AN INTEGRATED APPROACH FOR MINIMIZING DEFICIENCIES IN FUSARIUM DETECTION AT WHEAT GRAINS

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ABSTRACT

The toxigenic capacity of mycotoxins produced by genus Fusarium, and the fact that the prevalence of certain species is mainly determined by regional climatic conditions, raise the importance of defining workflows, which would minimize disadvantages of single methods of detection. For this, visual inspection and microscopy (isolation of the pathogen by PDA platting followed by screening based on colony morphology and structure), chromatography (LC-MS/MS for mycotoxins composition and quantity), and specific TaqMan PCR (for Fusarium genus and species F. graminearum, F. culmorum, F. avenaceum, and F. poae) were employed to investigate this infection at seeds of 32 winter wheat cultivars (Triticum aestivum L.) in use in Albania. The detection efficacy of TaqMan PCR depending on the matrix used to extract the template DNA (hyphae, flour), and on the method of extraction (silica binding, CTAB or SDS-based) were also assessed, and wheat flour was suggested as the proper matrix. The McNemar calculation, used to verify the amplification efficacy using TaqMan dual probe and signal detection in FAM channel as compared to electrophoresis, proved the high sensitivity of the protocol. 13 cultivars were found infected by F. graminarium; 16 by F. culmorum; 13 by F. avenaceum, and none of them by F. poae based on PCR. The comparison of the sensitivity of TaqMan PCR to detect at genus level compared to species level proved mismatches, which varied from 26% for F. graminarium to 15% for F. avenaceum, to 5% for F. culmorum, implying the need for further improvement of the protocols. Typical mycotoxins were evidenced at 13 cultivars, and the colony micro-morphological characteristics (macroconidia, oval microconidia, conidiophores) proved their taxonomy at genus level. A comparison on the methods capacity for pathogen detection showed the ratio 27:14:13 for TaqMan: visual inspection: mycotoxins presence, respectively with results matching for 9 infected samples only, highlighting the value of an integrated approach, which would take advantage of each method either related to the detection limits, or to cost-efficiency.

Keywords: mycotoxins, colony morphology, PCR

INTRODUCTION

Fusarium is one of the most widespread, abundant, and important genera of soil microfungi recognized as pathogens of numerous plant species, among which wheat and other small grain cereals. The infestation of the ears appears to be the most serious, resulting in Fusarium head blight (FHB), also known as scab (Rios et al., 2012). The last is a disease complex in which several species co-exist, the predominant ones being F. graminearum, F. culmorum, F. avenaceum and F. poae (Bottalico et al., 2002; Waalwijk et al., 2004; Horevaj et al., 2011). As the prevalence of certain species in a region is primarily determined by climatic conditions, particularly temperature and moisture requirements, of great concern is considered the toxigenic capacity of mycotoxins produced by this genus, which varies greatly between species. Thus, F. culmorum, F. graminearum, F. langsethiae, F. poae, and F. sporotrichioides produce their own spectrum of trichothecenes, while F. avenaceum may produce moniliformin, beauvericin, and enniatins (Leslie et al., 2014). Among the mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), patulin (PAT), fumonisins (FUMs), and trichothecenes (TCs) like deoxynivalenol (DON) and T-2 toxin (T-2) are considered as the most concerning (Wokorach et al., 2021). In this context, several countries have established or proposed regulations for the control of mycotoxins in food and feed, with the permitted levels varying depending upon the intended end usage of the commodity (Ciko et al., 2024). The identification of Fusarium species locally takes importance in this context, yet remaining a difficult task due to the wide variation in morphological and nonmorphological characteristics, including virulence (Rios et al., 2012). To overcome this issue different methods have been applied, starting with the more traditional Blotter test with freezing and potato-dextrose-agar medium, which are also methods recommended by the Manual of Sanitary Seed Analysis to detect pathogens in wheat seeds (Brasil et al., 2009), and are commonly used. Following this methodology, the identification and measurement of fungal structure with its colony morphology, spore formation and spore characteristics are examined under microscope and compared with already published literatures, identification keys and reference books (Khan et al., 2023). Immunological Assays (LFIA, ELISA, FPIA), the Sensor-based (Surface Plasmon Resonance Sensor, Piezoelectric Sensors, Electrochemical Sensors, Colorimetric Sensors), and Chromatographic Techniques (TLC, GC, HPLC, HPLC-FLD, LC-MS/MS, UPLC-MS/MS, UHPLC-MS/MS, UFLC-MS/MS) on the other side, are among the most accurate techniques for identifying FHB mycotoxins at wheat flour. However, it was evidenced that none of them is capable of analyzing all mycotoxin categories at once, because of their chemical characteristics (volatile/non-volatile, co-elution, UV absorption, fluorescence) versus methods restrictions (matrix interferences, cross-reactivity of antibodies, selectivity and reproducibility of data, need for derivatization, etc) (Ciko et al., 2024). Other studies have tried to apply imaging systems to detect FHB or predict DON content levels in wheat kernels, seeds, or flour, and most of them were performed with hyperspectral imaging techniques (Bernardes et al., 2022). Among them, approaches such as the use of NIR reflectance to classify red and white wheat, the measurement of protein content, and the use of NIR spectroscopy of vomitoxin and ergosterol were reported (Polder et al., 2005). Also, RGB imaging of seed morphology, the X-ray computed tomography for evaluating seed shape in finer detail, and the invention of an automated light-weight free software for grain image analysis (GrainScan and SmartGrain as two examples of them (Leiva et al., 2022), and the photoluminescence method in the near ultraviolet and visible range was reported valuable ((Dorokhov et al., 2022). A special place in the range of molecular diagnostic tests for Fusarium species take those based on the polymerase chain reaction (PCR), which among others include assays specific for F. graminearum, F. poae, F. culmorum and F. avenaceum (Doohana et al., 1998). A set of primers targeting the gene encoding translation elongation factor 1-α (tef1-α)-Ef1 was reported useful to detect genus Fusarium (Kheseli et al., 2021), while Elsalam (2011) described the improvement of a single-seed DNA extraction protocol from germinated wheat seeds, and demonstrated its usefulness to provide DNA template for the F. graminearum specific loop-mediated isothermal amplification of the gaoA target gene fragment, as well as for the use with the group specific PCR primer pair Tox5-1/Tox5-2. Waalwijk (2004) reported the successful use of TaqMan methodology to amplify the F. avenaceum, F. culmorum, F. graminearum, F. poae gene fragments with species-specific primers. Meanwhile, the Real-time PCR (qPCR) is considered as the standard analytical method for species-specific, quantitative estimation of fungal biomass in host tissue. Several mycotoxin genotyping techniques have been developed for the fast detection of genes involved in mycotoxin production in both fungal cultures and plant material.

