Research Article

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Different diagnostic approaches for the characterization of the fungal community and *Fusarium* species complex composition of Italian durum wheat grain and correlation with secondary metabolite accumulation

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Abstract

BACKGROUND: The evolution of the fungal communities associated with durum wheat was assessed using different diagnostic approaches. Durum wheat grain samples were collected in three different Italian cultivation macro-areas (north, center and south). Fungal isolation was realized by potato dextrose agar (PDA) and by deep-freezing blotter (DFB). Identification of *Fusarium* isolates obtained from PDA was achieved by partial *tef1α* sequencing (PDA + *tef1α*), while those obtained from DFB were identified from their morphological characteristics (DFB + mc). The fungal biomass of eight *Fusarium* species was quantified in grains by quantitative polymerase chain reaction (qPCR). Fungal secondary metabolites were analyzed in grains by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Correlations between *Fusarium* detection techniques (PDA + *tef1α*; DFB + mc and qPCR) and mycotoxins in grains were assessed.

RESULTS: *Alternaria* and *Fusarium* showed the highest incidence among the fungal genera developed from grains. Within the *Fusarium* community, PDA + *tef1α* highlighted that *F. avenaceum* and *F. graminearum* were the most represented members, while, DFB + mc detected a high presence of *F. proliferatum*. *Alternaria* and *Fusarium* mycotoxins, principally enniatins, were particularly present in the grain harvested in central Italy. Deoxynivalenol was mainly detected in northern-central Italy.

CONCLUSIONS: The adoption of the different diagnostic techniques of *Fusarium* detection highlighted that, for some species, qPCR was the best method of predicting their mycotoxin contamination in grains.
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Supporting information may be found in the online version of this article.

Keywords: *Alternaria*; *Fusarium*; mycotoxins; wheat; qPCR; LC–MS/MS

INTRODUCTION

Durum wheat is one of the most important cultivated cereals in the EU[1] and, among EU countries, Italy is one of the top producers.[2] Italy is also the main pasta producer and exporter in the world, so a focus on the quality of the raw material is important in the Italian durum wheat supply chain.[3] The majority of Italian durum wheat production comes from southern regions, an area traditionally suited for growing durum wheat.[4] However, lately, durum wheat cultivation has expanded to the central-northern regions,[4] resulting in higher production but, at the same time, increasing the risk of fungal diseases.[5,6]

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These macro-areas are characterized by higher humidity, which promotes fungal development. This can reduce grain quality.[7-10]

A wide range of fungal communities are associated with wheat grains.[11] Some members of these communities can biosynthesize mycotoxins – secondary metabolites that highly dangerous to human health. Among them, the genera *Alternaria* and *Fusarium* are usually the main components of grain mycobiota in many cultivation areas.[7,12,13]

Many *Fusarium* species are associated with *Fusarium* head blight (FHB), a wheat disease resulting in yield losses and quality reduction due to grain mycotoxin contamination.[14,15] The distribution of *Fusarium* species is influenced by climatic conditions (especially during anthesis), agricultural practices, fungicide application, and cultivar susceptibility.[5,6,16-25] These aspects give dynamism to the *Fusarium* "consortium" associated with grain.[7,26,27]

The main FHB causal agent is the aggressive species *Fusarium graminearum sensu stricto* (hereafter *F. graminearum*), a member of the *Graminearum* clade of the *Fusarium sambucinum* species complex (FSAMSC).[21,28-31] The *Fusarium culmorum* species (belonging to the *Graminearum* clade of FSAMSC) is considered another aggressive FHB causal agent, even if its distribution is not as extensive as that of *F. graminearum*.[32] In recent decades, an increased incidence of less aggressive species, such as *Fusarium poae* (*Sambucinum* clade of FSAMC), members of the *Fusarium tricinctum* species complex (FTSC), *Fusarium proliferatum* (a member of the *Fusarium fujikuroi* species complex, FFSC), or members of the *Fusarium incarnatum-equiseti* species complex (FIESC), was detected in many cultivation areas, including Italy.[7,21,22,33-44] Finally, within FSAMC, other species such as *Fusarium sporotrichioides* and *Fusarium langsethiae* (*Sporotrichioides* clade) are considered important members of the community.[45]

Among the secondary metabolites biosynthesized by *Fusarium* species, type A (for example, T-2 and HT-2 toxin) and B [for example deoxynivalenol (DON) and nivalenol (NIV)] trichothecenes, are the most monitored for their occurrence and toxicity. In particular, DON (mainly produced by *F. graminearum* and *F. culmorum*) is considered the most common wheat mycotoxin and it is the cause of cytotoxicity, immunotoxicity, reproductive toxicity, possible carcinogenicity, teratogenicity, and mutagenicity in humans and animals.[46] For this reason, legal limits for this compound on cereal grains (for example 1750 μg kg^{-1} for unprocessed durum wheat) have been established by the EU.[47] For T-2 and HT-2 toxins (mainly produced by *F. sporotrichiodies* and *F. langsethiae*), being characterized by very high toxicity,[48,49] the EU also settled a maximum recommendation level on cereal grains (for example 100 μg kg^{-1} for unprocessed wheat).[50] Nivalenol (mainly produced by *F. graminearum*, *F. culmorum* and *F. poae*), despite not regulated, is another common contaminant of cereal grains with potential health implications.[51] Recently, other secondary metabolites, such as depsipeptides – enniatins (ENNs) and beauvericin (BEA) – and moniliformin (MON), mainly produced by members of the FTSC, have attracted the attention of the scientific community both for their potential negative impact on human health[52-54] and for their interaction with the plant and other *Fusarium* species.[55,56]

The constant change of the *Fusarium* 'consortium', and of the other fungal microorganisms composing the wheat grain mycobiota, determines the plethora of secondary metabolites that can accumulate. For this reason, it is essential to monitor the evolution of the fungal community in a certain cultivation area to assess the risk of mycotoxin contamination.

So far, many surveys have been conducted on durum wheat grains harvested in Italy.[5,7,8,10,22,33,57-60] They have differed in the extent of the area that was examined (single regions or selected regions as representatives of cultivation areas), the observed fungal genera (only *Fusarium* or also other fungal microorganisms), the fungal isolation method adopted (artificial growth media or deep-freezing blotter, DFB), the identification method of the isolated *Fusarium* species [morphological, molecular by polymerase chain reaction (PCR) with species-specific primers or by PCR following the amplification of target gene regions, the quantification of the *Fusarium* species in the grains (by real-time quantitative PCR, qPCR), and in the quantification of different types, and with different techniques, of secondary metabolites.

Given this context, the present study aimed to combine and compare all the different approaches adopted in the previous studies. Durum wheat samples were collected in three main cultivation macro-areas (north, center, and south). The fungal communities, isolated from kernels with potato dextrose agar (PDA) or DFB, were determined exclusively by the observation of morphological characteristics. With a particular focus on the *Fusarium* species, the isolates obtained using the PDA method were identified by partial *translation elongation factor 1α* (*tef1α*) sequencing, whereas those obtained with the DFB method were identified based on their morphological characteristics (microscopic analysis). The choice of these two approaches was made to deploy and compare the most commonly adopted method of 'fungal isolation + *Fusarium* identification' described in the previous surveys. The fungal biomass of eight *Fusarium* species in the kernels was quantified by qPCR. A wide range of fungal secondary metabolites was analyzed in grains by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Finally, for the most important *Fusarium* species, correlations between different isolation + *Fusarium* identification approaches, qPCR and the accumulation of the most important secondary metabolites in grains were determined.

MATERIALS AND METHODS

Durum wheat sampling and determination of durum wheat fungal community

The present survey was carried out on 70 durum wheat samples collected from 13 Italian regions (Fig. 1). All the samples were obtained from crops during the 2017–2018 season. Based on their origin, the samples were grouped in three macro-areas (north, center, and south Italy), typically characterized by different climatic conditions. Detailed information for each sample can be found in Table S1 in the supporting information. After harvest, samples (about 500 g each) were divided into three representative sub-samples (150 g each): one for mycobiota determination, one for DNA quantification in the grain by qPCR of eight *Fusarium* species associated with FHB, and one for fungal secondary metabolite quantification in the grain by LC–MS/MS analysis.

To determine the mycobiota of durum wheat grains, two methodologies were used: (1) fungal isolation on PDA; (2) fungal isolation on DFB.

Fungal isolation on PDA

The fungal community associated with durum wheat grains on PDA was isolated as described previously.[61] After 5 days of

