MYCOTOXINS BIOSYNTHESIS BY *FUSARIUM OXYSPORUM* AND *F. PROLIFERATUM* ISOLATES OF ASPARAGUS ORIGIN

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Abstract: The subject of this work was fumonisin B_1 (FB_1) and moniliformin (MON) biosynthesis by three isolates of *Fusarium oxysporum* and three isolates of *F. proliferatum* of asparagus spears origin. The cultures of fungi were grown on rice and asparagus media for 3 weeks at 20°C. Experiment was carried out in 3 replicates. FB_1 and MON occurrence was evaluated with high-performance liquid chromatography (HPLC) analyses. Analysis of variance was carried out to determine biosynthesis of FB_1 and MON by *F. oxysporum* and *F. proliferatum*.

FB_1 was found in the amount up to 2012.8 µg/g in cultures of *F. proliferatum* on rice and in a very small amount in two cultures on asparagus medium. *F. oxysporum* did not produce FB_1 on any of the media. MON was biosynthesized by two the same isolates of *F. oxysporum* in the amount up to 182.8 µg/g on rice and up to 743.3 µg/g on asparagus medium and one isolate (different on each medium) of *F. proliferatum*.

Key words: fumosin B_1 (FB_1), moniliformin (MON), biosynthesis, media

INTRODUCTION

Fungi of *Fusarium* genus are pathogens of many different plant species. In asparagus they infect underground part of plant and therefore first symptoms of the disease very often are not observed on the aboveground plant parts (Blok and Bollen 1995; Elmer *et al*. 1996; Baayen *et al*. 2000; Elmer 2000). They occur among others in the form of rusty spots in different parts of white spears or in underground parts of green spears of asparagus. The disease is caused the most often by *Fusarium oxysporum* f. sp. *asparagi*, *F. proliferatum* and more rarely by *F. subglutinans*, *F. redolens*, *F. merismoides*, *F. equiseti*, *F. dimerum*, *F. lateritum* and some others (Gossmann *et al.* 2001; Weber *et al*. 2006, 2007).

Mycotoxins: fumonisins, primarily fumonisin B_1 (FB_1), moniliformin (MON) and lower amounts of other toxins may be found in infected asparagus spears (Gossmann *et al.* 2001; Weber *et al.* 2006). It is worth to point out the lack of a close correlation between occurrence in spears of mycotoxins and *Fusarium* fungi. Sometimes mycotoxins are not detected in spears infected by *Fusarium* fungi (Weber *et al*. 2006). Therefore in the research work we tried to check the ability for mycotoxins biosynthesis by different isolates of two *Fusarium* species.

The aim of this work was to assess FB_1 and MON mycotoxins biosynthesis by three isolates of *F. oxysporum* and three isolates of *F. proliferatum* in the laboratory conditions on two different media: rice and asparagus medium.

MATERIALS AND METHODS

Three isolates of *F. oxysporum* and three isolates of *F. proliferatum* originated from asparagus spears were the subject of the work. Two media: rice and asparagus, were used. Rice medium (65 g of rice and 81 ml of distilled water) was prepared in 500 ml Erlenmayer flasks. After 16 hours medium was sterilized 15 minutes at 120°C. Asparagus medium consisted of: extract of 200 g white asparagus spears of Rolnik Company; 18 g of agar; distilled water to 1 litre capacity. Mixed 200 g of asparagus spears and 500 ml of distilled water were boiled for 15 minutes. On the next day supernatant of asparagus spears extract was mixed with 18 g of agar, supplemented to 1 litre with distilled water and boiled until agar dissolved. The medium was poured into Erlenmeyer flasks and sterilized for 15 minutes in 120°C. Then 25 ml of the medium was poured into each of Petri dishes.

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Experiment was performed in 3 replicates. The media were inoculated with 5 mm disks of PDA overgrown by mycelium of particular fungal isolates. The cultures of *F. oxysporum* and *F. proliferatum* were grown at 20°C and those on rice were shaken daily to prevent clumping of the colonized kernels.

Extraction and purification of mycotoxins

After 3 weeks samples (10 g) of fungal cultures were homogenized for 3 minutes in 20 ml of methanol-water (3:1, v/v) and filtered through Whatmann no. 4 filter paper according to the method described by Sydenham *et al.* (1993). The supernatant was then divided into two equal subsamples for FB_1 and MON analyses. The fraction used for FB_1 analysis was adjusted to pH 5.8–6.5 using 0.1 M KOH. A SAX cartridge was attached to the SPE manifold unit (Supleco, Bellefonte, PA USA) and conditioned at a flow rate of 2 ml/min successively with 5 ml of methanol followed by 5 ml of methanol-water (3:1, v/v). An aliquot (10 ml) of the filtered subsample extract was applied at the top of conditioned cartridge at a flow rate of 2 ml/min, washed with 8 ml methanol-water (3:1, v/v), immediately followed by 3 ml of methanol. FB_1 was eluted at a flow rate of 1 ml/min from the column to a glass collection vial, with 10 ml of 1% acetic acid in methanol. The eluate was evaporated to dryness at 40°C under a stream of nitrogen. Dry residue was stored at 4°C until high-performance liquid chromatography (HPLC) analyses were performed.

