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Factors affecting fusarin C mycotoxin production by *Fusarium moniliforme* isolates of Taiwan

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Abstract. The effects of various nutritional and environmental factors on fusarin C biosynthesis in the defined medium were performed on isolates of *Fusarium moniliforme* collected from corn fields in Taiwan. The parameters of temperature (28°C to 35°C), aeration (100 r. p. m.), pH (3.5 to 4.5), and sugar concentration (30 to 40%) were important for optimal biosynthesis of fusarin C. Of the three carbohydrates tested, all at a concentration of 40g/l, sucrose producing 464 μg/ml of mycotoxin was the best carbon source. On glucose medium 163 μg/ml of mycotoxin were produced. Starch medium was the poorest carbon source with only 7 μg/ml of fusarin C. Our *F. moniliforme* isolates produced a lower amount of fusarin C in liquid cultures as compared with North American strains.

Key words: Carbohydrate; Fusarin C; *Fusarium moniliforme*; Mycotoxin; Temperature.

Introduction

Fusarium monilforme Sheldon is one of the predominant pathogenic fungi found on cereal crops worldwide (Marasas *et al.*, 1984b). Besides causing severe crop loss, this organism produces mycotoxins which cause mycotoxicoses in various livestock animals resulting in severe loss in weight and reduction of productivity (Kriek *et al.*, 1981a; Kriek *et al.*, 1981b; Marasas *et al.*, 1984a). It is also suspected to be related to human esophageal cancer (Marasas *et al.*, 1979; Marasas *et al.*, 1981).

Of the mycotoxins produced by *F. moniliforme*, a mutagenic compound, fusarin C, has been isolated from a North American strain of *F. moniliforme* by Wiebe and Bjeldanes (1981). Later, this mycotoxin was found to be produced by the isolates of *F. moniliforme* from South Africa (Gelderblom *et al.*, 1983), China (Cheng *et al.*, 1985), Canada (Farber and Sanders, 1986a) and Europe (Thrane, 1988).

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In Tseng *et al.* (1990), we reported on *F. moniliforme* isolates capable of producing fusarin C. However, the yields of the toxin were less than that produced by other strains of *F. moniliforme*. Recently Farber and Sanders (1986b) indicated several factors influencing the production of fusarin C by North American *F. moniliforme* in MYRO medium. This paper reports on the effects of various nutritional and enviromental factors on fusarin C biosynthesis in *F. moniliforme* isolates from Taiwan.

Materials and Methods

Organism and Culture Inoculum

Two isolates of *F. moniliforme* were collected from corn with noticeable pink discoloration on the kernels. These were collected from fields of the Luchu (isolate PCA 5-1) and Tsoying (isolate PCA 5-3) districts of Taiwan. The cultures were isolated by single spore isolation technique, and the Nelson, Toussoun and Marasas system was adopted for identification (Nelson *et al.*, 1983). Both isolates are also known as fusarin C producers (Tseng *et al.*, 1990).

Spore inocula were produced on potato dextrose agar plates (at 25°C for 7 days) by the tested isolates of *F. moniliforme*. Plates containing spores were rinsed with sterile distilled water. A concentration of 2.3×10^5 spores per ml was added to a 250-ml Erlenmeyer flask containing 50 ml of the defined medium. This medium is used in all experiments unless otherwise indicated.

Defined Medium

A modified medium, originally developed by Farber and Sanders (1986a), was initally used to induce fusarin C production by both *F. moniliforme* PCA 5-1 and PCA 5-3. The medium consisted of the following ingredients (in grams per liter): $(NH_4)_2HPO_4$, 1; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 2; NaCl, 5, and Sucrose, 40.

In the preliminary test for fusarin C production, triplicate flasks containing the defined media (50 ml/flask, pH 6.2) were inoculated with the fungi and incubated at 28°C for 7 days at 100 r. p. m. in a controlled environment incubator shaker (New Brunswick Scientific Co., Inc., Edison, N. J.). The above culture conditions were also used for all subsequent experiments unless otherwise indicated.