The genetic potential of Fusarium spp. isolates to produce fumonisins and trichothecenes was determined by the PCR-based molecular analysis using Tri13F/Tri13DONR and FUM1F/FUM1R specific primer pairs (Otipa et al.,

2022). Others (Goral et al., 2022) have found relationships between the weather conditions, the locations and the amount of *F. culmorum* DNA and trichothecene genotypes. They also proved significant correlations between disease incidence, fungal biomass (quantified as the total amount of fungal DNA or DNA trichothecene genotypes) and toxins (DON, 3AcDON and NIV) concentrations. DNA extraction procedures seem to have an important role in the effectiveness of eliminating PCR inhibitors, too. Cankar (2006) and Fredlund (2008) demonstrated that varying extraction techniques used on Fusarium head blight (FHB) infected wheat led to notable variations in the quantity of DNA identified. A study conducted by Brandfass (2008) has shown that enhancing the quantity of sample material led to enhanced reproducibility of DNA extraction. Brunner (2009) recommended utilizing a reference gene to standardize the fungal DNA content to minimize variability caused by DNA extraction. Also, the consistency of the standard calibration curve is a significant aspect that might impact the accuracy of qPCR experiment, and so does the precise DNA quantification, while the slope, y-intercept, and linearity of this curve are crucial parameters. For the early infection identification, the qPCR test must be capable of quantifying quantities of fungal DNA.

Based on the importance of establishing an efficient workflow for the detection of Fusarium species at wheat grains, and knowing the complexity of factors which may interfere with the results especially when seeds are infected naturally, here we present an integrated approach based on the use of microscopic evaluation of colony morphology and structure, the LC-MS/MS identification of mycotoxins at wheat flour, and the genus and species-specific qPCR-based detection. Thirty-two winter wheat cultivars in use in Albania were used in the study, aiming to investigate the presence of FHB infection locally, to identify four main Fusarium species, to analyze the efficacy of different methods of detection, and based on them to establish a workflow worth following thereafter. The group has previously investigated local wheat cultivars for their response to abiotic stresses such as high temperature (Bacu et al., 2024a), herbicide treatments (Bacu et al., 2024b; Kokojka et al., 2021), salinity (Bacu et al., 2020a; 2020b), and drought (Ibro et al., 2019).

MATERIALS AND METHODS

Seeds from thirty-two winter wheat cultivars (Triticum aestivum L.) in use in Albania were the plant material under investigation, namely: Nogal, Urma, Dajti, Krajlica, Vittoria, Frenetik-1, UBT-2, Artiko, Toborzo, Nogal Croatia, Basilic, Local wheat of Lushnja, Frenetik-2, UBT2/2, Komolica, Artiko, Isja, Local wheat of Korca, Artillo, Sirtaki, MV Tambor, UBT-1, Ciari, Dajti Kagjinas, Eli, Dajti Korca, Progres Korca, Sacramento Korca, Fondate, Basilio, Urma Adelajde, Victtoria. They were offered by the Institute of Plant Genetic Resources, Agricultural University of Tirana, Albania, and from the Centre for Agricultural Technology Transfer of Korca, the Ministry of Agriculture and Rural Development of Albania. The workflow of methodologies followed in this study is summarized at Figure 1, and each of them is described in details in the following subsections.

LC-MS/MS evaluation of mycotoxins present in wheat seeds

Mycotoxin extraction: 5 g ±0.02 g milled wheat grain was acidified with 0.1M HCl, were mixed, and allowed to stand in the dark. Then, Acetonitrile was added to the samples, which were shaken before QuEChERs was added, and immediately vortexed and shaken following (Mcelhinney et al., 2015). After centrifuging a portion of supernatant was transferred, and the extraction solution were evaporated at 40˚C under N_2 gas (Mbisana et al., 2023). Reside was dissolved with ACN: H_2O: Ac. Ac (35:64.5:0.5), filtered with 0.2µm PTFE membrane filter and placed in a pp-vial (Ciko et al., 2024).

LC-MS/MS condition: Liquid chromatography separation was carried out on a Shimadzu 8040 liquid chromatography system. The analytical column was an Acquity HSS C18 (100 mm × 2.1 mm, 1.8 µm particle size). Separation was achieved using a binary gradient comprising of 0.5% acetic acid aqueous solution with 5mM ammonium acetate (solvent A), and Methanol with 0.5% acetic acid and 5mM ammonium acetate (solvent B) at a flow rate of 0.35 ml/min (SSH EN 17194:2019). The eluent gradient profile was as follows: 0 min: 10% B; 0.05 min: 10% B; 1 min: 10% B; 6 min: 100% B, 9 min: 100% B, 9.01 min: 10% B and 12 min: 10% B (Liao et al., 2013). The temperature of the column was 40°C and the injection volume was 2µL (Ciko et al., 2024).

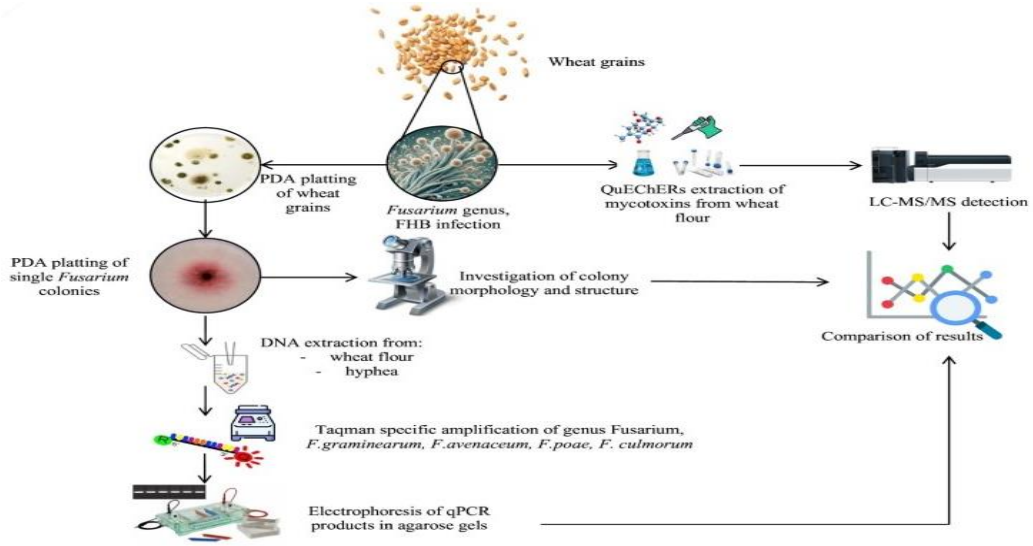


Figure 1. Schematic presentation of the workflow followed for the detection of Fusarium infection at wheat seeds. Prepared using ChemDraw/Revvity Signals Software.

