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Effectiveness of Species- and Trichothecene-Specific Primers in Monitoring *Fusarium graminearum* Species Complex in Small Grain–Pea Intercropping Systems

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Abstract: Tracking the distribution of *Fusarium* species and the detection of changes in toxin production provides epidemiological information that is essential for *Fusarium* head blight (FHB) management. Members of *Fusarium graminearum* species complex (FGSC) were characterized using species and trichothecene-specific primers. Associations between members of the FGSC, cereal crop species (wheat, rye, triticale, and oat), seeding time (winter and spring), type of cultivation (monocrop and intercrop) and chemotype grouping were investigated with multiple correspondence analysis and multiple regression modeling. We found that triticale and oat were more related to isolates classified into *F. graminearum* s. *lato* than with other isolates. In contrast, wheat and rye were more associated with *F. graminearum* s. *stricto*. Cereal crop species affected the frequencies of *F. graminearum* s. *stricto* ($p = 0.003$) and *F. graminearum* s. *lato* ($p = 0.08$) and unidentified isolates with morphological characteristics like those of FGSC members ($p = 0.02$). The effectiveness of species-specific primers was 60.3% (Fg16F/R) and 76.2% (FgrF/FgcR), and the effectiveness of primer sets for the trichothecene genotyping of the *Tri5* and *Tri3* genes was 100% and 90.6%, respectively. The decrease in *Fusarium*-damaged kernel values in the wheat–pea intercropping system indicated that intercropping systems have the potential to control FHB.

Keywords: *F. graminearum* species complex; small grains; intercropping; sequence characterized amplified region (SCAR); trichothecene genotyping

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1. Introduction

Fusarium head blight (FHB) is one of the most economically important wheat diseases. The causal agents of FHB include representatives of the *Fusarium graminearum* species complex (FGSC), *Fusarium culmorum*, *Fusarium poae*, *Fusarium cerealis*, *Fusarium langsethiae*, *Fusarium sporotrichioides*, *Fusarium avenaceum*, *Fusarium tricinctum* species complex; and the nontoxigenic species *Microdochium nivale* and *Microdochium majus* [1]. FGSC, also known as *F. graminearum* s. *lato*, comprises 16 phylogenetically distinct species: *Fusarium austroamericanum*, *Fusarium meridionale*, *Fusarium boothii*, *Fusarium mesoamericanum*, *Fusarium acaciae-mearnsii*, *Fusarium asiaticum*, *Fusarium graminearum* sensu *stricto*, *Fusarium cortaderiae*, *Fusarium lousianense*, *Fusarium brasilicum*, *Fusarium gerlachii*, *Fusarium nepalense*, *Fusarium aethiopicum*, *Fusarium ussurianum*, F. sp. NRRL 34461, and *Fusarium graminearum* (Gulf Coast) [2]. The prevalence of species composing FGSC differs in different parts of the world [3].

Representatives of the FGSC are B-type trichothecene producers [2,4]. However, different types of B-type trichothecene mycotoxins are produced by different FGSC members [2].

F. aethiopicum, *F. vorosii*, *F. boothii* and *F. nepalense* are reported to produce only 15-AcDON, and *F. graminearum* s. *stricto* and *F. asiaticum* produce 3-AcDON, 15-AcDON, and NIV. In contrast, the other FGSC members are mainly NIV and/or 3-AcDON producers [2]. The chemotype group determines the potential of *Fusarium* species to cause infection. DON chemotypes are reported to be 10–24 times more toxic to plants than are NIV chemotypes [4]. However, the relationship between mycotoxin accumulation and the level of FHB infection is not straightforward and is highly dependent on environmental conditions and the specific plant response [4,5].

Monitoring the changes in the distribution of *Fusarium* species and the detection of alterations in toxin production provides epidemiological information that is essential for FHB management [6–8]. Sequence-based identification of *Fusarium* species is considered to be the most reliable method for distinguishing *Fusarium* species. However, there are obstacles to using it in routine screening of the presence of *Fusarium* species in cereal crops since it usually requires editing more than translation elongation factor 1-α (*TEF1*), DNA-directed RNA polymerase II largest (*RPB1*) and/or second largest subunit (*RPB2*) sequences for queries showing similarity at or below 99.4% [9]. In addition, the effectiveness of species-specific primers for trichothecene production-related genotyping of FGSC is less than 100% since the structure of the genes used for primer development can differ between isolates with different origins [10]. Interspecific crossing should not be overlooked as a factor that hinders the routine identification and trichothecene genotyping of *Fusarium* spp. since interspecific crosses yield hybrids with 100% sequence identity across different genes between multiple *Fusarium* species [11].

There is also variability in the reports of the relationship between the TRI (trichothecene) genotype and chemotype, which opens the question of the applicability of methods for trichothecene production-related chemotyping and genotyping [12]. Villafana et al. [12] pointed out that chemotype data obtained by in vitro induction of trichothecene production could be considered inappropriate for the prediction of the risk of toxins produced in field conditions. Consequently, the enhancement in the screening power of methods used in routine surveillance of FHB methods and their mycotoxin production potential has yet to be improved. The enhancement of the applicability of the methods used for the identification of *Fusarium* spp. is becoming increasingly important for routine screening of *Fusarium* populations infecting crops cultivated as part of intercropping systems. Interactions between simultaneously grown crops can cause specific agroecological conditions and, as such, can influence the distribution and predominance of different *Fusarium* species. Data on how intercropping systems affect economically important pathogens are scarce but also encouraging since they support long-term ecosystem-based strategies for the prevention of pathogen damage through a combination of cultivation techniques.

