APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1982, p. 371–375
0099-2240/82/080371-05$02.00/0
Vol. 44, No. 2

New Process for T-2 Toxin Production

DANIEL CULLEN,* EUGENE B. SMALLEY, AND RODNEY W. CALDWELL
Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706

Received 8 February 1982/Accepted 16 April 1982

Strains of *Fusarium* produced high levels of T-2 toxin when cultured on certain media absorbed into vermiculite. Modified Gregory medium was nutritionally complex (2% soya meal, 0.5% corn steep liquor, 10% glucose) and, when inoculated with the appropriate fungal strain, yielded maximum T-2 toxin within 24 days of incubation at 19°C. On Vogel synthetic medium N (H. J. Vogel, Microb. Genet. Bull. 13:42–43, 1956) supplemented with 5% glucose, optimal toxin levels were synthesized after incubation for 12 to 14 days at 15°C. *Fusarium tricinctum* T-340 produced 714 and 353 mg/liter on modified Gregory medium and Vogel synthetic medium N plus 5% glucose, respectively. Improved analytical procedures were developed and involved aqueous methanol extraction, purification by liquid-liquid partitions, and gas-chromatographic quantitation.

T-2 toxin [4B,15-diacetoxy-8a-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-en-3a-ol], one of a family of related 12,13-epoxytrichothecenes, is commonly associated with cereals molded with *Fusarium tricinctum* (Corda) Sacc. (1, 12, 14). Episodes of farm animal toxicoses caused by trichothecene-contaminated feeds are well documented (8, 11, 12, 14). Some strains of *F. tricinctum* also produce zearalenone, an estrogenic polyketide that causes hyperestrogenism in swine (4).

In culture, trichothecenes are often produced on autoclaved cereals with long-term (4 to 6 weeks), low-temperature (8 to 12°C) incubations (1–4, 10, 14, 16). Such natural substrates sometimes yield high levels of T-2 toxin (up to 1 g/kg), but purification is complicated by many interfering substances (J. R. Bamburg, Ph.D. Thesis, University of Wisconsin, Madison, 1968). Surface fermentations or shake cultures of nutritionally complex media such as Gregory medium (GM) (6) or peptone-supplemented Czapeks-Dox medium (CZ) (16, 17) require less stringent purification although, as for cereal substrates, bulky Fernbach or Erlenmeyer flasks are used (1–3, 14, 16, 17). Generally, yields are low in liquid media (14).

The availability of more rapid and convenient methods of toxin analysis would facilitate certain types of investigations. For example, extensive geographical surveys involving toxicity testing of many *F. tricinctum* strains are needed (13). Furthermore, with the recent identification of the heterothallic perfect state of *F. tricinctum, Gibberella tricincta*, the possibility of genetic studies of toxin production now exist (5). In preliminary studies, (D. Cullen and E. B. Smalley, Phytopathology 71:212, 1980), we demonstrated modest T-2 toxin production by a process initially designed for the commercial production of zearalenone which utilized a medium absorbed into vermiculite as the substrate (P. H. Hidy, U.S. patent 3,580,811, 1971).

We report here two new culture media and improved analytical procedures suitable for rapidly and conveniently evaluating *Fusarium* strains for T-2 toxin production.

MATERIALS AND METHODS

Source and maintenance of cultures. Strain T-340 was obtained from the Fusarium Research Center, Pennsylvania State University, University Park, and identified as *F. tricinctum* (Corda) Sacc. *Fusarium sporotrichioides* Sherb. strain T-2 (NRRL 3299) was isolated from corn in France and was used extensively in early trichothecene studies (2, 14). Strain T-2-1 is a valine and isoleucine auxotrophic mutant derived from strain T-2 by UV irradiation. All cultures were maintained as single spore clones and stored in sterile soil at 4°C. For inoculum, infested soil particles were sprinkled onto *Schizophyllum* complete agar (15) 5 days before use.

Media improvement. The ability of various media to support toxin production was evaluated in a standard test which involved placing 5 g of vermiculite (2-3-4 Terralite; Grace Co., Cambridge, Mass.) into petri plates (100 by 10 mm), adding 19 ml of test medium by pipette to the vermiculite, autoclaving, and inoculating with 5-mm agar plugs from *Schizophyllum* complete agar plates previously inoculated with strain T-340. All incubations were conducted at 90% relative humidity to prevent drying.

GM (6), prepared without calcium carbonate (i.e., 2% [wt/vol] soya meal and 0.5% corn steep liquor), was supplemented with 3, 10, 20, and 30% glucose. Twenty plates were prepared for each glucose level and incubated at 19°C. At 4-day intervals, duplicate plates from each glucose level were analyzed for T-2 toxin.