Parameters for Induction Fusarin C

a. Sugar concentration: The glucose, sucrose and starch concentrations in the defined medium were varied from 10-50 g/l to observe effects on fusarin C production. A sugar concentration of 40 g/l was used for all other experiments.

b. Temperature: Flasks containing 50 ml of defined medium were inoculated and incubated at 10, 20, 28, 35, and 40°C to monitor effects on fusarin C production. In all other experiments, 28°C was the temperature of incubation.

c. pH value: The pH of the defined medium was adjusted with 1N HCl or 1N NaOH from 3.5 to 8.5. This was done to examine the effect of pH change on toxin production. All other experiments were set at pH 6.2.

d. Aeration: The effects of various aeration conditions on toxin production were investigated. Inoculated flasks incubated in controlled environment incubator shakers were shaken at either 25, 50, 100, 150, or 200 r. p. m. For other experiments, 100 r. p. m. was employed.

e. Level of inoculum: Various concentrations of spore suspension from 2.3×10^4 spores/ml to 2.3×10^8 spores/ml were added to flasks containing 50 ml of medium. In all other experiments, a concentration of 2.3×10^7 spores/50 ml of medium was used.

f. Volume of medium: The volume of medium added to flasks was varied from 12.5 ml to 150 ml. The effect of this on fusarin C production was observed. For all other experiments a volume of 50 ml medium/flask was employed.

Extraction and Analysis of Fuasrin C

Liquid culture media (50 ml in 250-ml Erlenmyer flasks, unless otherwise stated) were blended with a homogenizer (Blender 7011 Model, Waring Products, New Hartford, Connecticut, USA) at full speed for 2 min and then passed through a Büchner glass funnel. The filtrates were extracted twice with an equal volume of ethyl acetate. The combined extracts were evaporated to dryness under vacuum at 45°C and resuspended in 2 ml chloroform. One milliliter of the extract was transferred into a small vial (brown color) with Teflon-lined caps, evaporated to dryness under a stream of nitrogen, and stored at -20°C for further analysis. All experiments were performed under fluorescent lighting to prevent the photodegradation of fusarin C to long-wave UV light.

Thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used to analyze and confirm the presence of fusarin C as previously described (Tseng *et al.*, 1990). The extract was initally applied to a TLC plate (Silica gel 60, E. Merck). After being spotted or streaked with 50 or 500 μl of the extract, it was developed with chloroform-methanol (9:1, v:v). Standards and positive samples were identified by the presence of a bright yellow spot under visible light. The band corresponding to authentic fusarin C on TLC plate was scraped and eluted with chloroform. The eluant was evaporated to dryness under a gentle stream of nitrogen and served as purified extract. HPLC analyses were performed on a Waters Associates Instrument (Model ALC/GC-204) equipped with a M-6000 A pump, a U6K universal injector and a model 440 UV detector (365 nm). Separations were carried out on a LiChrosorb RP-18 column (4 mm × 25 cm; 10 μm, E. Merck) with methanol-chloroform (1:19) as the mobile phase at a flow rate of 1.0 ml/min. Samples were spiked with standard fusarin C to confirm the identity of the peak eluting in the position of fusarin C. The authentic fusarin C was kindly donated by R. Vleg-

gaar, National Chemical Research Laboratory Council for Scientific and Industrial Research, Pretoria, South Africa. Mass spectrometry was used as an additional test to confirm the presence of fusarin C as reported by Tseng *et al.* (1990).

Results and Discussion

The preliminary test for fusarin C production did demonstrate that the *F. moniliforme* incubated at 28°C was able to produce fusarin C. Thus, all subsequent experiments were performed at 28°C. Fusarin C from the culture media was quantified by comparing the peak heights of fusarin C in HPLC chromatograms with those of standard fusarin C compounds. HPLC analysis of a purified extract of PCA 5-3 showed a peak eluting the same position as fusarin C (Fig. 1).