Calibration curve: Five point extracted matrix calibration curve (control, 1*LOQ, 2*LOQ, 5*LOQ, 10*LOQ) were prepared by fortifying negative samples (5g) with the appropriate volume from working standard solutions. A calibration line was fitted using linear least squares regression with 1/X fit.

Evaluation of Analytical Results*:* was completed according to SANTE/11312/2021.
Calculation of results: The concentration of the analyte in the analyzed sample is determined as follows:

$$C = (y - a) / b$$

Where: b – the slope of the calibration curve; a – the y-intercept of the calibration curve.

Isolation, purification and identification of the seed borne myco flora
The potato dextrose agar (PDA) was used in this method for the isolation of mycoflora. Wheat seeds from 32 cultivars were sterilized for 2 min using a water-ethanol (95%), sodium hypochlorite (7%) solution (82:10:8% vol), and rinsed with sterile water for 1min. After that seeds were placed on PDA supplemented with streptomycin sulphate (0,16g/L) into 10 Petri dishes (90mm diameter) containing 10 seeds each. The dishes were incubated at 22°C in the dark for 5 days, and after that a combination of visual and stereomicroscope examination were carried out on each sample (10 replicates per sample) to assess fungal development. In order to obtain monoconidial cultures, from the colonies identified as belonging to Fusarium genera based on colony visual inspection, hyphae were isolated and plated on PDA plates for 5 days at 22°C for the fungal growth.
Identification of Soil-borne fungal microbes
Cultured fungi were placed on slides after being stained with lactophenol-cotton blue to identify the fungal structures. Fusarium colonies were identified based on their pigmentation, and morphological traits such as the presence of macroconidia, microconidia, sporodochia, and chlamydospores, which were compared with already published literatures (Khan et al., 2023). Hyphea from colonies classified as belonging to Fusarium genus were kept frozen at -20ºC until used for DNA extraction.
DNA extraction for PCR detection of Fusarium
Three extraction protocols were employed to obtain Fusarium DNA to be used as template for qPCR reactions: extraction from wheat flour, extraction from hyphae using a CTAB-based protocol, and extraction from hyphae using silica binding methodology.
DNA extraction from wheat flour*:*
Milled wheat grain was used to extract DNA from different cultivars following (Mohammadi et al., 2012) [extraction buffer 500µL; 100 mM Tris/HCl pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl, supplemented with 1% (w/v) SDS]. The sample was vortexed vigorously for 1 minute, potassium acetate (100µL pH=5.2) was added, the solution was vortexed 1 minute until it turned milky white. Tubes were centrifuged at 15700rcf for 10 minutes at

4ºC, and from the upper layer the DNA was precipitated with 350µL of isopropanol at -20ºC for 30 minutes. The DNA was then pelleted by centrifugation at 15700rcf for 5 minutes at 4ºC, the pellet was washed with 300µL of pre-chilled 70% (v/v) ethanol and centrifuged at 15700rcf for 5 minutes at 4ºC. The pellet was dried at room temperature and DNA samples were dissolved in 50µL of TE buffer and treated with 3µL of RNase (10mg/mL).

CTAB DNA Extraction from hyphea:

Hyphae (10mg) from single fungi colonies grown in vitro on PDA media were used to extract DNA following the CTAB extraction protocol according to Conlon (2022).

DNA extraction from hyphae using silica gel binding (gSYNC DNA mini kit of Geneaid Biotech Ltd., GS100): The DNA was extracted from hyphae using gSYNC DNA mini kit (Geneaid Biotech Ltd., GS100), which utilizes Proteinase K and chaotropic salts to lyse cells and degrade proteins, facilitating DNA binding to the silica gel membrane of a spin column.

PCR primers

Five primer pairs were used to amplify gene fragments specific for Fusarium genus, and species F. graminearum, F. culmorum, F. avenaceum and F. poae, respectively (Tab1).

Table 1. Primer pairs used for the specific amplification of Fusarium elongation factor EF1α, TRI6, Fcul, JIA and OPT18 gene fragments for the detection of genus Fusarium, F. graminearum, F. culmorum, F. avenaceum, and F. poae, respectively.

Oligo name	Type of primer	Primer Sequence (5'-3')	Expected product (bp)	Reference
EF1α	FusEF (forward)	CTGGGTTCTTGACAAGCTCA	96	Sohlberg et al., 2022
	FusER (reverse)	CGGTGACATAGTAGCGAGGA		
	FusEP (probe)	FAM-TACCACGCTCACGCTCGGCT-TAMRA		
Tri6	Tri6_10F (forward)	TCTTTGTGAGCGGACGGGACTTTA	245	Horevaj et al., 2011
	Tri6_4R (reverse)	ATCTCGCATGTTATCCACCCTGCT		
Fcul	Fcul F (forward)	CACCGTCATTGGTATGTTGTCACT	400	Góral et al., 2022
	Fcul R (reverse)	CGGGAGCGTCTGATAGTCG		
JIA	JIAF (forward)	GCTAATTCTTAACTTACTAGGGGCC	500	Waalwijk et al., 2004
	JIAR (reverse)	CTGTAATAGGTTATTTACATGGGCG		
OPT18	OPT18F (forward)	GATGCCAGACCAAGACGAAG	472	Brandfass et al., 2006
	OPT18 R (reverse)	GATGCCAGACGCACTAAGAT		

TaqMan qPCR for the Fusarium elongation factor EF1α gene

qPCR amplification was performed in the Mx3000P QPCR System (Stratagene, La Jolla, CA, USA), and data were collected and analyzed using the MxPro QPCR software (Stratagene). The Fusarium spp. qPCR runs were carried out according to (Sohlberg et al., 2022). For this, qPCR reactions (20 µL), which contained 10 µL of Forget-Me-Not EvaGreen qPCR Master Mix (Biotium, Inc., Fremont, CA), 5 µL sample DNA, 1 µL of each 5 mM forward and reverse FusE PCR primers (Eurofins Genomics), 1 µL of 2 mM FusP of the hydrolysis probe (Eurofins Genomics) and 2 µL of 30 nM ROX (Biotium, Inc., Fremont, CA) were prepared in duplicate for all samples. The reaction conditions used in this study consisted of an initial denaturation step for 10 min at 95°C (segment 1), followed by 39 cycles of 95°C for 10 s, annealing for 45 s at 57 °C and extension for 1 s at 72°C (segment 2). Amplicons were loaded onto a 1.5% (w/v) agarose gel stained with 6x DNA Loading Dye Buffer and compared with DNA ladder 100bp (GeneRuler, Thermo Fisher Scientific) to confirm the size of the amplified product.