In Serbia, the predominant causal agent of FHB in wheat is *F. graminearum* s. *stricto* 15-AcDON chemotype [10,13,14]. Relevant investigations conducted in Serbia have mainly focused on factors influencing the distribution of *Fusarium* spp. infecting wheat cultivated under conventional production conditions. However, there are still few reports on the distribution of *Fusarium* spp. causing FHB on other cereal crop species such as rye, triticale and oat. We hypothesized in this study that the complexity of the interactions between cereal and legume crops could affect the level of *Fusarium* infection in cereals. It was also suspected that the simultaneous cultivation of different crops could cause stratification of the FGSC population in the same growing area. Consequently, the main objectives of this study were to test (1) the level of *Fusarium*-damaged kernels (FDKs) when small grains were intercropped with pea; (2) the effectiveness of species- and trichothecene-specific primers in the routine surveillance of *Fusarium* species infecting not only wheat but also rye, triticale and oat; and (3) the possibilities of identifying FGSC population stratification in small areas with different cereal crop species.

2. Materials and Methods

The study was conducted on 53 *F. graminearum* isolates originating from seed samples collected in 2019 from the same field trial where FHB-susceptible varieties of cereal crops (wheat, rye, triticale and oat) were cultivated as monocrops and intercropped with pea. (Tables S1 and S3). The varieties used in the study were Ilina and Nataša (wheat), Odisej (triticale), Savo (rye), Jadar and Dunav (oat) and Kosmaj and Junior (pea).

A field trial was conducted in 2018/2019 growing season at the experimental field of the Institute of Field and Vegetable Crops Novi Sad, Serbia. Winter seeding and spring seeding were made on 25 October 2018 and 6 March 2019, respectively. The harvest of all varieties (seeded in winter and spring) was on 11 July 2019. Immediately after the harvest, the seed samples were processed for further analysis for the presence of the pathogens. A field trial was arranged in accordance with a randomized block design, with four replications. The plot size of each replicate was 5 m^2. The crops were cultivated without the use any fertilizers or pesticides. The seeding ratio of the cereal crop–pea intercropping system was 30:70%. The soil type was a slightly carbonated loamy chernozem, and soybean was the previous crop species. The sowing preparation included ploughing, disk harrowing and cultivating.

The average temperature (14.2 °C) and total precipitation (125.4 mm) in May gave rise to suitable conditions for *Fusarium* head blight infection of cereals. In addition, the average temperatures at the end of May and at the beginning of June (approximately 20 °C) were favorable for further disease development. Climatic conditions at the time of anthesis were favorable for FHB in all maturity groups.

After shelling, all the samples were initially examined using the seed health testing method recommended by the International Seed Testing Association [15]. Forty seeds were examined from each plot by plating 10 seeds in Petri dishes, which were replicated four times. The seeds were surface sterilized (1.0% NaOCl for 1 min) and rinsed twice with sterile distilled water. The first isolation of fungi was performed on 2% water agar media amended with streptomycin sulfate and then transferred to potato dextrose agar PDA, V8 Juice Agar (HiMedia, Einhausen, Germany) and water agar for morphological identification to the species level [16].

Grain infection/contamination with *F. graminearum* clade members and *Alternaria* spp. was estimated for each plot and expressed as FDKs (*Fusarium*-damaged kernels) and ACKs *(Alternaria*-contaminated kernels) as follows: FDKs (%) = (Number of seeds on which *Fusarium* species occurred/Total number of seeds per plot) × 100. ACKs (%) = (Number of seeds on which *Alternaria* species occurred/Total number of seeds per plot) × 100.

Prior to DNA extraction, all *Fusarium* isolates were purified by single-spore isolation. DNA was extracted using the procedure of Möller et al. [17]. Briefly, 10% cetyltrimethylammonium bromide (CTAB), proteinase K, and SEVAG (chloroform:isoamyl alcohol, 24:1, *v*/*v*) were used for DNA isolation. The DNA was precipitated using isopropanol, resuspended in 0.1X Tris-EDTA (TE) buffer and then stored at −20 °C.

2.1. Identification of Fusarium Species Using Species-Specific PCR (SCAR Analysis)

All isolates that were previously identified as *F. graminearum* at the morphological level were subjected to molecular identification using sequence-characterized amplified region (SCAR) analysis (Table 1). The isolates were analyzed with the species-specific primer pair Fg16F/R [18] and classified into the SCAR groups defined by Carter et al. [19]. SCAR-type polymerase chain reaction (PCR) products reported by Carter et al. [19] include type 1 (420 bp), type 2 (510 bp), type 3 (540 bp), type 4 (580 bp), type 5 (520 bp), and type 6 (400 bp). PCR products of 420 bp and 400 bp are associated with *F. graminearum* s. *stricto* [20–25], and PCR products of the remaining SCAR groups are produced by other members of the FGSC. Isolates that were not identified using the Fg16F/R primer pair were subjected to identification with the FgrF/FgcR primer pair reported to be highly specific to all lineages of the *F. graminearum* clade, with the exception of lineage 5 [26].

Table 1. Primer sets and PCR conditions for screening of the FGSC.

