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# Production of Fusarin C on Cereal and Soybean by *Fusarium moniliforme*

C. W. BACON,* D. R. MARIJANOVIĆ, W. P. NORRED, AND D. M. HINTON

*Toxicology and Mycotoxin Research Unit, Richard B. Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, Georgia 30613*

Received 10 April 1989/Accepted 5 August 1989

**Two isolates of *Fusarium moniliforme* were compared with respect to production of a mutagenic compound, fusarin C, on seven corn varieties as well as on soybean, wheat, rye, barley, and a liquid culture medium. The isolates were originally obtained from corn and barley. Both isolates produced fusarin C on seed of all five crops within a 21-day period, and one isolate produced the largest amount on oats. Soybean was the poorest substrate for both isolates. Although the quantity of fusarin C produced on grain was isolate dependant, specific substrate requirements for each strain were suggested. The isolates differed in their ability to grow and produce fusarin C on corn with different moisture contents (16, 20, 24, and 28%). One isolate was more xerotolerant and grew at 16% moisture but did not produce the mutagen.**

*Fusarium moniliforme* Sheldon is a major fungal parasite of several economically important members of the family *Gramineae*, which include such cereal crops as corn, rice, wheat, barley, and sorghum (2, 5). This fungus is distributed worldwide, occurring from the humid and subhumid temperate zones to the subtropical and tropical zones, and contaminates many foodstuffs used for human and animal consumption (5, 8). It is the most prevalent fungus associated with corn (16).

In addition to its role as a plant pathogen, *F. moniliforme* has been associated with human esophageal cancer (19) and mycotoxicoses of farm animals (15, 18). The chemical identity of specific mycotoxins associated with these toxicoses is not known. Nevertheless this fungus is known to produce several mycotoxins (3, 13, 17). Recently Bjeldanes and Thomson (4) reported that culture extracts of this fungus were highly mutagenic in the Ames bacteriological assay, and Gelderblom et al. (11) later isolated and characterized a mutagenic compound, fusarin C, from a *F. moniliforme* isolate cultured on corn. It was subsequently established that fusarin C also induces mutagenesis in mammalian cells in vitro (6, 7). In addition to mutagenic activity, fusarin C has been shown to have immunosuppressant effects (6), and the fungus-infected corn has the same effect in poultry (D. Marijanović, W. P. Norred, W. L. Ragland, C. W. Bacon, P. C. Stancel, and P. Holt, Abstr. Annu. Meet. United States-Japan Natural Resources Joint Panel of Toxic Microorganisms, Washington, D.C., p. 27, 1988). Fusarin C inhibits macrophage activation and macrophage-mediated cytotoxicity of mouse tumor cells (6, 9). The concentration of fusarin C required to inhibit the immune response was lower than that required to induce mutations (6); therefore the precise manner of suppressing lymphocyte proliferation by fusarin C is not clear. These latter studies suggest that fusarin C might also play an indirect role in carcinogenesis. Thus its role in animal and human toxicity may be more important and complex than initially considered.

Fusarin C has been reported to occur naturally on corn kernels from China (7), South Africa (22), the United States (25), and other parts of North America (10). Depending on the substrate used to screen isolates of *F. moniliforme* for fusarin C production, 81 to 95% of all isolates produced this substance (10). Presently there is little information to indicate the moisture requirements for the production of fusarin C on corn and whether *F. moniliforme* can produce fusarin C on other cereal grain and foodstuffs. In this study we investigated the formation of fusarin C on varieties of corn, corn of various moisture levels, seed of several cereal grains, a variety of soybeans, and a liquid medium. We were especially interested in comparing the production of fusarin C by a strain of this fungus isolated from corn with the production by a strain isolated from barley to determine whether prior host-pathogen combinations influence subsequent mutagen production on a different grain.

## MATERIALS AND METHODS

**Organisms.** One *F. moniliforme* Sheldon strain, M3783, was originally isolated from barley and was obtained from J. M. Farber, Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario. The other strain, RRC374, was isolated from corn that was obtained from J. Oliver, Department of Plant Pathology, University of Georgia, Athens. Stock cultures of both fungi were maintained on Difco potato dextrose agar at 4°C.

