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Pathogenic and Genetic Characterization of *Fusarium verticillioides* Strains Collected from Maize and Sorghum Kernels

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Abstract: Commercial and sanitary qualities of cereals are important and contribute to the determination of the final application and destination of the grain and, thus, farmer profit. Although considered a minor crop in Italy, in the last years, the cultivation area of sorghum (*Sorghum bicolor* L. Moench) has been increasing and is often adjacent or subsequent to the most common maize (*Zea mays* L.) fields, with the risk of sharing mycotoxigenic fungi that are common to both cereals, such as *Fusarium verticillioides*. The present work aims to clarify if a fungal physiological characteristic such as fumonisin production and some plant growth parameters such as effects on germination, root and shoot development and on tissue necrosis may be common and shared among *F. verticillioides* strains belonging to maize and sorghum, especially if present on adjacent fields at the same time. Sorghum kernels are commonly less colonized and contaminated by *F. verticillioides* and fumonisins than maize, and, at least in part, this aspect could be explained by the higher frequency of low-producing strains obtained from sorghum compared to those obtained from maize. Moreover, although no clear pathogen specificity was found, the results relating to the parameters analyzed suggest a host preference by *F. verticillioides* isolated from maize that affects maize seedlings with greater severity as well as *F. verticillioides* strains from sorghum versus sorghum. Five microsatellite markers showed minor differences between the two sets of strains but could not reliably discriminate between hosts. The resulting data suggest that in adjacent maize and sorghum crops, the population of *F. verticillioides* selected during sorghum cultivation may not represent a particular mycotoxigenic or pathogenic issue for maize cultivation.

Keywords: *Fusarium verticillioides*; maize; sorghum; fumonisins; genetic diversity

1. Introduction

Cereals are increasing in importance due to the increased worldwide demand for human food, livestock feed, raw materials for industrial production and sources of biomass for energy production [1]. In recent years, these aspects led farmers to increase the cultivation of cereals, making the presence of maize (603,000 ha in 2020) [2] and sorghum (52,000 ha in 2020) [2] in Italy more frequent in crop succession as well as in adjacent fields and areas. Since these cereals share some common pathogens, the growth of maize and sorghum plants in close proximity, or in rotation, could increase the occurrence of diseases and mycotoxin accumulation, as well, due to shared pathogens, increasing the risk to reduce yield and quality production [3]. *Fusarium verticillioides* is an important fungal pathogen with a wide range of cereal hosts [4]. Although *F. verticillioides* is the main pathogen for maize in Italy, being responsible for Fusarium ear rot, this species is also an inhabitant of sorghum and is responsible for diseases in this plant species such as Sorghum grain mold and stalk and root rot [5–8]. Cereal colonization by *F. verticillioides* is frequently associated with the accumulation of fumonisins (FBs) produced in the field or during storage [9], representing a threat for its consumption as food and feed [10]. The occurrence of FBs

in maize kernels has been largely documented, while the level detected in sorghum is generally low; on the other hand, the co-occurrence of different mycotoxins in sorghum samples (aflatoxin, fumonisin, trichothecenes) is not uncommon [11,12]. The generally low colonization of *F. verticillioides* on sorghum has been associated with limited systemic growth in plant tissues [13] and production of fungitoxic compounds (i.e., phytoalexins) that inhibit pathogen growth [14]. In addition, some positive grain characteristics, such as hardness and polyphenol accumulation in the pericarp, contribute to resistance to fungal infection [15]. These traits are not usually associated with commercial maize hybrids, which, in fact, are more susceptible to disease and mycotoxins. Therefore, this study was conducted to evaluate possible differences in pathogenicity, FB production and genetic variability among strains of *F. verticillioides* that colonize maize and sorghum. Since these cereals cultivated in succession or in proximity could pose a risk of *F. verticillioides*-related disease and FBs occurrence, the comprehension of these aspects can help their management.

2. Materials and Methods

2.1. Fusarium spp. Isolation

Maize ears (Kefrancos; KWS Italia) and sorghum panicles (KSH4G04, KWS Italia) were randomly collected at physiological maturity (BBCH 89, fully ripe; approximately 3 kg of kernels per three samples, each) from adjacent fields in Rovigo (Italy). Samples were homogenized and ground to flour with a Grindomix GM200 Laboratory Mill (Retsch Italy, Torre Boldone, Italy), and serial dilutions were spread on Potato Dextrose Agar (PDA (Difco, Detroit, MI, USA) plates to estimate fungal incidence [16]. Five plates for each dilution were made. Plates were incubated for seven days at 25 °C in the dark, and developed colonies were counted to estimate fungal colonization (CFU·g^{-1}). Colonies were morphologically identified [17], and *F. verticillioides* isolates were subcultured to obtain single spore cultures [18]. Approximately 100 and 70 single-spore isolates were obtained from maize and sorghum kernels, respectively. Twenty-five *F. verticillioides* isolates belonging to maize and sorghum samples (hereafter named M- and S-strains), were randomly selected for subsequent analyses. Other *Fusarium* spp. (*F. proliferatum*, *F. oxysporum*, *F. semitectum*, *F. nygamai*, *F. equiseti* and *F. crookwellense*) were morphologically identified according to Leslie and Summerell [17].