Primer Set	Nucleotide Sequence (5′–3′)	Size of Amplified PCR Fragment (bp)	PCR Conditions
Fg16F Fg16R	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	420 (SCAR 1) 400 (SCAR 6)	initial denaturation: 95 °C, 3 min denaturation, annealing and elongation (38 cycle): 95 °C, 30 s 62 °C, 20 s 72 °C, 45 s final extension: 72 °C, 5 min
FgrF FgcR	GTTGATGGGTAAAAGTGTG CTCTCATATACCCTCCG	500	initial denaturation: 94 °C, 85 s denaturation, annealing and elongation (25 cycles): 95 °C, 35 s 53 °C, 30 s 72 °C, 30 s final extension: 72 °C, 5 min

(PCR) Polymerase chain reaction; (FGSC) *Fusarium graminearum* species complex.

PCR mixtures with the Fg16F/R primer pair were prepared using the method described by Demeke et al. [27]. Briefly, 25 ng of purified DNA was used as a template in a 25 µL reaction mixture containing 1X PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 1.5 mM $MgCl_2$, deoxynucleotide triphosphates (0.2 mM each), primers (0.4 µM each) and 0.75 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR reaction mixtures (25 µL) with FgrF/FgcR contained 25 ng of purified DNA, 1X PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 2 mM $MgCl_2$, deoxynucleotide triphosphates (0.2 mM each), primers (0.5 µM each), and 1.25 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling parameters for both primer pairs are given in Table 1. PCR was performed in a Veriti 96-well thermal cycler (AB Applied Biosystems, Waltham, MA, USA). The PCR products were separated by electrophoresis in 1.5% agarose gels containing 1 µg mL^{-1} ethidium bromide. Normalization of 25-line gels was obtained by using a 100-bp DNA ladder (Bio-Lab, Conyers, GA, USA).

2.2. Trichothecene Production-Related Genotyping of F. graminearum Isolates

Trichothecene genotyping of *F. graminearum* isolates and classification into either 3- or 15-AcDON was performed using the primer sets Tri315F/Tri315R and Tri303F/Tri303R [22]. PCR was performed on a 25 µL reaction mixture containing 25 ng DNA, 1X PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.3)), 2 mM $MgCl_2$, deoxynucleotide triphosphates (0.2 mM each), primers (0.5 µM each) and 1.25 units of Taq DNA polymerase. The amplification reactions were performed in a Veriti 96-well thermal cycler (AB Applied Biosystems). The thermal cycling parameters for each primer pair as well as the size of amplified PCR fragments are presented in Table 2. Isolates that were not confirmed as 3- or 15-AcDON producers were analyzed with a qualitative Tri-5-specific PCR assay with a Tri5F/R primer pair specific to trichothecene producers according to the methods of Sanoubar et al. [28]. The PCR conditions for the Tri5F/R, Tri315F/Tri315R and Tri303F/Tri303R primer pairs are given in Table 2.

2.3. Statistical Analysis

Multiple linear regression (stepwise regression) was used to analyze whether seeding time, type of cultivation and level of contamination of competing *Alternaria* species influenced the *Fusarium* spp. infection level of cereal crop grain. Seeding time (winter and spring) and type of cultivation (monocropping and intercropping) were considered categorical variables, while the level of grain contamination by *Alternaria* spp. was a continuous variable. The data were analyzed using stepwise regression, since this method

was reported to be suitable to address multicollinearity [29]. Regression models were also followed with the variance inflation factor (VIF) to detect multicollinearity. VIFs are measures of how much the variance of an estimated regression coefficient increases if predictors are correlated and are 1 if there is no correlation between factors. If the VIF exceeds 5, the regression coefficient for that term is not strongly estimated. The alpha level to enter and alpha level to remove the influencing factors in the stepwise multiple regression were set by default to 0.15. Bursac et al. [30] reported that an alpha level of 0.05 to enter and to remove the influencing factors in stepwise multiple regression could fail to identify important variables. Spearman's coefficient of correlation was used to characterize the relationships between grain infected with *F. graminearum* and grain contaminated with *Alternaria* spp.

Table 2. Primer sets and PCR conditions for trichothecene genotyping of the FGSC.

Primer Set	Nucleotide Sequence (5′–3′)	Size of Amplified PCR Fragment (bp)	PCR Conditions
Tri303F/ Tri303R	GATGGCCGCAAGTGGA GCCGGACTGCCCTATTG	586	initial denaturation: 94 °C, 2 min denaturation, annealing and elongation (30 cycles): 94 °C, 30 s 60 °C, 1 min 72 °C, 2 min final extension: 72 °C, 10 min
Tri315F/ Tri315R	CTCGCTGAAGTTGGACGTAA GTCTATGCTCTCAACGGACAAC	864	
Tri-5F Tri-5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	544	initial denaturation: 95 °C, 3 min denaturation, annealing and elongation (38 cycles): 95 °C, 30 s 62 °C, 20 s 72 °C, 45 s final extension: 72 °C, 5 min

Multiple linear regression was also used to identify whether different crop species (wheat, rye, triticale and oat), seeding time (winter, spring), type of cultivation (monocropping, intercropping) and grain contamination by *Alternaria* spp. influenced relative frequencies of FGSC members. Factorial plots were used to illustrate how a response variable relates to one or more categorical variables. The relative frequencies of FGSC members were calculated with respect to the total number of *F. graminearum* isolates. The relative frequencies of members of FGSC per small grain crop were calculated via a two-way relative frequency table, where the counts of *F. graminearum* s. *stricto*, *F. graminearum* s. *lato* and unidentified *Fusarium* species for each crop were divided by the total number of isolates per crop (Table S2). Associations between qualitative variables (seeding time; type of cultivation; cereal crop species; presence/absence of *F. graminearum* s. *stricto*, *F. graminearum* s. *lato*, and unidentified members of the F. graminearum clade and chemotype group) were estimated using multiple correspondence analysis (MCA).