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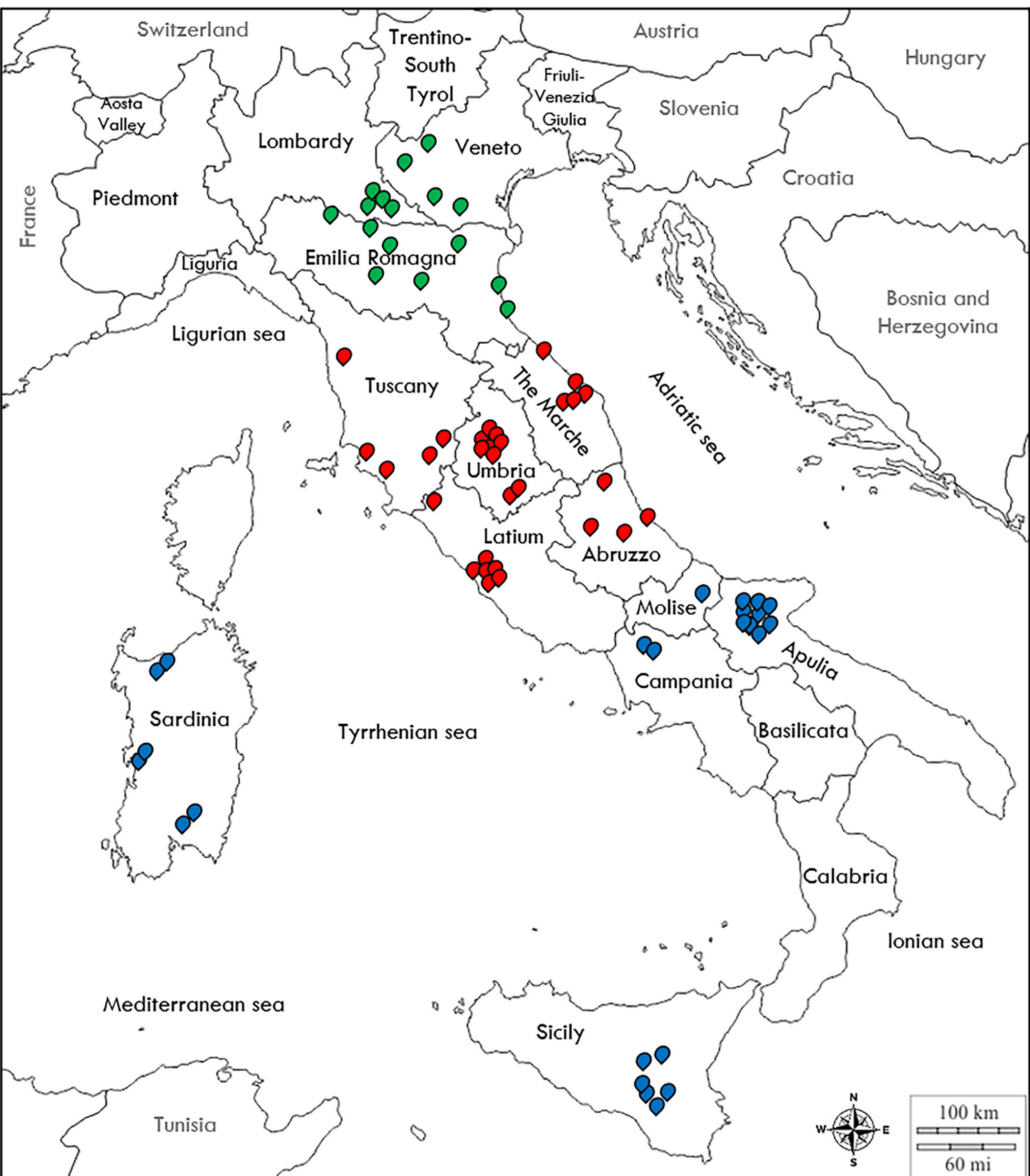

Figure 1. Map of Italy showing the 70 sampling locations (tags). Durum wheat samples were ascribed to three macro-areas: north (green tags), center (red tags) and south (blue tags).

incubation (22 °C in the dark), the fungal colonies deriving from kernels were observed both under a stereomicroscope (SZX9, Olympus, Tokyo, Japan) and with an optical microscope (Axiophot, Zeiss, Oberkochen, Germany) for genus identification.

Fungal isolation on DFB

The fungal species associated with durum wheat grains were isolated using the DFB method[62] as described previously.[10] After 7 days of incubation, the fungal colonies developed from every single kernel were observed under a stereomicroscope

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(SZX9, Olympus) and an optical microscope (Bx41TF, Olympus) for genus identification.

Identification of *Fusarium* species developed from durum wheat grains

To identify the *Fusarium* species obtained with the PDA or DFB isolation methods, the two approaches commonly adopted in previous studies were used as follows: (1) the species obtained with the PDA method were molecularly identified; (2) the species obtained with the DFB method were morphologically identified.

Molecular identification of the Fusarium species isolated with the PDA method

After the observations described above, all the isolates morphologically identified as belonging to the genus *Fusarium* were transferred into new plates containing PDA and placed at 22 °C in the dark. After 15 days, the cultures were assigned to specific morphotypes based on colony morphology (color and shape) and on the morphology of reproductive structures observed under the optical microscope (Axiophot, Zeiss). This selection resulted in a subset of representative isolates of each morphotype for each durum wheat sample. After obtaining single-spore cultures, the representative isolates were placed into new PDA plates at 22 °C in the dark for 15 days.

The preparation of isolates for DNA extraction was realized as described previously,[7] while DNA extraction was carried out using the method already outlined.[61] The DNA extracted from *Fusarium* isolates was subject to partial *tef1α* gene amplification with EF1 and EF2 primers[63] followed by purification and sequencing carried out by an external service (Genewiz Genomics Europe, Leipzig, Germany). The sequences obtained were verified and edited using Chromatogram Explorer Lite v4.0.0 (Heracle Biosoft Srl 2011, Arges, Romania) and analyzed by comparison with those deposited in the NCBI Basic Local Alignment Search Tool (BLAST) database[64] and with those deposited in the *Fusarium* Multilocus Sequence Typing (MLST) database (http://www.westerdijkinstitute.nl/fusarium/; https://fusarium.mycobank.org/).[65,66] The species identification was based on >99.4% similarity between the query and reference sequences.[67]

The abundance of each *Fusarium* species involved in the *Fusarium* complex in each of the three investigated macro-areas was calculated as the total number of isolates belonging to the morphotype from which the identified representative isolate was sub-sampled.

Morphological identification of the Fusarium *species isolates with the DFB method*

All the isolates morphologically identified as belonging to the genus *Fusarium* were identified based on the morphology of reproductive structures: shape and size of macroconidia; presence, absence and shape of microconidia; branching type of conidiogenous cells (monophialides or polyphialides); and ability to produce chlamydospores. The identification was carried out by direct observation under the optical microscope (Bx41TF, Olympus) or/and after growth on PDA and carnation leaf agar (CLA), following the methods described previously.[68-70] When the identification could not be realized only by the microscopic observation, single-spore cultures were also plated on Spezzieller Nährstoffarmer agar (SNA), on which morphological characteristics were observed as previously described after an incubation time of 21 days at 24 °C (12 h light).

Fungal biomass quantification of eight *Fusarium* species in durum wheat grains by qPCR

DNA was extracted from pure fungal cultures of eight selected *Fusarium* spp., and from healthy durum wheat grain to determine the standard curves for qPCR analyses. In detail, the fungal strains used to obtain standard curves (Table S2[7,57,58,61,71]) were grown on PDA for 1 week at 22 °C in the dark and then DNA from their mycelium was extracted as previously described.[61] DNA from healthy durum wheat grain was extracted following a method outlined previously.[16] The quality and concentration of the DNA were determined by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 (Thermo Fisher Scientific). Dilution series from 0.05 pg to 5 ng of fungal strains DNA and from 5 pg to 50 ng of wheat grain DNA, with a dilution factor of 10, were used to plot standard curves in each qPCR set, using two technical replications for each assay. Standard curves, line equations, R^2 values, reaction efficiencies and limits of detection (LOD) were calculated.[16] qPCR analyses were carried out using species-specific primers indicated in Supporting Information, Table S3.[72,73] qPCR assays were performed in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with a previously described protocol.[7]

About 100 g of each durum wheat sample were finely ground with a blender and 4 g of ground grains were used for total DNA (durum wheat DNA and potential fungal DNA) extraction using a previously described method.[16] The DNA quality and concentration were estimated as previously described and the concentration of each sample was adjusted to 25 ng μL^{-1} for qPCR analysis. Quantitative PCR assays were performed as previously described. A dissociation curve was obtained at the end of the qPCR program to monitor the presence of primer-dimers and/or non-specific amplification products. Two technical replicates per sample were used in each assay. The fungal biomass of each investigated *Fusarium* species was expressed as the ratio of the detected DNA (pg) to the total durum wheat grain DNA (ng).

Detection and quantification of secondary metabolites by LC–MS/MS

Sub-sampled durum wheat kernels were finely ground (<0.5 mm) by a blender (IMETEC, Azzano San Paolo, Milan, Italy) and 5 g of each milled sample was extracted for 90 min using 20 mL of acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a 1 + 1 dilution using acetonitrile/water/acetic acid (20/79/1, v/v/v). Finally, 5 μL of the diluted extracted were directly injected as previously described.[74] Quantification was based on external calibration using a serial dilution of a multi-analyte stock solution. Results were corrected for apparent recoveries (thus taking into consideration both matrix effects as well as recoveries of the extraction) that had been determined during method validation.[74] As part of ongoing assurance the trueness of the method is monitored by analyzing samples deriving from a proficiency testing scheme organized by BIPEA (Gennevilliers, France) with a rate of satisfactory z-scores of $-2 < z < 2$ of >95% for the 1700 results submitted so far. Limits of detection and quantification were determined following the EURACHEM guide.[75]

Statistical analysis

Data regarding the mycobiota composition and the presence of *Fusarium* species were analyzed using a generalized linear model (GLM) with a Poisson error and a log link. A scale parameter was added to account for over/under-dispersion (quasi-Poisson model); isolation method and macro-areas were used as the

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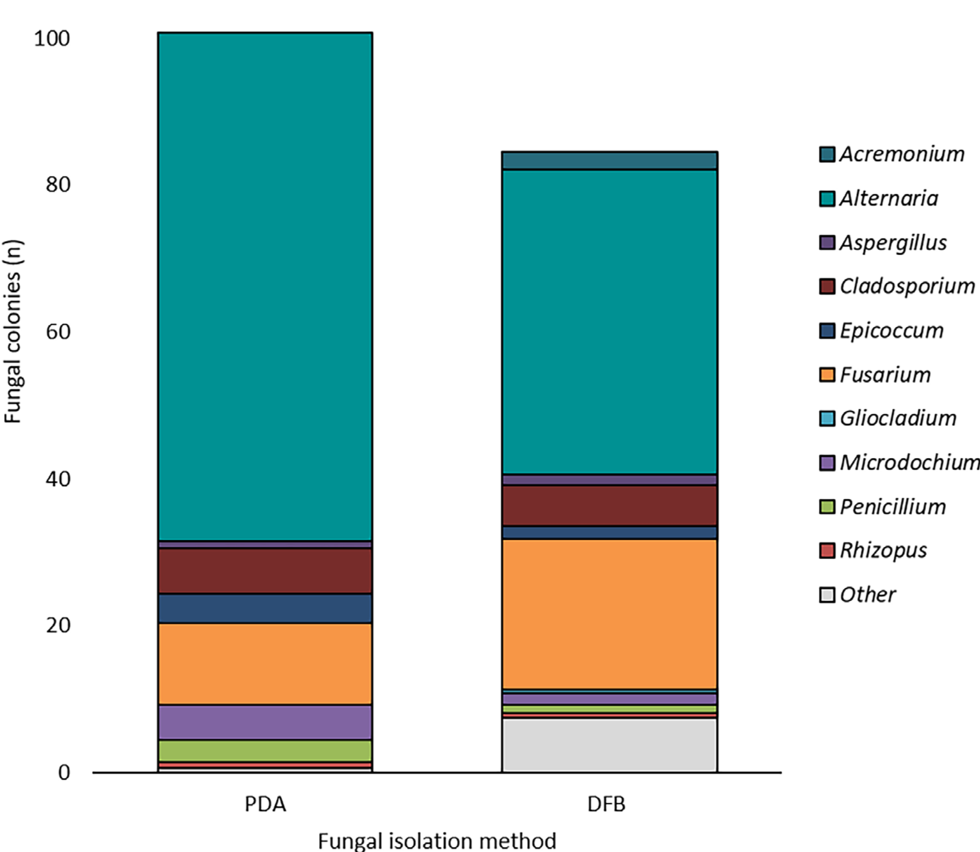