**Culture conditions.** Corn, barley, wheat, oats, and soybean seeds used in this study were of whole sound seed-grade quality and did not contain fusarin C. The corn used consisted of four open-pollenated varieties (Hickory King, Reid's Yellow Dent, Hasting Prolific, and Trucker's Favorite), one variety of popcorn (South American), and two sweet corn varieties (Aristogold and Golden Cross Bantam). The growing medium was prepared by placing 25 g of seed of the desired variety and 25 ml of distilled water in a pint Mason jar and autoclaving the jars for 30 min on 2 successive days.

Fungi used for inoculations were grown on potato dextrose in petri dishes for 14 days at 25°C. A water suspension of conidia ($10^5$ spores per ml) was prepared from these dishes, and 0.5 ml was used to inoculate each jar of grain. The inoculated grain was incubated in darkness for 21 days at 24°C.

The liquid medium and procedure of Farber and Sanders

* Corresponding author.

(10) were used to compare the production of Fusarin C under laboratory conditions.

**Moisture content of corn.** The water content of autoclaved Reid's Yellow Dent and Golden Cross Bantam corn was determined by the official air oven method (24) on triplicate samples of corn. The moisture content of the corn was adjusted by adding sterile water to produce moisture contents of 16, 20, 24, and 28%. The corn was then mixed thoroughly with the water, sealed, and stored for 2 weeks at 24°C. The moisture content of the corn, determined again at the end of the 2-week period, remained at the initial level. Fifty grams of this corn was placed in sterile 250-ml flasks and inoculated with conidial suspensions (0.5 ml) of each strain. The flasks were closed with styrofoam plugs that were covered with polyethylene wrap, and the inoculated corn was incubated at 24°C for 21 days.

**Fusarin C extraction.** Fusarin C was extracted by adding 100 ml of acetonitrile to each culture and by grinding for 1 min with a Brinkmann homogenizer (model PT10-35). The extract was filtered, and the residue was reextracted with 50 ml of acetonitrile. The acetonitrile fractions from a culture were combined and evaporated to dryness under reduced pressure at 30°C. The residue was dissolved in 100 ml of methylene chloride and transferred to a separatory funnel, and then 50 ml of distilled water was added. This solution was shaken by hand for 30 s, the water was discarded, and the methylene chloride was filtered through prepleated filters (0.189-mm thickness) containing 1 g of sodium sulfate. The methylene chloride was evaporated to dryness under reduced pressure at 30°C. The residue was suspended in 5 ml of methylene chloride-methanol (97:3, vol/vol) (21) and stored at −20°C under nitrogen until analyzed.

To prevent decomposition, all extractions and analyses were carried out under gold fluorescent lighting as recommended by Scott et al. (21). The methylene chloride-methanol fraction was subjected to a preliminary cleanup as described previously (23), except a silica gel disposable prepacked column (Sep-Pak; Waters Associates, Inc.) was used, 1 ml of the methylene chloride-methanol extract was applied to the column, and 3 ml of methylene chloride-methanol (9:1, vol/vol) was used to elute fusarin C.

**Fusarin C determination.** Thin-layer chromatography was used to determine the presence of fusarin C. The methylene chloride-methanol was evaporated, the residue was suspended in 5 ml of methanol, and 0.025 ml was applied to prepared silica gel plates (Eastman Kodak chromagram, 13181) and developed in $CHCl_3$-$CH_3OH$ (9:1, vol/vol). The presence of fusarin C was established under visible light by comparison with a standard as a bright yellow spot. Fusarin C was separated by using preparative thin-layer chromatography. For this analysis, 0.1 ml of the methanol extract was applied to the plates; after developing, the fusarin C band was scraped off the plates. The silica gel was eluted with two 5-ml portions of $CHCl_3$-methanol (80:20, vol/vol). The silica gel was removed by centrifugation, and the $CHCl_3$-methanol was evaporated. The residue was suspended in 2 ml of methanol, and fusarin C was assayed at 360 nm (21). Because fusarin C is not stable, it should be noted that fusarin C concentrations reported in this work are not absolute (10, 23).

High-pressure liquid chromatography of the extracts was performed by the method of Farber and Sanders (10) with a Beckman model 100A liquid chromatograph with a Hitachi model 100-40 variable-UV-wavelength detector set at 358 nm and a Hewlett Packard 3390 A integrator.