To provide more information on which means are significantly different and to estimate how much they differ, we used a general linear model and Tukey's pairwise comparisons with 95% confidence.

The effectiveness of the primer pairs was expressed as the percentage of isolates producing PCR products in relation to analyzed isolates for a given primer set. The analysis was performed using Minitab 17 Statistical Software (2010) (trial version) and XLSTAT 2019 (trial version) in Microsoft Excel and package 'ggplot2' in R software (RStudio Team, 2020).

3. Results

The dominant fungal species isolated from the collected seeds were FGSC members and *Alternaria* spp. (Table S3). On average, triticale had the lowest ACKs (22.5%), but the FDKs (11.8%) was as high as the FDKs of wheat (13.8%). The highest level of ACKs was recorded for oat (43.1%), followed by the lowest level of FDKs (0.6%) (Figure 1). A significant negative correlation between average grain infection with the *F. graminearum* clade and *Alternaria* spp. was observed (r = 0.567, p = 0.022).

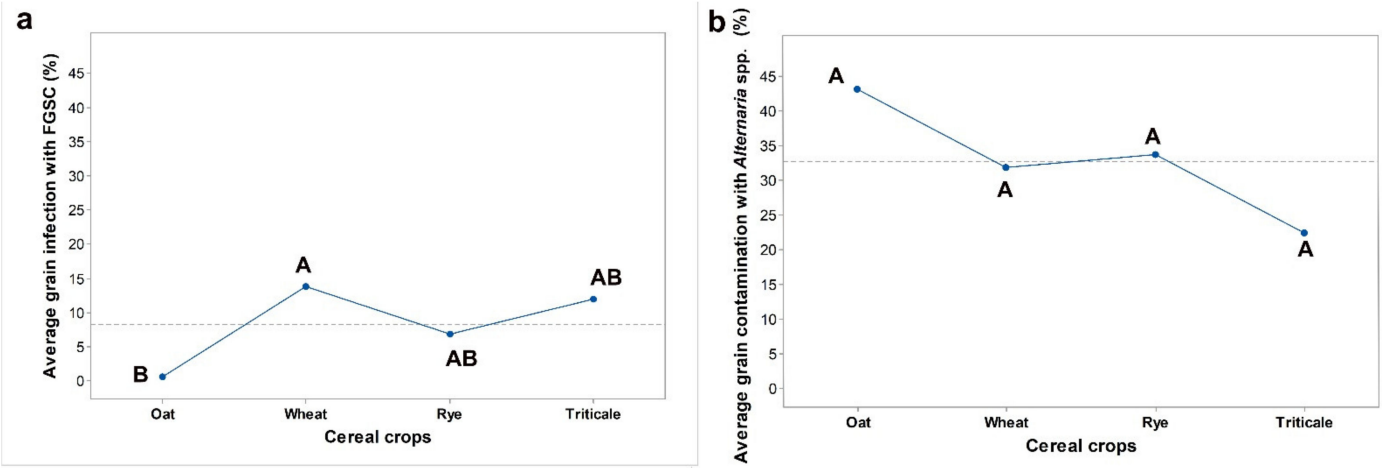

Figure 1. Average FDKs (**a**) and ACKs (**b**) on wheat, rye, triticale and oat cultivated as monocrops and intercropped with pea. Tukey's pairwise comparisons of means were performed with 95% confidence. The means that do not share the same letter are significantly different.

Regression analysis indicated that FDKs differed significantly among cereal crop species (p = 0.028) and that they were affected by the type of cultivation (p = 0.027). The seeding time (winter and spring) did not affect FDKs significantly at an alpha level of 0.05 (p = 0.096) but seeding time should not be neglected either since the value was less than the setup limit of 0.15 introduced by entering and removing the influencing factors in the regression model (Figure 2). The coefficient of determination for the regression model comprising seeding time and type of cultivation as independent variables and FDK value as the dependent variable was 84.3%. The VIFs were equal to or less than 1.75, indicating that the regression coefficients were strongly estimated and that the regression model was reliable.

3.1. Effectiveness of Species- and Trichothecene-Specific Primers for the Characterization of the FGSC in Small Grain–Pea Intercropping Systems

The most frequent member of the *F. graminearum* population (32 out of 53 isolates) was *F. graminearum* s. *stricto* (Table 3, Table S1). Sequence characterized amplified region (SCAR) analysis with the Fg16F/R primer pair assigned all *F. graminearum* s. *stricto* isolates to SCAR Group 1, giving a PCR product of 420 bp. The members of the FGSC that did not produce a PCR product with Fg16F/R (21 out 53 isolates) were subjected to analysis with the FgrF/FgcR primer pair (Table S1). The isolates that produced PCR products with FgrF/FgcR accounted for 30.2% of the population and were assigned as *F. graminearum* s. *lato*. The effectiveness of the FgrF/FgcR primer pair was 76.2% since it resulted in the identification of 16 out of 21 isolates (Table 3). Isolates that did not produce a PCR product with any primer pair accounted for 9.4% of the *F. graminearum* population. The morphological features of the unidentified isolates could not be distinguished from those of other members of the FGSC (Table 3).