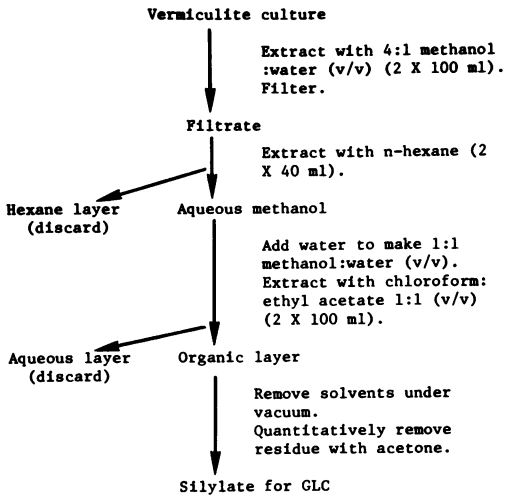

FIG. 1. Extraction and purification procedure for trichothecenes. GLC, Gas-liquid chromatography.

Vogel synthetic medium N (VN) (H. J. Vogel, Microb. Genet. Bull. 13:42–43, 1956) and CZ (16, 17) were supplemented with 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0% peptone. Glucose was added to each peptone level to final concentrations of 1, 3, and 5%. Plates were analyzed for T-2 toxin after 14 days of incubation at 19°C. This time period (14 days) was previously determined to be optimal in preliminary time-course experiments in which VN and CZ were supplemented with the following concentrations of peptone and glucose (percent peptone/percent glucose): 0/1, 0/5, 1/1, 1/5.

Optimal temperatures for T-2 toxin production were determined for two media which had supported high toxin levels in initial experiments. Standard vermiculite test plates containing VN supplemented with 5% glucose (VNG) and GM supplemented with 10% glucose (GMG) were prepared and incubated for 14 and 24 days, respectively. Three replicate test plates per temperature-medium combination were analyzed.

In cereal substrate tests, 100 g of autoclaved corn or rice (50% [wt/wt] moisture content) in 500-ml Erlenmeyer flasks was inoculated and incubated at 24°C for 2 weeks, followed by 8°C for 4 weeks (2, 14).

Surface fermentations on GM (1, 6) and peptone-supplemented CZ (16, 17) were tested by inoculating 200 ml of medium in 2,400-ml Fernbach flasks and incubating for 3 weeks at 8 to 10°C. Entire cultures (mycelium and filtrate) were lyophilized and analyzed for trichothecenes (9).

Standards. T-2 toxin was prepared by L. C. Wu (formerly visiting scientist, University of Wisconsin, now with Campbell Soup Co., Napoleon, Ohio). Diacetoxyscirpenol was purchased from Makor Biochemicals (Jerusalem, Israel). Deoxynivalenol was kindly supplied by R. F. Vesonder (Northern Regional Research Laboratories, Peoria, Ill.). HT-2 and T-2 tetraol were prepared by alkaline hydrolysis of T-2 toxin (18). Zearalenone was prepared as described by Hidy (Hidy, U.S. patent 3,580,811, 1971).

Analysis. Trichothecene extraction and purification from cereal cultures were done by the method of Ikedobi et al. (9). This procedure was modified for toxin analysis of vermiculite cultures (Fig. 1). Without drying, cultures were transferred to 500-ml Erlenmeyer flasks and twice extracted with aqueous methanol by agitating for 10 min on a rotary shaker (200 rpm). After filtration through Whatman no. 1 filter paper, nonpolar lipids were removed with two *n*-hexane (or Skellysolve B) partitions. Trichothecenes were precipitated with the addition of water and extracted with two chloroform-ethyl acetate partitions. After the removal of solvents under vacuum, the residue was taken up in 10 ml of acetone.

Before quantitation by gas-liquid chromatography, trimethylsilyl (TMS) derivatives of the trichothecenes were prepared by drying a sample of purified extract under nitrogen and adding 100 μl of silylation reagent (Derivisil; Regis Chemical Co., Morton Grove, Ill.) in a Pierce Reactivial (Pierce Chemical Co., Rockford, Ill.). Silylation was complete after 10 min at 60°C.

