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Production of Trichothecene Mycotoxins by *Fusarium* Species in Shake Culture

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Twelve T-2 toxin-producing isolates and four fusarenon-X-producing isolates of *Fusarium* species were examined for their ability to produce trichothecene mycotoxins in shake culture and jar fermentation. T-2 toxin producers such as *Fusarium solani*, *F. sporotrichioides*, and *F. tricinctum* produced T-2 toxin and neosolaniol in semisynthetic medium. *F. solani* M-1-1 produced the largest amount of the mycotoxins in a nutrient medium consisting of 5% glucose (or sucrose), 0.1% peptone, and 0.1% yeast extract in either shake culture or jar fermentation at 24 to 27 C for 5 days. None of the isolates produced significant amounts of fusarenon-X in shake cultures.

Dozens of cytotoxic 12,13-epoxytrichothecenes, such as nivalenol, fusarenon-X, T-2 toxin, and neosolaniol, can be produced by several strains of *Fusarium* species (3, 4, 6, 7). These mycotoxins are divided into type-A (T-2 toxin, neosolaniol, diacetoxyscirpenol, HT-2 toxin) and type-B (fusarenon-X, nivalenol) trichothecenes according to the structural variation in the C-8 position and the classification differences in the mycotoxin-producing fungi (Fig. 1).

Various methods for the production of trichothecene mycotoxins have been reported (1, 2, 5), but none are satisfactory for a mass production of the toxins in either shake cultures or jar fermentations.

This paper reports the cultural conditions influencing the production of T-2 toxin, neosolaniol, and fusarenon-X by *Fusarium* species on three liquid media in shake cultures and jar fermentors.

MATERIALS AND METHODS

Organisms. Known T-2 toxin-producing *Fusarium* isolates used in this study were *Fusarium solani* M-1-1 and M-1-2, *Fusarium sporotrichioides* M-1-4, M-1-5, and NRRL 3510, *Fusarium roseum* "Avenaceum" M-11-1, and *Fusarium roseum* 70-K-11. These isolates have been reported to produce T-2 toxin by stationary culture using Czapek medium supplemented with peptone (PSC medium) (3, 4, 6, 7). In addition, four isolates of *Fusarium tricinctum* (A-R-5, A-R-6, A-R-7, and A-R-8), isolated in 1973 from "blackened rice grains" produced in Hokkaido, were also employed. These isolates were found to produce T-2 toxin under the same culture conditions (unpublished data). Four isolates of *Fusarium* species capable of producing high yields of fusarenon-X in stationary culture (4, 7) were also selected for study. Stock cultures of the organisms were maintained on sterile soil-peat moss-perlite (2:1:1) at 4 C and were subcultured on a potato-dextrose agar slant at 27 C for 2 weeks and used for inoculum.

Medium. The following three media were studied. Medium A contained glucose, 10 g; peptone, 1 g; yeast extract, 1 g; and water, 1 liter; medium B consisted of glucose, 40 g; corn steep liquor, 1 g; yeast extract, 2 g; and water, 1 liter (pH 5.4); and medium C contained Czapek-Dox medium supplemented with 10 g of peptone per liter (PSC medium) (5).

Culture conditions. Erlenmeyer flasks (500 ml) containing 250 ml of the medium per flask were stoppered with cotton plugs and autoclaved for 20 min at 120 C. Media were inoculated with spores and incubated at 27 C for 5 days on a rotary shaker operating at 150 rpm. Four flasks were used for each isolate. As a reference to stationary culture, *F. solani* M-1-1 and *F. nivale* Fn-2B were cultured in medium C at 27 C for 2 weeks.

Inoculum for the jar fermentor was produced by subculturing the mold at 24 C for 4 days in 1 liter of medium A with 5% glucose. The inoculum was aseptically transferred to a 50-liter jar fermentor containing 27 liters of the following medium: sucrose, 5%; peptone, 0.1%; yeast extract, 0.1%; and tap water to volume. The fermentation was carried out at 23 C for 5 days at 240 rpm and with 0.05% of silicone antiform reagent.