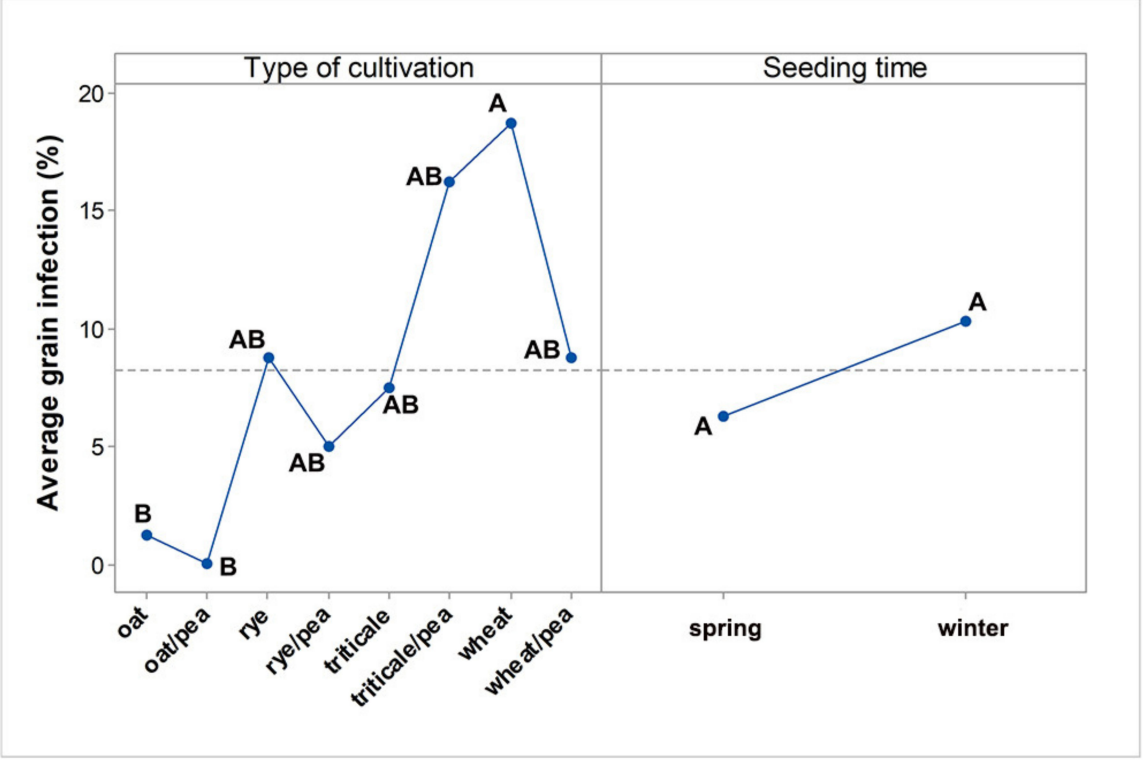

Figure 2. Factorial plots indicating effects of type of cultivation and seeding time on the FDKs of cereal crop species. Tukey's pairwise comparisons of means were performed for 95% confidence levels. The means that do not share the same letter are significantly different.

Table 3. Molecular genotypes within the *F. graminearum* population and the effectiveness of primer pairs.

			Species Level		*Tri5*	*Tri3*
FGSC	**Number of Isolates**	**Morphology of FGSC**	***F. graminearum* s. *stricto* (Fg16F/R)**	***F. graminearum* s. *lato* (*FgrF*/*FgcR*)**	**Tri5F/R**	**Tri315F/R (15-AcDON)**
F. graminearum s. *stricto*	32	+	+	n/a	n/a	+
F. graminearum s. *lato*	13	+	-	+	+	+
F. graminearum s. *lato*	3	+	-	+	+	-
Unidentified	2	+	-	-	+	-
Unidentified	3	+	-	-	+	+
Effectiveness of primer pairs			60.3%	76.2%	100%	90.6%

FGSC (*F. graminearum* species complex); (+) indicates that the PCR product is present; (-) indicates that the PCR product is missing; n/a indicates that the primer pair was not used for identification of the isolates shown to be *F. graminearum* s. *stricto*

Trichothecene genotyping of the FGSC isolates was performed on the *Tri3* and *Tri5* genes using the primer sets Tri315F/R, Tri303F/R and Tri5F/R (Table S1). All *F. graminearum* s. *stricto* isolates were shown via the Tri315F/R primer pair to be 15-AcDON producers. Only 9.4% of the isolates were not identified as 15-AcDON producers; all of them belonged to *F. graminearum* s. *lato* or were not identified, although they exhibited morphological features similar to those of members of the FGSC (Table 3). Isolates that were not identified as 15-AcDON producers were subjected to genotyping with Tri303F/R; none of them were shown to be 3-AcDON producers. Further characterization with the Tri-5F/R primer pair confirmed that the unidentified isolates are trichothecene producers (Table 3). When testing

these isolates with the Tri-5F/R primer pair, all isolates produced high-intensity DNA bands on agarose gels (designated Tri5-3), except for three isolates originating from rye. These isolates from rye were identified as *F. graminearum* s. *lato* and produced PCR bands fainter than the Tri5-3 bands (designated Tri5-2 and Tri5-1) (Figure 3).

