

Occurring *Fusarium* spp. in sorghum (*Sorghum bicolor* L. Moench) grains

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RESEARCH ARTICLE

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ABSTRACT

In our study, we examined mycotoxin-producing fungi found in sorghum grains (*Sorghum bicolor* L. Moench). During the experiment, the internal infection of grains was examined on 3 different selective culture media, as individual literature differs as to which selective culture media are the most suitable for the isolation of *Fusarium* species under laboratory conditions (Leslie and Summerell, 2006). During our experiments, we also used molecular methods to identify the selected *Fusarium* species down to the species level. In the case of *Fusarium* species our experiment shows that the best media is Nash & Snyder media. During the morphological identification we worked with 18 *Fusarium* monosporal cultures and we detected *Fusarium avenaceum*.

KEYWORDS

mycotoxin-producing, fungi, phytopathology, molecular identification, selective media

INTRODUCTION

Because of the unpredictability of the weather, farmers must stand on more than one foot in the development of the forage base; thus, the economic importance of high-yielding second crops,

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such as sorghum, has increased, as has their production area. Because summers are becoming hotter and drier by the year, the fact that it adapts well to high temperatures and high light intensity and is capable of high dry matter production is advantageous to sorghum cultivation. It has excellent drought tolerance and regeneration ability due to its abundant lateral root system and large root mass, and it can be grown in areas with poor soil quality (Sorghum ID).

More than 35% of the world's sorghum production is grown directly for human consumption since, unlike other cereals, it has many beneficial effects on the human body (Astoreca et al., 2019). Sorghum has a lot of benefits, like lowering the risk of diabetes, heart disease, and colic. Because of this, its use has grown in recent years, and it is now an important part of the long list of alternative foods (Serna-Saldivar, 2016).

In addition to food use, about 48% of the world's sorghum production also served as an important feed for ruminants, pigs, cattle, fish, and poultry. Depending on the quality of the grain, the animal species, and their growth stage, it can be incorporated into the rations in different amounts. Sorghum is also grown for green fodder, and it has a lot of potential because of its fast growth and high-quality green fodder yield (Peerzada et al., 2017).

Mycotoxins are secondary metabolic products produced by filamentous fungi that are chemically very stable and are only slightly damaged by processing. When they get into the consumer's body, they pose a number of human and animal health risks (cancers, reproductive biological disorders, etc.). *Fusarium*, *Alternaria*, *Aspergillus*, *Penicillium*, *Claviceps*, and *Stachybotris* are all types of fungi that can produce mycotoxins (Varga et al., 2009).

MATERIAL AND METHODS

During our experiment, we worked with sorghum varieties belonging to two genotypes from four settlements (Kapolc, Hedrehely, Gyékényes, and Dány). The sorghum grains were surface sterilized with a 10% NaOCl solution for 2 min and after we rinsed the grains with distilled water, and then the grains were examined on 3 different media. The media used in the experiment were the Nash & Snyder, Rose Bengal (RbGU), and Czapek-Dox media (Leslie and Summerell, 2006). We examined in 10 replicates and 10 biological replicates of the sorghum grains, which means from one settlement we worked with 100 grains.

The grains placed on the culture medium were incubated at room temperature, and after the 7th day, the fungal colonies that grew out of the grains were examined. For genus-level identification, PDA (potato-dextrose) medium was used, onto which mycelium grown from grains was isolated. After 3 days, the outgrown fungal colonies were placed under UV light for 7 days to induce fungal conidia formation. After the UV light we classified them to different genera made the microscopic determination so we examined the different conidia.

For the precise identification of *Fusarium* species, the preparation of monospore cultures is unavoidable (Geiser et al., 2004); therefore, prior to the molecular genetic tests, we prepared monospore cultures from the *Fusarium* genera selected for each settlement. For the preparation of the monospore cultures, we isolated the *Fusarium* species on SNA media to indicate conidial growth. We used sterilized PDA media for the monospore cultures; after the sterilization, exactly 10 ml of PDA were poured per 90-mm petri dish. For the main inoculum, we applied 6 ml of distilled water to the fungi and shaken them in small circular motions before placing the 6 ml in a sterile tube. After we made dilutions from 10 to 10, from 10⁻¹ to 10⁻⁶. We put 100 µl



of each dilution in the 10 ml PDA and spread them with a sterile rake. After we incubated at 25 °C in the dark for 7 days, DNA was extracted from the monospore cultures using the Zymo Research DNA kit (Quick-DNA Fungal/Bacterial Miniprep Kit). During species-level identification, primers specific for 7 *Fusarium* species were used (Table 1) (*Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Fusarium subglutinans*, *Fusarium graminearum*, and *Fusarium poae*) (Hornok et al., 2005; Mulé et al., 2004; Nicholson et al., 1998; Parry and Nicholson, 1996; Turner et al., 1998). Negative and positive controls were used during gel electrophoresis.

