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Production of fusarin C mycotoxin by *Fusarium moniliforme* isolates of Taiwan

Tsung-Che Tseng[1], Chun-Shiang Chung and Ishien Li
Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China

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Abstract. Tweleve isolates of *Fusarium moniliforme* collected from corn in Taiwan were screened for the ability of production of fusarin C mycotoxin on corn. The toxin was analyzed based on thin-layer, gas and high-performance liquid chromatographies, and it was further confirmed by mass spectrometry. Results showed that more than 83% of tested isolates was able to produce fusarin C when growing on corn cultures in ranging from 50 to 4,830.2 μg/kg dry weight. The isolate of PCA 5-1 is the best fusarin C producer. All of the toxin producers synthesized a great amount of fusarin C over a 4-week period at 32°C. This is an original report of subtropic *F. moniliforme* isolates being able to produce fusarin C.

Key words: Fusarin C; *Fusarium moniliforme*.

Introduction

Fusarium moniliforme Sheldon occurs on a great variety of crop plants and is one of the prevalent fungi associated with corns worldwide (Marasas *et al.*, 1984a). This organism produces mycotoxins which cause human and animal diseases (Kriek *et al.*, 1981a and 1981b; Marasas *et al.*, 1979, 1981 and 1984b). Of the toxins previously known to be produced by this fungus, e.g. zearalenone (Nelson *et al.*, 1973), moniliformin (Cole *et al.*, 1973) and toxin which causes a fatal disease in horses (equine leukoencephalomalacia) (Wilson *et al.*, 1973), none have been proved to be mutagenic. Until a highly mutagenic and suspect carcinogenic compound, fusarin C, was first isolated from American strain of *F. moniliforme* (Wiebe *et al.*, 1981).

Production of fusarin C by *F. moniliforme* and *Fusarium* spp. is reported from various countries (Bjeldanes and Weib, 1980; Gelderblom *et al.*, 1983; Cheng *et al* ., 1985; Farber and Sanders, 1986a, 1986b). Recently, it has been proved that *Fusarium* species (*F. culmorum, F. graminearum, F. sporotrichiodes, F. poes, F. tricinctum* and *F. avenaceum*) isolated from European crops and soils are able to produce fusarin C (Thrane, 1988). There is little information in the literature to indicate whether subtropic isolates of *F. moniliforme* are capable of producing fusarin C. The objective of this work was to screen for the production of fusarin C in local isolates of *F. moniliforme* in Taiwan.

Materials and Methods

Organisms

Tweleve isolates of *F. moniliforme* collected from corns which were noticed pink discoloration either on kernels or stems in fields of various districts of Taiwan, including Hsinying (Isolates PCA 2-1 & 2-2), Tainan (PCA 2-3, PCA 3-1), Yungkang (PCA 3-2), Luchu (PCA 5-1), Tsoying (PCA 5-3), Kaohsiung (PCA 5-4), Fengshan (PCA 5-5), Pingtung (PCA 5-6), Hsinyuan (PCA 6-1) and Neipu (PCA 6-2). All cultures were isolated by single spore isolation technique (Hansen, 1946), and the Nelson, Toussoun and Marasas sys-

[1]Corresponding author.

tem was adopted for identification (Nelson *et al.*, 1983).

Screening for Fusarin C Producing Strains

Inoculation. All isolates were grown on 2% malt extract agar (Difico Laboratories) for 7 days at 26°C. The whole slant was then transferred into a 250-ml Erlenmeyer flask which contained 50 g of autoclaved whole corn grain with 20 ml distilled water as a solid substrate. Triplicate corn cultures were inoculated and incubated at various temperatures and incubation periods. All experiments were performed under fluorescent lighting to prevent the sensitivity of fusarin C to U V light.