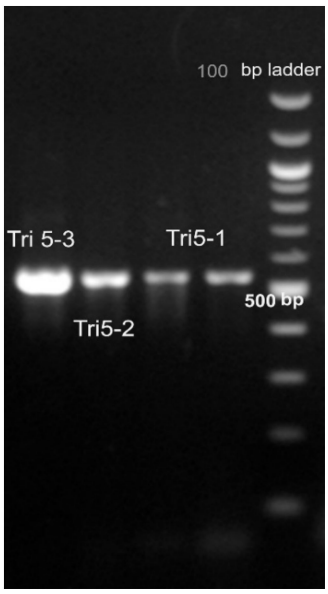

Figure 3. Qualitative *Tri-5*-specific PCR assay giving *Tri-5* DNA bands with three intensity levels.

3.2. Stratification of FGSC Members among Different Cereal Crops

The isolates originating from wheat seed samples were identified predominantly as *F. graminearum* s. *stricto* (77.3%). Only 13.6% and 9.1% of FGSC members isolated from wheat were identified as *F. graminearum* s. *lato* or were unidentified, respectively (Figure 4). A similar proportion of *F. graminearum* s. *stricto* was found on rye, where *F. graminearum* s. *stricto* accounted for 72.7% and *F. graminearum* s. *lato* accounted for 27.3% of isolates. The largest proportion of *F. graminearum* s. *lato* and unidentified isolates of FGSC was found on triticale (63.2%) (Figure 4). Only one isolate originated from oat was identified as *F. graminearum* s. *lato*. A relatively large proportion of *F. graminearum* s. *lato* and unidentified isolates on triticale drew our attention. Multiple stepwise regression analysis showed that cereal crop species affected the frequencies of *F. graminearum* s. *stricto* ($p = 0.003$), *F. graminearum* s. *lato* ($p = 0.08$) and unidentified isolates ($p = 0.02$). This implies the possibility that stratification of FGSC members occurs among different cereal crop species in the same growing area (Figure 4).

The results obtained by the molecular characterization of the *F. graminearum* population were further subjected to MCA to investigate the association of members of the FGSC with the type of cultivation (monocropping, intercropping), seeding time (winter, spring) and chemotype group (Figure 5).

MCA indicated that the first two dimensions contributed to 40.49% of the overall variability. The contribution of the first and second dimensions was 23.09% and 17.40%, respectively (Table S4). The isolates of *F. graminearum* s. *stricto* were closely associated with wheat and rye that were sown in winter. *F. graminearum* s. *stricto*, *F. graminearum* s. *lato* and unidentified FGSC isolates showed dissimilar profiles since, according to the analysis results, *F. graminearum* s. *stricto* was positioned in the opposite quadrant from the *F. graminearum* s. *lato* and unidentified isolates; indeed, *F. graminearum* s. *lato* was more associated with triticale intercropped with pea. *F. graminearum* s. *lato* and unidentified isolates were only related to trichothecene producers; this is in contrast to *F. graminearum* s. *stricto*, which was associated with 15-AcDON producers. The quality of association between variable categories and a particular axis was presented with squared cosine (cos2). Squared

cosine showed that not all points were equally well explained by the two dimensions. For some items, more than two dimensions were required to adequately represent the data (Table S4). Red-colored variables had the highest degree of association with the first two dimensions in contrast to blue-colored ones. (Figure 5).

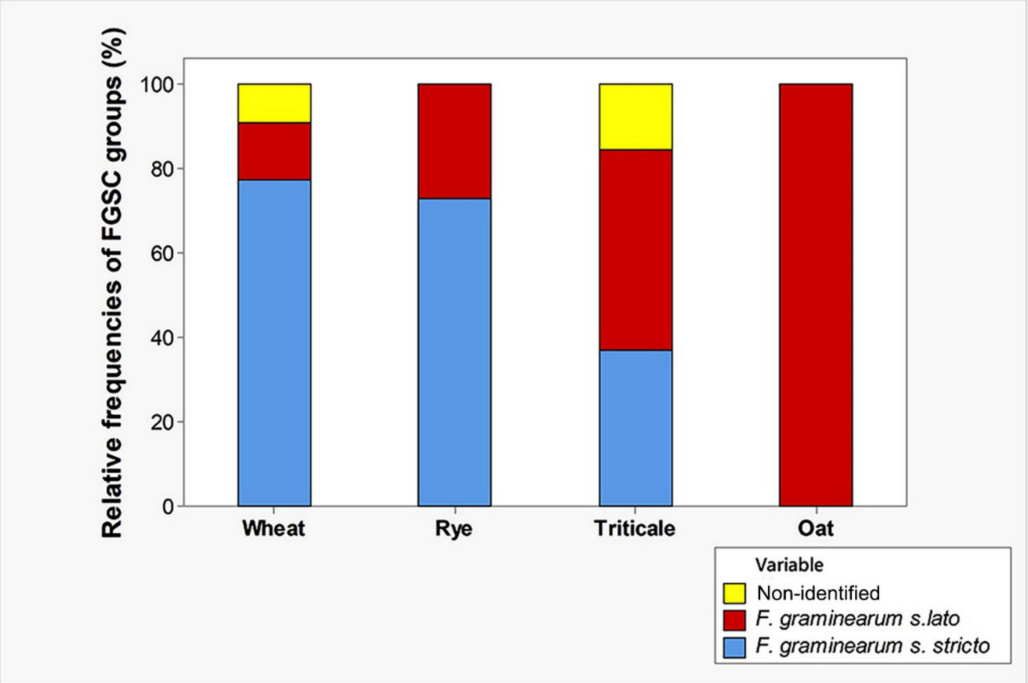

Figure 4. Associations among wheat, rye, triticale, oat and *F. graminearum* species complex (FGSC) groups.