The glucose, sucrose, and starch concentrations in the defined media were evaluated for their ability to support fusarin C production by both *F. moniliforme* PCA 5-3 and PCA 5-1. Increasing concentrations of sugars from 10 to 40 g/l led to increased amounts of fusarin C produced by both isolates (Table 1). At a concentration of 40 g/l, sucrose and glucose were the best carbohydrate source for fusarin C production. Starch was the poorest. Similar results have been reported by Farber and Sander (1986a). Attempts to stimulate the toxin production by increasing sugar concentration to 50 g/l had the reverse effect. It suppressed mycotoxin production. This is similar to the work of Miller *et al.* (1983) who found that increasing glucose concnetrations led to decreased mycotoxin yields.

The effects of pH and temperature on fusarin C biosynthesis are shown in Table 2. The optimum temperature for *F. moniliforme* PCA 5-3 was 35°C. Above

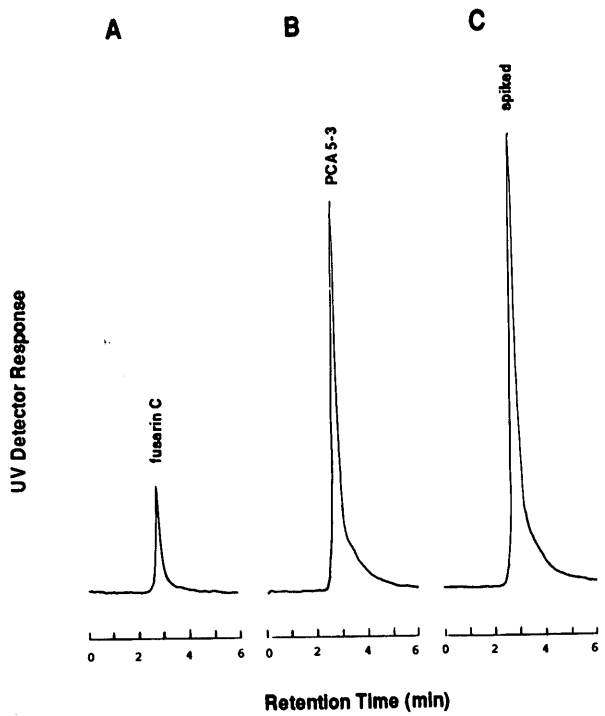

Fig. 1. L. C. chromatograms of authentic fusarin C (18ng) (A), purified extract of *F. moniliforme* PCA5-3 in a defined medium (50 ml/flask, pH6.2), incubated at 28°C, 7 days, aeration at 100 r.p.m. (B), and purified spiked with fusarin C (C).

Table 1. *Effects of cabohydrates on fusarin C biosynthesis by* ***F. moniliforme*** *in defined medium*

Carbohydrate (%)		Fusarin C (μg/L)	
		Isolate PCA5-3	Islate PCA5-1
Glucose	10	0.26*	0.12
	20	49.21	26.54
	30	129.40	107.42
	40	163.32	152.15
	50	86.17	75.26
Surcose	10	0.49	0.32
	20	29.10	40.42
	30	368.21	144.76
	40	463.56	156.21
	50	134.72	70.72
Starch	10	0.46	0.66
	20	1.65	1.20
	30	4.90	2.76
	40	7.35	5.95
	50	2.55	1.34

*Average of triplicate determinations.

Table 2. *Effects of temperature and pH on biosynthesis by* ***F. moniliforme*** *PCA5-3 in defined medium*

Temperature (°C)	Fusarin C (μg/L)	pH	Fusarin C (μg/L)
10	0.33*	3.5	275.82
20	16.82	4.5	449.45
25	171.81	5.5	216.27
28	320.16	6.2	207.32
35	418.64	7.5	87.24
40	10.30	8.5	ND**

*Average of triplicate determinations.
**Not detectable.

this temperature a drastical drop in the toxin production occurred. This contrasts with production of fusarin C in MYRO medium at 28°C by *F. moniliforme* (Farber and Sanders, 1986a). Other investigators using differed Fusarium species have also reported optimal mycotoxin production in liquid culture at 28°C (Miller *et al.*, 1983; Farber and Sanders, 1986b). Compounds such as diacetoxyscripenol and zearalenone have been found to produce greater amounts at lower temperatures (8-15°C) (Bergers *et al.*, 1985; Smalley *et al.*, 1970). The optimum pH for fusarin C production in the culture medium was 4.5. Decreasing the pH value of the culture medium from 7.5 to 4.5 resulted in an approximately 5-fold increase in fusarin C production. This differs from the optimum pH value of 3.2 for *F. monilifomre* M3783 reported by Farber and Sanders (1986a).