The fraction used for MON analysis was defatted with *n*-hexane (3×50 ml), concentrated and later purified in glass columns containing 1.5 g of the Florisil gel (Merck 60–100 mesh, No 12 994; Merck, Darmstadt, Germany) according to the method described by Kostecki *et al*. (1995). The gel was activated for 1.5 h at 110°C prior to column preparation and the columns were conditioned with 5 ml acetonitrile and washed with 5 ml chloroform. The extract was applied at the top of the column and washed with 5 ml chloroform followed by 5 ml acetonitrile. Finally, MON was eluted using 5 ml of water. After solvent evaporation, the toxin residue was dissolved in 5 ml methanol to be quantified. All solvents used for toxin extraction and purification were of analytical grade and were supplied by Sigma-Aldrich (Stenheim, Germany).

Quantitative analysis of mycotoxins

Solvents used for mycotoxin determination by HPLC were also from Sigma-Aldrich, but were of HPLC grade. Phthaldialdehyde (OPA), Sigma-Aldrich, was used for FB_1 analyses.

FB_1 was quantified according to the method described by Shephard *et al.* (1990) and Sydenham *et al.* (1990). The FB_1 standard (1 ng/μl in methanol-water at 1:1, v/v) was prepared and stored at 4°C. The OPA reagent (20 mg per 0.5 ml methanol) was prepared and diluted with 2.5 ml 0.1 M disodium tetraborate ($Na_2B_4O_7 \times 10\ H_2O$), then combined with 25 μl 2-mercaptoethanol. The mixture was stored up to 1 week at room temperature in a dark, capped amber vial. The FB_1 standard (25 μl) or spear extracts (200 μl) were derivatized with 225 or 200 μl of the OPA reagent. After 3 min, the reaction mixture (10 μl) was injected in an HPLC column. Methanol-sodium dihydro-gen phosphate (0.1 M in water) solution (77:23, v/v) was adjusted to pH 3.35 with o-phosphoric acid after filtration through an 0.45 μm Waters HV membrane and used as the mobile phase with the flow rate of 0.6 ml/min (Waters Division of Millipore, Milford, MA, USA). A Waters 2695 apparatus, with a C18 Nova Pak column (3.9×150 mm) and a Waters 2 475 fluorescence detector (λ_{Ex} = 335 nm and λ_{Em} = 440 nm) were used to quantify the metabolite. The FB_1 retention time was 7.35 min. MON content was preliminarily estimated on a Merck 5 554 silica gel thin-layer chromatography plate (Merck) with 2-propanol-butanol-water-ammonium hydroxide (12:4:1:1, v/v/v/v) as the developing solvent, according to the method described by Goliński *et al.* (1999). The colour of spots was developed with 3-methyl-2-benzo-thiazolinonehydro-chloride (MBTH) (Chełkowski *et al.* 1990). The intensity of dark spots on the chromatogram was compared with that of the metabolite standard. A more precise quantification was made by HPLC using a Waters 501 apparatus (Waters Division of Millipore) with a C18 Nova Pak column (3.9×300 mm) and a Waters 486 UV detector (λ_{max} = 229 nm). Acetonitrile-water solvent (15:85, v/v) buffered with 10 ml 0.1 M K_2HPO_4 in 40% *t*-butyl-ammonium hydroxide in 1 l of solvent (Sharman *et al.* 1991) was used as the mobile phase at a flow rate of 0.6 ml/min. The MON retention time was 11.5 min with 90% recovery and detection of 25 μg/g.

Statistical analysis

Results were subjected to analysis of variance (Bogartz 1994) to evaluate the yield of FB_1 and MON on two media by *F. oxysporum* and *F. proliferatum* isolates using t-Student test.

RESULTS

FB_1 was found in a a high amounts in all three cultures of *F. proliferatum* on rice (894.9–2012.8 μg/g) and in a very low amounts in two cultures on asparagus medium (0.014–0.036 μg/g) (Table 1)

Table 1. *In vitro* biosynthesis of fumonisin B_1 (FB_1) by *F. oxysporum* (F.o.) and *F. proliferatum* (F.p.)

Species	Isolate	FB_1* in μg/g of medium		Significance of differences **
		rice	aspara-gus	between media
F.o.	SE – 10 nz_1	34.5 a	0.004 a	–
	30 na	6.9 a	0.001 a	–
	43 na	0.2 a	0.002 a	–
F.p.	SE – 9 nz_4	984.8 b	0.014 b	+
	53 nb	2012.8 c	0.036 c	+
	76 nd	894.9 b	0.001 a	+
Control		2.4 a	0.000a	–

* in columns means followed by the same letters are not significantly different

** lack of significant differences (–); occurrence of significant differences (+)

On both media in cultures of *F. oxysporum* FB_1 did not occur in quantities significantly higher than in control sample. Significant differences between media were found only in case of *F. proliferatum* (all isolates).