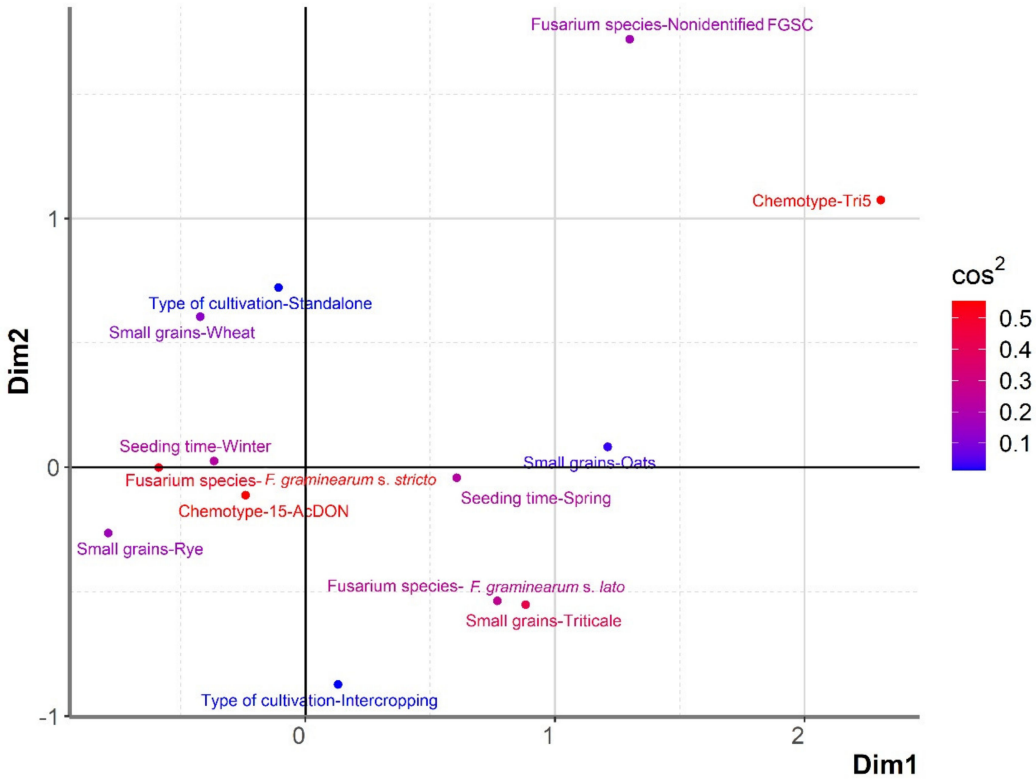

Figure 5. Multiple correspondence analysis (MCA) between cereal crop species (wheat, rye, triticale,

and oat); seeding time (winter and spring); type of cultivation (monocrop and intercrop); the presence of *F. graminearum* s. *stricto*, *F. graminearum* s. *lato*, unidentified (FGSC) members and chemotype groups (15- AcDON and trichothecene producer indicated only by expression of Tri5 gene). Squared cosine (cos2) indicated the quality of association between variable categories and a particular axis. Red-colored variables indicates the highest degree of association with the first two dimensions. Dim1 refers to the first dimension of MCA analysis. Dim2 refers to the second dimension of MCA analysis.

4. Discussion

Despite the importance of trichothecene production-related genotyping and the high accuracy of methods for the identification of *Fusarium* species in routine surveillance, the data on efficacy of species- and trichothecene-specific primers in the characterization of FGSC members is still needed for particular crops and regions to better capture the efficacy of the primers. This study tested the potential of species- and trichothecene-specific primers in monitoring *Fusarium graminearum* populations infecting not only wheat but also rye, triticale and oats and found that the development of species- and trichothecene producer-specific primers with increased discriminating power for routine usage is still needed.

F. graminearum s. *stricto* belongs to SCAR Group 1 and lineage 7/RAPD C [22] and can be distinguished from other phylogenetic species of the FGSC [21,23,25]. In the present study, *F. graminearum* s. *stricto* (SCAR Group 1) was the dominant member of the FGSC and was identified via the Fg16F/R species-specific primer pair. However, 39.6% of isolates originating from the same *F. graminearum* population were not identified with the Fg16F/R species-specific primer pair. Interestingly, compared with triticale, wheat and rye were more associated with *F. graminearum* s. *stricto*. A total of 72.6% of isolates that were not identified with Fg16F/R were identified with the FgrF/FgcR primer pair reported to be highly specific for all lineages of the *F. graminearum* clade except for lineage 5 (*F. acaciae-mearnsii*) [26]. These isolates were assigned as *F. graminearum* s. *lato* and were predominant on triticale together with unidentified isolates whose morphological characteristics were like those of members of the FGSC. Unidentified isolates were confirmed to be trichothecene producers only using the Tri5F/R primer pair and were morphologically distinct from other B-type trichothecene-producing *Fusarium* species (*F. culmorum*, *F. cerealis*, *F. poae*, and *F. equiseti*). Our results are congruent with those of Župunski et al. [10] and Karlov et al. [31] who reported that FgrF/FgcR primers had higher specificity in distinguishing members of the *F. graminearum* clade from other causal agents of FHB in wheat. However, Župunski et al. [10] did not test the applicability of Fg16F/R and FgrF/FgcR species-specific primer pairs neither on rye, triticale and oats nor on wheat grown in combination with pea as intercrops. Given that species-specific primers were developed on the basis of sequences of the IGS region (FgrF/FgcR) and from RAPD amplification products (Fg16F/R), our results indicate that the target sequences should be analyzed in more detail in *Fusarium* isolates originating form rye, triticale and oats in order to increase discriminating power of screening methods for routine surveillance of FGSC in intercropping systems.