DNA amplification by PCR was performed in the cases of *F. culmorum*, *F. graminearum*, *F. poae*, and *Fusarium avenaceum* set to denaturation at 95 °C for 2 min 5 cycles of 95 °C for 30s; 66 °C for 20s, 72 °C for 45s; 5 cycles of 95 °C for 30s, 64 °C for 20s, 72 °C for 45s; 25 cycles of 95 °C for 30s, 64 °C for 20s, 72 °C for 45s final extension at 72 °C for 5 min (Hornok et al., 2005). In the cases of *F. subglutinans*, *F. proliferatum*, and *F. verticillioides*, the amplification was performed set to: denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min (Mulé et al., 2004). A 2% agarose gel was prepared to detect amplified PCR products, and ethidium bromide was used for staining. Evaluation of the gel was performed in a Biorad gel documentation system using the Image Lab (Biorad) program.

RESULTS

In terms of the number of fungal colonies grown from grains on the three different media, the Nash-Snyder medium had the highest internal infection rate, followed by the Czapek-Dox

Table 1. Species-specific primers we used during the experiments

Primer name	Primer sequence	Species	Predicted size of the PCR product (bp)	References
C51F C51R	5'- ATGGTGAAGCTCGTCGTGGC-3' 5'-CCCTTCTTACGCCAATCTCG-3'	<i>Fusarium culmorum</i>	570 bp	Nicholson et al. (1998)
P82F P82R	5'-CAAGCAAACAGGCTCTTACC-3' 5'-TGTTCCACCTCAGTGACAGGTT-3'	<i>Fusarium poae</i>	220 bp	Parry and Nicholson (1996)
FG11F FG11R	5'-CTCCGGATATGTTGCGTCAA-3' 5'-GGTAGGTATCCGACATGGCAA-3'	<i>Fusarium graminearum</i>	280 bp	Nicholson et al. (1998)
AV-F AV-R	5'-CAAGCATTGTGCGCACTCTC-3' 5'-GTTTGGCTCTACCGGGACTG-3'	<i>Fusarium avenaceum</i>	345 bp	Turner et al. (1998)
SUB1 SUB2	5'-CTGTCGCTAACCTCTTTATCCA-3' 5'-CAGTATGGACGTTGGTATTATATCTAA-3'	<i>Fusarium subglutinans</i>	631 bp	Mulé et al. (2004)
PRO1 PRO2	5'-CTTTCCGCCAAGTTTCTTC-3' 5'-TGTCAGTAAGTCGACGTTGTTG-3'	<i>Fusarium proliferatum</i>	585 bp	Mulé et al. (2004)
VER1 VER2	5'-CTTCCTGCGATGTTTCTCC-3' 5'-AATTGGCCATTGGTATTATATCTA-3'	<i>Fusarium verticillioides</i>	578 bp	Mulé et al. (2004)



medium. On the rose bengal (RbGU) culture medium, when examining the internal infection values, fewer fungal colonies appeared (Fig. 1).

The largest number of fungi belonging to the *Fusarium* genus grew on Nash & Snyder's culture medium. In the case of the Czapek-Dox culture medium, we experienced the presence of fungi belonging to the genus *Fusarium* in the smallest number. In terms of their average distribution, in the case of the Nash-Snyder culture medium, we experienced the highest value (3 pieces) in the case of sample Gyékényes2, and the lowest value in the case of the culture medium was an average of 0.5 internally infected grains. In the case of the Czapek-Dox medium, the fewest *Fusarium* species grew, since we did not experience internal contamination caused by *Fusarium* species in the case of the 3 samples here (Fig. 2).

During our experiments, other mycotoxin-producing genera, such as *Alternaria* and *Aspergillus* species, appeared. Regarding *Alternaria* and *Aspergillus* species, the internal infection values were quite different. *Alternaria* species dominated in all three investigated selective media; however, *Aspergillus* species were present to the smallest extent in all three selective media.

During the macroscopic and microscopic examination of the monosporal pure cultures, 18 monosporal cultures of the selected *Fusarium* species were made. Table 2 shows the macroscopic and microscopic properties of each culture.