Extraction. A modified Farber and Sander's method (1986a) was used. Fifty grams of corn culture were removed and blended (Blender 7011 Model, Waring products) at full speed for 2 min in 100 ml of CH_2Cl_2-CH_3CN (1 : 1). The blended materials were then passed through a Büchner glass funnel, evaporated to dryness under vacuum at 30°C, and resuspended in 10 ml of 3% CH_3OH in CH_2Cl_2. A suspension (5 ml) was fractionated on a silica column (2.5×16 cm, kieselgel 60; 70-230 mesh ASTM, E. Merck), which was eluted with 60 ml of 10% CH_3OH in CH_2Cl_2. The eluant (orange colour) was evaporated to dryness under vacuum at 30°C, reconstituted in 1 ml of $CHCl_3$, and then applied to a Sep-Pak Silica Cartriges (part No. 51900, Waters Associates), previously rinsed with 2 ml methanol. It was then eluted successively with 5 ml each of n-hexane, ether and $CHCl_3$: CH_3OH_3 (19 : 1). The $CHCl_3$: CH_3OH eluate was collected and concentrated to 1 ml for further analysis.

Analysis of fusarin C: The extract was initially applied to a TLC plate (Silica gel 60, E. Merck). After being spotted or streaked with 50 or 500 μl of extract, it was 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. The band cornesponding 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 was served as purified extract. Gas chromatography (GC) and high-performance liquid chromotography (HPLC) were used for analytical analyses, while mass spectrometry (MS) was used to confirm the presence of fusarin C. A gas chromatograph (Hitachi Model 163) equipped with FID detector was used. Separations were carried out on a SE-30 column (3 mm× 1m, 10% Chromosorb W, 80-100 mesh) with nitrogen as carrier gas (30 ml/min), column temperature setting at 170°C and both injector and detector temperatures at 190°C. The eluant samples from TLC plate which contaimed suspected fusarin C were analyzed their trimethylsilyl ester derivatives by using Tri-Sil-TBT as silylation reagents (Pierce Chemical Co. Box 117, Rockford, I11), similarly as previous described by Tseng *et al.*, (1985). HPLC analyses were performed on a Waters Associates Instrument (Model ALC/G C-204) equipped with a M-6000 A pump, a U6K universal injector and a Model 440 UV detector (365 nm), it was also served for quantitative analysis. Separations were carried out on a LiChrosorb RP-18 column (4 mn×25 cm; 10 μm, E. Merck) with CH_3OH-$CHCl_3$ (1 : 19) as the mobile phase at a flow rate of 1.0 ml/ml. 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. Vleggaar, National Chemical Research Laboratory Council for Scientific and Industrial Research Pretoria, South Africa.

Mass spectrometry was done on TLC-purified extracts obtained from *F. moniliforme* PCA 5-1 growing in corn kernal as a substrate, to confirm the presence of fusarin C (EIMS molecular ion $M^+=431$). A Joel TMS (model D-100) mass spectrometer operated as the following settings: Ion source temperature 180°C, ionizing energy 75 eV, ionizing current 300 μA, electron multiplier voltage 1.3 KV. Use output range 0.03 V and scanning speed 10 cm/sec.

Results and Discussion

Preliminary experiments illustrated that the extracts of *F. moniliforme* isolates were identified to have fusarin C, based on the presence of a bright yellow spot on TLC plate under visible light with a R_f value range from 0.31-0.35 (Fig. 1). A similar result was also reported by Farber and Sanders (1986a).

A TLC-purified extract from *F. moniliforme* PCA 5-1 was further confirmed by the presence of fusarin C (molecular ion $M^+=431$, $C_{23}H_{29}NO_7$) (Fig. 2). Attempts were made to analyze fusarin C from purified extract of sample PCA 5-1 by gas chromatography. The TMS derivates of the sample showed a peak (Rt=3.0 min) in the same position as authentic fusaric C (Fig. 3). Upon spiking the extract with authentic fusarin C and then