Genotyping of trichothecene-producing *F. graminearum* isolates showed that all *F. graminearum* s. *stricto* isolates belong to the 15-AcDON chemotype. The effectiveness of the Tri315F/R primer pair in trichothecene genotyping is proven on isolates originating from many parts of the world including England and Wales [22], China [32], Poland [33], Turkey [34], Taiwan [23] and others. Neither of these indicated the lack of Tri315F/R effectiveness. However, in this study, 9.4% of the isolates yielded no PCR product for the *Tri3* gene and were identified as trichothecene producers only. We suspected that specificity in the structure of the respective gene of the FGSC isolates caused the lack of effectiveness of Tri315F/R. Knowing that the development of primers for trichothecene production-related genotyping was carried out on 305 isolates belonging to lineages 2, 6, 7, and 9 and originating from 9 regions (China, France, Germany, Italy, Nepal, USA, Sweden, Poland, and Hungary) [21], further testing of the universality of primers for trichothecene production-related genotyping is highly recommended on FGSC isolates with more divergent origins. This assumption is congruent with the report of Wang

et al. [35] who indicated that interaction between host and pathogen could result in high genetic diversity of the *Tri3* gene. de Kuppler et al. [4] conducted trichothecene genotyping of *F.graminearum* population using other primer pairs than that in this study, but also indicated on the absence of PCR products for limited number of isolates as well as slight variability in the size of PCR products for a given primer pair. This was explained by the high genetic diversity of *F. graminearum* at the field, crop and country levels.

The enhancement of the applicability of methods used for screening causal agents of FHB will be challenging, mainly because of interspecific crossings among FGSC members. Interspecific crossings have already been shown in the laboratory [36] as well as between *F. boothii* and *F. graminearum* under field conditions [11]. The necessity for an increment of the efficacy of trichothecene-specific primers in the characterization of FGSC members is additionally accentuated by the fact that trichothecene genotypes isolated from barley were more heterogenous when compared with those isolated from wheat [37]. Lee et al. [38] indicated that the lineage composition of *F. graminearum* could be host specific and variable among locations, but the data on that variability in small-scale production areas are still scarce.

Interest in the introduction of alternative cultivation practices and in the importance of underutilized crop species in the food industry has increased in the past few years and decades. There have been reports that oat–pea intercropping results in a decrease in *Ascochyta* infection of pea [39]. There are also reports on the reduction in the infection level of *Fusarium* species and *Uromyces viciae-fabae* on faba bean when intercropped with cereal crop species [40,41]. In this study, wheat–pea intercropping reduced the level of FHB infection of grain. This result is in accordance with the report of Drakopoulos et al. [42] who demonstrated that growing winter pea as an interval cover crop after silage maize, reduced DON and Ac-DON in spring wheat under no tillage. However, data on factors influencing the stratification of *F. graminearum* populations on different cereal crop species in the same growing area and possibilities for pathogen control in intercropping systems are still scarce. Knowing that the regulatory network for plant responses to abiotic and biotic stresses may function antagonistically or synergistically [43–45], we believe that the use of multiomics approaches in studies of plant–pathogen interactions and the enhancement of the discriminating power of methods used for the identification and chemotyping of *Fusarium* species will facilitate FHB management, especially in the nonconventional production of cereal crops.

5. Conclusions

This study tested the potential of species- and trichothecene-specific primers in monitoring *Fusarium graminearum* populations infecting wheat, rye, triticale and oat when cultivated as monocrops and intercrops with pea in small areas. We found a lack of effectiveness of species-specific and primers for trichothecene genotyping in molecular characterization of FGSC members infecting different cereal crops. Consequently, the development of species- and trichothecene producer-specific primers with increased discriminating power for routine usage is still needed.

The triticale was more related to isolates classified into *F. graminearum* s. *lato* than with isolates identified as *F. graminearum* s. *stricto*.

The decrement of FDK values in the wheat–pea intercropping system indicated that cereal-pea intercropping systems have the potential to control FHB, which should be investigated further in large-plot or commercial production.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture12060834/s1; Table S1: FGSC isolates used in the study with PCR products produced by FGSC specific primer and primer sets for molecular chemotyping; Table S2: Relative frequencies of *F. graminearum* s. *stricto*, *F. graminearum* s. *lato* and unidentified (FGSC) per crop; Table S3: Averages of grain infection with FGSC and *Alternaria* spp. in standalone and intercrop cultivation of wheat, rye, triticale and oat with pea in Serbia; Table S4: Amount of variance explained by each principal component in the MCA analysis.

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