A Varian gas chromatograph (model 1860) equipped with a flame ionization detector was employed. Injections (1 μl each) were made into glass columns (1.6 m by 2 mm) packed with 3% OV-17 on Chromosorb W (80/100 mesh) (Pierce Chemical Co.). A temperature program from 200 to 280°C (8°C/min) was used with the injector temperature at 280°C and the detector set at 300°C. The nitrogen and hydrogen flow rates were 30 and 20 ml/min, respectively. Quantitation was achieved by estimation of peak area relative to an internal standard, methyl behenate (Sigma Chemical Co., St. Louis, Mo.). Detector response to T-2 toxin was linear (r^2 = 0.99) between 0.05 and 2.0 μg. The limit of detection for T-2 toxin was 0.5 mg/liter. For mass spectroscopy, similar operating conditions were used with an Aerograph (model 2700) gas chromatograph connected to a DuPont mass spectrometer (model 21-491B).

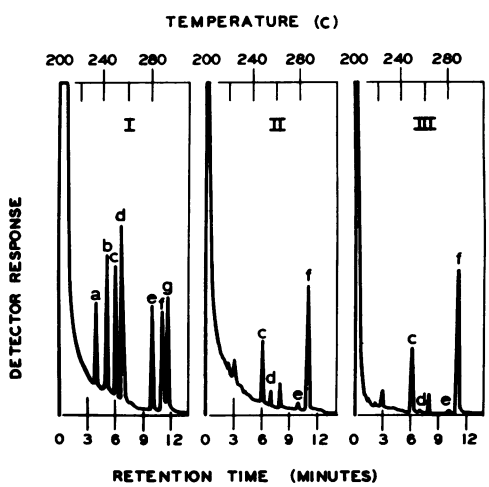

FIG. 2. Gas chromatograms of: I, standard toxins; II, GMG extract; III, VNG extract. Peaks correspond to: a, TMS-deoxynivalenol; b, TMS-T-2 tetraol; c, methyl behenate; d, TMS-diacetoxyscirpenol; e, TMS-HT-2 toxin; f, TMS-T-2 toxin; g, TMS-zearalenone.

TABLE 1. Effect of glucose concentration on T-2 toxin production by *F. tricinctum* strain T-340 after 24 days of incubation at 19°C

Medium[a]	T-2 toxin produced (mg/liter)[b] at glucose concn[c]:			
	2%	10%	20%	30%
PSC	131	307	224	176
GM	138	502	355	155

[a] PSC, CZ supplemented with 1% [wt/vol] peptone. GM was prepared without calcium carbonate.
[b] All values represent the means of two replications.
[c] All glucose concentrations are wt/vol.

RESULTS

Chromatograms of silylated extracts from GMG (II) and VNG (III) cultures are compared with those of standard toxins (I) in Fig. 2. In addition to TMS-T-2 toxin (peak f), TMS-diacetoxyscirpenol (peak d) and TMS-HT-2 (peak e) were detected, but at much lower levels. The mass spectra of these silylated derivatives were identical to those of pure standards. Zearalenone, T-2 tetraol, and deoxynivalenol were not detected.

Recovery efficiencies from GMG cultures spiked with 100 and 1 mg of T-2 toxin were 77 and 82%, respectively. The precision (reproducibility) of analysis was determined by separate analysis of 12 GMG plates inoculated with T-2-1 and T-340. The coefficients of variation were 25 and 13%, respectively. Further variations in toxin yield due to inconsistencies between incubators and batches of complex media components (corn steep liquor and soya meal) were also observed.

The highest T-2 toxin yields were obtained on GMG cultures incubated at 19°C for 24 days (Fig. 3; see Table 4). Glucose concentration strongly affected T-2 toxin production on GM and peptone-supplemented CZ (Table 1).

The combinations of peptone and glucose concentrations in CZ for optimal toxin yield (307 mg/liter) were 1 and 10%, respectively (Table 1).

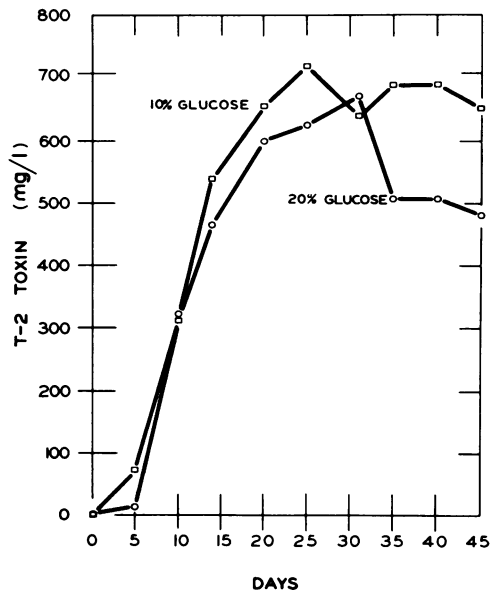

FIG. 3. Time course of T-2 toxin production by strain T-340 on GM supplemented with 10 and 20% glucose. Cultures were incubated at 19°C. Values represent the means of duplicate test plates.

