however, the level of fumonisins contamination on this matrix resulted low if compared with that detected in maize. Several studies have been conducted worldwide to investigate the natural occurrence of these compounds on wheat and derivates. In most cases, the level of detected fumonisins did not exceed the legal limits provided for maize. In South America, a two-years investigation was conducted by Palacios and co-workers (2011) on Argentinian durum wheat. In 2007, these compounds were detected on the 97% of analyzed samples in a range of 10-1246 μg/kg, while, in 2008, low levels of fumonisins were found with FB2 concentration major than FB1. Another survey conducted in the same country confirms low fumonisins concentrations on common and durum wheat that were detected in a range of 0.16-608 μg/kg and 0.15-1304 μg/kg, respectively (Cendoya et al., 2014b). On the contrary, a different situation was observed in Brazil. In fact, Mendes and co-workers (2015) observed a contamination of FB1 in the 54% of analyzed common wheat samples ranging from 958 to 4906 μg/kg. In USA, FB1 was detected in durum wheat samples with a concentration ranging from 5 to 2210 μg/kg. In Asia, few studies have been conducted on fumonisins contamination. Liu and co-workers (2012) and Li and collaborators (2015) observed a low incidence of these secondary metabolites as well as a low concentration. Low fumonisin levels were detected in several studies conducted in Italy. Cirillo et al (2003) confirm the higher fumonisins concentration on maize products while on wheat the median of FB1 and FB2 was equal to 210 μg/kg. A three-year survey (2008-2010) was also conducted on durum wheat cultivated in Emilia Romagna where low levels of FB1 were detected with a maximum of 33 μg/kg in 2010 (Amato et al., 2015). Same trend was observed on Italian spelt with a maximum FB1 level of 70 μg/kg (Castoria et al., 2005). On the contrary, in Serbia the 82% (2012) and the 92% (2013) of durum wheat samples were contaminated with a high level of these compounds (maximum value 5400 μg/kg). In our study, even if after artificial inoculation, the level of fumonisins accumulated in all the investigated fractions was lower if compared with the limits established by UE on maize cultures. In details, the total amount of FB1+FB2 was equal to 433.3, 456.7 and 296.7 in kernels, chaff and rachis respectively.

In conclusion, even if several studies showed low fumonisins contamination in wheat and wheat-based products, the Brazilian and the Serbian reports demonstrated that, under favorable climatic and environmental conditions, also wheat can be a suitable substrate for fumonisins production and consequently, it could be a main source of FB1 intake as already observed in Netherlands (Bakker et al., 2003).

Several hypotheses have been proposed to explain the lower fumonisin accumulation on wheat compared with maize. Busman and co-workers (2012) hypothesized that wheat kernels are less favorable substrate for the production of fumonisins compared with maize. In detail, as for the synthesis of other mycotoxins, also for these compounds, nutritional components of barley and wheat could act as inhibitors of their biosynthesis. For example, the production of patulin by *Penicillium roqueforti* is related to the protein/carbohydrates ratio of the substrate: high ratio was not suitable for the production of the latter mycotoxin. In this context, the high protein/carbohydrates ratio in wheat and barley compared with that present in maize could be the cause of the lower fumonisins production on these cereals. The same authors explain this phenomenon with the host specialization and adaptation (Marín et al., 1999). In fact, even if adapted to grow in other grains, *F. proliferatum* could be unable to adapt the secondary metabolism to produce fumonisins on this substrate. Other authors suggest that the presence of a different mycobiota on barley and wheat compared with that associated with maize could inhibit the biosynthesis of fumonisins by F. *proliferatum* or degrade the mycotoxins after being produced (Mendes et al., 2015). Another possible explanation could be the environmental factors such as temperature and humidity. On maize, *F. proliferatum* showed better performance at lower temperatures and in presence of a not excessive water stress if compared with *F. verticillioides*. In other words, *F. proliferatum* could be responsible for fumonisin contamination in maize in the earliest stages of kernels development when the temperatures are still mild, while, when water stress increased, this species could be replaced by *F. verticillioides* (Marín et al., 1999).

