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Fusarin C Production by North American Isolates of *Fusarium moniliforme*

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A liquid culture medium was developed to screen North American isolates of *Fusarium moniliforme* Sheldon and *Fusarium subglutinans* (Wollenw. and Reink.) Nelson, Toussoun, and Marasas for their ability to produce fusarin C. Parameters which were important for the optimal biosynthesis of fusarin C included pH (3.0 to 4.0), aeration, and sugar concentration (30 to 40%). Of seven sugars tested, sucrose and glucose were the best carbohydrate sources for mycotoxin production, resulting in levels of fusarin C of >60 ppm (>60 μg/g) in liquid culture (28°C; 7 days). A time-course study of fusarin C production was done over a 21-day period, during which time pH values, glucose concentrations, nitrogen levels, and fungal biomass were determined. Of the two *Fusarium* spp. studied, 13 of 16 isolates of *F. moniliforme* produced fusarin C in liquid medium (14 of 16 in corn), while none of the 15 isolates of *F. subglutinans* studied was found to produce the compound. Levels of fusarin C produced by *Fusarium* sp. isolates growing on corn ranged from 18.7 to 332.0 μg/g.

Fusarium moniliforme Sheldon, a ubiquitous fungus belonging to the section Liseola (Booth), is one of the principal fungi found on corn crops worldwide (11). The organism has been associated with human esophageal cancer (12, 13) and several animal mycotoxicoses (8–10). Of the toxins previously known to be produced by this fungus, none have proved to be mutagenic.

Recently, however, a highly mutagenic compound, fusarin C, was isolated from a culture of a North American strain of *F. moniliforme* growing on corn (17). The compound has been found to occur naturally in both hand-selected visibly *Fusarium* sp.-infected and healthy looking corn kernels in South Africa (5).

Currently, there is little information in the literature to indicate whether North American isolates are capable of producing fusarin C. Thus, the objectives of this work were to (i) develop a liquid culture medium capable of screening isolates for fusarin C, (ii) try to optimize production of the compound in liquid culture, and (iii) determine the levels of fusarin C produced by North American *Fusarium* sp. isolates growing in corn cultures.

MATERIALS AND METHODS

Organisms. Five *F. moniliforme* Sheldon Canadian isolates (M3782, W6, W84, W85, and W86) and seven Canadian *F. subglutinans* (Wollenw. and Reink.) Nelson, Toussoun and Marasas isolates were kindly donated by Randy Clear, Grain Research Lab Division, Agriculture Canada, Winnipeg, Manitoba. Canadian isolate M2041 (*F. moniliforme*) was obtained from A. V. Asselin, University of Guelph, Guelph, Ontario. The remaining eight Canadian isolates of *F. subglutinans* were a gift from Sandra Needham, Agriculture Canada, Ottawa, Ontario, while the remaining 10 *F. moniliforme* isolates (United States) were kindly donated by P. Nelson, Pennsylvania State University, University Park.

Media. In preliminary investigations, all isolates were screened by using three liquid culture media, both shaking and nonshaking, as follows (all medium components in grams per liter): (i) Czapek-Dox medium (1); (ii), glucose, 10, yeast extract, 1, and peptone, 1 (16); (iii) $(NH_4)_2HPO_4$, 1, KH_2PO_4, 3, $MgSO_4 \cdot 7H_2O$, 2, NaCl, 5, sucrose, 40 g, and glycerol, 10 (MYRO medium; 3).

For pre-enrichment of the strains prior to inoculating the fungi in the above three liquid culture media, an inoculation medium (3) was used (NH_4Cl, 3 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 2 g; KH_2PO_4, 2 g; peptone, 2 g; yeast extract, 2 g; malt extract, 2 g; glucose, 20 g).

In experiments where corn was used as a solid substrate, 50 g of whole grain kernels was added along with 20 ml of double-distilled water to 250-ml Erlenmeyer flasks. The flasks were autoclaved at 121°C for 15 min, cooled, and then inoculated.