2.2. Molecular Identification and Mating Type Determination

To molecularly confirm the identification of *F. verticilliodes* at the species level, the M- and S-strains were grown in 50 mL Potato Dextrose Broth (PDB Difco, Detroit, MI, USA) for 7 days (25 °C in the dark under 100 rpm rotatory shaking); mycelia were collected, washed with sterile water, squeezed and stored at −80 °C before DNA extraction. Genomic DNA was extracted from 60 isolates (30 M-strains and 30 S-strains) according to Doyle [19], and a molecular analysis was performed to confirm strain identity at the species level by PCR applying a specific species assay with VER1 and VER2 primers [20]. Twenty-five isolates from both cereals detected as *F. verticillioides* strains by PCR were selected for the analyses. Mating type identification for each strain was tested by multiplex PCR by using primers to amplify the MAT-1.1 and MAT-1.2 idiomorphs according to Steenkamp et al. [21]. All of the strains produced a fragment of ~200-bp or 800 bp for MAT-1.1 and MAT-1.2, respectively.

2.3. Total Fumonisin Production

Conidial suspensions for FBs production (FB_1, FB_2 and FB_3) and subsequent inoculations were obtained by growing *Fusarium verticillioides* strains (M- and S- strains) on Spezieller Nährstoffarmer Agar (SNA) plates for 15 days at 25 °C in the dark. Conidia were collected with sterile water and 0.02% Tween-20 (Sigma–Aldrich, Milan, Italy), and the concentration was adjusted to 10^6 conidia mL^{-1}. FBs production by selected strains was carried out according to Vismer et al. [12] with some modification. Briefly, twenty grams of maize flour with a low FBs content (<100 µg kg^{-1}) were put in glass Petri dishes and autoclaved for 1 h at 121 °C. To each plate, 20 mL of sterile water was added and left

to equilibrate overnight to obtain a moisture percentage of 50%. Plate inoculation was performed by three droplets of each fungal strain (50 µL of a 10^6 CFU mL^{-1}, each) equally outdistanced. Thirty plates per strain as replicates and three inoculations per plate were used to minimize small variations in starting inoculum and toxin production (Vismer et al. 2019). Plates were incubated at 25 °C for 21 days in the dark and then dried overnight at 60 °C. After drying, the 30 replicates for each strain were pooled, mixed well, and ground to powder in a Laboratory Mill (Retsch Italy, Torre Boldone, Italy) The mill was thoroughly cleaned following grinding of every different strain. Ground powder was stored at 4 °C until analyzed. FBs were extracted by suspending 5 g of maize flour in extraction buffer (VICAM Milford, MA, USA) according to the manufacturer's instructions. FBs content was then quantified by using the immunostrip Fumo-V Aqua test from VICAM (Milford, MA, USA). The extraction solution of a sample exceeding the maximum range of 100,000 ppb was further diluted 1:1 with extraction buffer. FBs content linearity in diluted samples was verified with internal controls and HPLC-certified samples in endowment to our laboratory.

2.4. Pathogenicity Assays

Fusarium verticillioides strains were analyzed on maize and sorghum seedlings for their capacity to inhibit the kernel germination and the root and leaf development and to produce necrotic lesions on detached leaves (DLA). Kernels were surface sterilized with H_2O_2 10% for 15 min (maize) or 5 min (sorghum) and rinsed thoroughly with sterile water. Thirty sterile kernels from maize and sorghum for each isolated strain (three replicate of ten kernels) were soaked for 30 min in a 10^6 mL^{-1} conidial suspension and placed into Petri dishes on sterile water-saturated filter paper according to Yates et al. [22]. Uninoculated kernels were soaked in sterile water and used as the control. After 7 days, pathogenetic abilities were estimated by determining the percentage of not-germinated kernels calculated as seed with radicle >5 mm long (germination inhibition; Gi%) and reduced length of roots (root inhibition; Ri%) and shoots (shoot inhibition; Si%) in comparison to the same tissues from the uninfected control.

DLA was performed according to Browne and Cooke [23]. Maize and sorghum sterile kernels were grown at 22 °C under a 16 h of photoperiod and 75% RH up to the fourth true leaf stage. Leaf portions of approximately 10 cm (15- and 21-day old seedlings for maize and sorghum, respectively) were detached from the second and third true leaves and immediately used in the experiment. Leaves were disinfected with ethanol 10% and placed into sterile Petri dishes on filter paper saturated with water, three leaves per Petri dish (150 mm diameter), five dishes as replicates. For each strain, to avoid the diffusion of inoculum, two SNA plugs of actively growing mycelium were applied to opposite parts of the adaxial surface of each leaf. Plates containing inoculated leaves were placed at 25 °C for two days in the dark and subsequently in the light to favor fungal pigmentation and obtain a better image contrast. Seven days after inoculation, leaves were digitalized, and area extension with necrosis or colonizing mycelium were measured through ImageJ 1.8.0 software.