Figure 2. Average number of fungal colonies (*n*) per durum wheat sample belonging to different fungal genera as visually and microscopically assessed after their development from durum wheat grains collected in Italy with two different isolation methods (potato dextrose agar, PDA; deep freezing blotter, DFB). Columns represent the fungal community composition expressed as the average number of isolates of different genera developed from 70 durum wheat samples with each method.

factors. Data were analyzed using a GLM, with the Poisson error and log link. Back-transformed counts with delta standard errors were derived and reported in figures and tables. Data about biomass and secondary metabolites were analyzed using a heteroscedastic linear model, with a different variance per macro-area (generalized least square, GLS, fitting).[76] The macro-area was included as a factor. Both for GLM and GLS fits, pairwise comparisons were performed using a general procedure outlined previously.[77] Canonical variate analyses were used to discriminate among macro-areas according to the content in metabolites; results were presented biplots.[78] The correlations were studied by using the Pearson correlation coefficient. All analyses were performed by using the R statistical environment,[79] together with the packages 'nlme',[76] 'emmeans',[80] and 'vegan'.[81]

RESULTS

Composition of the fungal community of durum wheat grains

The analysis of the fungal community in the grains by PDA and DFB isolation methods showed the presence of 'infected' kernels (from which fungal colonies developed) as well as of 'healthy' seeds (from which no fungal development was observed) (Table S4 in the supporting information). The PDA method showed a significantly ($P < 1 \times 10^{-4}$) higher presence of fungal colonies in comparison with the DFB method. Both methods, highlighted that the southern Italian samples were always those presenting the lowest average number of fungal colonies developing from grains ($P < 0.009$) (Supporting Information, Table S4).

The overall fungal community isolated was composed of fungal colonies belonging to the following genera: *Acremonium* (isolated only by DFB), *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Gliocladium* (isolated only by DFB), *Microdochium*, *Penicillium*, and *Rhizopus*. Colonies that could be not identified by visual observation were classified as 'other' (Fig. 2, Supporting Information, Table S5).

In both methods, *Alternaria* was the genus with the significantly higher ($P < 1 \times 10^{-4}$) average number of colonies than all other genera detected. *Fusarium* was the genus with the second highest average number of colonies developed from grains ($P < 0.03$) analyzed by PDA and DFB. All other genera showed, in both methods, an average number of colonies significantly lower than those belonging to *Alternaria* and *Fusarium* ones.

The two methods had different effects to promote the development of fungal colonies belonging to certain genera. In detail, the PDA method allowed to isolate a significantly higher average

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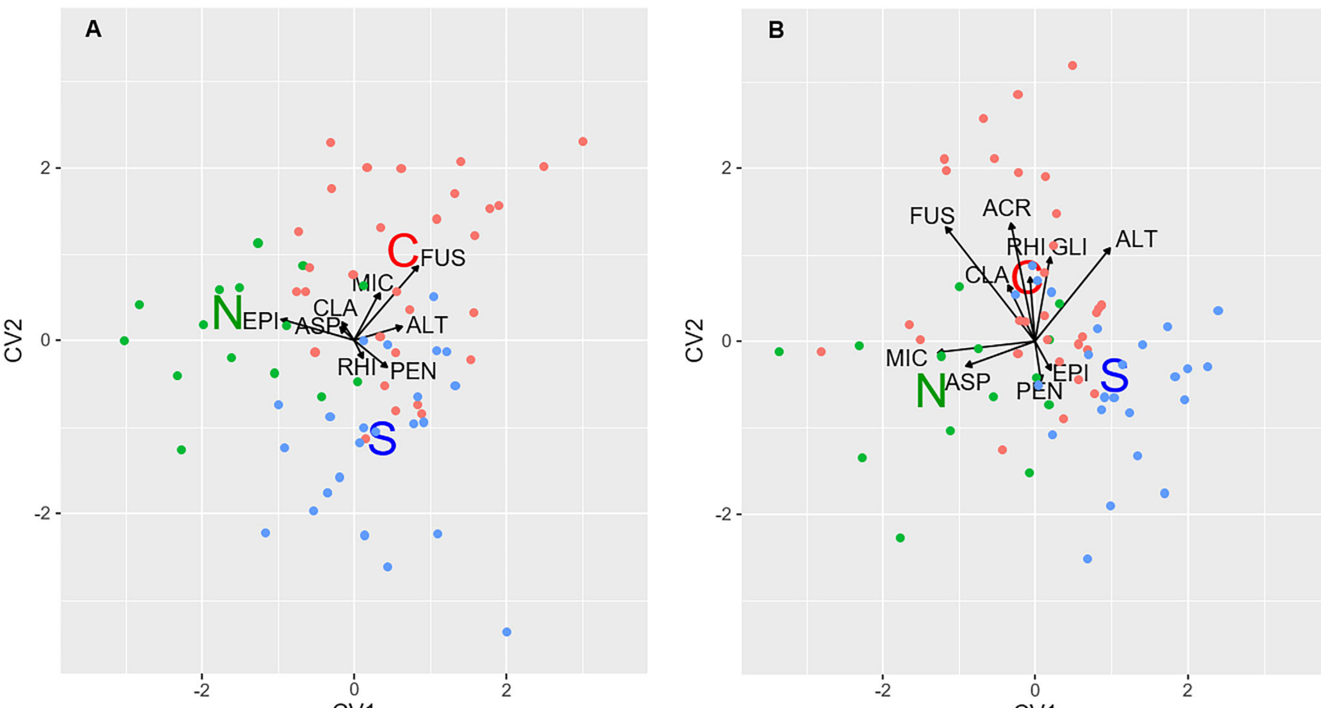

Figure 3. Biplot from the canonical variate analysis for the fungal genera isolated on potato dextrose agar (PDA) (A) and using deep freezing blotter (DFB) (B) in grain samples collected in the three Italian macro-areas. N: northern Italy, C: central Italy; S: southern Italy; ACR: *Acremonium*; ALT: *Alternaria*; ASP: *Aspergillus*; CLA: *Cladosporium*; GLI: *Gliocladium*; EPI: *Epicoccum*; FUS: *Fusarium*; MIC: *Microdochium*; PEN: *Penicillium*; RHI: *Rhizopus*. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area. In detail, green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

number of colonies belonging to *Alternaria* ($P = 1 \times 10^{-4}$), *Epicoccum* ($P = 0.01$), *Microdochium* ($P = 3 \times 10^{-4}$) and *Penicillium* ($P = 0.01$) genera, than DFB. Conversely, DFB promoted a significantly higher development of *Fusarium* species ($P = 1 \times 10^{-4}$), than PDA.

For some fungal genera, significant differences in the distribution across the three macro-areas were detected (Fig. 3(A), (B)). For example, in the DFB method, *Alternaria* were significantly higher ($P < 0.01$) in central and southern Italy than in northern Italy. A similar pattern, although not significant ($P > 0.08$), was also obtained with PDA. Durum wheat samples harvested in central Italy were also those with the highest number of fungal colonies belonging to the genus *Fusarium*. In detail, in DFB the average number of *Fusarium* colonies followed the pattern center ≥ north > south ($P < 1 \times 10^{-4}$), whereas, in PDA it was center > north ≥ south ($P < 1 \times 10^{-4}$). In both methods, southern Italy was the macro-area with the lowest number of *Fusarium* colonies developed from grains (Fig. 3(A), (B)).

Fusarium complex composition in durum wheat grains

The total number of *Fusarium* isolates developed from durum wheat grains with PDA and DFB and successively identified by partial *tef1α* sequencing (isolates obtained by PDA) and by morphological characteristics (isolates obtained by DFB), was significantly higher in DFB ($P = 1 \times 10^{-4}$) than PDA (Table S6 in the supporting information). Without distinguishing between isolation methods, the total *Fusarium* isolates subject to identification followed the pattern: center ≥ north > south ($P < 1 \times 10^{-4}$) (Table S6 in the supporting information).

The *Fusarium* community isolated on PDA from the durum wheat grains and identified by partial *tef1α* sequencing (hereafter PDA + *tef1α*) was composed of a total of 15 different species (Table S7, Fig. 4). *Fusarium avenaceum* and *F. gramineraum* were the overall most represented species followed by *F. poae*, *F. proliferatum*, and FIESC members. In detail, no significant differences ($P = 0.4$) were detected between *F. avenaceum* and *F. graminearum*, but a significant difference ($P < 0.001$) was found between *F. avenaceum* and the other three species mentioned above. Some species (*F. antophilum*, *F. nelsonii*, *F. brachygibbosum*, *F. pseudograminearum*, and *F. oxysporum*) were detected only with the PDA + *tef1α* approach.