We identified *Fusarium avenaceum* at 345 base pairs (Fig. 3) on samples 4, 11, 14, and 18 of the monosporal cultures, which were isolated from the grains of Gyékényes2 and Kapoly1.

DISCUSSION

During the examination of mycotoxin-producing fungi isolated from the grains of sorghum, the internal infection of the grains reached 100% in many cases under laboratory conditions, using different selective culture media. In all three selective mediums, it was seen that *Alternaria*

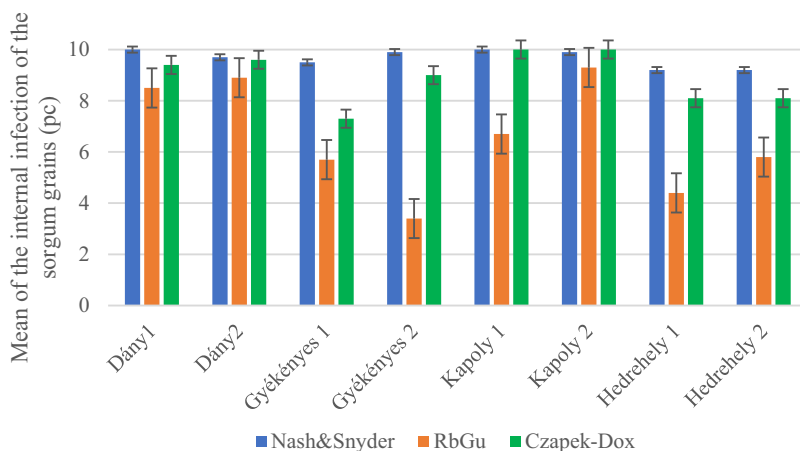


Fig. 1. The distribution of the number of fungal colonies grown on the three different selective media on the 7th day (I: 95% confidence interval)



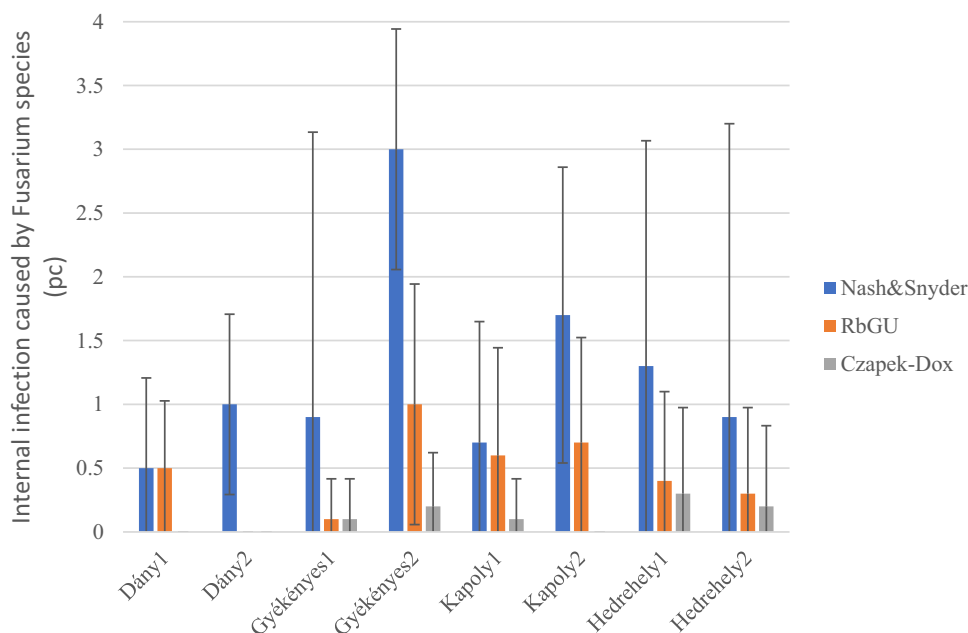


Fig. 2. Average distribution of *Fusarium* species for the three selective culture media on day 7 (I: 95% confidence interval)

species caused most of the internal infections in the grains. *Fusarium* and *Aspergillus* species also showed up, but in different amounts.

In the case of Nash and Snyder's culture medium, we experienced the highest internal infection (so the most mycelium grew from the grains on this culture medium), followed by the Czapek-Dox culture medium. When examining the internal infection values on the rose bengal (RbGU) medium, fewer fungal colonies appeared than on the two previously mentioned mediums, this may be explained by the fact that the rose bengal medium mostly favors the growth of *Fusarium* species (Leslie and Summerell, 2006).