Fusarin C standards were obtained as gums from L. F. Bjeldanes, University of California, Berkeley, and J. M. Farber, Health and Welfare Canada, Ontario, Canada. All standards were stored as gums under nitrogen at −20°C. Standards were also prepared by the liquid culture fermentation procedure of Farber and Sanders (10) with strain RRC374. The fusarin C prepared by this procedure was purified by column chromatography and thin-layer chromatography (21). The UV spectra were determined in methanol with an Aminco DW-2 spectrophotometer. Mass spectrometry was done on fusarin C purified by thin-layer chromatography and column chromatography with a Finnigan 4535/TSQ quadrupole mass spectrometer.

TABLE 1. Production of fusarin C on corn varieties by two strains of *F. moniliforme*[a]

| Corn variety | Fusarin C (mean ± SD), μg/g | |
|---|---|---|
| | M3783 | RRC374 |
| Hasting Prolific | 144.22 ± 10.12a | 64.22 ± 6.14a |
| Hickory King | 131.17 ± 9.70a | 77.31 ± 10.16a |
| Reid's Yellow Dent | 157.56 ± 12.24a | 66.02 ± 4.23a |
| South American popcorn | 75.31 ± 4.15b | 40.92 ± 5.71b |
| Trucker's Favorite | 145.20 ± 11.15a | 108.61 ± 3.83c |
| Aristogold | 187.10 ± 3.17c | 57.05 ± 4.10a |
| Golden Cross Bamtam | 194.46 ± 1.15c | 55.86 ± 5.23a |

[a] Means represent four replicates, and values not followed by the same letters within a column are significantly different ($P = 0.001$) by analysis of variance.

## RESULTS

The two strains of *F. moniliforme* differed quantitatively in their ability to produce fusarin C on corn (Table 1). Strain M3783 produced significantly higher levels of this metabolite on all corn varieties than did strain RRC374. South American popcorn was the poorest substrate for the production of fusarin C by both strains. Significantly greater amounts of fusarin C were accumulated by strain M3783 on the two sweet corn varieties, compared with that on the other varieties, but not with strain RRC374. On the average, strain M3783 accumulated 40% more fusarin C on the other corn varieties than the amount accumulated by strain RRC374.

Both strains produced fusarin C on the three cereal and soybean seeds (Table 2). Despite the large differences in the amount of fusarin C produced on each of these grains, there was an approximately 6- to 10-fold increase in fusarin C production over that produced on corn. Depending on the seed type, strain RRC374 produced a range of 33 to 95% less fusarin C than did strain M3783. There was no significant difference ($P = 0.001$) in the amount of this metabolite produced by the two strains on soybeans, and the amount

TABLE 2. Production of fusarin C by two strains of *F. moniliforme*[a]

| Substrate | Fusarin C (mean ± SD), μg/g | |
|---|---|---|
| | M3783 | RRC374 |
| Barley | 1,028.76 ± 5.61a | 115.77 ± 3.21a |
| Oats | 1,267.28 ± 4.21b | 103.26 ± 11.31a |
| Soybean | 98.72 ± 32.14c | 70.83 ± 71b |
| Wheat | 636.35 ± 4.17d | 426.32 ± 3.71c |

[a] Means represent three or four replicates, and values not followed by the same letters within a column are significantly different ($P = 0.001$) by analysis of variance.

TABLE 3. Effect of moisture content of corn on the production of fusarin C by two strains of *F. moniliforme*[a]

| Moisture content (%) | Fusarin C (mean ± SD), μg/g (dry wt) | | | |
|---|---|---|---|---|
| | Reid's Yellow Dent | | Golden Cross Bantam | |
| | M3783 | RRC374 | M3783 | RRC374 |
| 16 | ND | 10.18 ± 6.17a | ND | 12.17 ± 4.21a |
| 20 | 21.16 ± 7.21a | 29.19 ± 6.87b | 14.86 ± 8.84a | 31.22 ± 3.23b |
| 24 | 97.01 ± 5.76b | 144.22 ± 3.71c | 156.01 ± 9.21b | 146.82 ± 4.76 |
| 28 | 167.21 ± 8.71c | 60.23 ± 4.23d | 206.21 ± 3.71c | 50.89 ± 8.21c |

[a] Values are the means of three replicates, and values not followed by the same letter within a column are significantly different ($P = 0.001$) by analysis of variances. ND, None detected and no macroscopic growth observed during the 21-day incubation period.

produced was comparable with that produced on the varieties of corn. These results confirmed the results in Table 1, which indicated that strain M3783 produced more fusarin C on natural substrates than did strain RRC374. Strain M3783 produced the largest amount of fusarin C on oats, compared with that produced on other substrates.