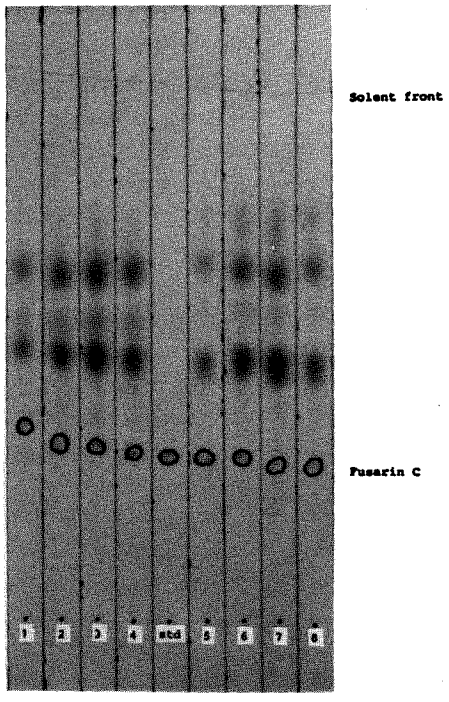

Fig. 1. Thin-layer chromatograms of extracts of *F. moniliforme* isolates. Developing solvents: $CHCl_3$-CH_3OH (9 : 1 : v : v); lanes 1-8, *F. moniliforme* PCA 2-3, PCA 3-1, PCA 3-2, PCA 5-1, PCA 5-3, PCA 5-4, PCA 5-5, PCA 6-1; Std=fusarin C.

Table 1. *Production of fusarin C by the isolates of* ***Fusarium moniliforme*** *growing on corn at various temperatures and incubation periods*

Isolates	Fusarin C (μg/kg dry weight)					
	28°C			32°C		
	2	3	4wk	2	3	4wk
PCA 2-1	82.6[a]	132.2	360.7	84.6	186.3	399.2
PCA 2-2	50.0	72.3	87.5	57.6	82.7	98.2
PCA 2-3	346.4	756.5	980.4	484.4	810.6	1080.2
PCA 3-1	735.3	1860.4	3240.3	890.3	2600.7	3980.4
PCA 3-2	220.3	237.3	936.2	380.2	841.5	900.8
PCA 5-1	790.2	1846.2	2802.1	690.3	1900.4	4830.2
PCA 5-3	996.1	2604.1	3420.7	930.2	2730.2	3612.0
PCA 5-4	460.5	1245.6	3243.6	710.0	2130.1	4210.0
PCA 5-5	302.8	822.1	1242.2	24.7	936.0	1510.6
PCA 5-6	ND[b]	ND	ND	ND	ND	ND
PCA 6-1	765.7	1488.4	2976.5	800.4	1610.5	3120.5
PCA 6-2	ND	ND	ND	ND	ND	ND

[a]Average of triplicate determinations.
[b]Not detectable.