Without peptone, CZ yielded very low toxin, e.g., 2.4 mg/liter when amended with 5% glucose (Table 2). Sucrose substituted for glucose gave no apparent increase in T-2 toxin.

T-2 toxin production was enhanced by decreasing peptone and increasing glucose concentrations in VN (Table 2). The maximum yield (288 mg/liter) at 19°C was observed with 5% glucose (VNG) and no peptone supplement. The nitrogen source under these circumstances was limited to ammonium nitrate (2 g/liter). In a separate time-course experiment, maximum T-2 toxin production (353 mg/liter) was obtained in 12 to 14 days on VNG.

Yields of T-2 toxin on GMG and VNG were compared with those on other substrates (Table

TABLE 2. Effect of peptone and glucose concentrations on T-2 toxin production by *F. tricinctum* strain T-340 after 14 days of incubation at 19°C

Medium	% Glucose	T-2 toxin produced (mg/liter)[a] at peptone concn:					
		0.0%	0.2%	0.4%	0.6%	0.8%	1.0%
CZ	1	19.8	56.9	113	108	124	45.0
	3	ND	103	145	146	129	123
	5	2.4	101	146	140	113	135
VN	1	92.5	57.0	36.0	11.6	25.4	18.3
	3	236	99.0	86.5	87.0	68.6	42.2
	5	288	192	145	120	100	88.8

[a] All values represent the means of duplicate cultures. ND, None detected.

TABLE 3. Production of T-2 toxin on various substrates by *Fusarium* species

Species and strain	T-2 toxin produced on[a]:					
	Autoclaved cereals (mg/g)		Surface fermentations (mg/liter)		Vermiculite (mg/liter)	
	Corn	Rice	GM	PSC	GMG	VNG
F. tricinctum T-340 (*val*$^+$ *ile*$^+$)	5.9	2.7	60.0	27.0	714	370
F. sporotrichioides T-2 (*val*$^+$ *ile*$^+$)	2.7	1.7	42.5	52.5	511	307
F. sporotrichioides T-2-1 (*val ile*)	ND	ND	0.18	0.14	8.9	ND

[a] Incubation conditions were: cereals, 24°C for 2 weeks followed by 15°C for 4 weeks; surface fermentations, 8 to 10°C for 21 days; GMG vermiculite, 19°C for 24 days; VNG vermiculite, 19°C for 14 days. PSC, peptone-supplemented CZ. All values represent the means of duplicate cultures. ND, None detected.

3). For all three strains, toxin yield was substantially higher on vermiculite-supported media as compared with similar surface fermentations. Little growth and no toxin production by auxotroph T-2-1 were detected on cereal substrates and VNG, but small quantities (8.9 mg/liter) were elaborated on GMG.

No significant increases in toxin production on GMG were observed at temperatures above or below 19°C (Table 4). Slight, but statistically insignificant, yield increases were observed with lower-temperature (15°C) incubations on VNG.

DISCUSSION

For routine screening of isolates, we have adopted GMG. Dozens of strains have been tested, and all isolates of *F. tricinctum*, *F. sporotrichioides*, and *F. poae*, including auxotrophic strains, grow on this medium. With the exception of certain auxotrophic isolates, all strains producing T-2 toxin on GMG also produce toxin in cereals and surface fermentations.

In addition to its usefulness in screening for toxic fusaria, the process might provide an inexpensive source of T-2 toxin for investigative purposes. Medium improvements such as alternative carbohydrate sources and strain selection would, quite probably, increase yields to grams per liter.

Although it yields lower amounts of T-2 toxin relative to GMG, VNG may have certain applications. For example, the effects of specific carbohydrates or nitrogen sources on trichothecene synthesis could be studied more precisely than in nutritionally complex media. Recently, we have synthesized heterokaryons between nontoxic and toxic auxotrophic *Fusarium* strains (D. Cullen, E. B. Smalley, and R. Dimond, manuscript in preparation). Since auxotrophs are unable to grow on VNG, nuclear associations in forced heterokaryons would be maintained. Dominance interactions relating to trichothecene production could then be studied. Finally, VNG may provide an efficient alternative to cereal substrates in the preparation of radiolabeled T-2 toxin (7).