Although strain M3783 produced more fusarin C on the natural products used in this study, RRC374 produced more of this compound than M3783 in a liquid medium (10). In liquid culture RRC374 and M3783 produced a mean value of 231 and 62 mg/liter, respectively. These two strains also differed in the color and intensity of the major pigment; M3783 produced a yellow pigment on both liquid medium and seed substrate, whereas RRC374 was pale yellow on liquid medium and purple to red on seed substrates.

The two strains differed in their ability to grow and produce fusarin C on corn when moisture content was varied (Table 3). Strain RRC374 was the more xerotolerant of the two, growing and producing more mutagen on corn at 16% moisture than that produced by M3783. There was an optimum moisture requirement difference for each strain; M3783 produced 36 and 24% more toxin than strain RRC374 at 28% moisture on Reid's Yellow Dent and Golden Cross Bantam corn, respectively. Strain RRC374 produced more fusarin C at 24% moisture on each corn variety than at the other moisture concentrations, indicating that this moisture concentration was within the optimum for this strain.

## DISCUSSION

Although the strains used in this study were originally isolated from different cereals (corn and barley) and were presumed to be pathogens of these two crops, both were capable of producing fusarin C not only on the cereal grain from which they were isolated but also on all corn varieties used as well as wheat, oats, and soybeans. *F. moniliforme* is a pathogen of all of these crops (5). Farber and Sanders (10) established that under laboratory conditions strains of *F. moniliforme* isolated from cereal grain and mixed feed ingredients produced fusarin C on corn. Our study confirms their work and establishes that, regardless of their origin, producing isolates are capable of synthesizing fusarin C on several autoclaved cereal grains and soybeans. However, the natural occurrence of fusarin C on grain other than corn, alone and in combination with other mycotoxins, has not been examined; based on work presented in this study, this should be determined.

The two strains used in this study produced amounts of fusarin C on autoclaved corn that were within the range reported by others (10, 12). This concentration was within the range reported as naturally occurring (22). The variation in amounts of fusarin C produced by each strain is not unusual, because variation among isolates for syntheses of other toxins is reported for many other species of fungi. Relative to corn, the best substrates for fusarin C production by two isolates were barley, oats, and wheat, suggesting a substrate effect. Because growth was not measured, it is not known how much of this effect was due to increased or decreased growth of either strain. However, growth is not the only requirement for maximum synthesis of a mycotoxin, as it has been established that the production of ochratoxin A on barley by *Aspergillus ochraceus* and *Penicillium viridicatum* depends on the level of protein in this grain (14). Further, two strains of *Aspergillus flavus* produce identical growth on barley and wheat, but the yield of aflatoxin produced by each strain is significantly different, indicating a substrate effect (20). The majority of other strains of *A. flavus* produce similar amounts of toxin on either grain, and the growth rates are not correlated with aflatoxin yield (20). In our study, both isolates consistently produced a large amount of fusarin C on wheat but were inconsistent for the other grains and soybeans. Of the fungus strains and grain combinations tested, M3783 on oats yielded the largest amount of fusarin C.

Under storage conditions, water limitation and temperature are the most important environmental determinants for growth. This study suggests that there are variations among isolates for water tolerance limits as strain M3783 did not grow at the lower moisture concentration, whereas strain RRC374 not only grew but also produced fusarin C on this same low moisture concentration. These differences were found for field corn and sweet corn, which suggests that this effect is due to moisture and not to substrate preference and/or the interaction of strain with substrate. We do not know how much variability exists in the *F. moniliforme* population with respect to these traits. There may exist strains of this fungus with lower moisture optima for growth and toxin synthesis than those used in this study. In addition to being a parasitic field fungus, *F. moniliforme* is also a storage fungus (1); especially for high-moisture stored corn, it has even been reported to grow anaerobically (23). Therefore it is important to determine whether fusarin C is produced in the field and how much, if any, is produced in storage. The reports on the natural occurrence of fusarin C indicate only its presence on mature harvested corn (7, 22, 25). Clearly, the ecological parameters associated with the bioproduction of fusarin C and its relationship to the growth and parasitism of grain by *F. moniliforme* and any postharvest interactions are unknown, but are the subjects of current research.

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