The *Fusarium* community isolated on DFB and identified by morphological characteristics (hereafter DFB + mc) comprised a total of 16 different species (Table S7 in the supporting information, Fig. 4). *Fusarium proliferatum* was the most represented species, followed by *F. globosum*, *F. avenaceum*/*F. acuminatum*, *F. verticillioides*, *F. graminearum*, and *F. culmorum*. In detail, *F. proliferatum* showed a significantly ($P < 1 \times 10^{-4}$) higher average number of fungal colonies developed from grain than the other species mentioned. The species *F. lateritium*, *F. globosum*, *F. langsethiae*, *F. subglutinans*, *F. verticillioides*, *F. semitectum*, and *F. chlamydosporum* were detected only with the DFB + mc approach. For *F. avenaceum* and *F. acuminatum*, accurate discrimination is possible only with the use of molecular identification. For this reason, isolates obtained by DFB and belonging to one of these two species were considered by DFB + mc as the *F. avenaceum*/*F. acuminatum* group. Finally, where morphological identification was not able to allow the isolates to be attributed to a certain *Fusarium* species they were considered as *Fusarium* spp.

Significant differences in terms of the average number of fungal colonies developed/identified were recorded for a total of three

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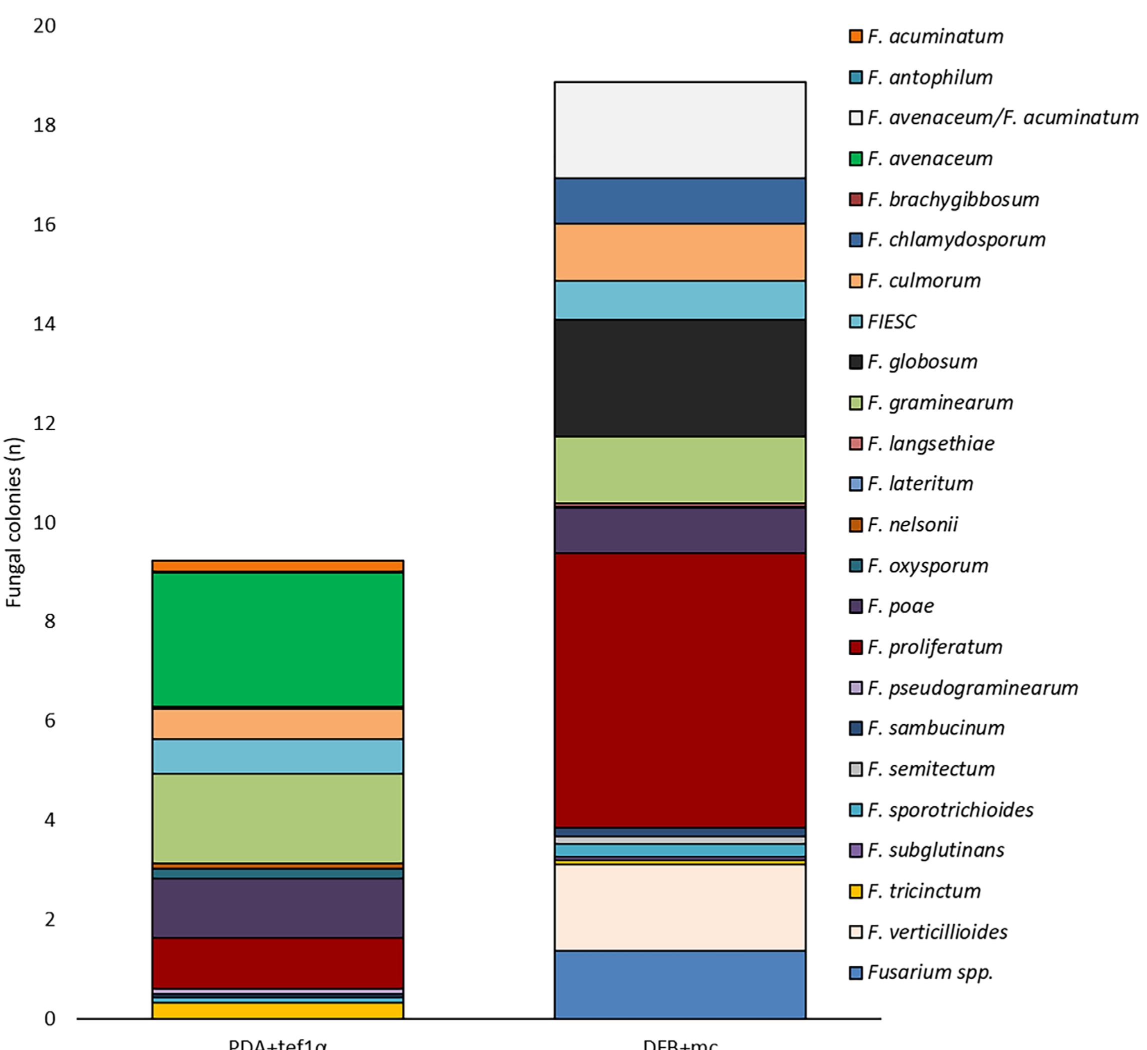

Figure 4. Average number of isolates (*n*) belonging to the different *Fusarium* species as identified by partial *translation elongation factor 1 α* (*tef1α*) sequencing or morphological observation after their isolation from durum wheat grains collected in Italy with two isolation methods (potato dextrose agar, PDA; deep freezing blotter, DFB). *Fusarium* isolated obtained with the PDA technique were identified by partial *tef1α* sequencing (PDA + *tef1α*), while those obtained with the DFB technique were identified by the observation of morphological characteristics (DFB + mc). Columns represent the *Fusarium* community composition expressed as the average number of isolates of different species developed from 70 durum wheat samples with each isolation method and following the identification approach.

species detected with both approaches. *Fusarium trincictum* showed a significantly higher number of colonies ($P = 0.02$) using the PDA + *tef1α* approach, while the number of fungal colonies belonging to *F. proliferatum* and *F. culmorum* was significantly higher ($P = 1 \times 10^{-4}$ and 0.02 respectively) with the DFB + mc approach.

The breakdown of the *Fusarium* complex into its single components (species) allowed the detection of differences in their distribution across the surveyed territories (Fig. 5(A), (B); Table S7 in the supporting information). For example, samples from northern and central Italy were characterized by the presence of *F. graminearum*, *F. poae*, *F. proliferatum*, and *F. tricinctum* as compared to southern Italian ones. Both isolation/identification approaches showed this for *F. proliferatum* ($P < 0.01$) (Fig. 5(A), (B)). *Fusarium graminearum* and *F. poae* showed a significant north ≥ center > south pattern adopting PDA + *tef1α* ($P < 0.002$) (Fig. 5(A)), while using DFB + mc the pattern was slightly different (center ≥ north ≥ south) ($P = 1 \times 10^{-4}$ for the center-south contrast and >0.05 for the center-north and north-south contrast), confirming, however, the lowest presence of these two species in southern Italy (Fig. 5(A), (B)). Central Italy was the macro-area where PDA + *tef1α* revealed the highest presence of *F. avenaceum* (Fig. 5(A)) and *F. tricinctum* in comparison to that recorded, for the first species, in Northern and Southern Italy ($P < 0.001$) or, for the second species, only in southern Italy ($P = 0.03$). Finally, the DFB + mc approach showed that southern Italian samples were characterized by a significantly ($P < 1 \times 10^{-4}$) higher presence of *F. culmorum* (Fig. 5(B)).

Fungal DNA accumulation in durum wheat grains

The R^2 values and efficiency of the qPCR reactions are summarized in Table S8 in the supporting information. The fungal DNA of eight *Fusarium* species present in the durum wheat grain

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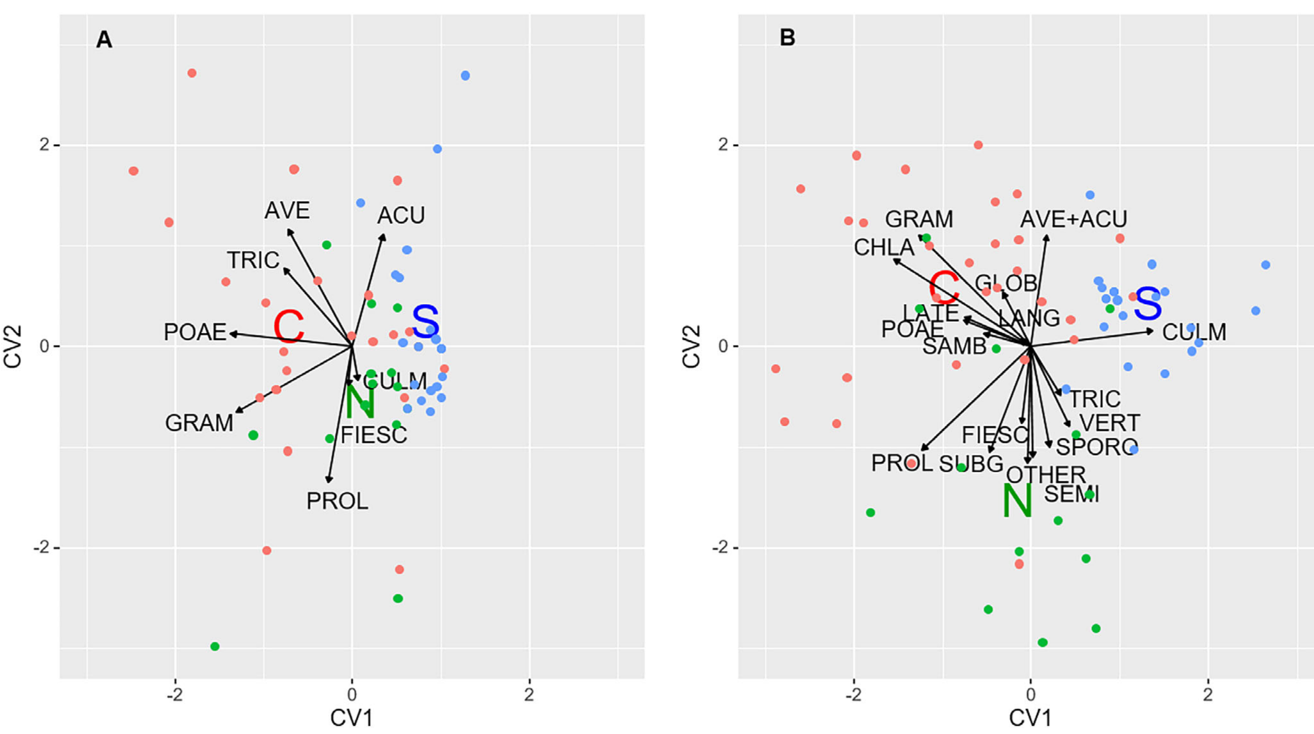