Analysis. Cultures were filtered, and the dry weight of mycelium was determined after drying the mycelial mat at 70 C for 24 h. Glucose content of the culture media was determined by the anthrone method. The crude toxin was prepared from culture filtrate according to the method previously reported (5), and the trichothecenes in the crude toxin were assayed by thin-layer chromatography with silica gel

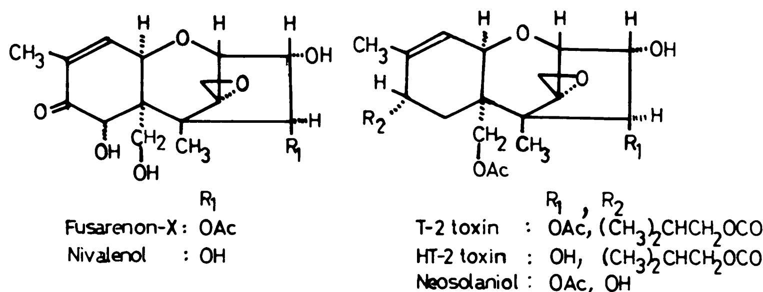

FIG. 1. *Structure of trichothecene mycotoxins.*

plates (Kieselgel G, 0.25 mm) developed in ethylacetate-*n*-hexane (3:1) (7). Plates were sprayed by 20% H_2SO_4 and heated at 100 C for 10 min, and the relative amount of T-2 toxin, neosolaniol, and fusarenon-X was determined quantitatively by visual comparison under ultraviolet light with known amounts of trichothecenes. Furthermore, T-2 toxin and neosolaniol were quantitated using gas-liquid chromatography; a derivative of T-2 toxin was made by mixing 3 mg of the crude toxin dissolved in 0.25 ml of anhydrous pyridine with 0.20 ml of hexamethyldisilazan and 0.05 ml of of trimethychlorosilane in a total volume of 0.5 ml and allowing the mixture to stand at room temperature for 24 h before use. For determination of neosolaniol content, the same amount of sample was mixed with 0.20 ml of trimethysilylimidazole and 0.05 ml of trimethylchlorosilane, and the silylation was accomplished by incubating the mixture at 60 C for 60 min followed by incubation at room temperature for 24 h before use. The gas-liquid chromatographic analyses were carried out on Shimazu gas chromatograph (model GC-4B) with the following conditions: coiled Pyrex column (3 mm by 1 m) packed with OV-17; operating temperatures—injection at 280 C, column at 240 C, and detection at 300 C; gas flow rate—nitrogen, 40 ml/min; air, 0.9 ml/min; and hydrogen, 0.7 ml/min.

RESULTS

Production of trichothecenes by shake culture. The 12 T-2 toxin-producing isolates were compared on the basis of mycelial growth, production of crude toxin, and production of the two trichothecenes in three kinds of culture media. The yields of mycelium, as well as the crude toxin, were higher when cultured in media B and C than in medium A (Table 1), whereas the thin-layer chromatographic determination revealed that the content of T-2 toxin and neosolaniol was relatively higher in the crude toxin from medium A than from media B and C. Among the 12 isolates tested, *F. solani* M-1-1 produced the largest amounts of T-2 toxin and neosolaniol in medium A. Fusarenon-X was produced in shake culture using medium B, but the yields were much lower than those obtained using stationary culture with media A and C (Table 2). Based on these preliminary results, *F. solani* M-1-1 and medium A were selected as standard isolate and medium for production of T-2 toxin and related mycotoxins.

Time course production of the trichothecene. Production of the fungal mat, crude toxin, and two trichothecenes by *F. solani* M-1-1 in medium A was found to be time dependent (Fig. 2). Changes in pH of the culture medium and consumption of glucose were also analyzed. Fungal growth was maximum on day 3 of incubation, and the glucose in the medium was completely consumed after 3 days of growth. The maximum amount of crude toxin was obtained on day 2 of incubation and it decreased thereafter. Thin-layer and gas-liquid chromatographic analyses revealed that biosynthesis of T-2 toxin preceded that of neosolaniol and that the maximum yield of T-2 toxin and neosolaniol, 20 and 7 mg/liter, respectively, was attained in 5 days of incubation. The yield of T-2 toxin was more than three times greater than that of neosolaniol during the whole course of shake culture fermentation.