PCR Reactions for TRI6, Fcul, JIA and OPT18 genes

Real-time PCR was carried out in 20 µL reactions, which contained Forget-Me-Not EvaGreen qPCR Master Mix (Biotium, Inc., Fremont, CA), 2 µL of 30 nM ROX, and 2.5 µL sample DNA/2.5 µL of 50 nM PMA dye (Biotium, Inc., Fremont, CA) (1:1). Both the forward and reverse primers for the TRI6, Fcul, JIA, and OPT18 genes were individually adjusted to a final volume of 1.5 µL. Concentration of each set of primer was 1.0 uM for TRI6 and Fcul, and 0.3uM for JIA and OPT18 gene respectively. All activities related to the preparation of the PCR reaction, dilution of DNA samples, and the preparation of standard curves were performed using Mx3000P QPCR System (Stratagene, La Jolla, CA, USA), and data were collected and analyzed using Eva Green melting curve option of the MxPro QPCR software (Stratagene). The PCR program for the TRI6 gene consisted of using the following cycling protocol: 95 °C 10min, 40 cycles of 9°C for 30s, 62°C for 3s and extension step at 72°C for 30s, followed by analysis of dissociation curves at 55 to 95°C. The PCR program for the Fcul gene consisted of using the following cycling protocol: 95°C 15 min, 40 cycles of 95°C for 15 s and 62°C for 1 min followed by analysis of dissociation curves at 60 to 95°C. The PCR program for the JIA gene consisted of using the following cycling protocol: Initial

denaturation 94°C/30s, 35 cycles of 94°C/30s, 60°C /20s, and extension step at 72°C/30s, followed by the analysis of dissociation curves at 55 to 95°C. Melt curve analysis to PCR products of TRI6, Fcul, JIA and OPT18 was performed in the segment 3 to quantify the thermal variation in fluorescence during the conversion of PCR products into single-stranded molecules across a temperature range. The collected data were subsequently utilized to generate a derivative melt curve plot. The peaks in this graph represented the highest rates of fluorescence change, which were used to estimate the melting temperature (Tm) of the PCR products.

Statistical Analysis

Statistical analysis of qPCR products was performed using the commercial software (SPPS version 20.0; SPSS Inc., Chicago, IL). The McNemar test (He et al., 2011) was used: a) to compare endpoint qPCR fluorescence, with the presence of 96bp band on agarose gels, for Fusarium genus detection; b) to assess the sensibility of the FusE PCR compared to species-specific PCR.

RESULTS & DISCUSSION

LC-MS/MS evaluation of mycotoxins at wheat grains

QuEChERs was the method followed for mycotoxin extraction from the wheat flour of 32 winter wheat cultivars (Triticum aestivum L.), which were further evaluated for the composition and concentrations of the analytes via LC-MS/MS (Tab 2). Nineteen out of thirty-two cultivars did not have mycotoxins presence; The rest contained: HT-2 (Trichothecenes class, Type A, known to be produced from F. poae, F. sporotrichioides and F. langsethiae); 15-ac DON and DON (Trichothecenes class, Type B, known to be produced from F. graminearum); Fusaron X (Trichothecenes class, Type B, produced from different Fusarium species); Enniatin B1, Enniatin B (Enniatins class, produced from several different Fusarium species); Ergometrine and Ergometrinine (Ergot Alkaloids class, produced from Claviceps purpurea). The concentration of mycotoxins in the samples were above the limit of quantification (LOQ), except DON (Ciko et al., 2024).

Table 2. LC-MS/MS qualitative and quantitative evaluation of mycotoxins present at wheat flour samples.

No	Cultivar	HT-2 µg/kg	15-ac DON µg/kg	Fusaron X µg/kg	Enniatin B1 µg/kg	Enniatin B µg/kg	DON µg/kg	Ergometrine µg/kg	Ergometrinine µg/kg
1	Nogal	-	-	-	-	-	-	-	-
2	Urma	24.65	-	-	-	-	-	-	-
3	Dajti	29.13	-	-	-	-	-	-	-
4	Krajlica	13.51	-	-	-	-	107.40	-	-
5	Vittoria	-	-	-	-	-	-	-	-
6	Frenetik-1	-	15.15	13.41	-	-		-	-
7	UBT-2	-	-	-	-	-	-	-	-
8	Artico	-	-	17.73	-	-	112.16	-	-
9	Toborzo	-	-	-	-	-	-	-	-
10	Nogal Croat	-	-	-	-	-	-	-	-
11	Basilic	-	-	-	-	-	-	-	-
12	Local Lushnja	-	-	-	-	-	-	-	-
13	Frenetic-2	-	-	-	-	42.13	209.06	-	-
14	UBT 2/2	-	13.02	-	-	13.86	652.10	-	-
15	Komolica	-	-	-	-	-	-	-	-
16	Artiko	-	-	13.45	43.36	24.48	-	-	-
17	Isja	-	-	-	-	-	-	-	-
18	Local Korca	-	-	-	-	-	-	-	-
19	Artillo	10.54	-	-	-	-	-	-	-
20	Sirtaki	-	-	-	-	-	-	-	-
21	MV Toborzo	-	-	-	-	-	131.30	-	-
22	UBT-1	-	-	-	-	-	-	-	-
23	Ciari	-	-	-	-	-	-	-	-
24	Dajti Kogjinas	-	-	14.56	-	-	-	-	-
25	Eli	-	-	-	-	26.19	-	-	-
26	Dajti Korce	-	-	-	-	95.00	-	-	-

27	Progres Korce	-	-	-	-	-	-	-	-
28	Sacramento Korce	-	-	-	-	-	-	-	-
29	Fondate	-	-	-	-	23.3	-	32.82	5.29
30	Basilio	-	-	-	-	-	-	-	-
31	Urma-Adelaide	-	-	-	-	-	-	-	-
32	Victtoria	-	-	-	-	-	-	-	-

Isolation of Fusarium colonies on PDA plates

The blotter paper and the agar plate are the most popular and frequently used methods for the detection of several fungi, which can produce mycelial growth during incubation, as described by the International Seed Testing Association. Nineteen colonies identified visually as belonging to Fusarium genera were grown in PDA plates, and colony morphology and structure were further investigated via stereomicroscopy (Tab 3, Fig 2, Fig 3). Their pigmentation, and morphological traits such as the presence of macroconidia, microconidia, sporodochia, and chlamydospores revealed notable morphological similarities among the isolates.

