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Effects of Temperature and Incubation Period on Production of Fumonisin B_1 by *Fusarium moniliforme*

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The kinetics of the production of fumonisin B_1 (FB_1) by *Fusarium moniliforme* MRC 826 in corn cultures was investigated as a function of fungal growth at various incubation temperatures. The growth rate of *F. moniliforme*, as measured by ergosterol concentration, was higher at 25°C than at 20°C, reaching a stationary phase after 4 to 6 weeks in both cases. FB_1 production commenced after 2 weeks during the active growth phase, continued to increase during the stationary phase, and decreased after 13 weeks. The overall maximal yield of FB_1 (17.9 g/kg, dry weight) was obtained in corn cultures incubated at 20°C for 13 weeks, but it was not significantly ($P > 0.05$) higher than the maximum yield (16.5 g/kg, dry weight) obtained at 25°C after 11 weeks. However, a significantly ($P < 0.05$) higher mean yield was detected at 25°C (9.5 g/kg, dry weight) than at 20°C (8.7 g/kg, dry weight). Production reached a plateau after 7 weeks of incubation at 25°C or 9 weeks of incubation at 20°C. The maximal production of FB_1 at 30°C was very low (0.6 g/kg, dry weight). FB_1 was also found to be heat stable, as there was no reduction in the FB_1 concentration after boiling culture material of *F. moniliforme* MRC 826.

Fusarium moniliforme Sheldon is one of the most prevalent fungi associated with basic human and animal dietary staples such as corn (10, 13–15). Culture material of *F. moniliforme* MRC 826, isolated from corn intended for human consumption in Transkei, southern Africa, proved to be highly toxic to a variety of experimental animals (6, 8, 9), has been shown to cause leukoencephalomalacia in horses (8) and to be hepatocarcinogenic in rats (7, 12), and has cancer-promoting activity in a short-term cancer initiation-promotion model in rats (3, 4). The latter bioassay was used in the isolation and purification of two new mycotoxins, fumonisins B_1 (FB_1) and B_2 (FB_2), from culture material of *F. moniliforme* MRC 826 (2). The structures of FB_1 and FB_2 have recently been elucidated (1), and FB_1 has been shown to have cancer-promoting activity in rats (2) and to cause leukoencephalomalacia in a horse (11).

The fumonisins may be of major importance in the etiology of several animal and human diseases. However, for a proper evaluation of the toxicological effects of these mycotoxins, sufficient amounts have to be purified for a variety of biological studies. At present, no information is available on experimental conditions for the optimal production of the fumonisins in culture. This paper reports the chemical determination of FB_1 in corn cultures of *F. moniliforme* and the influence of temperature and incubation period on the production of FB_1 in these cultures. In addition, the relationship between fungal growth and FB_1 production as well as the heat stability of this mycotoxin were also investigated.

MATERIALS AND METHODS

Fungal cultures. The strain of *F. moniliforme* used in all experiments was originally isolated from corn in Transkei, southern Africa, during 1975 and deposited in the culture collection of the South African Medical Research Council (MRC) as *F. moniliforme* MRC 826 (14). Lyophilized conidia were used to inoculate autoclaved (1 h at 121°C and 120 kPa on each of two consecutive days) corn (400 g of whole yellow corn kernels and 400 ml of water in 2-liter wide-mouthed glass fruit jars with a diameter of 11 cm and a cotton cover), and cultures (surface/volume ratio, approximately 2:5) were incubated in the dark at 20, 25, and 30°C. Triplicate jars were harvested weekly; their contents were weighed, lyophilized, and ground; and the concentration of FB_1 was determined as described below.

Chemical analyses. (i) Fumonisin B_1 standard. FB_1 was isolated and purified as described previously (2). For use as an analytical standard, FB_1 was further subjected to two successive column separations on silica gel and reverse phase (C_{18}) columns, using $CHCl_3$-CH_3OH-CH_3COOH (6:3:1) and CH_3OH-H_2O (3:1) as eluants, respectively. The purity of FB_1 was verified by nuclear magnetic resonance spectroscopy.