Influence of carbohydrates on mycotoxin production. Influence of carbohydrate sources on fungal growth and trichothecene production was examined. *F. solani* M-1-1 was cultured for 5 days in medium A, in which various kinds of carbohydrates were added at a 1% level. Glucose, sucrose, and galactose supported high production of the mycotoxins. Substantially fewer trichothecenes were produced with the other carbohydrates (Table 3).

The effect of glucose concentration on fungal growth and T-2 toxin production was examined. The amount of the crude toxin increased proportionally to the concentration of glucose up to the 5% level of glucose. The content of T-2 toxin in the crude toxin also increased with increasing glucose concentration. Gas-liquid chromatographic determinations of the trichothecenes indicated that the culture filtrate with 1% glucose contained 13.5 mg of T-2 toxin and 2.25

TABLE 1. *Comparative producibility of T-2 toxin and neosolaniol by Fusarium spp. in shake culture*

Culture media	Species	Strain	Mycelia (g/liter)	Crude toxin (mg/liter)	Trichothecenes[a]	
					T-2 toxin	Neo-solaniol
Medium A	*F. solani*	M-1-1	2.8	130	+++	++
	F. solani	M-1-2	2.7	123	++	+
	F. sporitrichioides	M-1-4	3.1	111	+	+
	F. sporotrichioides	M-1-5	2.8	129	+	+
	F. roseum "Scirpi"	M-8-1	3.0	69	+	+
	F. roseum "Avenaceum"	M-11-1	2.8	76	+	+
	F. sporotrichioides	NRRL 3510	3.8	73	±	±
	F. roseum	70-K-11	4.3	78	+	±
	F. tricinctum	A-R-5	4.8	67	+	+
	F. tricinctum	A-R-6	4.5	63	+	+
	F. tricinctum	A-R-7	4.7	56	+	+
	F. tricinctum	A-R-8	3.4	80	+	+
	F. solani[b]	M-1-1	3.3	111	+	+
Medium B	*F. solani*	M-1-1	8.7	145	+	±
	F. solani	M-1-2	7.6	133	–	–
	F. sporotrichioides	M-1-4	8.1	132	+	±
	F. sporotrichioides	M-1-5	8.8	121	+	–
	F. roseum "Scirpi"	M-8-1	8.3	131	+	+
	F. roseum "Avenaceum"	M-11-1	6.7	128	+	+
	F. sporotrichioides	NRRL 3510	10.2	131	+	–
	F. roseum	70-K-11	10.4	152	+	–
	F. tricinctum	A-R-5	10.3	151	–	–
	F. tricinctum	A-R-6	7.0	160	–	–
	F. tricinctum	A-R-7	9.3	155	–	–
	F. tricinctum	A-R-8	10.1	124	–	–
	F. solani[b]	M-1-1	7.5	182	++	++
Medium C	*F. solani*	M-1-1	10.8	341	+	+
	F. solani	M-1-2	10.4	348	–	–
	F. sporotrichioides	M-1-4	12.1	299	++	+
	F. sporotrichioides	M-1-5	12.0	331	++	+
	F. roseum "Scirpi"	M-8-1	10.4	398	+	+
	F. roseum "Avenaceum"	M-11-1	10.2	410	+	+
	F. sporotrichioides	NRRL 3510	14.0	414	–	–
	F. roseum	70-K-11	11.4	456	–	–
	F. tricinctum	A-R-5	9.0	463	–	–
	F. tricinctum	A-R-6	9.5	502	–	–
	F. tricinctum	A-R-7	13.7	356	–	–
	F. tricinctum	A-R-8	11.9	408	–	–
	F. solani[b]	M-1-1	8.7	378	+++	++

[a] Indicated as the relative amounts of trichothecenes.
[b] Cultured stationary at 27 C for 2 weeks.

mg of neosolaniol per liter, and with 5% glucose it was found to contain 35 mg of T-2 toxin and 7.5 mg of neosolaniol per liter.

Effects of peptone, yeast extract, and metal ions on T-2 toxin production by F. solani M-1-1. The influence of peptone and yeast extract concentrations on T-2 toxin production by *F. solani* M-1-1 was examined using medium A containing 5% glucose. Peptone and yeast extract, both at 0.1% levels were critical for high production of T-2 toxin by *F. solani*, but higher concentrations of these nutrients reduced toxin production.