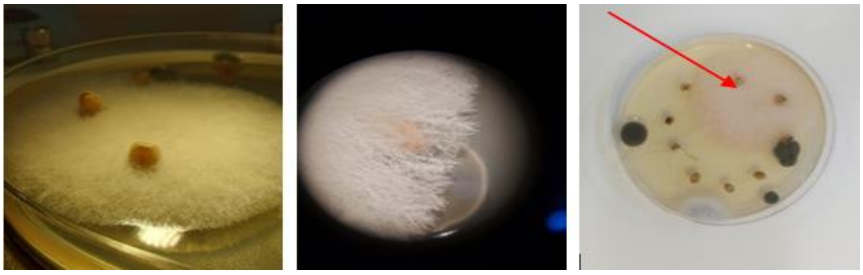


Figure 2. Microscopic examination of fungal colonies on PDA plates.

Table 3. Data on visual evaluation of suspected Fusarium colonies at PDA plates.

Cultivars with suspected Fusarium infection	No of seeds per PDA plate	Mean average no of colonies in PDA plates	Mean average no of suspected colonies	Colony color	Fusarium single-colony ID
Urma	10	6	1	Orange-white	2-I, 2-II
Dajti	10	6	1	white	3-I
Vittorio	10	9	1	white	6-II
Frenetik 1	10	5	1	pink	7-I
UBT-2	10	3	1	orange	8-I
Artico	10	2	1	yellow	9-I
Toborzo-1	10	3	1	white	10-I
Toborzo-2	10	6	1	white	10-II
Frenetik-2	10	8	1	orange	14-I, 14-II
Artiko	10	3	1	yellow	17-II
UBT-1	10	2	2	white	24-I, 24-I*
Ciari	10	2	2	orange	25-I
Ciari	10	4	1	white	25-II
Dajti-Kagjinas	10	6	1	white	26-I
Dajti-Kagjinas	10	8	1	yellow	26-II
Eli	10	8	1	yellow	27-II

Typically, the morphology was characterized by purplish-white mycelium, varying lengths of macroconidia, oval microconidia, and conidiophores of differing lengths, with the presence of chlamydospores also noted. Conidiophores were short, single, the clusters were well branched forming a flower shape (figure 3.a.), where is some cases they appeared in long chains (figure 3.c.). Macroconidia were curved, pointed at the tip, mostly three septate basal cells (figure 3b.). Microconidia were numerous, mostly non-septate, ellipsoidal shape. Chlamydospores were dispersed at the extremes.

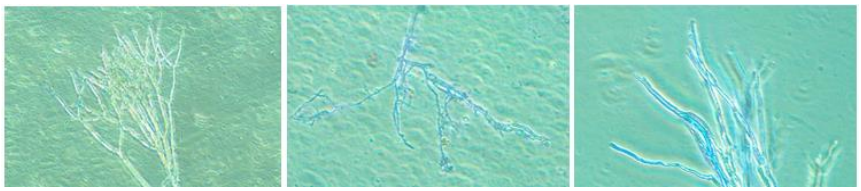


Figure 3. Microscopic investigation of the microstructure of Fusarium colonies grown on PDA (macroconidia, microconidia, and conidiophores). From left to right figures a, b, c.

Fungal colonies exhibited rapid growth in PDA medium within 4 days, with the white aerial mycelium often transitioning to reddish, purple, or pink coloration in the sclerotium when produced in abundance, or displaying a cream to yellowish-brown coloration that may eventually turn orange in the presence of abundant sporodochia (Fig 4).

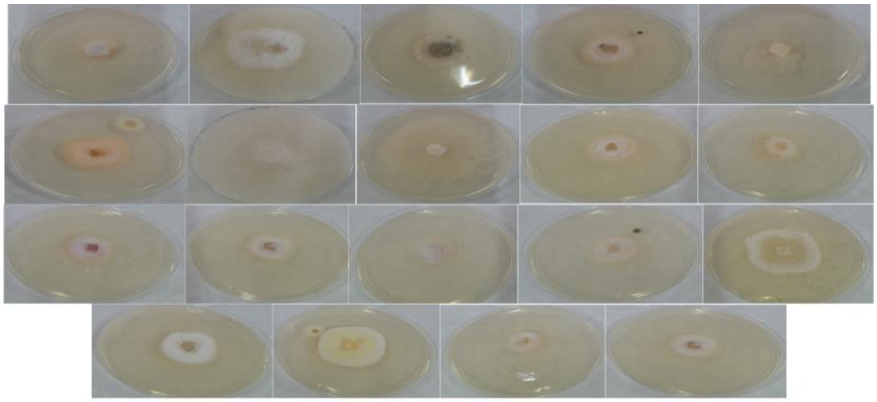


Figure 4. Single-colonies of Fusarium five days after being plated in PDA.

From the upper left side of the figure to the right are pictures of samples described in details at Table 3 as Fusarium colony ID.

PCR detection of genus Fusarium using TaqMan methodology

TaqMan methodology was employed to detect the presence of Fusarium infection at wheat cultivars by amplifying a fragment of the genus-specific elongation factor EF1-α gene. DNA templates were of three categories: 1. Extracted from hyphae of eighteen PDA plated single-colonies of Fusarium via silica-binding methodology; 2. Extracted from hyphae of eighteen PDA plated single-colonies of Fusarium via CTAB-based methodology; 3. Extracted from thirty-two wheat flour samples. Figures 5-7 represent results on the amplification of EF1α gene fragment from the above-described DNA templates.

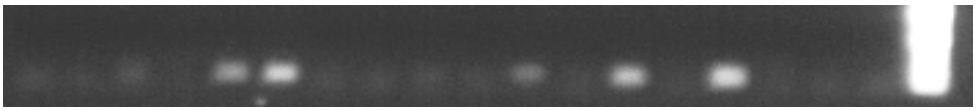
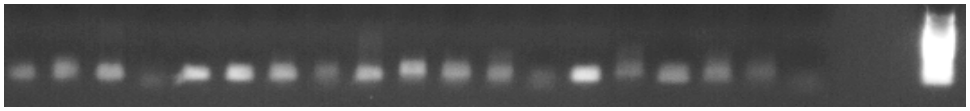


Figure 5. Amplicons of elongation factor EF1α (FusE primer pair) using template DNA extracted from hyphae of single-colonies via silica binding method. Samples from right to left: M-molecular ladder 100bp; Samples 2-I, 2-II, 6-II, 7-I, 8-I, 9-I, 10-I, 14-I, 14-II, 17-II, 24-I, 24-I*, 25-I, 25-I*, 25-II, 26-I, 26-II, 27-II.