Figure 5. Biplot from the canonical variate analyses for the different *Fusarium* species as identified by partial *translation elongation factor 1 α* (*tef1α*) sequencing (A) or observation of morphological characteristics (B) after their isolation from durum wheat grains collected in the three Italian macro-areas with two isolation methods (potato dextrose agar, PDA; deep freezing blotter, DFB). In detail, the *Fusarium* isolates obtained with the PDA technique were identified by partial *tef1α* sequencing (PDA + *tef1α*), while those obtained with the DFB technique were identified by the observation of morphological characteristics (DFB + mc). N: northern Italy, C: central Italy; S: southern Italy; ACU: *F. acuminatum* AVE: *F. avenaceum*; ACU + AVE: *F. avenaceum* + *F. acuminatum*; CULM: *F. culmorum*; FIESC: *Fusarium incarnatum equiseti species complex*; GLOB: *F. globosum*; GRAM: *F. graminearum*; LANG: *F. langsethiae*; LATE: *F. lateritium*; POAE: *F. poae*; PROL: *F. proliferatum*; SUBG: *F. subglutinas*; SAMB: *F. sambucinum*; SEMI: *F. semitectum*; SPORO: *F. sporotrichioides*; TRIC: *F. tricinctum*; VERT: *F. verticillioides*; OTHER: *Fusarium* spp. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area, in detail green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

samples is shown in Fig. 6 and detailed in Table S9 in the supporting information.

Among the eight *Fusarium* species analyzed, *F. graminearum* showed a significantly higher DNA level ($P < 0.01$) in the Italian durum wheat grains (Fig. 6, Table S9 in the supporting information). *Fusarium avenaceum* was the second most detected species and, even if this species showed a higher level than *F. culmorum*, no significant differences between these two species were found ($P = 0.06$). *Fusarium langsethiae* was similar to *F. culmorum* ($P = 0.13$) but significantly higher than the remaining species ($P < 3 \times 10^{-4}$). No significant differences ($P > 0.50$) were detected among *F. sporotrichioides*, *F. proliferatum*, and *F. poae*. Finally, all these species showed an accumulation level in grains significantly higher ($P < 0.04$) than *F. tricinctum* (Fig. 6, Table S9 in the supporting information).

The difference in the distribution across the three Italian macro-areas was detected for all species except for *F. langsethiae* ($P > 0.45$) (Fig. 7, Table S9 in the supporting information). Northern and central Italy were particularly characterized ($P < 0.02$) by the presence of *F. graminearum* and *F. poae* (Fig. 7). Central Italy was also marked by the accumulation of *F. avenaceum*, *F. proliferatum*, and *F. culmorum* DNA (Fig. 7). However, significant differences were detected only between the average DNA accumulation level of these three species recorded in central and northern Italy ($P < 0.02$). *Fusarium tricinctum* was found, without significant differences ($P = 0.80$), only in central and southern Italy. Finally, the presence of *F. sporotrichioides* was higher ($P < 0.04$) in the southern macro-area than in the central and northern macro-areas (Fig. 7).

Fungal secondary metabolites accumulation in durum wheat grains

The fungal secondary metabolites (μg kg^{-1}) as quantified by LC/MS–MS in the durum wheat grains collected across Italy are summarized in Tables S10–S14 and the secondary metabolites biosynthesized by *Fusarium* and by *Alternaria* were the most commonly present in the analyzed samples.

Considering all *Fusarium* secondary metabolites, central Italy was the macro-area with the significantly higher accumulation levels ($P < 0.03$) followed by northern and southern Italy (Fig. 8). Despite the conspicuous mycotoxin differences between north and south Italy, no significant differences ($P > 0.17$) were detected. Summarizing, *Fusarium* secondary metabolites followed the pattern: center > north ≥ south. Central Italy was also the macro-area with the highest levels of *Alternaria* secondary metabolites followed by southern ($P = 0.15$) and northern ($P = 0.03$) Italy (Fig. 8). The accumulation pattern of accumulation was therefore: center ≥ south ≥ north.

By focusing on the different groups of *Fusarium* secondary metabolites (Fig. 9(A); Table S9 in the supporting information) they were all present in the three macro-areas surveyed, except for zearalenone, which was not detected in southern Italy (Fig. 9(A), Table S9). The accumulation levels of trichothecenes were significantly higher in the central and northern macro-areas than in southern Italy ($P < 0.02$). Fumonisins, of which only the form B1 (FUMB1) was detected, were particularly present in central Italy, with an accumulation level significantly higher than southern Italy ($P < 0.04$) (Table S10). Depsipeptides showed no significant

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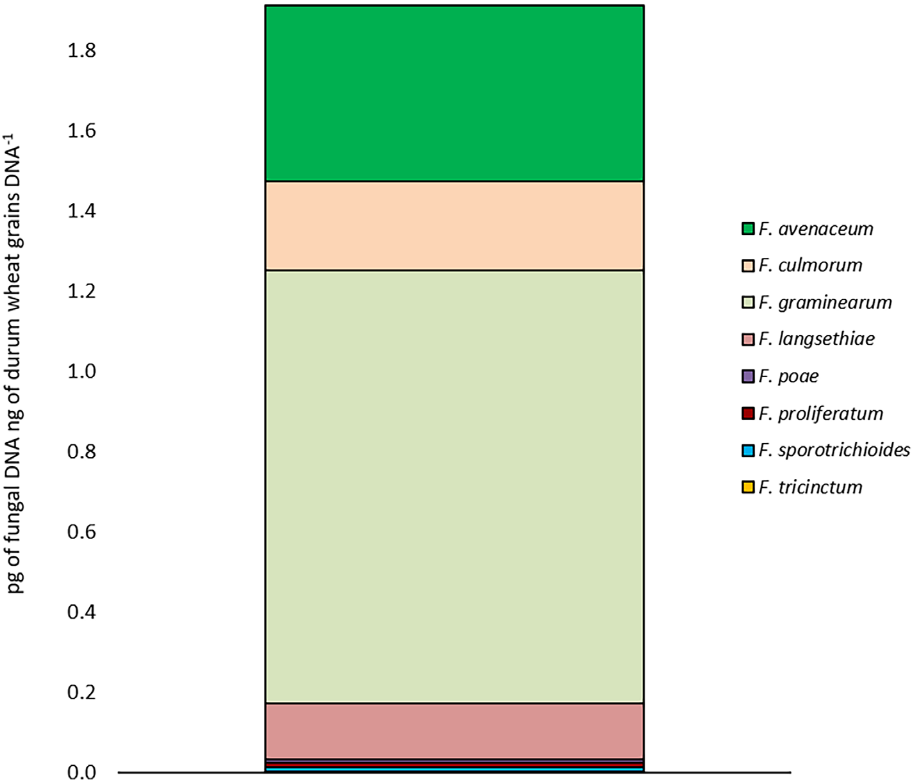

Figure 6. DNA amount of eight *Fusarium* species as detected by quantitative real-time polymerase chain reaction (qPCR) assays in durum wheat grains collected across Italy. Columns represent the *Fusarium* community composition expressed as the average of pg of each analyzed fungal species DNA/ng of durum wheat grains DNA in 70 durum wheat samples.

differences in the accumulation levels in grain from the three macro-areas ($P > 0.07$). Finally, other *Fusarium* secondary metabolites were present in significantly higher amounts in the grains from central Italy ($P < 0.03$) (Fig. 9(A)).

Among trichothecenes (Table S11 in the supporting information), DON was the compound with the highest detected levels. Considering the total average values and the percentage of positive samples, DON was particularly present in central Italy (721 μg kg^{-1}, 100%) and northern Italy (556 μg kg^{-1}, 100%) followed by southern Italy (81.9 μg kg^{-1}, 79%) (Fig. 9(B)). However, due to the high variation in contamination levels recorded within each macro-area, no significant differences ($P > 0.53$) in its distribution were found. In addition, DON exceeded the maximum admitted level for unprocessed durum wheat (1750 μg kg^{-1}; EU Commission Regulation),[47] in samples collected in central (three samples) and northern (one sample) macro-areas. Nivalenol also followed a similar, but not significant ($P > 0.46$), trend with the highest accumulation levels in central Italy (161 μg kg^{-1}, 90%) followed by northern (25.9 μg kg^{-1}, 94%) and southern Italy (14.4 μg kg^{-1}, 33%) (Fig. 9(B)). T-2 and HT-2 toxins were generally detected in low amounts and without significant distribution differences between macro-areas ($P > 0.72$). However, central Italy was the macro-area with the highest percentage of positive samples and two samples from this area exceeded the maximum level for the sum of T-2 + HT-2 toxins recommended for unprocessed wheat by the EU (100 μg kg^{-1}; EU Commission Recommendation).[50]

Focusing on depsipeptides (Table S12 in the supporting information), the ENNs analogs generally accumulated in the three macro-areas with the following gradient: ENB1 > ENB > ENA1 > ENB2 > ENA > ENB3. Considering the total ENNs (the sum of all the analogs), central Italy was the macro-area with the highest accumulation level (684 μg kg^{-1}) followed by northern Italy (461 μg kg^{-1}) and southern Italy (316 μg kg^{-1}) (Fig. 9(C)). However, no significant differences among the three macro-areas were detected ($P > 0.72$). Small amounts of BEA were also found, with the highest percentage of positive samples detected in northern and central Italy (100 and 93%, respectively) in comparison with southern Italy (21%).