Metal ions such as $MnSO_4$, (NH_4) $MoPO_4$, $CuSO_4$, $Fe_2(SO_4)_3$, $MgSO_4$, and $ZnSO_4$ did not significantly increase the yield of either mycelium or T-2 toxin at levels of 3 g/liter.

Mass production of trichothecenes by F. solani M-1-1 in shake culture and jar fermentation. *F. solani* M-1-1 was inoculated into forty 500-ml flasks, each containing 250 ml of medium A with 1 or 5% glucose, and, after

TABLE 2. *Comparative producibility of fusarenon-X by Fusarium spp. in shake culture*

Culture media	Species	Strain	Mycelia (g/liter)	Crude toxin (mg/liter)	Trichothecene[a] (fusarenon-X)
Medium A	*F. nivale*	Fn-2B	4.1	53	−
	F. episphaeria	Fn-M	4.2	44	−
	F. oxysporum	T-M-1	3.9	46	−
	F. oxysporum	T-M-2	4.0	51	−
	F. nivale[b]	Fn-2B	2.2	138	+++
Medium B	*F. nivale*	Fn-2B	7.6	115	++
	F. episphaeria	Fn-M	7.1	107	±
	F. oxysporum	T-M-1	8.5	147	+
	F. oxysporum	T-M-2	8.1	131	+
	F. nivale[b]	Fn-2B	6.7	205	+
Medium C	*F. nivale*	Fn-2B	12.3	267	+
	F. episphaeria	Fn-M	12.9	240	±
	F. oxysporum	T-M-1	14.5	216	−
	F. oxysporum	T-M-2	15.4	261	−
	F. nivale[b]	Fn-2B	5.3	379	++++

[a] Indicated as the relative amount of toxin.
[b] Cultured stationary at 27 C for 2 weeks.

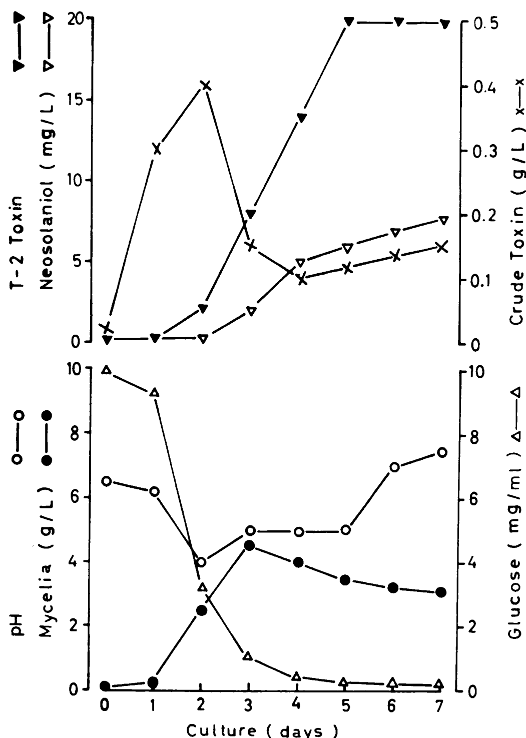

FIG. 2. *Time courses of fungal growth and trichothecenes production by F. solani M-1-1.*

shake culture at 27 C for 5 days with a rotary shaker operating at 150 rpm, the cultures were filtered. The yield of crude toxin was 4.4 g/10 liters from 5% glucose-medium A, and, after chromatography on a silica gel column with a mixture of *n*-hexane-acetone, 1.7 g of T-2 toxin and 0.5 g of neosolaniol were crystallized. With 1% glucose, the yield of crude toxin was 2.1 g/10 liters, from which 0.59 g of T-2 toxin and 0.14 g of neosolaniol were isolated (Table 4; Fig. 3). In the case of jar fermentation, *F. solani* M-1-1 was cultured in 27 liters of medium A containing 5% sucrose. After 5 days at 23 C, 25 liters of the culture filtrate was mixed with 250 g of powdered charcoal, and after mixing continuously for 60 min the charcoal was filtrated and washed with 5 liters of deionized water. The washed charcoal was then immersed in 5 liters of methanol for 3 h and 3 liters of methanol

TABLE 3. *Influence of carbohydrate source on trichothecene production by F. solani M-1-1*