TABLE 4. Effect of temperature on T-2 toxin production by *F. tricinctum* strain T-340

Medium	T-2 toxin produced (mg/liter)[a] at temp:					
	7°C	11°C	15°C	19°C	23°C	27°C
GMG	ND	350	690	714	280	ND
VNG	ND	220	370	353	145	ND

[a] GMG and VNG cultures were incubated for 24 and 14 days, respectively. All values represent the means of two replications. ND, None detected.

ACKNOWLEDGMENTS

This work was supported by the College of Agriculture and Life Sciences and the Environmental Toxicology Center, University of Wisconsin, Project no. 5110.

We thank Steve Vicen for the preparation of graphics.

LITERATURE CITED

1. **Bamburg, J. R., N. V. Riggs, and F. M. Strong.** 1968. The structure of toxins from two strains of *Fusarium tricinctum*. Tetrahedron 24:3329–3336.
2. **Burmeister, H. R.** 1971. T-2 toxin production by *Fusarium tricinctum* on solid substrate. Appl. Microbiol. 21:739–742.
3. **Burmeister, H. R., J. J. Ellis, and C. W. Hesseltine.** 1972. Survey for fusaria that elaborate T-2 toxin. Appl. Microbiol. 23:1165–1166.
4. **Caldwell, R. W., J. Tuite, M. Stob, and R. Baldwin.** 1970. Zearalenone production by *Fusarium* species. Appl. Microbiol. 20:31–34.
5. **El-Gholl, V. E., J. J. Ritchie, C. L. Schoulties, and W. H. Riding.** 1978. The identification, induction of perithecia, and pathogenicity of *Gibberella tricincta* n. sp. Can. J. Bot. 56:2203–2205.
6. **Gregory, K. F., O. N. Allen, A. J. Richer, and W. T. Peterson.** 1952. Antibiotics as agents for the control of certain damping off fungi. Am. J. Bot. 39:405–415.
7. **Hagler, W. M., C. J. Mirocha, and S. V. Pathre.** 1981. Biosynthesis of radiolabeled T-2 toxin by *Fusarium tricinctum*. Appl. Environ. Microbiol. 41:1049–1051.
8. **Hsu, I. C., E. B. Smalley, F. M. Strong, and W. E. Ribelin.** 1972. Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. Appl. Microbiol. 24:684–690.

9. **Ikedobi, C. O., I. C. Hsu, J. R. Bamburg, and F. M. Strong.** 1971. Gas-liquid chromatography of mycotoxins of the trichothecene group. Anal. Biochem. **42**:327–340.
10. **Joffe, A. Z., and B. Yagen.** 1977. Comparative study of the yield of T-2 toxin produced by *Fusarium poae*, *F. sporotrichioides*, and *F. sporotrichioides var. tricinctum* from different sources. Mycopathologia **60**:93–97.
11. **Mirocha, C. J., S. V. Pathre, B. Schauerhamer, and C. M. Christensen.** 1976. Natural occurrence of *Fusarium* toxins in feedstuff. Appl. Environ. Microbiol. **32**:553–556.
12. **Pathre, S. V., and C. J. Mirocha.** 1977. Assay methods for trichothecenes and their natural occurrence, p. 229–254. *In* J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), Mycotoxins in human and animal health. Pathotox Publishers, Park Forest, Ill.
13. **Smalley, E. B., A. Joffe, M. Palyusik, H. Kurata, and W. Marasas.** 1977. Panel on trichothecenes: research recommendations, p. 337–340. *In* J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), Mycotoxins in human and animal health. Pathotox Publishers, Park Forest, Ill.
14. **Smalley, E. B., and F. M. Strong.** 1974. Toxic trichothecenes, p. 199–228. *In* I. F. H. Purchase (ed.), Mycotoxins. Elsevier Publishing, New York.
15. **Snider, P. J., and J. R. Raper.** 1958. Nuclear migration in *Schizophyllum*. Am. J. Bot. **45**:538–546.
16. **Ueno, Y., N. Sato, K. Ishii, K. Sakai, H. Tsunoda, and M. Enomoto.** 1973. Biological and chemical detection of trichothecene mycotoxins of *Fusarium* species. Appl. Microbiol. **25**:699–704.
17. **Ueno, Y., M. Sawano, and K. Ishii.** 1975. Production of trichothecene mycotoxins by *Fusarium* species in shake culture. Appl. Microbiol. **30**:4–9.
18. **Wei, R., F. M. Strong, E. B. Smalley, and H. K. Schnoes.** 1971. Chemical interconversions of T-2 and HT-2 toxins and related compounds. Biochem. Biophys. Res. Commun. **45**:396–401.