2.5. Microsatellite Marker Analysis

Genetic similarity through microsatellite analysis was carried out by amplification of *Fv*-98, *Fv*-114, *Fv*-120, *Fv*-312 [24] and *Fv*-338 markers [25], the sequences of which are shown in Table 1. Markers were selected on the basis of high polymorphism produced and for their validation confirmed by two different studies [24,25]. Moreover *Fv*-114, *Fv*-120 and *Fv*-312 markers do not amplify *Fusarium thapsinum* and *Fusarium andiyazi* [25], common species in sorghum morphologically similar to *F. verticillioides* [5], providing a further confirmation of the appropriate classification of selected strains as *F. verticillioides*. Approximately 1 ng DNA was used to amplify selected markers by PCR amplification (0.3 mM dNTP, 0.2 µM of each primer, 1.5 mM MgCl$_2$ and 0.5 U of Taq DNA polymerase; total volume of 20 µL) in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The following amplification program was used: 5 min initial denaturation at 94 °C, followed by 35 cycles of 30 s

denaturation at 94 °C, 30 s annealing at 59 °C, and 30 s extension at 72 °C. PCR was completed with a 7 min final extension at 72 °C. The amplified fragments were separated by electrophoresis at 3.5 V/cm in a 3.5% (*wt/vol*) MS-8 agarose gel (Thermo Scientific, Milan, Italy) in 1× TAE buffer. The GeneRuler 100 bp DNA Ladder (Thermo scientific, Milan, Italy) was used. Fragment sizing was determined with the Gel Analyzer 19.1 software (www.gelanalyzer.com) on the basis of two independent electrophoretic separations. A marker was considered to be polymorphic if the amplified product was present at different lengths, present in some strains or absent in others. To avoid misinterpretation among close PCR fragments (bands), only those varying in size by 4 bp or more were considered different, and so bands were scored as presence (1) or absence of the band (0). Marker's profile was used to calculate diversity index, which included Shannon's index (*H'*; diversity of polymorphism in a population), Pielou's index (*J*; evenness of polymorphism distribution) and the Bray Curtis dissimilarity index (*BC*; difference of polymorphism between two populations). Diversity indexes were estimated according to their respective authors [26–28].

Table 1. Locus name, repeated motif and primer sequence used to amplify five polymorphic microsatellite loci in *Fusarium verticillioides*.

Locus Name	Repeated Motif	Primer Name and Sequence (5′–3′)
Fv-98	ATCC	*Fv*-98F AAACAAGATGCGGTCCATTC *Fv*-98R GGATCGGAGGAGAATCAACA
Fv-114	GTCT	*Fv*-114F CGAATGCCTTGATCTGCTTC *Fv*-114R GAGAATCCTGTTTGCGTGGT
Fv-120	TTG	*Fv*-120F GTAGCGCGGTAAGAAGATGC *Fv*-120R AGTCGAAGCCCAACTGAAGA
Fv-312	CAGA	*Fv*-312F TTTCCGAATTCCTGGATCTG *Fv*-312R GACGCAGTTTGCACAAGGTA
Fv-338	AGCAG	*Fv*-338F TAGACCAGGCAGACGAGACA *Fv*-338R TGTGAGTGGGTGAGAGTGGA

2.6. Statistical Analysis

Data belonging to FBs production and pathogenicity assays were analyzed for their differences by using *t*-test, ANOVA test or nonparametric Mann–Whitney U test. Each challenge among strains and cereals was considered a pathogenic interaction. Differences in strain frequencies between the two populations were analyzed with Yates's chi square test. Correlations between total FBs production for each strain and their pathogenic abilities were performed by calculating the Pearson's coefficients and their probabilities. Principal component analysis (PCA) performed on data obtained by pathogenicity assays was carried out by using Pearson's correlation matrix with the only correlated variables (FBs, mGi%, sGi%, mRi%, sRi% and sNa). Statistical calculations were performed in XLSTAT 2016 software (Addinsoft, Paris, France). Genetic distances on the basis of microsatellite profiles were obtained by using the Dice coefficient similarity matrix (for binary data). Resulting distances were applied to DendroUPGMA [29] to draw a dendrogram constructed according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm with 100 bootstrap. Numbers on the branches show bootstrap support greater than 30%.

3. Results

3.1. Incidence of Fusarium verticillioides and Related Species in Maize and Sorghum Samples

Quantification of *Fusarium* colonies in kernel (Table 2) revealed that maize samples were significantly more colonized than those of sorghum (16.66-fold; *p* < 0.001). *F. verticillioides*, *F. proliferatum* and *F. oxysporum* accounted for the majority of *Fusarium* species in kernels (99.0% for maize and 90.1% for sorghum) but with a strong prevalence of *F. verticillioides*. Sorghum kernels were also colonized at a low level (9.9%) by *F. semitectum*, *F. nygamai*, *F. equiseti* and *F. crookwellense*, while these species were almost absent (1.0%) in maize samples.

Table 2. *Fusarium* species from maize and sorghum samples and total fumonisin production by *F. verticillioides* strains.

	F. verticillioides		*F. proliferatum*		*F. oxysporum*		Other *Fusaria* *		*FBs* Content
	CFU g^{-1}	**%**	**CFU g^{-1}**	**%**	**CFU g^{-1}**	**%**	**CFU g^{-1}**	**%**	**µg kg^{-1}**
Maize	9757 ± 3063	88.8	461 ± 145	4.2	654 ± 205	6.0	110 ± 18	1.0	4050 ± 304
Sorghum	388 ± 68	58.8	186 ± 102	28.2	20 ± 18	3.0	65 ± 19	9.9	<200

Data represent fungal colonization of kernels (CFU g^{-1}) and the percentage of each species with respect to the total *Fusarium* spp. colonization. Fungal colonization value is the average ± standard error (SE) of three samples. * Indicates *Fusarium* species other than *F. verticillioides*, *F. proliferatum* and *F. oxysporum*.

3.2. Mating Type Determination

Determination of *F. verticillioides* mating type (Table 3) revealed that, among the M-strains, fifteen (60%) carried the MAT-1 allele and ten (40%) the MAT-2 allele. The same analysis conducted on S-strains shown that sixteen (64%) and nine (36%) were MAT-1 and MAT-2 strains, respectively. Statistical analyses revealed no differences in MAT frequencies between the two groups ($p = 0.77$).