(ii) Extraction and HPLC quantification. Culture extracts were prepared and partially purified by the maleyl derivatization method described previously (20). Briefly, culture material (5 g) was extracted with CH_3OH-H_2O (3:1; 50 ml). A sample (25 ml) was evaporated to dryness, taken up in an equal volume of CH_3OH-H_2O (1:3), and extracted twice with $CHCl_3$ (50 ml). Final purification of the aqueous phase was accomplished by using a Sep-pak C_{18} cartridge, eluting FB_1 in CH_3OH-H_2O (3:1; 10 ml). Purified extracts were treated with maleic anhydride and analyzed by high-performance liquid chromatography (HPLC) by the method of Siler and Gilchrist (18). HPLC analyses were performed on a Waters liquid chromatograph (Waters Associates, Milford, Mass.), using a reverse-phase cartridge (C_{18}; 10 μm) in a radial compression module (model RCM-100) and 0.05 M KH_2PO_4-CH_3OH (3:7; pH 3.5) as the mobile phase. The eluate was monitored at 230 nm. To determine whether the HPLC technique is quantitative at both low and high FB_1 concentrations, a standard curve was composed by analyzing

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different amounts (1, 2, 3, 4, and 5 μg) of pure FB_1 standard after maleylation. For determination of the percent recovery, control corn (5 g) was spiked in triplicate with 1.5 mg of pure FB_1 standard and analyzed as described above.

(iii) Ergosterol analyses. Ergosterol determinations were performed in duplicate on corn cultures of *F. moniliforme* MRC 826 incubated for 0, 2, 4, 6, 8, 10, 13, 15, and 17 weeks at 20, 25, and 30°C. The method of Seitz et al. (17) was used, with minor modifications. Samples (5 g) were extracted with methanol, the methanol extract was saponified for 30 min, and the mixture was extracted with petroleum ether (bp 60 to 80°C). After evaporation of the aqueous phase, the residue was dissolved in 2.5 ml of CH_2Cl_2-isopropanol (98:2) and a 100-fold dilution was used for HPLC analysis on a Waters liquid chromatograph, using a silica gel (Microsil) column (5.0 mm by 30 cm; 7 μm) and CH_2Cl_2-isopropanol (98:2) as the mobile phase. The eluate was monitored at 280 nm.

Stability to heat. Lyophilized culture material of *F. moniliforme* MRC 826 (500 g) was boiled in 500 ml of water for 30 min, after which it was oven dried at 60°C for 24 h. Both boiled and untreated materials were incorporated in rat mash (Epol Ltd., Johannesburg, South Africa) at a dietary level of 5% and fed to diethylnitrosamine-initiated male BD IX rats over a period of 4 weeks (3). Noninitiated rats receiving the untreated (nonheated) culture material (5%) served as the controls. The induction of gamma-glutamyl-transpeptidase-positive foci, a common marker for preneoplastic lesions (5), was used as endpoint for the assay. In addition, the boiled and untreated materials were analyzed for FB_1 by HPLC as described above.

Statistical analyses. All analyses were performed by using the general linear model analysis of variance procedure of the Statistical Analysis System program package. The Student-Newman-Keuls multiple comparison method was used to test for significant differences between main-effect means. Significance tests for differences between the yields at 20 and 25°C for the separate weeks were performed by using a significance level adjusted for the number of tests performed. The method used was Bonferroni inequality; i.e., an observed difference is deemed significant if its P value is less than $P' = 1 - (1 - P)^{1/n}$, where P is desired overall significance level and n is the number of weeks for which observations were taken.

RESULTS AND DISCUSSION

The analytical technique used for the quantification of FB_1 in culture material of *F. moniliforme* MRC 826 had a recovery of approximately 85%. The detection limit of the method was in the order of 10 μg/g, as reported by Sydenham et al. (20). HPLC chromatograms demonstrating the eluting position of FB_1 standard and a purified extract of a *F. moniliforme* MRC 826 corn culture are illustrated in Fig. 1. The standard curve indicated that, for the concentration range used, a linear relationship ($r = 0.9955$) exists between the recorder response and the amount of FB_1 analyzed. The method described is suitable for the determination of FB_1 in extracts of corn cultures of *F. moniliforme* MRC 826 as a result of the high quantities found to be produced by this strain in culture.