Inoculation procedure. All fungi were grown on 2% malt extract agar (Difco Laboratories) for approximately 10 days at 25°C. The whole slant was then transferred into a 250-ml Erlenmeyer flask containing 50 ml of double-distilled water and was homogenized (model PT 10-35 Polytron homogenizer; Brinkmann Instruments Canada, Ltd.) for around 15 s at low speed.

The homogenate (2.5 ml) was transferred into the inoculation medium, which was incubated at 28°C for 48 h in a New Brunswick incubator shaker at 220 rpm (model G-25 incubator shaker; New Brunswick Scientific Co., Inc.). The inoculation medium was homogenized again and 2.5 ml was transferred into all liquid culture media and corn cultures. Triplicate flasks containing liquid media were inoculated and incubated at 28°C for 7 days (shaking at 220 rpm) unless otherwise indicated. Duplicate corn cultures were inoculated and incubated for 21 days at 28°C in an atmosphere of 70% relative humidity. Flasks that were not analyzed immediately were frozen at −20°C. *F. moniliforme* M3783 was the strain used in all liquid culture (MYRO medium) experiments performed to optimize production of fusarin C. Owing to the known sensitivity of fusarin C to longwave UV light (6), all experiments were performed under "gold" fluorescent lighting (Westinghouse 6YT 126D) (P. M. Scott, G. A. Lawrence, and T. I. Matula, *in* Proceedings of the 6th International IUPAC Symposium on Mycotoxins and Phycotoxins, in press).

Extraction procedures. (i) Liquid culture. Liquid culture

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media (50 ml in 250-ml Erlenmeyer flasks unless otherwise stated) were extracted twice with an equal volume of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, evaporated to dryness under vacuum (Buchi Rotavapor-R; Brinkmann Instruments), and then reconstituted in 0.5 ml of chloroform. Depending on the analyses, 5 to 50 μl was transferred into 0.5-dram vials with Teflon-lined caps, evaporated to dryness under a stream of nitrogen, and stored at −20°C until analyzed.

(ii) Corn cultures. From 50 g of corn, 10-g portions were removed and blended (Kenmore 16 blender; Simpson Sears Ltd.) for 5 min in 100 ml of CH_2Cl_2-CH_3 CN (1:1) (15). The blended material was then passed through a Büchner sintered-glass funnel (150-ml capacity), evaporated to dryness under vacuum at 30°C, and resuspended in approximately 2.5 ml of 3% CH_3OH in CH_2Cl_2. A suspension (1 ml) was then applied to a silica gel column (kieselgel 60; 70/230-mesh ASTM; BDH Chemicals Canada Limited; column, 6.3 cm [length] by 1.5 cm [inside diameter]), which was eluted with 10% CH_3OH in CH_2Cl_2 (60 ml). The eluant was evaporated to dryness under a gentle stream of nitrogen, reconstituted in 0.5 ml of $CHCl_3$, and treated as in (i) above.

Analysis of fusarin C. In all experiments both thin-layer chromatography (TLC) and high-pressure liquid chromatography were used to confirm the presence or absence of fusarin C. For TLC analysis, TLC plates (silica gel 60; BDH Chemical Canada Limited), after being spotted with 5 to 10 μl of extract, were developed with $CHCl_3$-CH_3OH (9:1). Standards and positive samples were identified by the presence of a bright yellow spot under visible light (R_f value, 0.32 to 0.35). The high-pressure liquid chromatography system consisted of a Varian model 5000 liquid chromatograph with a VISTA 2050 variable UV wavelength detector set at 350 nm and a Varian 4270 integrator. The column (30 cm by 4.0 mm [inside diameter]) was packed with 5-μm reverse-phase monomeric Micro Pak MCH-5 (Varian Instruments Group). The mobile phase used in the isocratic system was methanol-water (70:30). Mass spectrometry was done on TLC-purified extracts obtained from *F. moniliforme* M3783 growing in MYRO medium and on several pooled extracts from various *F. moniliforme* strains growing on corn, to confirm the presence of fusarin C (mass spectrometric $M^+ = 431$). A VG Micromass ZAB-2F mass spectrometer operated at ×1,500 resolution, 70-eV electron energy, and 200°C ion source temperature was used.