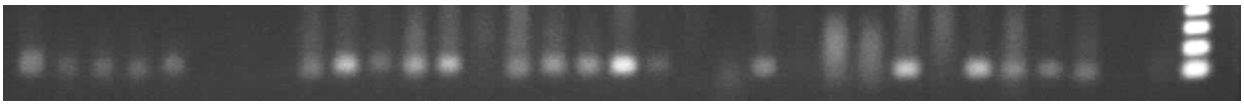
As seen (Fig 5) samples from cultivars Frenetic-1 (7-I), Artico (9-I), Frenetic-2 (14-I), Ciari (25-I, 25-I*) gave amplicons of the expected size. Thus, only four out of thirty-two cultivars were identified as infected.



2-I M

Figure 6. Amplicons of elongation factor EF1α (FusE primer pair) using template DNA extracted from hyphae of single-colonies via CTAB-based method. Samples from right to left: M-molecular ladder 100bp; Samples 2-I, 2-II, 6-II, 7-I, 8-I, 9-I, 10-I, 10-II, 14-I, 14-II, 17-II, 24-I, 24-I*, 25-I, 25-I*, 25-II, 26-I, 26-II, 27-II.

As seen (Fig 6) samples from cultivars Urma (2-II), Vittorio (6-II), Frenetik-1 (7-I), UBT-2 (8-I), Artico (9-I), Frenetik-2 (14-I, 14-II), Artiko (17-II), UBT-1 (24-I, 24-I*), Ciari (25-I, 25-I*), Dajti Kagjinas (26-I, 26-II), Eli (27-II) gave the expected amplicon. Thus, eleven out of thirty-two cultivars were identified as infected by Fusarium.



M

Figure 7. PCR products for elongation factor EF1α (FusE primer pair) using template DNA extracted from wheat flour. Samples from right to left: M-molecular ladder 100bp; Samples: Urma-Adelajde; Victoria, Nogal, Urma, Dajti, Krajlica, Vittoria, Frenetik-1, UBT-2, Artico, Toborzo, Nogal-Croatia, Basilic, Local Lushnja, Frenetik-2, UBT-2/2, Komolica, Artiko, Isja, Local Korca, Artillo, Sirtaki, MV Tambor, UBT-1, Ciari, Dajti Kagjinas, Eli, Dajti Korce, Progres Korce, Sacramento Korce, Fondate, Basilio.

Based on Fig 7 nineteen out of thirty-two samples gave the amplicon of the expected size. It is clear that except the disadvantage coming from the visual inspection of Fusarium colonies on PDA, which reduced the number of suspected infected cultivars from 32 to 13, the method chosen for DNA extraction had an important role as well. DNA extracted from same hyphae samples using silica binding versus CTAB methodology, proved to be templates of different quality (the ratio between the infected cultivars was 4:11, respectively). On the other side, the TaqMan dual probe and signal detection in FAM channel for less than 39 cycles according to (Sohlberg et al., 2022) (Fig 8) showed that nineteen out of thirty-two cultivars gave the expected amplicons, when the DNA template was extracted from the wheat flour.

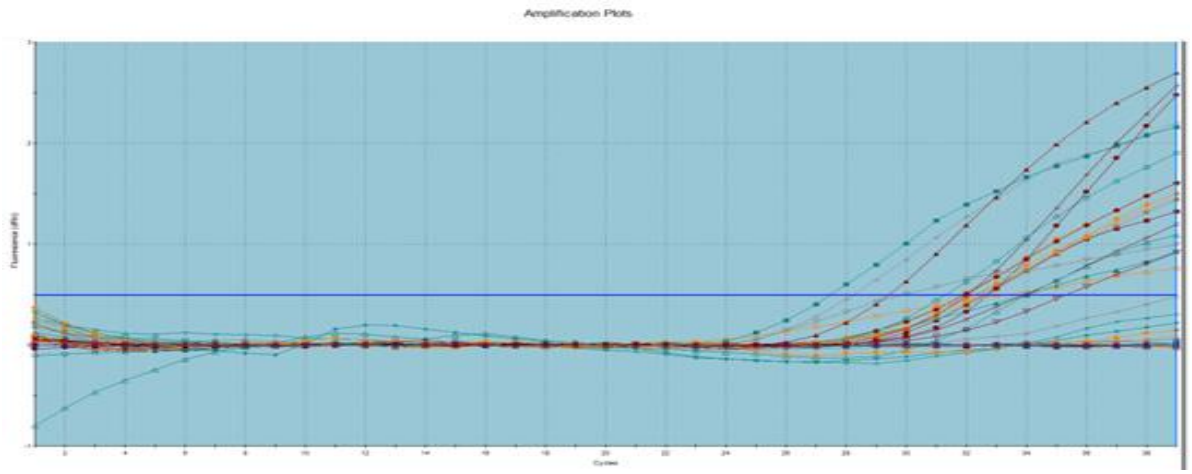
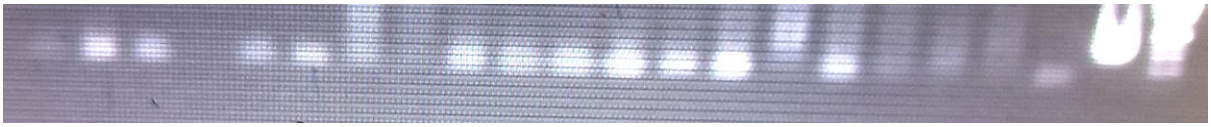


Figure 8. Using TaqMan dual probe and signal detection in FAM channel to assess the amplification of Fusarium elongation factor EF1α. Wheat flour positive samples (n=19) naturally contaminated with Fusarium spp.

qPCR detection of Fusarium species: F. graminearum, F. culmorum, F. avenaceum, and F. poae

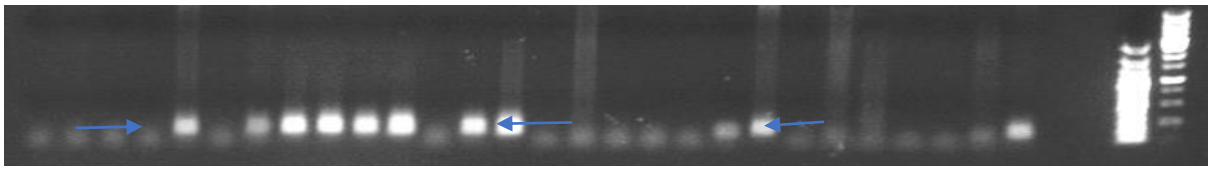
In order to investigate the presence of the most important species of Fusarium the DNA templates extracted from hyphae and flour samples were used to amplify gene fragments specific for F. graminearum (Fig), F. culmorum (Fig), F. avenaceum (Fig), and F. poae (Fig).



