APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1982, p. 967-970
0099-2240/82/040967-04$02.00/0
Vol. 43, No. 4

Production of Vomitoxin on Corn by *Fusarium graminearum* NRRL 5883 and *Fusarium roseum* NRRL 6101

RONALD F. VESONDER,* JOHN J. ELLIS, WILLIAM F. KWOLEK, AND DOUGLAS J. DEMARINI

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Two vomitoxin-producing isolates of *Fusarium* spp. were grown on cracked corn for 1 to 8 weeks at 15, 20, 25, 28, and 32°C. Maximum production of vomitoxin by *Fusarium graminearum* Schw. NRRL 5883 occurred at 30°C and 40 days, and that by *Fusarium roseum* Schw. NRRL 6101 occurred at 26°C and 41 days. These optimum production points were determined from response surface contour graphs in relation to temperature and time. Only small amounts of vomitoxin were produced at 15 and 20°C by each strain. A 133-μg quantity of vomitoxin, with an indicated purity of 95%, was isolated per gram of corn fermented with *F. graminearum* NRRL 5883.

3,7,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one (vomitoxin and 4-deoxynivalenol are generic names) was first detected in naturally moldy field corn in the United States (5) and in barley in Japan (3). *Fusarium graminearum* Schw. NRRL 5883 represents the predominant mold isolated from the corn, and *Fusarium roseum* Schw. NRRL 6101 represents that from barley. The latter strain, designated 117, was received from N. Morooka (Kagawa University, Miki-Tyo, Kagawa-Kan, Japan) in 1979 by the Agricultural Research Service Culture Collection. It was reported that this strain produced zearalenone on rice (2) and vomitoxin and 3-acetyl-deoxynivalenol on Czapek Dox medium supplemented with peptone (9). Since our initial discovery that vomitoxin can occur naturally in preharvest field corn (7), we have found that vomitoxin is the agent that causes swine to vomit and to refuse feed (6, 8, 9). Because of the occurrence in 1980 of vomitoxin in wheat from the provinces of Ontario and Quebec (H. L. Trenholm, J. I. Elliot, E. R. Farnworth, D. W. Friend, R. M. G. Hamilton, J. F. Standish, W. P. Cochran, H. Cohen, and G. A. Neish. Abstr. Annu. Meet. Am. Oil Chem. Soc. 72nd, New Orleans, La., abstr. no. 153, 1981), research activity continues in an attempt to determine the effect of vomitoxin on laboratory and farm animals and on birds in North America. For carrying on toxicological studies, as well as studies on methods of detection, substantial quantities of this toxin are necessary. Hence, the ability of these two *Fusarium* spp., isolated from two different cereal grains from different parts of the world at about the same time, to produce vomitoxin on corn was studied in relation to time and temperature. In addition, the ability of *F. graminearum* NRRL 5883 to elaborate vomitoxin on a variety of cereal grain and laboratory liquid media was studied further.

Fusarium spp. used in this study were grown on hay agar infusion slants to which a sterilized kernel of wheat was aseptically added to stimulate sporulation. These slants were incubated at 25°C for 7 days under alternating fluorescent light and darkness (12 h each). The inoculum for each strain was prepared by adding 5 ml of sterile water to each 7-day-old agar slant culture and suspending the spores and aerial mycelium with a sterilized inoculating loop. The spore suspensions from six 7-day-old slants were added to a sterile 100-ml Erlenmeyer flask and mixed. One-milliliter portions of this spore suspension (mostly aerial mycelium in the case of strain 6101) were added to 500-ml Erlenmeyer flasks each containing 50 g of sterilized cracked corn (free of trichothecenes [4]). The corn had been adjusted to 30% moisture and autoclaved for 30 min. The inoculated flasks were incubated statically at 15, 20, 25, 28, and 32°C and harvested at 7, 14, 21, 28, and 60 days for vomitoxin determinations. Both strains grew equally well on cracked corn. The corn that fermented with each *Fusarium* sp. at each time interval and temperature was blended with CH_3OH-H_2O (40:60) (4 ml/g, two times). Combined extracts from each *Fusarium* sp. were dried. The residue from each extract of fermented corn was adsorbed onto silica gel and added to the top of a silica gel column. Elution of the silica gel column was done with $CHCl_3$ and $CHCl_3$-CH_3OH (95:5) (7). Residues from the $CHCl_3$-CH_3OH eluates that did not lend themselves to quantitation by gas-liquid chromatography were rechromatographed on silica gel to remove interfering sub-

TABLE 1. Production of vomitoxin on corn by *F. graminearum* NRRL 5883

Day	Vomitoxin (micrograms per gram) produced at:				
	15°C	20°C	25°C	28°C	32°C
7	18	26	44	66	37
14	28	50	180	259	362
21	27	62	204	396	250
28	25	75	131	325	233
60	22	120	123	625	90

TABLE 2. Production of vomitoxin on corn by *F. roseum* NRRL 6101

Day	Vomitoxin (micrograms per gram) produced at:				
	15°C	20°C	25°C	28°C	32°C
7	3	14	21	12	23
14	2	15	74	84	26
21	9	19	150	150	35
28	20	20	90	113	29
60	11	15	100	170	16

stances. Quantitation for vomitoxin was done by gas-liquid chromatography with the internal standard method (7). The production data obtained from each of 25 fermentations for strains 5883 and 6101 at the different time intervals and temperatures are shown in Tables 1 and 2, respectively.