Table 3. Mating type determination and in vitro FBs production (µg kg^{-1}) on maize substrate.

		Maize		Sorghum	
	Strain	**FBs µg kg^{-1}**		**Strain**	**FBs µg kg^{-1}**
MAT-1	M06	20,300		S04	24,800
	M17	13,250		S09	21,900
	M22	11,170		S25	16,400
	M14	7700		S02	2490
	M15	6880		S08	1350
	M04	6430		S07	1340
	M02	2950		S05	830
	M09	2380		S12	310
	M18	1820		S10	300
	M11	830		S21	270
	M13	700		S23	240
	M12	570		S03	230
	M05	240		S22	230
	M01	220		S24	230
	M25	200		S11	200
	-	-		S20	200
MAT-2	M19	29,600		S16	106,300
	M24	19,400		S06	61,200
	M08	16,780		S13	7950
	M23	9640		S17	4100
	M16	5570		S15	2180
	M21	5140		S01	1930
	M07	4050		S14	590
	M10	3100		S18	290
	M03	850		S19	250
	M20	740		-	-

3.3. Fumonisin Quantification

Preliminary quantification of mycotoxin in cereal samples (3 kg of maize and sorghum kernels per three samples, each) collected at the same time from adjacent fields and used for fungal isolation revealed a higher FBs contamination for maize (4050 µg kg^{-1} on average) with respect to sorghum samples (<200 µg kg^{-1} on average).

FBs determination of monoconidial strains carried out on producing the toxins under in vitro conditions showed a great variability among strains (Table 3). In particular, three M-strains (M05, M01 and M25) produced low amounts of FBs (<500 µg kg^{-1}), while ten and twelve M-strains produced medium (500–5000 µg kg^{-1}) and high amounts (>5000 µg kg^{-1}),

respectively. Eleven, eight and six S-strains produced low, medium and high amounts of FBs, with the maximum amount produced by S16 with 106,300 µg kg^{-1}. The frequency of low FBs-producing strains was higher (p = 0.027) for strains isolated from sorghum (11/25) than strains isolated from maize (3/25). Concerning the FBs production by the two *F. verticilliodes* populations (Figure 1), statistical analyses revealed the presence of one (M19: 29,600 µg kg^{-1}) and five strains (S16: 106,300; S06: 61,200; 21,900, S04: 24,800 and S25: 16,400 µg kg^{-1}) with outlier values for maize and sorghum, respectively. FBs production, as a whole, did not determine significant differences (p = 0.651) between the two-average production (average 6820 ± 1543 µg kg^{-1} for M-strains and 10,244 ± 4810 µg kg^{-1} for S-strains). In contrast, the same analysis, performed omitting the outlier values, differentiated (p = 0.002) maize strains (average 5867 ± 1269 µg kg^{-1}) from sorghum ones (average 1275 ± 432 µg kg^{-1}).

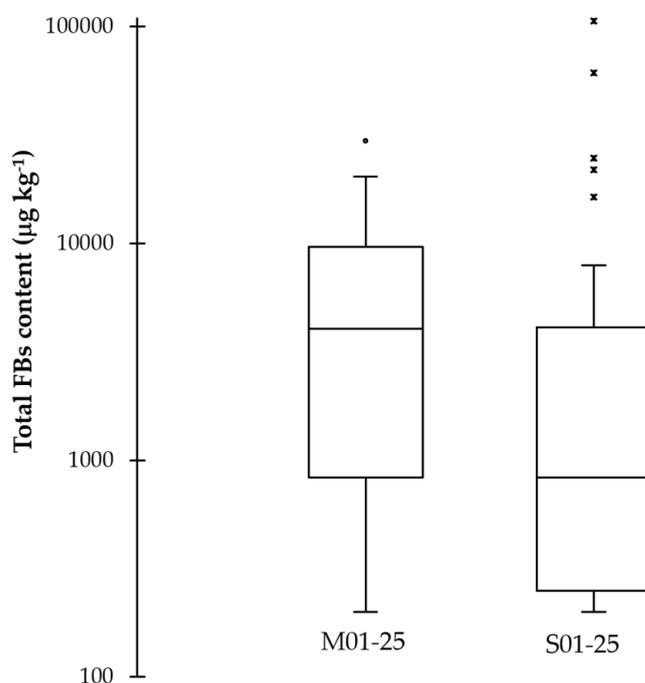**Figure 1.** Box plot of average FBs content (µg kg^{-1}) produced in vitro on a maize-based medium by strains isolated from maize (M01-25; 6820 µg kg^{-1}) and sorghum (S01-25; 10,244 µg kg^{-1}). Quartile values are reported for M- and S-strains, respectively (Q1: 830 and 250 µg kg^{-1}; Q2: 4050 and 830 µg kg^{-1}; Q3: 9640 and 4100 µg kg^{-1}). One dot and five asterisks represent outlier values calculated on the basis of p < 0.05.