M M

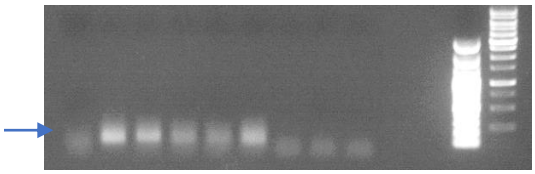
Figure 9. Amplicons of *F. graminearum* (Tri6 primer pair) using DNA extracted from wheat flour as template. Samples from right to left: M-molecular ladder 1kbp, M-ladder 100bp; Samples: Nogal, Urma, Dajti, Krajlica, Frenetik-1, Nogal-Croatia, UBT-2/2, Komolica, Artiko, Isja, Artillo, Sirtaki, MV Tambor, UBT-1, Dajti Korce, Progres Korce, Sacramento Korce, Fondate, Basilio, Urma-Adelajde;

Thirteen out of thirty-two samples (Fig 9) produced the expected amplicon of Fusarium graminearum.



M M

Figure 10. PCR products of *F. culmorum* using DNA extracted from hyphae and from wheat flour as template. Samples from right to left: M-molecular ladder 1kbp, ladder 100bp; Empty, Empty, Samples: Nogal[f], Urma[f], Urma-I, Urma-II, Dajti[f], Krajlica[f], Vittorio, Frenetik-1[f], Frenetik-1, UBT-2, Toborzo-1-I, Toborzo-1-II, Nogal Croatia[f], Frenetik-2, UBT-2/2[f], Komolica[f], Artiko, Artiko[f], Isja[f], Artillo[f], Sirtaki[f], MVToborzo[f], UBT-1-I, UBT-1[f], UBT-1-II, Ciari-1-I, Ciari-1-II, Ciari-II



M M

Figure 11. PCR products of *F. culmorum* using DNA extracted from hyphae and from wheat flour as templates. Samples from right to left: M-molecular ladder 1kbp, ladder 100bp, Empty, Empty, 26-I, 26-II, 27-II, 28M, 29M, 30M, 31M, 32M, 33M.
26-Dajti Kagjinas, 27-Eli, 28-Dajti Korce, 29-Progres Korce, 30-Sacramento Korce, 31-Fondate, 32-Basilio, 33-Adelajde
Samples named with I, II correspond to template DNA extracted from hyphae of different single-cell colonies of the same PDA plate; Samples named with * correspond to replicates taken from the same colony.

Fifteen out of thirty-two cultivars produced the expected F. culmorum amplicon (Fig 11).

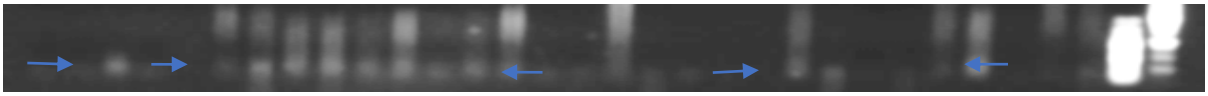


Figure 12. PCR products of *JIA* gene from *F. avenaceum* using DNA extracted from hyphea and from wheat flour as template.
Samples from right to left: M-molecular ladder 1kbp, ladder 100bp; 1^f, 2-I, 2-II, 2^f, 3^f, 5^f, 6-II, 7-I, 7^f, 8-I, 9-I, 10-I, 10-II, 11^f, 14-I, 14-II, 15^f, 16^f, 17-II, 17^f, 18^f, 21^f, 22^f, 23^f, 24^f, 24-I, 24-I*, 25-I, 25-I*, 25-II.
1-Nogal, 2-Urma, 3-Dajti, 5-Krajlica, 6-Vittoria, 7-Frenetik-1, 8-UBT-2, 9-Artico, 10-Toborzo-1, 11-Nogal Croat, 12-Bisilic, 13-Local Lushnja, 14-Frenetic-2, 15-UBT-2/2, 16-Komolica, 17-Artiko, 18-Isja, 20-Local Korca, 21-Artillo, 22-Sirtaki, 23-MV Toborzo, 24-UBT-1, 25-Ciari.
Samples named with I, II correspond to template DNA extracted from hyphae of different single- colonies of the same PDA plate; Samples named with * correspond to replicates taken from the same colony.

Thirteen out of thirty-two cultivars were infected by F. avenaceum (Fig 12).

PCR-based detection of Fusarium genus and species - Summary of the findings

27/32 cultivars tested were infected by at least one of the four Fusarium species investigated in this study; 19/19 of hyphae chosen from PDA grown colonies belonged to Fusarium genus; 27/32 DNAs extracted from flour were infected by Fusarium genus; 13/32 samples were infected by F. graminarium; 16/32 samples were infected by F. culmorum; 13/32 samples were infected by F. avenaceum; None of samples was infected by F. poae; 16/32 cultivars were infected by more than one Fusarium sps; F. graminarum and F. culmorum had the highest incidence (50% of samples), and F. poae the lowest (0%).

Table 4. Comparison between qPCR fluorescence-based and electrophoresis-based detection of the 96bp amplicon of EF1α gene (TP-True Positives, FP-False Positives, FN-False Negatives, TN-True Negatives); DNA templates were extracted from flour matrixes.

McNemar Calculation					
Gene under study	**Matrix**	**Contingency values**	**FusE_qPCR**	**96 pb ladder detection**	**Results**
FusE	flour	TP	Positive with FusE qPCR	Positive with gel electrophoresis	19
		FP	Negative with FusE qPCR	Positive with gel electrophoresis	1
		FN	Positive with FusE qPCR	Negative with gel electrophoresis	1
		TN	Negative with FusE qPCR	Negative with gel electrophoresis	3

A total of 19 wheat flour (Sensitivity=0.95) samples were correctly identified as positive for the FusE gene by both FusE qPCR dual probe and electrophoresis detection of 96 bp amplicons. One sample was FP and one sample was FN. Three samples were correctly assessed as negative for the FusE gene by both FusE qPCR dual prove and agarose gel electrophoresis.

Table 5. Comparison of the efficiency of TaqMan protocols on the amplification of specific gene fragments of Fusarium genus and species (PPV-Positive Predictive Values; NPV-Negative Predictive Values).