Response surface contour graphs of constant yields were prepared from these data for each strain studied with methods for fitting a second-degree equation (1). The contour graphs for strains 5883 and 6101 are shown in Fig. 1 and 2, respectively.

The yield contours in Fig. 1 and 2 in relation to time and temperature show the predicted yields of vomitoxin derived from the equations. The multiple correlation coefficient between observed and predicted yields for strain 5883 was $R = 0.87$, significant ($P = 0.01$), and that for strain 6101 was $R = 0.85$ ($P < 0.01$). The yields of vomitoxin under these laboratory conditions were four times greater at 25°C than at 20°C. Optimum production of vomitoxin by strain 5883 occurred at 30°C and 40 days, with a predicted yield of 362 μg per g of fermented corn. For strain 6101, maximum production occurred at 26°C and 41 days, with a predicted yield of 189 μg per g of fermented corn.

The *Fusarium* isolate from corn produced almost twice as much vomitoxin on cracked corn as did the *Fusarium* isolate from barley under these fermentation conditions. Strain 6101 did not sporulate well but grew predominantly in the vegetative state on the corn substrate.

For testing our model response surface contour graphs for the prediction of vomitoxin production, 1 kg of corn was fermented as described above at 28°C for 30 days with *F. graminearum*. A yield of 250 μg of vomitoxin per g of corn was predicted see Fig. 1. For vomitoxin isolation, the fermented corn was extracted with CH_3OH-H_2O as described previously. The extract was concentrated to 200 ml. Lead acetate (200 mg) was added, and the mixture was stirred for 5 min. This mixture was extracted with ethyl acetate. This extraction procedure was repeated two more times. The ethyl acetate extracts were pooled (centrifugation was necessary to separate

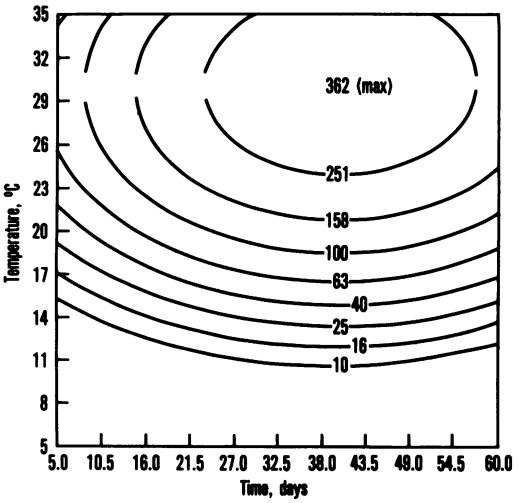

FIG. 1. Contour graph for *F. graminearum* NRRL 5883 (predicted yield).

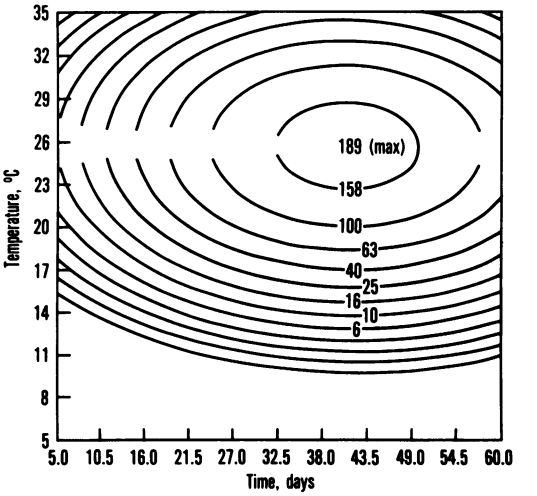

FIG. 2. Contour graph for *F. roseum* NRRL 6101 (predicted yield).

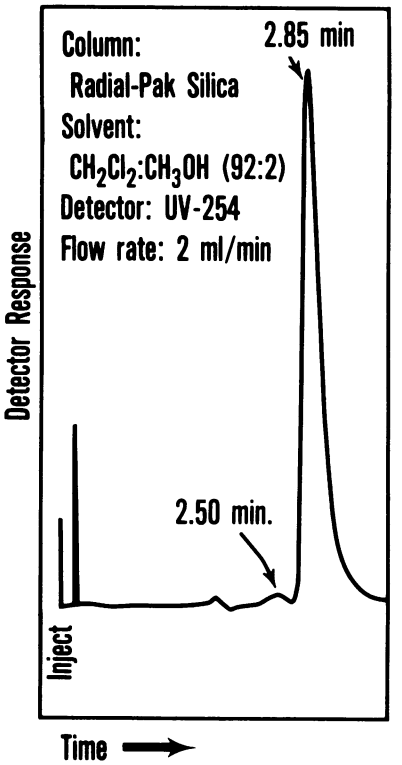

FIG. 3. Radial pressure liquid chromatogram of 96% pure vomitoxin.