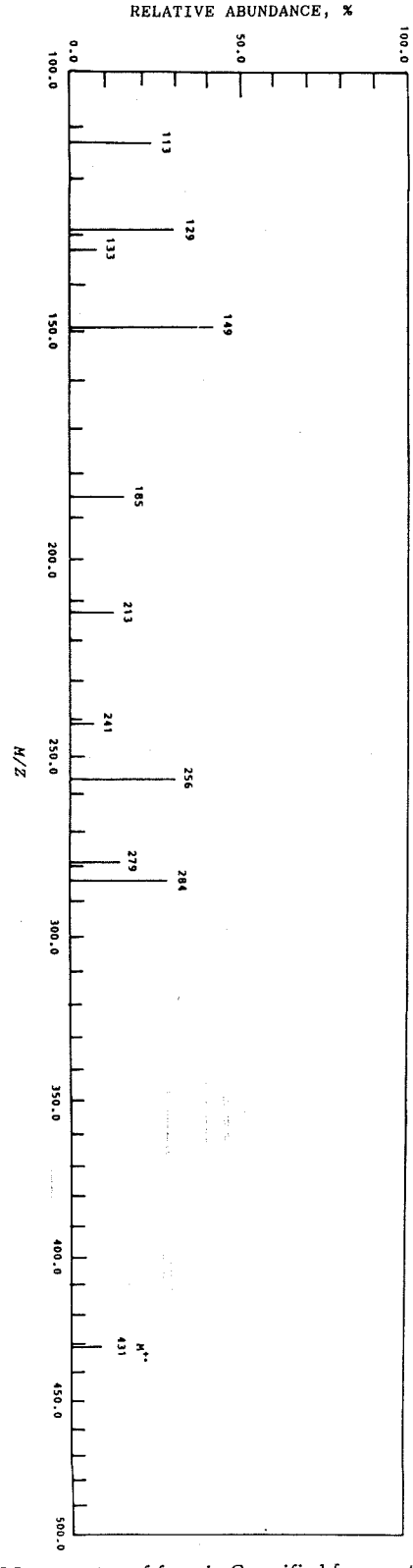

Fig. 2. Mass spectra of fusarin C purified from extract of sample PCA 5-1.

analyzed by GC, we again detected an increased single symmetrical peak in the position of fusarin C. The limit of detection was about 0.01 μg/g. This analytical method which developed from our laboratory will be an useful technique for fusarin C analysis both in cultural extracts as well as in cereal products.

The fusarin C content of samples from *F. moniliforme* isolates was quantified by comparing the peak heights of fusarin C peaks in HPLC chromatograms with those of standard fusarin C solutions. HPLC analysis of purified extract of PCA 3-2 showed a peak eluting in the same position as fusarin C (Fig. 4).

The amounts of fusarin C produced by the twelve isolates of *F. moniliforme* are shown in Table 1. Ten out of twelve tested isolates were able to produce fusarin C when growing on corn in amounts ranging from 50 to 4830.2 μg/kg dry weight. Isolate of PCA 5-1 is the best fusarin C producer. The effect of temperature on fusarin C biosynthesis in the current study is significant. All of the toxin producers except PCA 3-2 produced higher amount of fusarin C over a 4-week period at 32°C as compared with those observed at 28°C for 4 weeks and else treatments.

Previous study also performed with corn cultures demonstrated that the 14 isolates of North American *F. moniliforme* to produce fusarin C at 28°C (3 weeks), ranging from 18.7 to 332 μg/g dry weight (Farber and sanders, 1986a). Also, Gelderblom *et al.*, (1984) reported that 20 strains of *F. moniliforme* were able to produce fusarin C on corn in amounts ranging from 63 to 724 μg/g dry weight, when the corn cultures were incubated for 2 weeks at 25°C, followed by 2 weeks at 15°C. Apparently the Taiwanese isolates of *F. moniliforme* used in this study produced the lowerest amounts of fusarin C as compared with the South African and North American strains, also optimal temperature for the production of fusarin C by our isolates is higher than others in the previous reports. The significance of

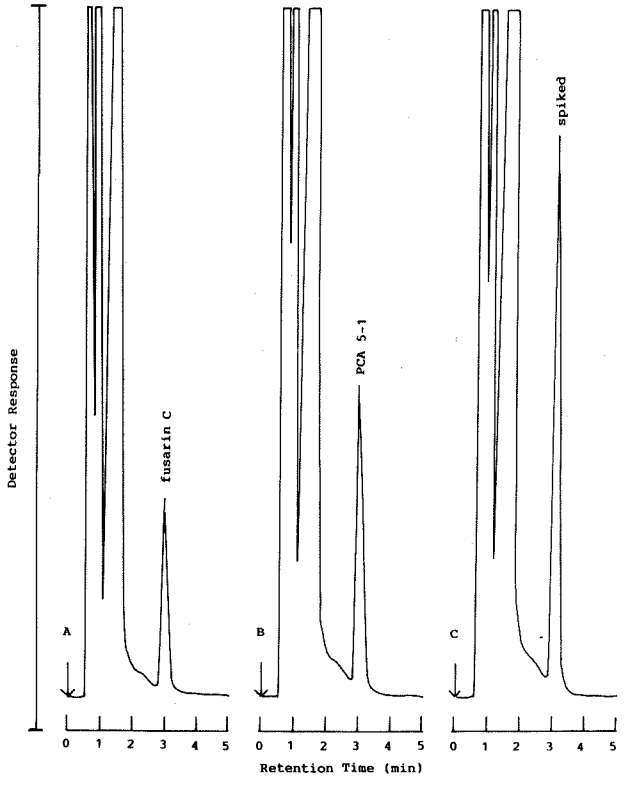

Fig. 3. Gas chromatograms of the TMS derivatives of fuarin C standard (0.034 mg/ml) (A), the purified (B) and spiked with the purified extracts of sample PCA 5-1 (C).