MON was biosynthesised by two isolates of *F. oxysporum* on both media and one isolate (different on each medium) of *F. proliferatum* on asparagus medium (Table 2). Significant differences between media were found in all isolates of *F. oxysporum* and two isolates of *F. proliferatum*.

Table 2. *In vitro* biosynthesis of moniliformin (MON) by *F. oxysporum* (F.o.) and *F. proliferatum* (F.p.)

Species	Isolate	MON * in µg/g of medium		Significance of differences **
		rice	aspara-gus	between media
F.o.	SE – 10 nz$_1$	0.0 a	82.2 a	+
	30 na	171.6 b	743.3 b	+
	43 na	182.8 b	460.0 b	+
F.p.	SE – 9 nz$_4$	3.3 a	35.8 a	–
	53 nb	143.9 b	15.3 a	+
	76 nd	27.8 a	362.3 b	+
Control		0.0 a	25.8 a	–

* in columns means followed by the same letters are not significantly different

** lack of significant differences (–); occurrence of significant differences (+)

DISCUSSION

F. proliferatum and other *Fusarium* species of Liseola section produce fumonisins (Chulze *et al*. 1996) associated with cancer-promoting activity (IARC 2002). The above species have been demonstrated to be important contaminants of maize (Sydenham *et al*. 1993; Doko and Visconti 1994; Sanchis *et al*. 1995), asparagus spears (Gossmann *et al*. 2001; Weber *et al*. 2006) and some other plant species. Range of temperature and other environmental conditions conductive to growth usually is wider than that for FB_1 production and depends on the isolate of fungus species (Marin *et al*. 1999a, b).

Ability of 9 isolates of *F. proliferatum* originated from asparagus to biosynthesis of FB_1 and FB_2 was tested by Logrieco *et al*. (1998). Level of FB_1 varied from 744–2504 µg/g. Similar level of FB_1 was found in our work only on the rice medium. The occurrence of FB_2 varied from 118–946 µg/g (Logrieco *et al*. 1998). Liu *et al*. (2007) evaluated the ability of biosynthesis of FB_1 and FB_2 on corn and asparagus spears for 50 isolates of *F. proliferatum* originated from asparagus crowns. All isolates on corn and 96% isolates on asparagus spears produced fumonisins. Similar to our work biosynthesis of fumonisins on corn (FB_1 = 10.0–11499 µg/g and FB_2 = 2.0–6.6 µg/g) was higher than on asparagus spears (FB_1 = 0.2–781.6 µg/g and FB_2 = 0.1–40.3 µg/g). Amount of FB_1 on rice in our work appeared to be similar to that on corn in work of Liu *et al*. (2007).

Low biosynthesis yield of FB_1 by *F. proliferatum* on asparagus medium in agreement with low level of the mycotoxin occurrence in field asparagus spears. On the other hand high biosynthesis yield of MON both by *F. oxysporum* and *F. proliferatum* on rice and asparagus medium correlate with high level of the mycotoxin present in field asparagus spears sampled from the field (Weber *et al*. 2006).

CONCLUSIONS

1. FB_1 and MON biosynthesis depends on isolate and species of *Fusarium* genus as well as on type of medium.
2. Of the two examined species, only *F. proliferatum* isolates produced FB_1 mostly on rice medium.
3. MON biosynthesis was found both for *F. oxysporum* and *F. proliferatum* at similar level on rice and asparagus media.

ACKNOWLEDGEMENTS

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POLISH SUMMARY

TOKSYNOTWÓRCZOŚĆ IZOLATÓW *FUSARIUM OXYSPORUM* I *F. PROLIFERATUM* POCHODZĄCYCH ZE SZPARAGA

Celem pracy była ocena wytwarzania mikotoksyn FB_1 i MON przez 3 izolaty *Fusarium oxysporum* i 3 izolaty *F. proliferatum* na dwóch różnych pożywkach. Izolaty grzybów pochodzące z wypustek szparaga hodowano na namoczonym i wysterylizowanym ryżu oraz na wysterylizowanej pożywce szparagowej. Na obydwu pożywkach doświadczenie przeprowadzono w 3 powtórzeniach, przez 3 tygodnie, przy 20°C. Oznaczanie mikotoksyn w obu pożywkach przeprowadzono, po uprzedniej homogenizacji i ekstrakcji, metodą wysokosprawnej chromatografii cieczowej (HPLC). FB_1 stwierdzono w bardzo dużej ilości we wszystkich kulturach *F. proliferatum* na ryżu i w bardzo małej ilości w kulturach dwóch izolatów tego gatunku na pożywce szparagowej. W kulturach *F. oxysporum* nie zanotowano FB_1 w ilościach większych niż w kontroli. Na obydwu pożywkach MON wytwarzały te same dwa izolaty *F. oxysporum* i jeden (inny na każdej pożywce) izolat *F. proliferatum*. Uzyskane wyniki wskazują, że wytwarzanie poszczególnych mikotoksyn zależy od izolatu i gatunku grzyba, rodzaju *Fusarium* oraz od rodzaju pożywki.