The analysis also revealed the presence of 17 compounds classified as 'other *Fusarium* secondary metabolites' (Table S13 in the supporting information). Due to the high level of variation within each macro-area, no significant differences in their distribution across the surveyed territory were detected. However, central Italy was the macro-area where the vast majority of 'other *Fusarium* secondary metabolites' showed the highest presence both in terms of positive samples and total average levels. For some compounds (aminodimethyloctadecanol, antibiotic Y, butenolid, chlamidospordiol, and chlamidosporol) southern Italy, together with central Italy, was the macro-area showing the highest presence (Fig. 9(D)). Conversely, for some other compounds (aurofusarin, culmorin, MON and 15-hydroxyculmorin), northern Italy, in conjunction with the center, revealed the highest

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Figure 7. Biplot from the canonical variate analyses for the eight *Fusarium* species detected using real-time quantitative polymerase chain reaction (qPCR) directly in grain samples collected from the three Italian macro-areas. N: northern Italy, C: central Italy; S: southern Italy; AVE: *F. avenaceum*; CULM: *F. culmorum*; GRAM: *F. graminearum*; LANG: *F. langsethiae*; POAE: *F. poae*; PROL: *F. proliferatum*; SPORO: *F. sporotrichioides*; TRIC: *F. tricinctum*. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area; green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

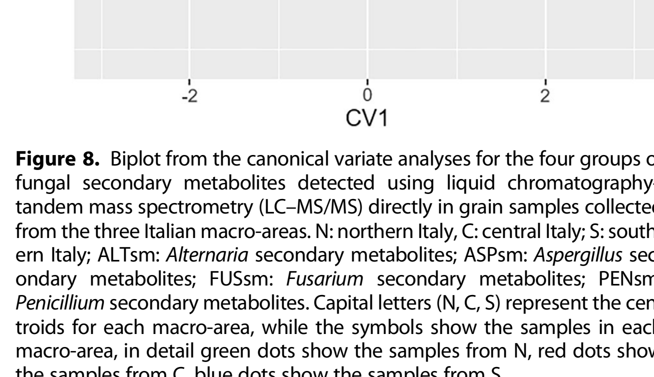

Figure 8. Biplot from the canonical variate analyses for the four groups of fungal secondary metabolites detected using liquid chromatography–tandem mass spectrometry (LC–MS/MS) directly in grain samples collected from the three Italian macro-areas. N: northern Italy, C: central Italy; S: southern Italy; ALTsm: *Alternaria* secondary metabolites; ASPsm: *Aspergillus* secondary metabolites; FUSsm: *Fusarium* secondary metabolites; PENsm: *Penicillium* secondary metabolites. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area, in detail green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

accumulation in the grains (Fig. 9(D); Table S13 in the supporting information).

Finally, a focus on *Alternaria* secondary metabolites showed the presence of nine compounds in all three macro-areas (Table S14 in the supporting information) but no significant differences in their distribution across the surveyed territory were detected. However, a higher presence of each single compound in central Italy was recorded in particular for alternariol (AOH), altertoxin-I (ATXI), macrosporin, and tentoxin (TEN) (Fig. 10).

Correlations between the different approaches

Correlations (r) between the levels of the eight *Fusarium* species detected with the three approaches adopted in this study (PDA + *tef1α*; DFB + mc; qPCR) were calculated (Table 1).

There was a significant ($P < 2.02 \times 10^{-5}$) positive association between the average number of *F. avenaceum*, *F. graminearum* and *F. poae* colonies detected with PDA + *tef1α* and DFB + mc, and the fungal DNA found in the grains by qPCR (Table 1). For these three species, a significant ($P < 1.26 \times 10^{-10}$) positive association also existed between the colonies detected with PDA + *tef1α* and DFB + mc approaches (0.669; 0.724; 0.677) (Table 1). For *F. proliferatum*, the positive correlation with qPCR was significant ($P = 0.009$) only for DFB + mc (0.303). For this last species, the correlation level (0.146) between PDA + *tef1α* and DFB + mc was not significant ($P = 0.14$). The absence of significant correlation ($P > 0.25$) between PDA + *tef1α* or DFB + mc and qPCR was detected for *F. sporotrichioides* (−0.102; −0.007), *F. tricinctum* (0.137; −0.044) and *F. langsethiae* (not detected with PDA + *tef1α*; 0.122).

For the eight *Fusarium* species a correlation (r) between the levels detected by each single approach (qPCR; PDA + *tef1α*; DFB + mc) and the presence in the grains of two selected secondary metabolites typically biosynthesized by them was also calculated (Table 1). Starting with *F. avenaceum*, a significant ($P < 1.6 \times 10^{-6}$) positive association was detected between MON or ENNs in the grains and detection by qPCR (0.673; 0.479), PDA + *tef1α* (0.741; 0.544) or DFB + mc (0.537; 0.505). Similarly, the positive association was significant ($P < 2.39 \times 10^{-9}$) between DON and *F. graminearum* detected with qPCR, PDA + *tef1α* or DFB + mc (0.762; 0.727; 0.641). Concerning NIV, the presence of a significant positive correlation ($P < 2.17 \times 10^{-5}$) was detected for the three different approaches adopted for *F. graminearum* detection (0.484; 0.578; 0.730). The same mycotoxin was significantly associated ($P < 0.01$) with *F. poae* identified with qPCR, PDA + *tef1α* or DFB + mc (0.328; 0.292; 0.335). *F. tricinctum* levels were significantly correlated ($P = 9 \times 10^{-7}$) to MON only when quantified by qPCR (0.548) and to ENNs ($P < 0.001$) when detected by qPCR (0.371) and PDA + *tef1α* (0.364). Fumonisin form B1 and fusaric acid showed a significant correlation ($P < 0.01$) with

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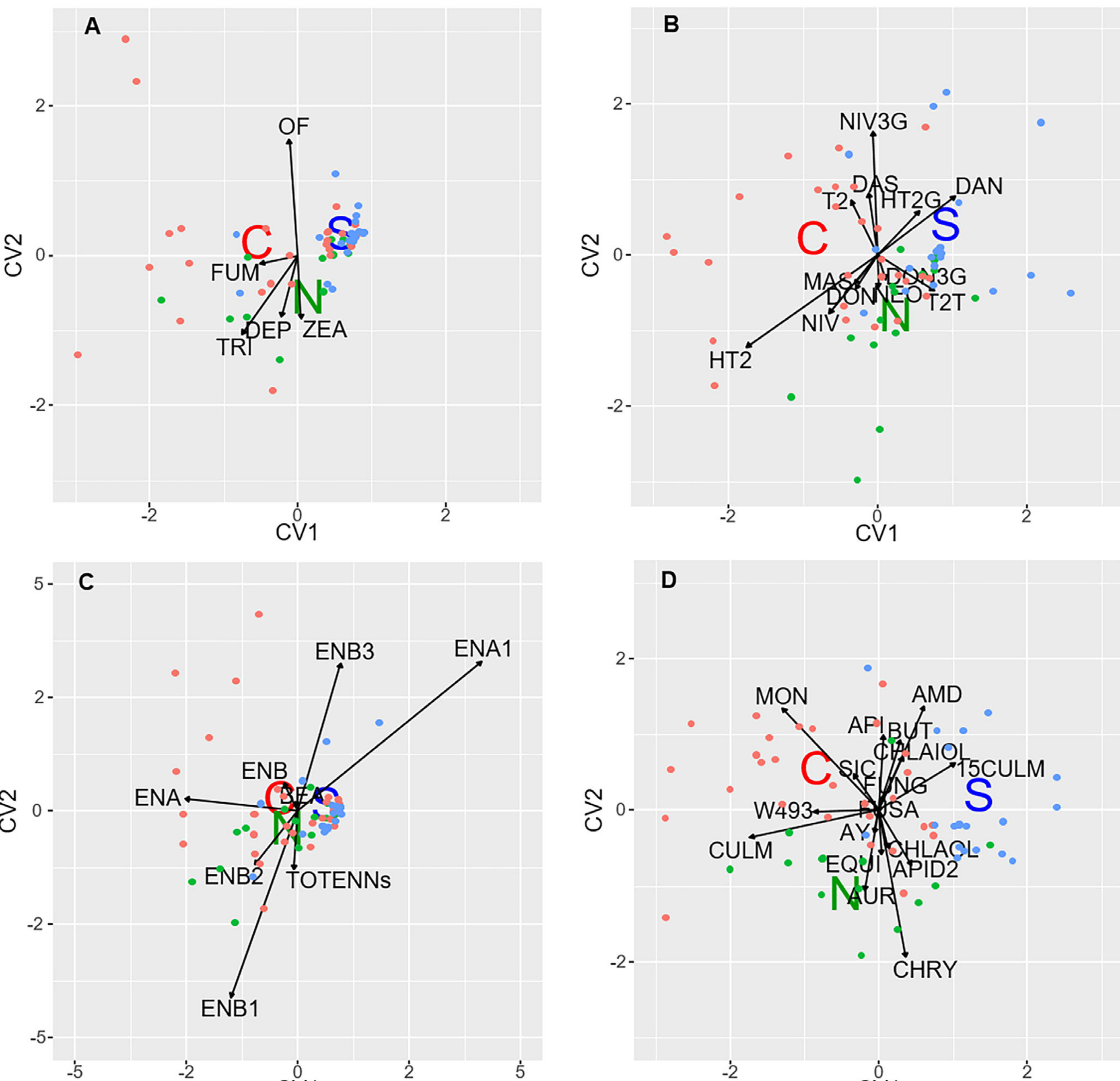