When various concentrations of spore inoculum were added to flasks containing 50 ml of the defined medium, it was observed that increass in spore inoculum led to increass in fusarin C production by *F. moniliforme* PCA 5-3. The medium containing 2.3×10^4, 2.3×10^5, 2.3×10^6 and 2.3×10^7 spores per 50 ml of medium supported the production of 161.01, 409.56, 700.91, 848.69, and 1741.48 μg of fusarin C per ml, respectively.

The greatest concentration of fusarin C was found in the flask containing 150 ml of medium (Table 3). Extracts from stationary cultures inoculated with *F. moniliforme* PCA 5-3 contained approximately 6-fold less fusarin C than the shake cultures at 100 r. p. m. These observations suggest that increasing the volume of liquid medium in the shaker flask or lowering the speed of the incubator shaker, conditions under which decreased amounts of O_2 would be present, led to an increase in fusarin C production. Similar aeration effects on mycotoxin production have been reported by Miller *et al.* (1983) and Shih *et al.* (1974). Recently Jackson *et al.* (1989) reported that zinc regulates ammonium assimilation, carbohydrate synthesis, and fusarin C biosynthesis in submerged cultures of *F. moniliforme*. However, the mechanism by which zinc regulates these metabolic processes is still unknown.

In this report, we have shown that the parameters of temperature (28°C to 35°C), aeration (100 r. p. m.), pH (3.5 to 4.5), and sugar concentration (30 to 40%) are important for optimal fusarin C biosynthesis. Of the three carbohydrates tested (all at 40 g/l), sucrose with 464 μg/ml of fusarin C produced was the best carbon source followed by glucose with 163 μg/ml by the PCA 5-3 isolate, and starch, the poorest, with 7 μg/ml of mycotoxin. Our *F. moniliforme* isolates produced a lower amount of fusarin C in liquid cultures as compared with North American strains (Farber and Sanders, 1986a). These results may be due to the geographical origin of the fungi; however, genetic differences still can not be ruled out. The differences in the effects of pH, temperature, aeration, and carbohydrate levels on the production of fusarin C by *F. moniliforme* in liquid medium possibly suggest that the fungi are producing fusarin C in response to different environmetal stresses, such as nutrient limitation. Further investigations will study the effects of metals on fusarin C biosynthesis by our *F. moniliforme* isolates as well as the mechanism by which they regulate toxin production.

Table 3. *Effects of aeration and culture volume on fusarin C biosynthesis by* ***F. moniliforme*** *PCA5-3 in defined medium*

Aeration (r.p.m.)	Fusarin C (μg/L)	Culture volume (ml)	Fusarin C (μg/L)
Nonshaking	57.62*	12.5	195.24
25	81.49	25.0	167.42
50	144.16	50.0	345.01
100	362.54	75.0	424.03
150	50.24	100.0	665.82
200	20.16	150.0	876.08

*Average of triplicate determinations.

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探討影響台灣 *Fusarium moniliforme* 菌株產生 fusarin C 眞菌毒素之因子

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本文旨在探討從本省玉米田中分離之 *F. moniliforme* 菌株，接種於特定培養基(Defined medium)，在改變其營養成份及培養環境下，觀察其合成 fusarin C 之能力。結果顯示，溫度(25°C～35°C)，通氣(100 r. p. m.)，酸鹼度(pH 3.5～4.5)和醣類(30～40%)爲合成毒素之重要因子。若以三種碳水化合物而言(培養基中含量皆爲 40 g/L)，發現蔗糖(464 μg/ml)爲最佳碳源促進產毒者，其次爲葡萄糖(163 μg/ml)，而澱粉(7 μg/ml)最差。很明顯地，台灣收集之 *F. moniliforme* 菌株在液體培養基下之產毒量，與北美洲之菌株比較，顯著偏低。