For the *Fusarium* species we examined, the Nash and Snyder's culture medium was the best for the fungi to grow, as had already been shown in many publications (Booth, 1971; Leslie and Summerell, 2006).

Regarding the monospore cultures, we found that the color distribution of the colonies was almost the same, and the conidia of each genotype had different shapes and numbers of segments. The explanation for this can be that each genotype of sorghum showed the presence of different *Fusarium* species.

During the PCR amplification, we identified the species *F. avenaceum* in two samples, Kapoly1 and Gyékényes2, considering the seven species-specific primer pairs we tested. Regarding the other primer pairs, the DNA of the *Fusarium* species did not match the primer pairs we examined. Several international studies confirm that among the *Fusarium* species isolated from grain sorghum, the appearance of *F. avenaceum* identified by us was the least frequent (Leslie et al., 1990). In the research conducted with different sorghum samples in the



Table 2. Macro- and micromorphological examination of the isolated Fusarium species

Sample		Pigmentation of the mycelia	Shape of the macroconidia	Colour
1.	Gyékényes1	heavily pigmented	3-sectioned, straight	brown
2.	Gyékényes1	heavily pigmented	3-sectioned, straight	brown
3.	Gyékényes1	heavily pigmented	small, club-shaped	brown
4.	Kapoly2	heavily pigmented	segmented, straight, narrowing towards the end	brown
5.	Gyékényes2	nonpigmented	5-branched, tapering towards the tip	white (no pigmentation)
6.	Gyékényes2	slightly pigmented	divided by a transverse wall, straight	pink
7.	Hedrehely1	slightly pigmented	3-part, club-shaped	violet
8.	Gyékényes2	slightly pigmented	It is divided into 3 parts, one of its apex cells is narrowed	violet
9.	Hedrehely2	slightly pigmented	short, club-shaped	violet
10.	Hedrehely2	slightly pigmented	long, straight	violet
11.	Kapoly2	heavily pigmented	divided by a transverse wall, short, straight	brown
12.	Gyékényes2	slightly pigmented	tapering towards the end	pink
13.	Hedrehely2	heavily pigmented	short, club-shaped	brown
14.	Gyékényes1	nonpigmented	5-branched, tapering towards the tip	white (no pigmentation)
15.	Dány1	nonpigmented	long, oval towards the end	white (no pigmentation)
16.	Dány2	nonpigmented	medium, tapering towards the end	white (no pigmentation)
17.	Gyékényes1	slightly pigmented	small, club-shaped	violet
18.	Gyékényes1	slightly pigmented	short, club-shaped	pink

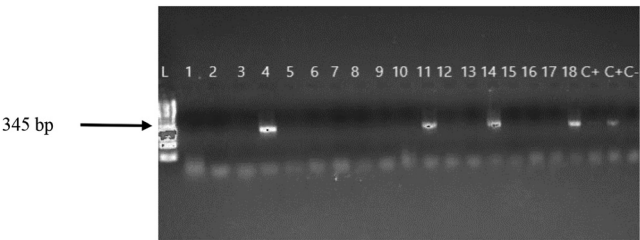


Fig. 3. 1.5% agarose gel run with *Fusarium avenaceum* species specific primer with DNA extracted from monosporal cultures

state of Karnataka, the appearance of *F. graminearum* and *F. verticillioides* species was observed, and in Argentina, *F. proliferatum*, *F. subglutinans*, and *F. verticillioides* were dominant (Pena et al., 2019; Nagaraja et al., 2016).



Evaluating the results, further studies of the internal infection of sorghum grains would be necessary, possibly examining the level of internal infection of the grains on several selective media. For a more precise identification of *Fusarium* species, further molecular genetic studies would be needed, using additional primer pairs in addition to the 7 primer pairs we examined, for example primer pairs of the EF1 α (TEF gene region) or RPB2 genes (Geiser et al., 2004; Ponts et al., 2020). After the identification and sequencing of the PCR products, a more precise match should be obtained in order to identify them at the species level.

However, it is important to emphasize that special attention needs to be paid to the species *F. avenaceum*, as they can produce enniatins (ENN), which can be classified as "emerging mycotoxins" (Gautier et al., 2020). As these toxins are currently not regulated (EC 1881/2006), the examination of grain sorghum samples for mycotoxin contamination can be an important step in the future in terms of increasing the human and animal feeding rate (Astoreca et al., 2019; Peerzada et al., 2017).

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