Figure 9. Biplot from the canonical variate analyses for the five groups of *Fusarium* secondary metabolites (A), for trichothecenes (B), depsipeptides (C), and other *Fusarium* secondary metabolites (D), detected using liquid chromatography–tandem mass spectrometry (LC–MS/MS) directly in grain samples collected from the three Italian macro-areas. N: northern Italy, C: central Italy; S: southern Italy; AMD: aminodimethyloctadecanol; API: apicidin; APID2: apicidin D2; AUR: aurofusarin; AY: antibiotic Y; BEA: beauvericin; BUT: butenolide; CHLAIOL: chlamidospordiol; CHLAOL: chlamidosporol; CHRY: chrysogin; CULM: culmorin; DAN: deacetylneosolaniol; DAS: deacetoxyscirpenol; DEP: Depsipetides; DON: deoxynivalenol; DON3G: deoxynivalenol-3-glucoside; ENA: enniatin A; ENA1: enniatin A1; ENB: enniatin B; ENB1: enniatin B1; ENB2: enniatin B2; ENB3: enniatin B3; TOTENNs: total enniatins (EA + EA1 + ENB + ENB1 + ENB2 + ENB3); EQUI: equisetin; FUM: Fumonisin B1; FUNG: fungerin; FUSA: fusaric acid; HT2: HT2 toxin; HT2G: HT-2- glucoside; MAS: monoacetoxyscirpenol; MON: moniliformin. NEO: neosolaniol; NIV: nivalenol; NIV3G: nivalenol-3-glucoside; OF: other *Fusarium* secondary metabolites; T2: T2 toxin; T2T: T2-tetraol; *SIC*: siccanol; TRI: trichothecenes; W493: w493; ZEA: zearalenone; 15CULM: 15-hydroxyculmorin. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area, in detail green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

F. proliferatum levels detected by all three different approaches. The highest levels of correlation were recorded between qPCR and FUMB1 (0.599). The levels of T-2 and HT-2 toxins were significantly correlated ($P < 3 \times 10^{-4}$) only with *F. langsethiae* DNA detected by qPCR (0.418 and 0.784) and not with the colony number recorded with the other two methods. Finally, there was an absence of significant correlation ($P > 0.274$) for *F. culmorum* and *F. sporotrichioides* with all three approaches and the levels of DON and NIV or T2 and HT-2 toxins, respectively (Table 1).

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Figure 10. Biplot from the canonical variate analyses for *Alternaria* secondary metabolites detected using liquid chromatography–tandem mass spectrometry (LC–MS/MS) directly in the grain samples collected from the three Italian macro-areas. N: northern Italy, C: central Italy; S: southern Italy; AOH: alternariol; AME: alternariol monomethyl ether; ALS: altersetin; ALTSOL: altersolanol; ATXI: altertoxin-I; INFECTO: infectopyrone; MACRO: mascrosporin; TEN: tentoxin; TEA: tenuazonic acid. Capital letters (N, C, S) represent the centroids for each macro-area, while the symbols show the samples in each macro-area, in detail green dots show the samples from N, red dots show the samples from C, blue dots show the samples from S.

DISCUSSION

Several mycotoxigenic fungi can seriously compromise grain quality. In Italy, this threat to food safety[82] is strictly related to climatic conditions.[5,7] To assess the evolution of the fungal community associated with Italian durum wheat grain, with reference to *Fusarium* species, the present investigation, conducted on samples harvested in three different durum wheat cultivation macro-areas (north, center, south of Italy), was realized using different diagnostic methods. Two different approaches to 'fungal isolation + *Fusarium* identification' (PDA + *tef1α* or DFB + mc) were adopted. The DNA of eight *Fusarium* species was also quantified directly in the kernels by qPCR and a wide range of fungal secondary metabolites was analyzed in grains by LC–MS/MS.

The fungal composition of fungal communities, assessed at the genus level by visual observation, was investigated with two of the most common methods used for the isolation of seed-borne fungal pathogens (PDA and DFB).[83] The results obtained show that southern Italy had the lowest levels of fungal colonies confirming that the expansion of durum wheat into central-northern Italy increased the occurrence of fungal microorganisms as a consequence of more favorable climatic conditions for their development.[6]

Concerning the overall presence of some fungal genera, differences between the two isolation methods were found. In this survey, PDA seemed to favour the development of *Alternaria*, *Epicoccum*, *Microdochium*, and *Penicillium*, whereas DFB appeared to promote *Fusarium*. Some genera (*Acremonium* and *Gliocladium*) were isolated only with DFB. A previous survey, conducted on durum wheat grains, did not reveal any effect of the two different isolation techniques on *Fusarium* spp. development.[7] Conversely, other researchers described the higher efficiency of DFB in promoting *Fusarium* spp. development compared to PDA.[83,84] *Alternaria* spp. has previously been described as enhanced by DFB.[7] The differences recorded in this survey, where this genus was more present in PDA, could be attributed to the different localization of the *Alternaria* species 'on the seed' as a contaminant agent[85] (in this case it could be enhanced by DFB without surface disinfection) or 'in the seed' as an infectious agent[86-89] (in this case it could be enhanced by PDA following surface disinfection).

In this survey, *Alternaria* was detected as the main component of the durum wheat grain fungal communities as previously reported in many cultivation areas[12,86,90-94] including Italy.[7,57,95] In detail, previous research conducted in three different Italian regions suggested a certain geographical effect on its distribution, underlining that central Italy showed the highest level of *Alternaria* incidence.[7] Similarly, the present survey showed that *Alternaria* was particularly present in the central macro-area followed by the southern one.

Alternaria spp. is a well-known mycotoxigenic genus.[94,96,97] During the present survey, the presence of *Alternaria* secondary metabolites such as AOH, alternariol monomethyl ether (AME), altersetin, altersolanol, ATXI, infectopyron, macrosporin, TEN, and tenuazonic acid (TeA) was also detected. Resembling the distribution of the producing fungal genus, central Italy was the macro-area with the highest accumulation of these compounds in durum wheat grains as previously reported in other surveys conducted in a region (Umbria) that belonged to this macro-area.[7,59] Based on these results, Central Italy appears to be particularly affected by the presence of *Alternaria* as well as its secondary metabolites. Monitoring these compounds is extremely important because, for some of them, such as AOH, AME, and TeA, some evidence of toxic effects in humans was reported.[98,99] For this reason, a preliminary draft of the EU Commission Recommendation, 2019 on the monitoring of three *Alternaria* mycotoxins (AOH, AME, TeA) in various food categories, including cereal-based foods for infants and young children, is available.[100]

Fusarium was the second most abundant fungal genus of the durum wheat mycobiome evidenced by the isolation analyses. It is also commonly associated with wheat grain across many cultivation areas,[27,34,35,38,42,90,101-105] including Italy.[7,8,10,22,57,58,60,73]

Some of the previous Italian surveys[7,8,10] showed that *Fusarium* incidence of *Fusarium* increased from southern durum wheat cultivation areas to central and northern ones. The present survey emphasizes the high *Fusarium* presence recorded in central Italy. A previous survey conducted in samples harvested in a single region representative of each macro-area showed the pattern of *Fusarium* incidence as north > center > south,[7] this investigation, conducted in samples harvested in several regions of each macro-area, showed that, for the considered season, the central macro-area could be considered to have the highest *Fusarium* abundance. This finding was also confirmed by the total *Fusarium* DNA detected by qPCR in grains as well as by *Fusarium* secondary metabolites analyzed by LC–MS/MS. In particular, the presence of some groups of these compounds, such as trichothecenes, zearalenone, fumonisins and other *Fusarium* secondary metabolites, was significantly higher in the central and northern macro-areas than in the southern one.

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Table 1. Correlations (r) between the different approaches adopted in this study for the detection of eight *Fusarium* species in durum wheat grains collected in Italy (qPCR; PDA + partial *translation elongation factor 1α* sequencing; DFB and morphological characteristics) and between these different approaches and the levels of the two most representative secondary metabolites of each of the eight species detected in durum wheat grains. Color intensity in the background of the r-value shows the goodness of correlation (from −1 to +1 on the base of the color scale shown at the bottom of the table)

F. avenaceum[d]

Species detection approach/ secondary metabolites	qPCR[a]	PDA[b]	DFB[c]	MON	ENNs
qPCR	1				
PDA	0.453	1			
DFB	0.486	0.669	1		
MON	0.673	0.741	0.537	1	
ENNs	0.479	0.554	0.505	—	1

F. culmorum

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	DON	NIV
qPCR	1				
PDA	0.433	1			
DFB	0.222	0.314	1		
DON	−0.095	−0.132	0.125	1	
NIV	0.028	−0.133	−0.026	-	1

F. graminearum

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	DON	NIV
qPCR	1				
PDA	0.676	1			
DFB	0.613	0.724	1		
DON	0.762	0.727	0.641	1	
NIV	0.484	0.578	0.730	-	1

F. langsethiae

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	T2 toxin	HT-2 toxin
qPCR	1				
PDA	nd**	1			
DFB	0.122	nd	1		
T2 toxin	0.418	nd	−0.010	1	
HT-2 toxin	0.784	nd	0.026	-	1

F. poae

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	BEA	NIV
qPCR	1				
PDA	0.671	1			
DFB	0.661	0.677	1		
BEA	0.369	0.126	0.093	1	
NIV	0.328	0.292	0.335	-	1

F. proliferatum

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	FUM B1	Fusaric acid
qPCR	1				
PDA	0.167	1			
DFB	0.303	0.146	1		
FUMB1	0.599	0.277	0.209	1	
Fusaric Acid	0.552	0.574	0.283	-	1

F. sporotrichioides

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	T2 toxin	HT-2 toxin
qPCR	1				
PDA	−0.102	1			
DFB	−0.007	−0.092	1		
T2 toxin	−0.044	−0.026	0.052	1	
HT-2 toxin	0.011	−0.037	0.101	—	1

F. tricinctum

Species detection approach/ secondary metabolites	qPCR	PDA	DFB	MON	ENNs
qPCR	1				
PDA	0.137	1			
DFB	−0.044	0.329	1		
MON	0.548	0.169	−0.010	1	
ENNs	0.371	0.364	0.196	—	1

−1 +1

Abbreviation: BEA, beauvericin; DON, deoxynivalenol; ENNs, total enniatins; FUMB1, fumonisin B1; MON, moniliformin; NIV, nivalenol.

[a] Quantitative polymerase chain reaction assay.