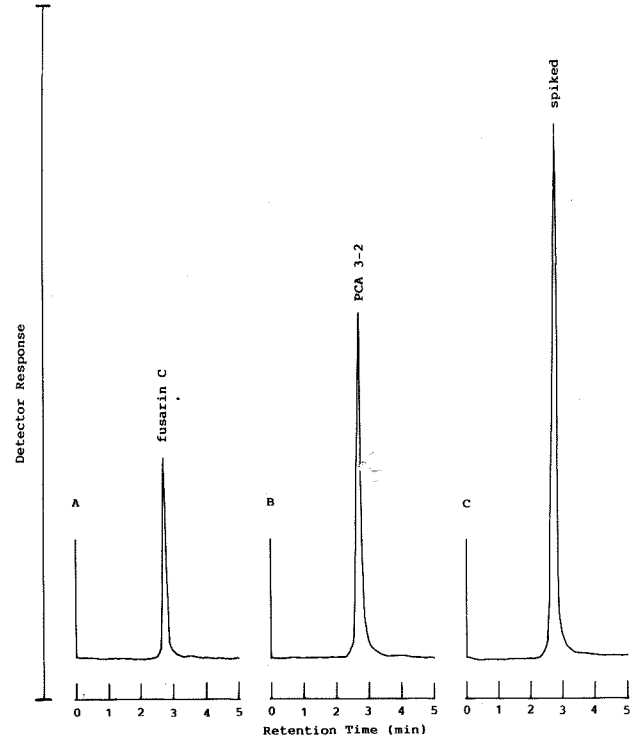

Fig. 4. LC chromatograms of 1 μl fusarin C standard (0.027 mg/ml) (A), the purified (B) and spiked with the purified extracts of sample PCA 3-2 (C). Detector response at 365 nm; sensitivity at 0.1 AUFS.

this is unclear at present. Farber and Sander (1986a) have indicated that slight changes in aeration, temperature, and pH can have drastic effects on the biosynthesis of fusarin C by *Fusarium* spp. However, the difference due to the geographical origin of the fungi still can not be ruled out.

In summary, this is an original report of Taiwanese isolates of *F. moniliforme* being able to produce fusarin C. Since fusarin C is a highly mutagenic metabolite and it is suspected to be potentially carcinogenic to humans (Gelderblom *et al.*, 1984), the further investigations will include to screen local *Fusarium* spp. instead of *F. moniliforme* which may produce the toxin and to study on parameters which will affect on fusarin C biosynthesis.

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台灣 *Fusarium moniliforme* 菌株產生 Fusarin C 眞菌毒素之研究

曾聰徹　鐘春香　李宜賢

中央研究院植物研究所

探討台灣玉米栽培地區收集之 *Fusarium moniliforme* 菌株，並篩選其在生體外 (*In vitro*) 產生眞菌毒素 Fusarin C 之能力。毒素之檢測係採用薄層色層分析，氣相色層分析以及高壓液相分析方法，並使用質譜儀加以確定。實驗結果，發現 83% 以上之被試菌株，在玉米培養基中，皆具產毒能力，其產毒量介於 50 至 4,830.2 μg/kg dry weight 之間，其中，菌株編號 PCA 5-1 爲 Fusarin C之最佳產毒者。同時發現所有產毒菌，其最適產毒之條件爲培養於 32°C，4 星期。這是從亞熱帶地區所分離之 *F. moniliforme*，首次發現具產生 Fusarin C 眞菌毒素之研究報告。