Liquid culture experiments. (i) Sugar concentration. The glucose and sucrose concentrations in MYRO medium were varied from 5 to 40 g/liter. In additional experiments various carbohydrates (sucrose, glucose, fructose, maltose, galactose, lactose, and glycerol, all at 40 g/liter) were evaluated for their ability to support fusarin C production by *F. moniliforme* M3783 in MYRO medium. The sucrose concentrations of MYRO medium for all other experiments was 40 g/liter.

(ii) pH. The effects of varying the initial pH of MYRO medium were investigated. Media were adjusted with either 1 N NaOH or 1 N HCl. The initial pH of MYRO medium for all other experiments was 6.0 to 6.2.

(iii) Fluctuating time/temperature conditions. The length of incubation and the storage temperature were altered to observe the effect on fusarin C production. Six different time/temperature sequences were studied (1, 2, and 4 weeks at 28°C; 2 weeks at 28°C/1 week at 11°C; 2 weeks at 28°C/2 weeks at 11°C; 1 week at 28°C/1 week at 11°C/1 week at 28°C).

Time-course production of fusarin C. *F. moniliforme* was inoculated into 100 ml of MYRO medium in 250-ml Erlenmeyer flasks, and the flasks were shaken at 220 rpm and 28°C for periods of up to 21 days. On each sampling day (days 2, 4, 6, 8, 11, 12, 14, and 21) three flasks were removed and 30-ml samples were transferred into 99-ml dilution bottles, which were then stoppered with rubber stoppers and covered with Parafilm. From this sample the pH, dry weight, glucose, and ammonium levels were determined. The remaining 70 ml was extracted twice with an equal volume of ethyl acetate and treated as previously described under extraction procedure for liquid culture.

The glucose oxidase-peroxidase method was used to determine glucose levels (enzyme kit from Sigma Chemical Co.), and the indophenol blue reaction was used to determine the levels of NH_4^+ (7). For the dry weight determination, 2.5 ml of diluted culture was passed through a washed and preweighed 13-mm-diameter 0.45-μm-pore size membrane filter (type HAWPO 1300; Millipore Corp.). The filter was then washed with 2.5 ml of sterile distilled water, dried to a constant weight, and weighed.

Fusarin C standard. The standard preparations of fusarin C (mass spectrometric $M^+ = 431$) used were stored as gums at −4 to −2°C. The first standard was kindly donated by L. F. Bjeldanes, University of California, Berkeley, while the last two were from P. M. Scott, Health and Welfare Canada, Ottawa, Ont. Owing to stability problems observed with these various standards (Scott et al., in press), quoted fusarin C concentrations should not be considered as absolute values. In some instances, toxin concentrations between replicates within an individual experiment varied by 10 to 20%, probably owing to the heat and light instability of the compound, as well as to the difficulties in dispensing an optimal and equivalent inoculum per flask (14).

RESULTS

Preliminary experiments demonstrated that, of the liquid culture media and incubation temperatures tested, MYRO medium incubated at 28°C supported the greatest production of fusarin C. Thus, all subsequent experiments with liquid culture media were performed in MYRO medium at 28°C.

Increased amounts of sucrose in liquid culture medium led to increased amounts of fusarin C produced by *F. moniliforme*. MYRO medium containing 5, 10, 20, 30, and 40 g of sucrose per liter supported the production of 2.0, 16.7, 29.3, 44.2, and 40.4 μg of fusarin C per ml, respectively. Similar experiments repeated with various levels of glucose (substituted for sucrose in MYRO medium) gave identical results except that 40 rather than 30 g of glucose per liter appeared to support slightly greater fusarin C production. Of seven carbohydrates tested (all at 40 g/liter), sucrose (64.4 μg/ml) and glucose (61.4 μg/ml) were the best and lactose (1.7 μg/ml) and glycerol (0.41 μg/ml) were the worst substrates for fusarin C production (data not shown).

The effect of initial pH on fusarin C biosynthesis is shown in Table 1. Decreasing the initial pH value of MYRO medium from 7.5 to 5.9 resulting in an approximately 17-fold increase in the production of fusarin C. Of all initial pH values tested, the best yield of fusarin C occurred at pH 5.9.