The fumonisins have recently been found to occur naturally in corn in South Africa (20) and the United States (21). These findings emphasize the importance of screening human and animal foodstuffs for the presence of these mycotoxins. However, to assess human exposure, the heat stability of the fumonisins is of major importance. In the present study, no difference in the FB_1 concentration of boiled and untreated culture materials could be detected by HPLC (Table 1). In addition, culture material also retained its cancer-promoting (gamma-glutamyl-transpeptidase-positive) activity in diethylnitrosamine-initiated rats (Table 1). The data indicate that FB_1 is not destroyed by cooking and could therefore enter the human food chain.

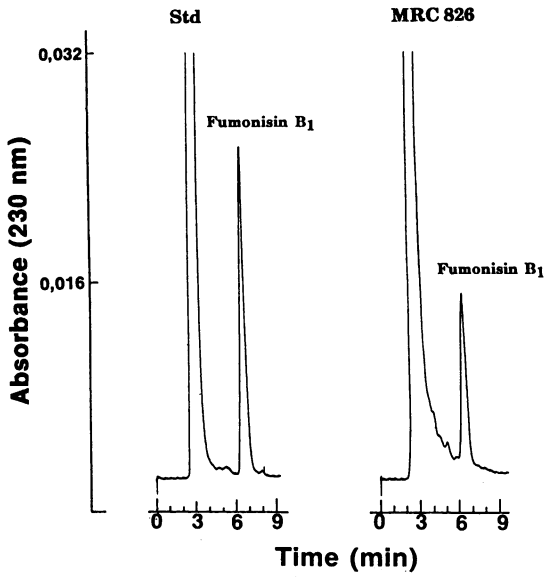

FIG. 1. HPLC-eluting chromatograms of FB_1 standard (Std) and a purified extract of culture material of *F. moniliforme* MRC 826.

The optimum incubation temperature for growth of *Fusarium* spp. in culture has been reported to be alternating at 25°C day/20°C night, although they also grow well at constant temperatures between 20 and 25°C (16). As incubation of fungal cultures at alternating temperatures is impractical, we investigated the production of FB_1 at constant temperatures of 20, 25, and 30°C. The ergosterol content of the fungal cultures was used as an indication of fungal growth, since it is a useful indicator of fungal invasion of grains (17), measuring both viable and nonviable fungal biomass.

Kinetics of the growth of *F. moniliforme* MRC 826 in corn

TABLE 1. Effect of heat treatment on FB_1 concentration and cancer-promoting activity of corn culture material of *F. moniliforme* MRC 826 in diethylnitrosamine (DEN)-initiated rats

MRC 826 sample	FB_1 concn (g/kg, dry wt)[a]	DEN initiation[b]	Mean GGT activity (foci/cm^2)[c]
Untreated	2.3 ± 0.6	−	ND
Untreated	2.3 ± 0.6	+	4.85 ± 0.92
Boiled[d]	2.5 ± 0.2	+	3.45 ± 0.71

[a] Values represent means ± standard deviations of triplicate determinations.

[b] Indicates whether or not the rats to be tested for cancer-promoting gamma-glutamyl-transpeptidase activity had been initiated with DEN (200 mg/kg). The promoting treatment commenced 1 week after initiation. Boiled and untreated culture material was incorporated in rat mash at a dietary level of 5% for a period of 4 weeks, five rats per treatment.

[c] Values represent means ± standard deviations of five rats. GGT, Gamma-glutamyl-transpeptidase; ND, none detected.

[d] Boiled in water for 30 min and dried at 60°C for 24 h.