Target	**Metric**	**Value**	**qPCR TaqMan Efficacy of detection**
FusE	Sensitivity	0.95	Fusarium genus identified in 95% of cases
	Specificity	0.75	
	PPV	0.95	
	NPV	0.75	
TRI6_gene	Sensitivity	0.74	F. graminearum identified in 74% of cases

	Specificity	1	
	PPV	1	
	NPV	0.38	
	Sensitivity	0.93	
	Specificity	NA	
	PPV	1	
Fcul_gene	NPV	0	F. culmorum identified in 93% of cases
	Sensitivity	0.85	
	Specificity	NA	
	PPV	1	F. avenaceum identified in 85% of cases
JIA_gene	NPV	0	
F. poae			No amplified products

The findings from the method comparison regarding the presence of Fusarium spp, and species identification reveal that the qPCR TaqMan dual probe assay exhibits different sensitivity levels for various Fusarium species. The qPCR TaqMan assay detected F. graminearum in 74% of instances, demonstrating moderate effectiveness in species identification. The assay demonstrated greater reliability in identifying F. culmorum, achieving a detection rate of 93%, indicating superior sensitivity for this species. Moreover, the qPCR TaqMan assay identified F. avenaceum in 85% of cases. These findings highlight the efficacy of qPCR TaqMan as a diagnostic method for species-specific identification of Fusarium spp., with its performance differing based on the species under examination and taking in consideration the effect of non-Fusarium spp DNA present in wheat flour.

Comparison of the efficacy of methods for Fusarium detection at wheat grains

The efficiency of the detection of Fusarium based on mycotoxins presence, microscopic evaluation, and qPCR amplification of specific genes was assessed for each cultivar under study (Tab 6). For 9 out of 32 cultivars results match for the three methods applied, with the TaqMan being able to identify Fusarium infection at 27 cultivars. Mycotoxins were present at 14 cultivars only, with one category (ergometrine, ergometrinine) not belonging to Fusarium products. The visual/microscopic investigation led to the conclusion that 14 cultivars were infected, however the comparison with those which contained mycotoxins showed important mismatches (10 out of 14).

Table 6. Alignment of results on Fusarium presence at wheat seeds as revealed by mycotoxins presence, PDA platting and microscopy, and specific qPCR. Colored boxes represent positive samples.

Cultivars	Mycotoxins presence	Microscopic evaluation	qPCR TaqMan
Nogal			
Urma			
Dajti			
Krajlica			
Vittoria			
Frenetik-1			
UBT-2			
Artico			
Toborzo			
Nogal Croat			
Basilic			
Local Lushnja			
Frenetic-2			
UBT 2/2			
Komolica			
Artiko			
Isja			
Local Korca			
Artillo			
Sirtaki			
MV Toborzo			
UBT-1			

Ciari			
Dajti Kogjinas			
Eli			
Dajti Korce			
Progres Korce			
Sacramento Korce			
Fondate			
Basilio			
Urma-Adelaide			
Victtoria			

CONCLUSIONS

- FHB is a disease complex in which several species co-exist, and since the prevalence of certain species in a region is primarily determined by climatic conditions, the establishment of a reliable workflow for the investigation of the presence of the most important ones (the more toxigenic) in wheat cultivars naturally infected became a major goal of the study. To achieve this, isolation of the pathogen by PDA platting followed by screening based on colony morphology and structure were seen as a classical, cost-effective approach, which conducted simultaneously with chromatography (LC-MS/MS for mycotoxins composition and quantity), and specific TaqMan qPCR were employed to investigate 32 winter wheat cultivars (Triticum aestivum L.).
- As previously reported the amount of Fusarium DNA can be used as a predictor of mycotoxin concentration in grains. However, the last is strongly dependent on the material analyzed, and no relationship has been found for naturally infected samples with low-toxin content (Xu et al., 2003). In our study, mycotoxins typical for the genus Fusarium were found at 13 cultivars; Meanwhile the macro and micro-morphological characteristics of fungal colonies (macroconidia, oval microconidia, conidiophores) on PDA classified 14 of them as infected by the same pathogen. Independently that the detection based on LC-MS/MS failed compared to PCR (14:27 cultivars) regardless the high sensitivity of both, one should consider that the presence of pathogen in the seeds does not necessarily imply the mycotoxins accumulation at the detection levels. Yet, the presence of certain toxins considered as highly toxigenic should be identified, and it is PCR-based methods which can elucidate the genetic potential of Fusarium spp. isolates to produce fumonisins and trichothecenes even when their quantity is below the detection limits. The fact that results from the three detection methods matched for 9 cultivars only, highlights the importance of using an integrated approach, which overcomes their disadvantages related to the detection limits, or to the cost-efficiency.
- Since the predominant Fusarium species associated with FHB in small-grain cereals in Europe are F. graminearum, F. culmorum, F. avenaceum and F. poae, their presence was investigated based on the PCR amplification of specific gene fragments of each. Results showed that 13/32 cultivars were infected by F. graminarium; 16/32 by F. culmorum; 13/32 by F. avenaceum, and none of them was infected by F. poae; In summary, 16/32 cultivars were infected by more than one Fusarium sps with F. graminarum and F. culmorum having the highest incidence (50%).
- The assessment of the detection efficacy of TaqMan qPCR depending on the matrix used to extract the template DNA (hyphae, flour), and on the method of extraction (silica binding, CTAB, SDS-based) showed that the use of hyphae from the suspected Fusarium colonies reduced the number of the suspected infected cultivars to 14/32, exposing this way one important restriction of the PDA platting and microscopic evaluation of the pathogen identity compared to the use of wheat flour as a matrix for DNA extraction (27/32). Also, using silica binding versus CTAB-based method to extract and purify DNA gave different amplification efficiencies (5:16, respectively). Therefore, flour is recommended as the proper matrix for DNA extraction.
- The sensitivity of TaqMan PCR was assessed using the McNemar calculation, which compares the dual probe and signal detection in FAM channel as compared to electrophoresis banding patterns, and proved the high sensitivity of method (95%). Also, the comparison of the sensitivity of TaqMan PCR applied for

detection at genus level compared to specific-PCRs used at species level proved mismatches, which varied from 26% for F. graminarium to 15% for F. avenaceum, to 5% for F. culmorum, implying the need for further improvement of the protocols.

- In conclusion, for the reliable detection of genus Fusarium at naturally infected wheat seeds the use of TaqMan qPCR on DNA templates from wheat flour is recommended. Meanwhile, the presence of highly toxigenic mycotoxins determined via LC-MS/MS, and the investigation of the micromorphology of Fusarium at subspecies level via PDA platting and stereomicroscopy, could be the methods of choice. This way, an integrated approach would provide the necessary data either for regulated practices of detection, or for research goals.

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