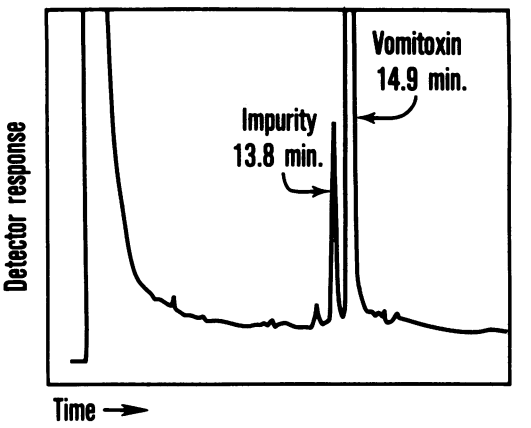

FIG. 4. Gas-liquid chromatogram of trimethylsilyl ether derivative of 96% pure vomitoxin.

ethyl acetate from the supernatant), dried over sodium sulfate, and filtered. The ethyl acetate extract was reduced to a volume of ca. 80 ml. Hexane was added to the extract to make an 80% solution. The ethyl acetate-hexane solution was passed through a silica gel (50 g) column, and the column was subsequently eluted with ethyl acetate-hexane (80:20). Vomitoxin was eluted in crude form (666 μg) with this solvent.

The crude vomitoxin was passed through a silanised silica (EM Reagents) gel column in CH_3OH-H_2O, and 180 μg of impure vomitoxin (ca. 85% pure by gas-liquid chromatography) was recovered. The impure vomitoxin was then chromatographed on a high-performance preparative chromatograph (model 500, used with Prep Pak-500/Silica; Waters Associates, Inc.) with the solvent system ethyl acetate-hexane (80:20). Vomitoxin (133 μg; mp, 144 to 150°C) eluted off with four column volumes under these conditions, with a purity of 95 to 96%, as analyzed by radial pressure liquid chromatography and gas-liquid chromatography (Fig. 3 and 4, respectively).

Analytically pure vomitoxin (mp, 152 to 153°C) was obtained by crystallization from ethyl acetate and hexane, but only after heavy losses. This fermentation for vomitoxin production was effective with the molds used, but the procedure was tedious. Several cereal grains were used as substrates for vomitoxin elaboration by *F. graminearum* NRRL 5883. Vomitoxin was produced on all of the solid substrates tested, namely, corn, corn grits, corn meal, rice, wheat, rye, oats, and barley. Various mycological media were used for testing vomitoxin production by strain 5883. The following did not produce vomitoxin: Czapek Dox, Czapek Dox supplemented with corn steep liquor, Fries media, Yeast Malt Extract media, GAN media, and Malt Extract media with 5 and 10% glucose. A small amount of vomitoxin was detected on Yeast Malt Extract media (less than 5 ppm [5 μg/ml).

We thank M. Woodman of Water Associates, Inc., for radial pressure liquid chromatography analyses of vomitoxin and guidance in the use of the Water 500 high-performance preparative chromatograph.

LITERATURE CITED

1. **Cochran, W. G., and G. Cox.** 1964. Experimental design, 2nd ed. p. 335–369. John Wiley & Sons, Ltd., London.
2. **Ishii, K., M. Sawano, Y. Ueno, and H. Tsunoda.** 1974. Distribution of zearalenone-producing *Fusarium* species in Japan. Appl. Microbiol. **27**:625–628.
3. **Morooka, N., N. Uratsuji, T. Yoshizawa, and H. Yamamoto.** 1972. Studies on toxic substances in barley infected with *Fusarium* spp. J. Food Hyg. Soc. Jpn. **13**:368–375.
4. **Vesonder, R. F., A. Ciegler, H. R. Burmeister, and A. H. Jensen.** 1979. Acceptance by swine and rats of corn amended with trichothecenes. Appl. Environ. Microbiol. **38**:344–346.

5. **Vesonder, R. F., A. Ciegler, and A. H. Jensen.** 1973. Isolation of the emetic principle from *Fusarium*-infected corn. Appl. Microbiol. **26**:1008–1010.

6. **Vesonder, R. F., A. Ciegler, A. H. Jensen, W. K. Rohwedder, and D. Weisleder.** 1976. Co-identity of the refusal and emetic principle from *Fusarium*-infected corn. Appl. Environ. Microbiol. **31**:280–285.

7. **Vesonder, R. F., A. Ciegler, R. F. Rogers, K. A. Burbridge, R. J. Bothast, and A. H. Jensen.** 1978. Survey of 1977 crop year preharvest corn for vomitoxin. Appl. Environ. Microbiol. **36**:885–888.

8. **Vesonder, R. F., and C. W. Hesseltine.** 1980/81. Vomitoxin: natural occurrence on cereal-grains and significance as a refusal and emetic factor to swine. Process Biochem. **16**:12–15.

9. **Yoshizawa, T., and N. Morooka.** 1974. Studies on the toxic substances in the infected cereals. III. Acute toxicities of new trichothecene mycotoxins: deoxynivalenol and its monoacetate. J. Food Hyg. Soc. Jpn. **15**:261–269.