3.4. Effect of Fusarium verticillioides Strains on Seed Germination

F. verticillioides strains belonging to maize and sorghum were tested on kernels to evaluate the differential effects on seed germination. Germination effects may have little impact on fumonisin contamination of the ear, but susceptibility of young tissues could result in increased environmental inoculum and an increased infection during ear ripening. Results were reported as percentage of germination inhibition of maize (mGi%) and sorghum (sGi%) germination compared to the uninfected control (Supplementary Materials S1). The germination of maize and sorghum seeds infected with M-strains was significantly affected by ten (22.7% average inhibition) and eleven strains (31.8% average inhibition), respectively, with respect to uninfected seeds. With regard to the test conducted with S-strains, only seven strains affect maize seed germination (16.7% average inhibition), while five strains were able to significantly reduce sorghum seed germination (28.0% average inhibition). Moreover, the comparison of data revealed that only six strains (M07, M13, M22, M24, S06 and S16) were able to reduce germination in both cereal species. Regarding seed germination, S-strains were found to be more pathogenic on sorghum germination (p ≤ 0.001),

but no preference for M-strains was evidenced ($p = 0.563$). No correlation was observed between the ability of the strains to produce FBs and their effect on seed germination ($p = 0.328$ for maize; $p = 0.097$ for sorghum).

3.5. Effect of Fusarium verticillioides Strains on Root Development

Root length development was evaluated to identify a detrimental effect by *F. verticillioides* infection on roots. Results were reported as percentage of inhibition of root development of maize (mRi%) and sorghum (sRi%) compared to roots from the uninfected control. Concerning the root evaluation after infection with M-strain, 56% of strains reduced root length on maize seedlings, and 36% of strains reduced root length on sorghum seedlings, with an average inhibition of 60.0 and 36.5%, respectively. Regarding S-strains, 65.4% of strains reduced root length on maize seedlings (23.5% reduction) and 72% on sorghum seedlings but with a lower inhibition (17.0% reduction). From the comparison of data, four M-strains (M03, M04, M07 and M16) and ten S-strains (S01, S04, S05, S06, S08, S09, S12, S15, S24 and S25) significantly reduced root length in both plant species. Conversely, six M- and two S-strains did not affect root development (Supplementary Material S2). With respect to root development, the inhibition of root length by M-strains was higher in both maize ($p = 0.001$) and sorghum plants ($p \leq 0.0001$) when compared to S-strains. A weak but positive correlation was observed between FBs and the effect on maize root length (rho = 0.105; $p = 0.022$); however, the same correlation was not found on sorghum roots (rho = 0.004; $p = 0.767$).

3.6. Effect of Fusarium verticillioides Strains on Shoot Development

Shoot length development was evaluated to identify a detrimental effect by *F. verticillioides* infection on shoots. Results were reported as percentage inhibition of maize and sorghum shoot development (mSi% and sSi%, respectively) compared to shoots from the uninfected control. Shoot length measured after infection with M-strains indicates that, on average, a significant inhibition for maize and sorghum seedlings was attributed to sixteen and six strains (on average mSi: 44.8% and sSi: 42.5%), respectively. Similarly to the effect on roots, a large number of S-strains reduced shoot length (60% of strains of maize, 68% of strains of sorghum), with an average inhibition value calculated for significant interactions as 46.7% (mSi%) and 35.4% (sSi%), respectively. From the comparison of the data, 12% of M-strains (M02, M06 and M24) and 44% of S-strains (S01, S02, S03, S04, S10, S11, S15, S16, S19, S23 and S24) significantly reduced the length of the shoots in both plant species. Conversely, ten strains did not affect root development (Supplementary Materials S3). No differences were observed between M- and S-strains with respect to their ability to reduce shoot development in maize ($p = 0.838$) and sorghum ($p = 0.541$). No correlation was observed between shoot development and FBs production for both maize (rho = 0.057; $p = 0.696$) and sorghum (rho = 0.089; $p = 0.497$).

3.7. Effect of Fusarium verticillioides Strains on Detached Leaf Assays (DLA)

Necrotic area (NA) was evaluated through DLA to identify a detrimental effect by *F. verticillioides* infection on leaves. Results were reported as necrotic extension area for maize (mNA) and sorghum (sNA) (Supplementary Materials S4). Results from DLA assays showed that necrotic areas produced by M-strains were generally larger ($p \leq 0.0001$) on maize (average 131.8 mm^2; range from 4.9 to 350.5 mm^2) than on sorghum leaves (on average 26.5 mm^2; range from 0.0 to 129.1 mm^2). Moreover, while all M-strains produced at least a small necrotic area on maize leaves, twelve produced little or no symptom on sorghum. Similarly, fifteen S-strains produced significant necrotic areas on maize leaves (average 103.2 mm^2; range from 13.5 to 151.4 mm^2), while only five of them significantly affected sorghum leaves (average 39.8 mm^2; range from 0.0 to 95.4 mm^2), and twenty strains produced no symptoms. Five M-strains (M02, M03, M06, M10, M12 and M20) and two S-strains (S23 and S25) significantly affected both cereals. On average, M-strains ($p \leq 0.0001$) and S-strains ($p \leq 0.0001$) produced a larger necrosis on maize leaves rather