Of six time/temperature sequences tested (Table 2), the largest amounts of fusarin C were produced over a 2-week period at 28°C, while at longer incubation times at 28°C (4 weeks), decreased amounts of fusarin C were observed. Fluctuating temperatures between 11 and 28°C for periods of up to 3 or 4 weeks did not result in increased levels of fusarin C as compared with that observed at 28°C for 2 weeks.

In a fermentation study done over 21 days, the largest

TABLE 1. Effect of initial pH on production of fusarin C by *F. moniliforme* in MYRO medium

pH		Fusarin C (μg/ml)[a]
Initial	Final	
7.5	5.8	3.1
7.0	5.6	3.9
6.5	4.8	26.6
6.3	4.1	39.2
5.9	3.2	52.0

[a] Average of triplicate determinations.

amounts of fusarin C were produced between days 2 and 6, with production stopping until a slight increase occurred between days 12 and 14 (Fig. 1). Between days 14 and 21 more than half of the fusarin C present disappeared. The pH dropped rapidly from around 6.2 to 3.0 within 48 h and then remained constant throughout the fermentation. Glucose decreased fairly rapidly between days 4 and 8, but was never completely utilized by the fungus. Ammonium had virtually disappeared by day 6 (Fig. 1) but subsequently increased and then decreased (data not shown), probably owing to fungal lysis.

Although 13 of 16 *F. moniliforme* isolates produced fusarin C while growing in liquid medium (MYRO, 28°C, 1 week), none of the 15 isolates of *F. subglutinans* tested was found to produce the compound in either liquid medium or corn. In only one instance (isolate M1554) was fusarin C detected in corn extracts and not in MYRO medium extracts by TLC. The concentration of fusarin C produced by the various *F. moniliforme* isolates ranged from 18.7 to 332 μg/g of corn (Table 3). Although most of the positive isolates of *F. moniliforme* were originally recovered from corn, fungi isolated from wheat, barley, and oats were also found capable of producing the compound.

DISCUSSION

High sugar concentrations resulted in greater toxin yields, and thus reduced sugar levels were not an important stimulus for the synthesis of fusarin C, as is the case for synthesis of deoxynivalenol (14), another *Fusarium* sp. mycotoxin. Our results are similar to fermentations to produce two other *Fusarium* sp. mycotoxins (neosolaniol and T-2) where around half of the original sugar concentration was present in the medium when toxin production had started (17; Fig. 1). Nitrogen limitation in the medium also was not an important stimulus for fusarin C production, as at least half of the nitrogen source available in MYRO medium was still present when fusarin C production was initiated. This is in contrast to work done on bikaverin (2) and gibberellin (4) biosyntheses where secondary metabolite synthesis did not begin until all nitrogen was depleted from the growth medium.

TABLE 2. Effect of fluctuating time/temperature conditions on production of fusarin C by *F. moniliforme* in MYRO medium

Conditions of incubation	Fusarin C (μg/ml)[a]
1 wk, 28°C	43.9
2 wk, 28°C	69.1
4 wk, 28°C	18.7
2 wk, 28°C/1 wk, 11°C	40.2
2 wk, 28°C/2 wk, 11°C	34.1
1 wk, 28°C/1 wk, 11°C/1 wk, 28°C	46.4

[a] Average of triplicate determinations.