[b] Potato dextrose agar and partial *translation elongation factor 1α* sequencing.

[c] Deep freezing blotter and morphological characteristics.

[d] In DFB *F. avenaceum* was considered as *F. avenaceum*/*F. acuminatum* group.

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In addition to *Alternaria* and *Fusarium*, other fungal genera were isolated from durum wheat grains. For example, *Epicoccum* and *Gliocladium* (also known as *Clonostachys*) are endophytes of wheat, for which antagonistic effects against *Fusarium* species were reported.[106,107] *Cladosporium* is another endophyte genus reported to be negatively correlated with *Fusarium* species.[108] The wheat endophytic *Acremonium* species showed mutual exclusion relationship in wheat with the pathogenic genus *Puccinia*.[109] Conversely, *Microdochium*, a well-known head-blight agent in wheat,[110] even if a non-toxigenic genus, can affect grain gluten quality.[111] Finally, *Aspergillus* and *Penicillium*, other mycotoxigenic genera, were also found. This highlights that the fungal communities associated with durum wheat grains are a reservoir of pathogenic (toxigenic and non-toxigenic) as well as endophytic non-pathogenic fungal genera, which could compete with or prevent FHB and other diseases, representing a source of potential biocontrol agents in wheat.[108]

In this investigation, the different *Fusarium* spp. isolated with the PDA method were identified by partial *tef1α* region sequencing (PDA + *tef1α*), while those isolated with the DFB method were identified by morphological characteristics (DFB + mc). Considering the species detected with both approaches, the most noteworthy difference was the highest presence of fungal colonies belonging to *F. proliferatum* species identified by the DFB + mc approach. The highest development of this species obtained with DFB + mc might have been caused by the absence of seed disinfection for the DFB isolation technique and by the absence of other fast-growing *Fusarium* or non-*Fusarium* species, which usually occur in PDA.[7] The DFB + mc approach also led to the identification of several species that were not detected by PDA + *tef1α* such as *F. langsethiae* and other species (*F. globosum*, *F. verticillioides*, and *F. subglutinans*) that belong to the FFSC, like *F. proliferatum*. This could be related to the same reason explained for *F. proliferatum*, leading to hypothesize that the isolation of FFSC members and of *F. langsethiae* was favored by the DFB technique. However, in this study DFB was coupled with morphological characterization. For this reason, it was not possible to attribute the presence of these species only to the isolation technique because the identification method might have also played a role in their highest/exclusive presence with respect to the PDA + *tef1α* method.

Both the approaches adopted confirmed that the *Fusarium* complex comprised a plethora of species and that more than 20 different *Fusarium* species can be associated with durum wheat grains. Like inter-genera diversity, intra-genus diversity could represent an interesting aspect to be further analyzed in terms of potential mutual exclusion between *Fusarium* species in a wheat spike.[45,112]

The PDA + *tef1α* approach indicated that the major *Fusarium* complex components of the Italian durum wheat, for the analyzed season, were *F. avenaceum* and *F. graminearum*, while the DFB + mc approach showed that the major component was *F. proliferatum*.

In the present survey, the presence of *F. avenaceum* was particularly high in the central macro-area. This result was also confirmed by qPCR carried out directly in the grains. Previous research conducted in a single region of central Italy (Umbria) showed that, for several years, *F. avenaceum* was the dominant member of the FHB community of wheat and barley.[57,58,113] This macro-area could be considered particularly favorable for the development of this species. The high presence of *F. avenaceum* (FTSC member) in central Italy detected in this study could be the explanation for the high accumulation of ENNs and MON, secondary metabolites typically produced by FTSC members, found in the grains harvested in central Italy. It should be mentioned that the accumulation of ENNs, MON and of other secondary metabolites (such as aminodimethyloctadecanol, antibiotic Y, butenolid) in Central Italy may also have been caused by *F. tricinctum*, another FTSC member detected only in this macro-area and in southern Italy.

Typically, *F. avenaceum* was reported to be the main FHB causal agent in northern America and northern Europe.[42,102-104,114-118] However, in recent decades, *F. avenaceum*, together with other FTSC members, also increased in temperate areas,[34,35,38,39,90,113,119] suggesting that this species is highly adaptable to a wide range of climatic conditions.[120] This flexibility of *F. avenaceum*, and its widespread presence across the world, makes it necessary to monitor contamination with its secondary metabolites in cereal grains as well as to increase knowledge regarding the toxic properties of these compounds against humans and animals. The secondary metabolites produced by FTSC members, particularly the most widespread and studied ENNs and MON,[39,121-125] have recently attracted the scientific community's attention, resulting in two EFSA scientific opinions on the risks to human and animal health related to their presence in feed and food.[53,54] In both cases, given the overall lack of toxicity data, no conclusions have been drawn about toxic exposure and for this reason, no legal maximum levels have been established yet.[53,54] However, considering the increasing evidence of the worldwide occurrence of grains contaminated with ENNs and MON, together with the potential risk of health hazards associated with chronic exposure[52,126] as well as the co-occurrence and possible synergisms with other secondary metabolites,[127] the risk connected with ENNs and MON should not be underestimated.

The present study has also confirmed the relevance of *F. graminearum*, particularly in the northern and central Italian macro-areas. This survey, corroborated by qPCR results, highlighted that this species has returned to play a dominant role, in particular in northern and central Italy, after several years in which it was not reported as the main member of the FHB complex.[7,8,57,61,113,119] Both the isolation techniques, as well as qPCR, confirmed that southern Italy was the Italian cultivation macro-area with the lowest *F. gramineraum* level, as already detected in previous investigations.[7,10,33] As a confirmation of this, the type B trichothecenes, first of all DON, typically biosynthesized by *F. graminearum*, were detected at very low levels in the grains harvested in the southern macro-area. Conversely, in the north and central areas, DON was commonly found, with 7.5% of samples exceeding the maximum admitted EU levels for unprocessed durum wheat.

Fusarium proliferatum, was the main component of *Fusarium* communities detected with the DFB + mc approach. This species was already detected as one of the most important members of the *Fusarium* group in Italy using the DFB isolation technique.[7] The PDA + *tef1α* approach used in this study also confirmed a remarkable incidence of *F. proliferatum* in the analyzed material. Surprisingly, qPCR analysis reported a very low presence of *F. proliferatum* DNA probably because the biomass of this species in the grains was very low and in many cases below the LOD of qPCR analysis. This could be attributable to the fact that this species is not particularly able to infect and colonize wheat grains. Conversely, the conditions that the fungus encountered during isolation, in particular in DFB, strongly promoted its development from the kernels in which it was present in very small amounts. *Fusarium proliferatum*, is traditionally considered to be one of the most important causal agents of *Fusarium* ear rot in maize, a

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disease that can result in mycotoxin contamination of grain due to the ability of this fungal species to biosynthesize fumonisins.[128] The maximum admitted levels of these compounds have been set by the EU for maize and derived products.[47] However, *F. proliferatum* has been detected in a wide range of crops including other cereals such as wheat.[129-131] Up to now, fumonisin accumulation has never been a particular problem in wheat, due to the low levels detected in this food matrix.[7,59,90,128,132-136]

The low levels of these compounds in wheat grains could be explained by hypothesizing that wheat grains are not as conducive to fumonisin biosynthesis as in maize.[132,137] However, in the presence of favorable climatic conditions, even wheat could be subject to a remarkable fumonisin accumulation as already observed in Brazil and Argentina.[138,139] As a confirmation of this, fumonisin accumulation recorded in the present study, even if not very high, showed an increase in comparison to what was previously detected in durum wheat in Italy.[7,59] This suggests the importance of monitoring *F. proliferatum* as well as fumonisins in durum wheat grains, as this could represent an additional source of fumonisin ingestion and a possible risk for consumers.

In this study, the simultaneous adoption of three different techniques (PDA + *tef1α*; DFB + mc and qPCR) for the detection of eight *Fusarium* species, allowed the accuracy of the two 'isolation + *Fusarium* identification' approaches to be defined when estimating the amount of fungal biomass accumulating in the grains. Correlations showed that, for certain species, such as *F. avenaceum*, *F. graminearum*, and *F. poae*, both 'isolation + *Fusarium* identification' approaches could be considered good predictors of fungal biomass accumulation in grains.

The adoption of the three different techniques (PDA + *tef1α*; DFB + mc, and qPCR) for the detection of eight *Fusarium* species as well as the quantification of mycotoxins by LC–MS/MS also allowed us to define the accuracy of the three techniques when predicting grain mycotoxin contamination. Correlations revealed that for the dominant members of the FHB communities, such as *F. avenaceum* and *F. graminearum*, all three identification approaches (PDA + *tef1α*; DFB + mc and qPCR) were good predictors of ENNs and MON (for *F. avenaceum*) as well as of DON and NIV (for *F. graminearum*) contamination. This was not the same for *F. proliferatum*, for which the qPCR method showed the highest levels of correlation with $FUMB_1$. Similarly, qPCR was revealed to be the best predictor in the case of T2 and HT-2 toxin contamination by *F. langsethiae*, which probably encountered the greatest difficulty developing in isolation conditions, being a fungus characterized by a very slow growth rate and which might have been overgrown during isolation processes by other rapidly growing species.[140]

To summarize, examining the composition of the fungal communities, the *Fusarium* complex and the fungal secondary metabolites associated with durum wheat harvested in Italy revealed that *Alternaria* and *Fusarium* species, with their mycotoxins, were particularly present in the central and central-northern cultivation macro-areas, respectively. Within the *Fusarium* communities, *F. avenaceum* (with ENNs and MON contamination) and the re-emerging species *F. graminearum* (with DON contamination) were mainly localized in the central and central-northern cultivation macro-areas, respectively. *Fusarium proliferatum* was also particularly abundant and fumonisin levels in durum wheat, even if low, were higher than in previous investigations. The adoption of different techniques for *Fusarium* detection (PDA + *tef1α*; DFB + mc and qPCR) also highlighted that, for certain species, qPCR was the best method for predicting their mycotoxin contamination in grains.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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