Another important role in mycotoxin production is played by temperature. The optimum temperature and a_w reported for inducing fumonisin *in-vitro* production ranged from 20 to 25°C and 0.95–0.99, while no production was observed at ≤0.93 a_w and 10°C (Cendoya et al., 2014a; Marín et al., 2010; Medina et al., 2013; Mogensen et al., 2009). In addition, in an *in-vitro* study, Cendoya and co-workers evaluated the *FUM* gene expression at different temperatures. The authors observed a slower mycotoxins production at 15 °C suggesting that the kinetics of fumonisins production could be influenced by the temperature (Cendoya et al., 2017).

Substrates and environmental characteristics can influence not only the total amount of fumonisins but also the prevalence of an analogue compared with another. In our study, in all investigated fraction, FB1 was the main mycotoxin accumulated while FB2 and FB3 showed a lower concentration. Cendoya et al. (2014a) observed that the optimal temperature for FB1 and FB2 production were different: the production of FB2 was favor by temperature

lower than 25 °C while at 30 °C the FB1 analogue represented the main fumonisin produced. This highlights that *in vitro* studied do not consider several factors that can influence the mycotoxin production in open field such as competition with other species and climatic fluctuations. In addition to agro-climatic condition, also the single isolate toxigenic ability can influence the amount of fumonisins in a specific field or geographic area as already observed in other studies conducted on several members of GFSC (Beccari et al., 2020; Covarelli et al., 2012; Mendes et al., 2015; Palacios et al., 2015). For this reason, a development of the present research would be to test the ability of a major number of *F. proliferatum* strains to produce fumonisins under natural conditions, considering the key role played by the aggressiveness of the single isolate.

This was a preliminary study aimed to evaluate, in open field conditions, the real risk connected with fumonisins accumulation in wheat cultivated in Northern Italy climatic conditions. Even if the amount of these compounds was largely lower than the limit established by the EU for maize, the hypothetical co-occurrence of fumonisins and other toxic secondary metabolites should be not underestimated. In addition to sanitary issues, the high presence of black point symptoms on kernels causes a reduction of grain quality making it unsuitable for industrial transformation. Future studies will examine the *F. proliferatum* strains in different environmental conditions and on different durum wheat variates to have more detailed data of the real risk connected with the fumonisins accumulation on durum wheat cultivated in Italian climatic conditions.

Collaboration

The HPLC-FLD analysis was carried at the DISTAL Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna with the technical support of Dr. Mara Mandrioli.

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Final conclusions

Fusarium head blight (FHB) is one of the most destructive cereal diseases spread worldwide. *Fusarium* species distribution and predominance are influenced by many factors such as environmental and climatic conditions, especially at local level (microscale) (Xu, 2003). These variables can play a key role not only in the infection and colonization process, but also in the distribution and prevalence of each species and, consequently, in the mycotoxin contamination of cereal grains (Pancaldi et al., 2010; Scala et al., 2016). Although the principal species responsible for FHB in Europe are *F. graminearum*, *F. culmorum* and *F. poae* (Bottalico and Perrone, 2002), other "secondary" species have become increasingly important contributors to FHB.

The dynamism of FHB complex across a region or a season triggered not only a quantitative but also a qualitative modification of cereal mycotoxins contamination.

In this contest, a continuous and extensive monitoring of cereal crops in a specific cultivation area is essential to follow the FHB complex evolution as well as to predict the phytosanitary condition of the grains. For this reason, a two-years survey was conducted to evaluate the FHB community composition on Italian durum wheat, one of the most important cultivated cereal species.