than on sorghum ones (Figure 2). However, based on the overall average NA values, it was not possible to detect any difference between maize leaves colonized by M- or S-strains (Figure 2). The bars corresponding to the NA values on sorghum leaves (Figure 2) also contain the effect of twelve M-strains and twenty S-strains unable to cause symptoms on these tissues. No correlation was observed between FBs production and necrotic area development in maize (rho = 0.017; *p* = 0.363) and sorghum (rho = 0.022; *p* = 0.878).
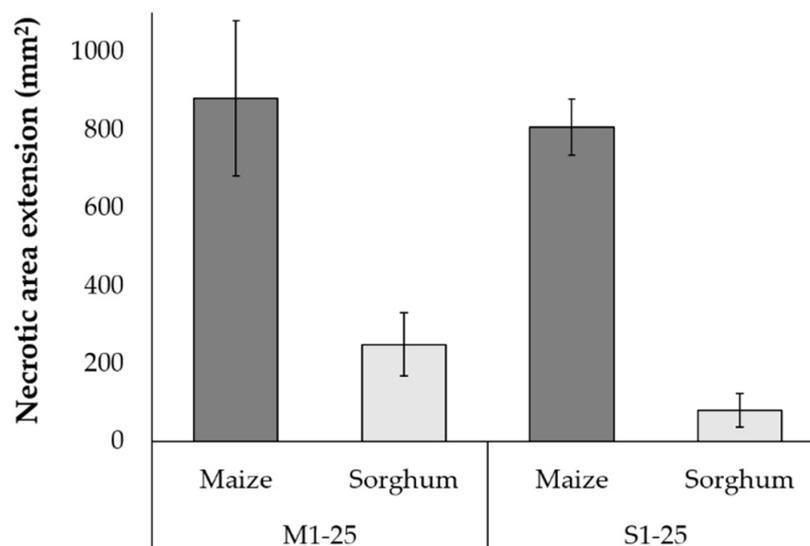

Figure 2. Necrotic areas (mean ± SE) on maize and sorghum leaves challenged with *F. verticillioides* isolated from maize (M1-25) and sorghum (S1-25). Null interactions for challenged sorghum were twelve and twenty for M- and S-strains, respectively.

3.8. Clusterization of Fusarium verticillioides Strains Based on Pathogenic Parameters

FBs production, inhibition of seed germination, root and shoot development and leaf necrosis were the pathogenic parameters considered for the characterization of the strains. Principal component analysis (PCA) was performed to visually investigate these different characteristics and the similarity between the variable analyzed (Figure 3). No significant correlation was found between the inhibition of shoot development in both cereals and maize necrotic areas with the other considered parameters (Figure 3). Cluster analysis showed that germination and roots inhibition in maize and sorghum (sGi%; mGi%; mRi%; sRi%) were positively correlated with FBs production with different correlation coefficients. Positioning of *F. verticillioides* strains on the biplot, resulting from PCA, showed that most of the M-strains were located in the same direction of variables (quadrant I, II and IV), while S-strains were mainly located in or near the quadrant III.

In Figure 3, quadrant I includes only M-strains characterized by a high FBs production and a low inhibition of maize seed germination. Quadrant II consists of strains with a low production of fumonisins and with a small inhibitory effect on maize germination and sorghum root development but with significant necrotic areas on sorghum (ten M-strains; two S-strains). Quadrant III includes 16 out of 25 S-strains characterized by low pathogenic effects for each considered parameters. Quadrant IV consists of strains from both populations (seven S-strains; six M-strains) characterized by high FBs production and maize seed germination inhibition but low inhibitory effects on sorghum.

Figure 3. Biplot of principal component analysis computed from fumonisin production (FBs), percentage inhibition of germination of maize (mGi%) and sorghum (sGi%), root inhibition of maize (mRi%) and sorghum (sRi%) and sorghum necrotic area (sNa) of *F. verticillioides* strains isolated from maize (M-strains) and sorghum (S-strains). Shoot development in both cereals and maize necrotic areas were not correlated to the other considered parameters.

3.9. Microsatellite Marker Analysis

F. verticillioides strains belonging to maize and sorghum were analyzed for their microsatellite profiles to verify a closer similarity among *F. verticillioides* isolated by different hosts. Five microsatellite markers were analyzed by PCR, and all fungal strains amplified a single band (Supplementary Materials S5). Data in Table 4 show that for *Fv*-98, *Fv*-114, *Fv*-312 and *Fv*-338 markers, the S-strains presented generally higher values in terms of polymorphism and diversity index (Shannon's index H' and Pielou's evenness index J') than M-strains. On the contrary, polymorphisms identified for *Fv*-120 marker were higher for the M-strains. Minimum and maximum values calculated for Bray Curtis dissimilarity values (BC) were produced by *Fv*-98 (BC 0.5) and *Fv*-114 (BC 0.84), respectively. Polymorphic profile for each marker and strain are reported in Supplementary Materials S6 and S7.

Table 4. Microsatellite polymorphism for *Fusarium verticillioides* isolated from maize and sorghum.

Microsatellite Marker	Strains	Polymorphic Bands/Tot	H' Index	J' Index	BC Dissimilarity
Fv-98	M1-25	3/7	1.040	0.946	0.50
	S1-25	6/7	1.223	0.679	
Fv-114	M1-25	5/9	1.332	0.828	0.84
	S1-25	6/9	1.555	0.868	
Fv-120	M1-25	9/11	2.027	0.926	0.56
	S1-25	6/11	1.658	0.925	
Fv-312	M1-25	6/10	1.432	0.799	0.68
	S1-25	9/10	1.921	0.875	
Fv-338	M1-25	6/12	1.457	0.813	0.68
	S1-25	11/12	2.123	0.885	

Table shows for each marker the number/total of polymorphic bands, Shannon's index (H' index), Evenness (J' index) and Bray Curtis (BC) dissimilarity calculated on the basis of microsatellite markers for M- and S-strains.