Carbohydrates (1% in medium A)	Final pH	Mycelium (g/liter)	Crude toxin (mg/liter)	Trichothecenes (mg/liter)	
				T-2 toxin	Neosolaniol
Starch	5.74	3.3	104	10	5
Sucrose	5.03	3.3	151	20	10
Lactose	5.81	4.0	117	10	3
Maltose	5.10	3.9	85	10	3
D-Glucose	5.34	3.0	128	20	10
D-Mannitol	5.52	3.8	84	10	3
D-Sorbitol	5.12	3.6	73	10	2
D-Galactose	4.65	3.5	200	30	10
D-Xylose	4.82	3.6	132	10	3
D-Fructose	4.78	4.1	132	10	5
Glycerol	4.77	4.4	77	5	1

TABLE 4. *Production of trichothecenes by F. solani M-1-1 in shake culture and jar fermentation*

Culture	Medium (%)	Crude toxin (g/10 liters)	Yields of trichothecenes (g/10 liters)		
			T-2 toxin	Diacetoxy-scirpenol	Neosolaniol
Shake culture (A)	Glucose (1) Peptone (0.1) Yeast extract (0.1)	2.1	0.59		0.14
Shake culture (B)	Glucose (5) Peptone (0.1) Yeast extract (0.1)	4.4	1.70		0.50
Jar fermentation (C)	Sucrose (5) Peptone (0.1) Yeast extract (0.1)	3.0	0.81	0.23	0.76

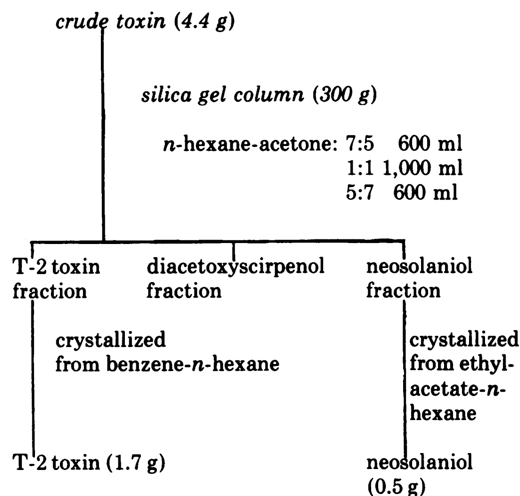

FIG. 3. *Procedure for isolation of T-2 toxin and neosolaniol from crude toxin obtained by shake culture of F. solani M-1-1 growing in 5%-glucose medium A.*

for 1 h. This procedure resulted in 6 g of the crude toxin. The crude toxin was mixed with 10 g of silica gel, and the mixture was placed on top of silica gel column (350 g of Kieselgel, 5 by 70 cm). Development with a mixture of *n*-hexane-acetone resulted in 0.81 g of T-2 toxin, 0.23 g of diacetoxyscirpenol, and 0.76 g of neosolaniol.

DISCUSSION

Medium A with 5% glucose or sucrose, 0.1% peptone, and 0.1% yeast extract apparently provided all necessary ingredients for the production of high levels of T-2 toxin and neosolaniol. None of the various metal ions examined increased the mycotoxin production.

The productivity of *Fusarium* spp. of T-2 toxin differed markedly from each other, and *F. solani* M-1-1, which was first isolated from moldy bean hulls (5), was found to produce the largest amount of T-2 toxin either in the shake culture, jar fermentator, or stationary culture.

T-2 toxin was predominantly produced by *F. solani*, and neosolaniol, which lacked isovaleroyl residue at C-8, was synthesized in the later stage of cultivation. This presumably indicated T-2 toxin to be a precursor of neosolaniol.

In the case of a sationary culture of *F. solani* on PSC medium (5), the purification procedure of trichothecenes from the crude toxin required two-step column chromatography, whereas pure trichothecenes were obtained by one-step column chromatography from the crude toxins of shake culture and jar fermentation. This means that the "crude toxin" in these culture systems contained a somewhat smaller amount of non-trichothecene compounds in comparison to that of stationary culture on PSC medium.

For these reasons, the shake culture on medium A with 5% glucose appears to be suitable for production of T-2 toxin as well as for screening T-2 toxin producers.

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