In both surveyed years, *F. proliferatum* was the principal species detected with DFB method. In the second year of investigation, in addition to *F. proliferatum*, *F. avenaceum*, a member of FTSC, was one of the principal species associated with FHB community in Italy even if some regional differences were observed. In particular, the presence of members of *F. tricinctum* species complex (FTSC) and *F. proliferatum* was variable according to different climatic conditions. In detail, the increased presence of *F. avenaceum* and other closely related species might be due to climate change and/or agricultural practices confirming previous studies (Gräfenhan et al., 2013; Karlsson et al., 2017; Vogelgsang et al., 2019; Cowger et al., 2020).

The present study confirms a "dynamism" within the FHB community already observed in Italy and the consequent increasing of the risk connected with the simultaneous presence of several mycotoxigenic fungi and, therefore, a wide range of secondary metabolites. This evidence highlights the importance of these species in the fungal community despite their relative weak virulence in comparison to *F. graminearum* considered one of the main FHB agents (Beccari et al., 2016, 2017, 2018).

These data confirm the risk connected with the gradual expansion of durum wheat cultivation along the peninsula. In addition, this survey highlights as the climatic conditions of South Italy are suitable to obtain a durum wheat raw material of high quality for the food industries and the final consumers.

Focusing on these two predominant species, two different studies were conducted: the first one aimed to understand the genetic diversity and mycotoxin potential of the FTSC strains mainly isolated from cereals, the second one focused on the pathogenetic role and the real mycotoxins risk connected with the presence of and *F. proliferatum* on Italian durum wheat. The GCPSR analysis based on *TEF1, RPB1* and *RPB2* sequences of 117 FTSC isolates allowed to detect five unnamed phylospecies new to science (i.e., *Fusarium* spp. FTSC 11 - 15) in addition to other already known species such as *F. avenaceum*, *F. tricinctum* and *F. acuminatum*. In details, the novel unnamed species FTSC 12 was the second most common species in Italian analyzed collection confirming the importance of a molecular approach in addition to the morphological identification. Fifty-nine isolates comprising 10 FTSC phylospecies were selected to assess their ability to produce mycotoxins and other secondary metabolites *in-vitro* on rice showing a significant production of secondary bioactive metabolites such as MON and ENNs and other less known secondary metabolites for which limited data concerning acute and chronic toxicity are available. In conclusion, considering the increased detection of FTSC members on cereals worldwide, the present study significantly increases our knowledge of FTSC species diversity and mycotoxin potential associated to FHB on Italian cereals. In addition, MON and ENNs toxin levels in cereals and other food and feed are currently not regulated by the European Food Safety Authority, for this reason, the toxin data reported in the present study should provide a robust framework for improving our understanding of the risk they pose to human health and food security (EFSA, 2014, 2018).

The present work allowed to detect an increased *F. proliferatum* frequency among FHB community on Italian durum wheat. In detail, in two investigations conducted, *F. proliferatum* was recorded on all investigated areas and regions confirming its high spread distribution and its adaptability to different agroclimatic conditions. However, despite its high frequency, the level of fumonisins was found low in both years. The preliminary study conducted aimed to evaluate, in open field conditions, the real risk connected with fumonisins accumulation in wheat cultivated in Northern Italy climatic conditions. Kernels produced by artificially inoculated heads showed the typical black-point symptoms associated to *F. proliferatum*, while browning was also observed on chaff and rachis. HPLC–

FLD analyses allowed to detect fumonisins (FB1, FB2 and FB3) in all investigated fractions (kernels, rachis and chaff) even if the amount of these compounds was largely lower than the limit established by the EU for maize. These data confirm previous studies that showed a lower fumonisin accumulation on wheat compared with other substrate such as maize. However, the hypothetical co-occurrence of fumonisins and other toxic secondary metabolites should be not underestimated.

In conclusion, the present study significantly increases our knowledge on the dynamism and the complexity of Italian FHB community, the genetic diversity, the mycotoxin potential inside the FTSC and the real risk connected with fumonisin produced by *F. proliferatum* on wheat in open field conditions.

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