3.10. Fusarium verticillioides Clusterization Based on Microsatellite Markers

F. verticillioides strains belonging to maize and sorghum were genotyped using five microsatellite markers, and these data were analyzed by UPGMA to cluster together strains with similar microsatellite pattern (Figure 4). The low bootstrap support did not allow us to identify different clusters. However, genetic relationships obtained by microsatellite analyses showed that M-strains tended to group separately from S-strains, but without a statistical support. The cophenetic correlation coefficient [30] was calculated, and a value of 0.767 indicated a good representation of the similarity matrix.
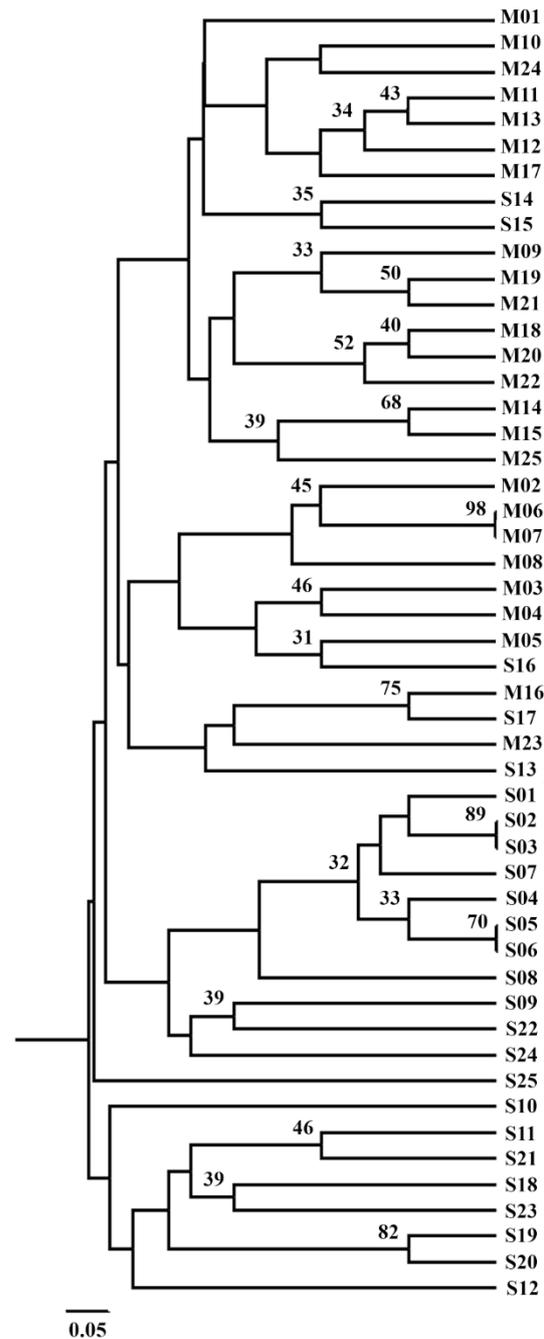
Figure 4. UPGMA clusterization based on the Dice similarity index calculated for M- and S-strains on the basis of genetic profile defined by five microsatellite markers. Numbers on the branches show bootstrap support greater than 30%.

4. Discussion

Sorghum and maize were infected with a pool of fungal pathogens and mycotoxigenic species belonging to *Fusarium* genus. Since *Fusarium verticillioides* in Italy represents the most common mycotoxigenic species of maize, this study aimed to elucidate if the *Fusarium* inoculum isolated from maize and sorghum can reciprocally represent a pathogenic and toxigenic issue. To date, maize and sorghum rotation or succession have been studied for agronomical purpose [31,32], and very few studies have investigated pathogenic aspects [33,34]. The isolation of *Fusarium* species from maize and sorghum kernels, grown in adjacent fields, shows that *F. verticillioides* is the most common species in these grains [35–37]. In particular, in this study, *F. verticillioides* represents 88.8% of *Fusarium* spp. in maize kernels and 59.3% of that in sorghum kernels. Moreover, while *F. verticillioides*, *F. proliferatum* and *F. oxysporum* represent almost all the *Fusarium* species occurring in maize, the kernels in sorghum have been colonized by other less abundant *Fusarium* species, usually present in this cereal worldwide [38,39]. A further difference is represented by fungal colonization, which is, in our samples, 16.5-fold higher in maize than in sorghum and may contribute to the different FBs content in the collected samples. Random selection of the two *F. verticillioides* populations isolated from maize and sorghum samples led us to determine the mating types for sexual reproduction [40–42] and revealed a very similar MAT1:MAT2 proportion, demonstrating that any potential pathogenic trait could be randomly transferred between these Fusaria populations. Indeed, through sexual reproduction, fungi recombine their genetic material and increase the diversity in the offspring, which may provide some benefits for the survival of species under different environmental conditions. A previous study [43] conducted in Brazil on *F. verticillioides* isolated from maize and sorghum samples reported instead a quite different MAT1:MAT2 proportion equal to 5:17 and 14:7 for strains from maize and sorghum, respectively.