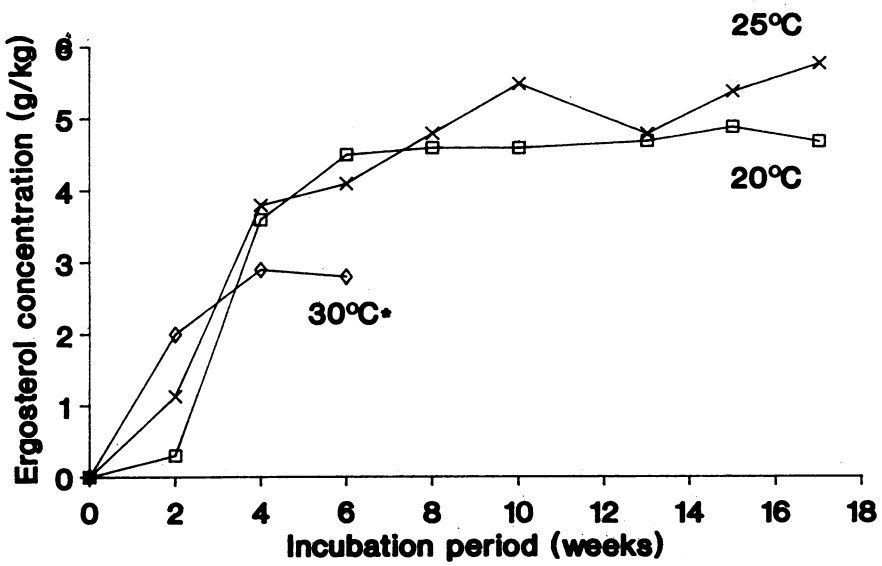

FIG. 2. Time course of growth as measured by ergosterol concentration of *F. moniliforme* MRC 826 in corn cultures at 20, 25, and 30°C. *Cultures not further analyzed during the remainder of the experiment.

cultures at 20, 25, and 30°C are presented in Fig. 2. During the first 2 weeks of incubation, cultures at 30°C grew significantly better than those at 20°C ($P < 0.01$) and 25°C ($P < 0.05$), while after 4 weeks the cultures incubated at 20 and 25°C had higher ergosterol concentrations than those incubated at 30°C; only that obtained at 25°C, however, was significant ($P < 0.05$). After 6 weeks, significantly ($P < 0.05$) higher levels of ergosterol were detected in cultures incubated at 20 and 25°C than at 30°C, and no further increase occurred in the cultures incubated at 30°C. Although the initial growth rate was higher at 25°C than at 20°C, the maximum growth rates were similar, reaching a stationary phase at 4 to 6 weeks. In this experiment, the optimum conditions for growth of *F. moniliforme* MRC 826 prevailed in cultures incubated at 20 and 25°C.

As our culture vessels and incubation conditions allowed evaporation, changes in the moisture content of cultures during incubation almost certainly influenced both growth and FB_1 production. This is evident from the data concerning the weight loss (Fig. 3) of the cultures incubated at the different temperatures. From 2 weeks onward, the cultures at 30°C had lost significantly more weight than those at 25°C ($P < 0.05$) and 20°C ($P < 0.01$). The weight loss between cultures incubated at 20 and 25°C also differed significantly ($P < 0.01$) from 2 weeks onward. This difference increased markedly with time, and the weight loss after 4 weeks at

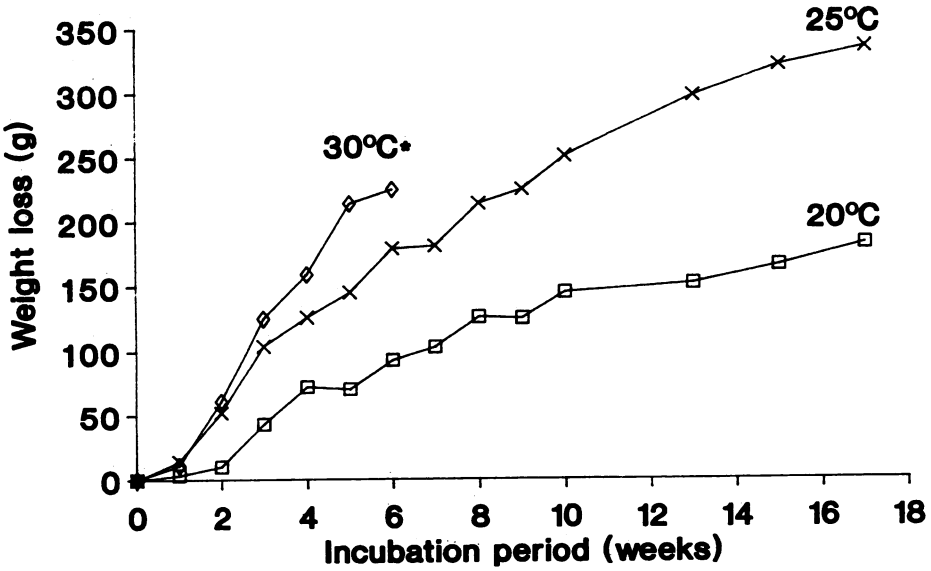

FIG. 3. Time course of weight loss of corn cultures of *F. moniliforme* MRC 826 at 20, 25, and 30°C. *, Cultures not further analyzed during the remainder of the experiment.