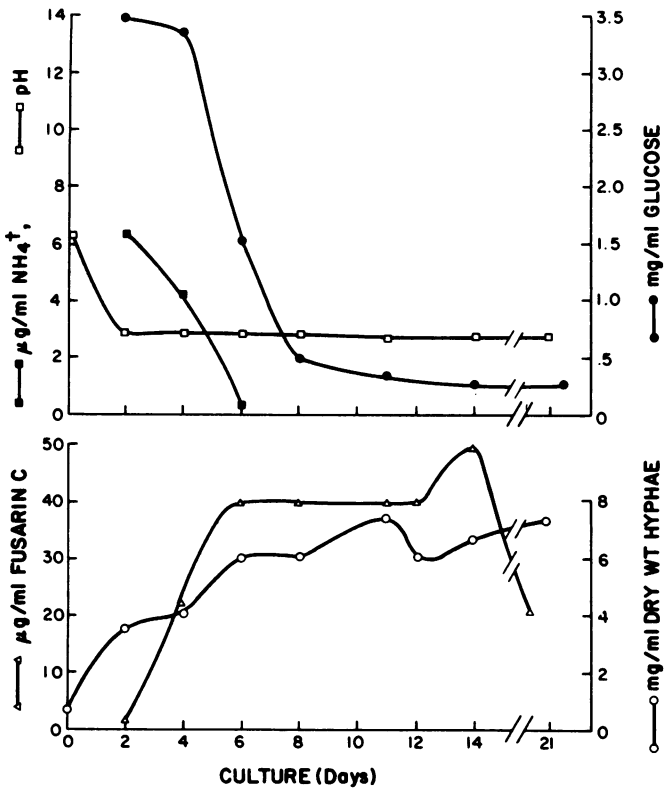

FIG. 1. Time-course production of fusarin C by *F. moniliforme* in MYRO medium relative to pH, dry weight, glucose, and NH_4^+ levels in the medium.

Another important parameter (besides pH and high levels of carbohydrate) governing the production of fusarin C by *F. moniliforme* appeared to be the O_2 or CO_2 level in the producing cultures. Extracts from still cultures (MYRO medium) inoculated with *F. moniliforme* M3783 contained

TABLE 3. Production of fusarin C by North American isolates of *F. moniliforme* growing on corn

Strain no.	Source	Fusarin C (μg/g)[a]
M1127	Sweet corn, ear rot	200.0
M1365	Moldy corn	ND[b]
M1368	Moldy corn	18.7
M1385	Corn, chicken feed	286.0
M1397	Corn, chicken feed	285.0
M1413	Corn, chicken feed	44.5
M1470	Cracked corn, chicken feed	234.0
M1548	Chicken feed	27.2
M1554	Oats, horse feed	180.0
M1680	Chicken feed	43.7
M2041	Wheat	123.0
M3783	Barley	332.0
W6	Corn	87.5
W84	Corn	ND
W85	Corn	28.9
W86	Corn	49.9

[a] Average of duplicate determinations.
[b] ND, Not detected.

approximately 400-fold less fusarin C than did shake cultures at 220 rpm (data not shown). No attempt was made to optimize the production of fusarin C by regulating the speed of the incubator shaker. However, increasing the volume of liquid in the shaker flask or increasing the inoculum level (data not shown), conditions under which decreased amounts of O_2 would be present, led to increases in fusarin C production. Similar aeration effects on mycotoxin production have been observed by investigators working with *Aspergillus* (15) and *Fusarium* (14) sp. mycotoxins.

Experiments performed with corn cultures demonstrated the inability of 15 strains of *F. subglutinans* to produce fusarin C. Similarly, Gelderblom et al. (5) observed that five South African strains of *F. subglutinans* failed to produce the compound. Also, Gelderblom et al. (5) found that all 20 strains of *F. moniliforme* studied were able to produce fusarin C while growing on corn in amounts ranging from 0.63 to 724 μg/g (dry weight) of corn. Generally, the North American strains used in this study produced lower amounts of fusarin C than the South African fungi, with two North American strains not producing any. However, the results demonstrate a widely varying potential for fusarin C production in North American cereal grain isolates. Isolates obtained from corn, wheat, barley, and oats were found capable of producing the compound, and thus the ability of various *Fusarium* sp. isolates to produce fusarin C on these as well as other grains should be investigated.

In summary, from the data presented it is evident that pH, aeration, and carbohydrate levels are important factors in the optimal biosynthesis of fusarin C in liquid culture by *F. moniliforme*. This is the first report of Canadian isolates of *F. moniliforme* being able to produce fusarin C. Further studies are in progress to verify the ability of Canadian isolates of other species of *Fusarium* to produce fusarin C as well as to observe whether fusarin C can be produced naturally in corn under field conditions.

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