With respect to the average amount of fumonisins produced by the *F. verticilliodes* strains analyzed, no difference was found between the two populations. Yet, the percentage of low-producing strains ($<500\ \mu g\ kg^{-1}$) was higher for sorghum (44%) than for maize (12%). Moreover, due to lateral flow sensitivity ($\geq 200\ \mu g\ kg^{-1}$), we were unable to determine for some strains whether no fumonisin was produced at all or the production was at a very low amount; nonetheless, the percentage of putative no-producing strains found in maize appeared not so different from other studies conducted in Italy [44] and worldwide [45,46]. On the contrary, six S-strains (24%) were found to be characterized by a high fumonisin production ($>5000\ \mu g\ kg^{-1}$), whereas in a study carried out in India, only a little number of *F. verticilliodes* strains isolated from sorghum (around 6%) were characterized by a similar fumonisin production [45]. As previously observed for maize [44], present data, also considering the outlier values on fumonisins, support findings that *F. verticillioides* strains collected from sorghum in a single location might show a great variability in fumonisin production. Data obtained with this study showed a weak but positive correlation between reduced maize root development and mycotoxin content; however, no other correlations have been identified between fumonisin biosynthesis and pathogenesis in the different tissues analyzed of maize and sorghum. With respect to pathogenic aggressiveness between sorghum and maize strains, a clear infection pattern was not identified, and the ability to produce symptoms was different for each strain and tissue challenged by infection, and different strains were able to induce different symptoms. Seed germination has been less influenced by *F. verticillioides* strains both from maize and sorghum, even if seed decay and germination reduction are symptoms observed under stressful conditions [44,47,48]. In particular, our data suggest that a limited number of strains may affect germination of maize and sorghum seeds, although in general at a low level. Only six strains (four M and two S) were able to significantly reduce seed germination in both cereals, while 42% of M-strains significantly affected maize or sorghum germination, and the percentage decreased to 24% for S-strains. Furthermore, maize strains affected germination of both cereals with similar severity, and strains belonging to sorghum demonstrated to be more pathogenic on sorghum seeds. Conversely to germination, root and shoot development

appeared more influenced by pathogens, with an increased number of strains able to reduce tissue development. Although total significant pathogenic interactions for S-strains (66% for root and shoot as a whole) were higher than those for M-strains (45%), the latter generally showed a higher severity, mainly due to the negative impact on root development. As previously suggested, at least for root development in maize, a role could be played by the higher fumonisin production determined in M-strains.

One of the most significant parameters, however, could be represented by necrotic areas produced on leaves. All strains were able to produce at least one small necrotic lesion on maize, while less than half of the strains induced symptoms in sorghum. These results highlight a greater susceptibility to *F. verticillioides* infection of maize rather than sorghum, which could represent an important aspect to be taken into account in the planning of crop successions. The resistance to fungal colonization in sorghum has been associated with both the constitutive presence [49] and induced accumulation of phenolic acids, condensed tannins and flavonoids [50–52] and to antimicrobial peptides [53], all together responsible for the reaction of the plant to biotic and abiotic stress [54,55]. Moreover, sorghum appears more resistant than maize to other stress conditions such as drought; in fact, in conditions of dehydration during the flowering stage, a higher drought resistance has been associated with a greater capacity of the antioxidant–enzymatic system of sorghum with respect to that of maize [56]. The features in pathogenicity of the *F. verticillioides* strains were analyzed through the principal component analysis (PCA), and the pathogenic parameters analyzed (fumonisin production, seed germination, root and shoot development and leaf necrosis) between and within strains highlighted similarities and differences among M- and S-strains. PCA distribution showed that the totality of the M-strains appeared distributed in the three quadrants that contained the considered variables.

Although the M-strains showed a large variability in terms of pathogenic capacity, the same was not supported by data in terms of genetic variability. In fact, four out of five microsatellite markers analyzed (*Fv*-98, *Fv*-114, *Fv*-312 and *Fv*-338) resulted as more polymorphic for S-strains than M-strains. Although obtained by exploring different genomic traits, these results showed that in our collection area of the *F. verticillioides* strains obtained from sorghum had a higher genetic diversity than those isolated from maize, as was similarly observed in Brazil [43], highlighting that strains coming from both local or distant regions can share similar peculiarities. This was also manifested by the higher degree of polymorphism detected in the collected S-strains. The higher diversity for S-strains demonstrated by Shannon's index (that measures the diversity of species in a community) and Pielou's evenness (that measures diversity along with species richness) suggested a good distribution of markers within groups. Furthermore, Bray Curtis dissimilarity (used to quantify the differences in species composition and abundance between two populations) calculated for each marker showed that, with the exclusion of *Fv*-98 (BC 0.50), more than half of the strains were different between the two collected groups with a maximum dissimilarity value of 0.84 for *Fv*-114. The higher genetic diversity observed for S-strains could suggest that the analysis based on the similarity calculated by the biplot would discriminate the strains between two host cereals, but this diversity was not sufficient to achieve this purpose. The resulting dendrogram seemed to differentiate strain isolates from maize and sorghum in most cases, but the bootstrap support for key nodes never exceeded 30%; hence, typical genetic profiles was not identified. The observation that no pattern was specific for *F. verticillioides* collected from maize and sorghum may suggest that the genetic population feature could be the result of an active genetic recombination occurring in the field, as observed in the extensive area of cultivation of cereals affected by this fungus [57]. Moreover, no similarities were observed among pathogenicity and microsatellite clusterization. Our findings were not surprising since, as previously reported [44], even using different markers the genetic variability in *F. verticillioides* was not correlated to pathogenicity. The pathogenicity and mycotoxin parameters shown by *F. verticillioides* strains isolated from sorghum, which were generally characterized by a lower FBs production, indicate that such strains should not pose a

substantial risk for fumonisin accumulation in maize kernels; on the other hand, an active genetic recombination cannot be excluded that could select, over time, the development of more aggressive or toxigenic strains.

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