TABLE 2. Time course of FB_1 production by *F. moniliforme* MRC 826 in corn cultures at 20 and 25°C

Wk	FB_1 concn (g/kg, dry wt)[a]		
	20°C	25°C	P[b]
0	ND	ND	NS
1	ND	ND	NS
2	0.4 ± 0.25 a	1.2 ± 0.38 a	NS
3	3.5 ± 0.91 ab	6.7 ± 1.10 b	<0.05
4	6.1 ± 0.62 bc	9.3 ± 3.26 bc	<0.01
5	5.4 ± 1.08 bc	11.6 ± 1.00 cd	<0.01
6	7.4 ± 3.78 c	10.3 ± 1.28 c	<0.05
7	8.9 ± 0.79 cd	14.6 ± 3.21 de	<0.01
8	11.2 ± 2.10 d	11.7 ± 1.18 cd	NS
9	15.3 ± 0.85 e	11.5 ± 1.55 cd	<0.01
10	15.2 ± 1.59 e	12.7 ± 0.33 cd	NS
11	16.2 ± 0.62 e	16.5 ± 2.36 e	NS
13	17.9 ± 1.84 e	15.4 ± 1.47 de	NS
15	14.3 ± 1.40 e	12.3 ± 0.76 cd	NS
17	8.1 ± 3.27 cd	9.1 ± 0.71 bc	NS
Mean	8.7	9.5	<0.05

[a] Values represent means ± standard deviations of triplicate determinations. Means in a column followed by the same letter do not differ significantly ($P > 0.05$). ND, Not detected.

[b] Denotes significance of differences between means in rows (20 and 25°C). NS, Not significant.

25°C was identical to that of the cultures at 20°C after 8 weeks. The weight loss observed in these cultures cannot be ascribed solely to a loss in moisture content as fungal growth did occur (Fig. 2) and some carbon dioxide must have been produced. However, the weight loss in the cultures at 30°C between 4 and 6 weeks of the incubation treatment can only be ascribed to evaporation as no fungal growth occurred during this period (Fig. 2).

As the moisture content undoubtedly limited both fungal growth and FB_1 production at 30°C, data for FB_1 production at 30°C were not included in the statistical evaluation of the growth-dependent production at different temperatures.

Production of FB_1 at 20 and 25°C commenced at 2 weeks and continued to increase during the stationary phase, confirming the inverse relationship reported previously (19) between the rate of mycelial growth and the biosynthesis of many secondary metabolites of fungi. The concentration of FB_1 started to decrease at both temperatures after 13 weeks. Since FB_1 is heat stable, the decrease in the FB_1 concentration after incubation periods longer than 13 weeks probably stems from enzymatic cleavage of the molecule or a conversion to other related compounds or both.

The initial rate of FB_1 production was faster at 25°C than at 20°C (Table 2), with FB_1 production significantly ($P < 0.01$ to 0.05) higher at 25°C than at 20°C between weeks 3 and 7. An apparent maximum FB_1 yield (17.9 g/kg, dry weight) was obtained at 13 weeks at 20°C (Table 2), but this was not significantly ($P > 0.05$) higher than the maximum yield obtained after 11 weeks at 25°C (16.5 g/kg, dry weight). However, the mean FB_1 yield over the total incubation period was significantly ($P < 0.05$) higher at 25°C than at 20°C, with production reaching a plateau after 7 weeks of incubation at 25°C in contrast to 9 weeks at 20°C.

It can be concluded that, under the conditions of the present experiment, the optimal incubation regimen for FB_1 production by *F. moniliforme* MRC 826 in cultures on corn, in terms of cost effectiveness, is 7 weeks at 25°C. However, the effect of various moisture levels, at different incubation temperatures, on the production of FB_1 and other fumonisins needs to be investigated.

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