Zearalenone and Trichothecene Production in Soybeans by Toxigenic Fusarium

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ABSTRACT

Several Fusarium isolates known to produce zearalenone or T-2 toxin were tested for their toxigenic potential on heat-sterilized whole and cracked soybeans, on soybean meal, and on rice. Moisture concentration levels and particle sizes of substrate were varied to determine effects on the amount and type of toxin produced. Only one of the three Fusarium isolates known to produce zearalenone, Fusarium roseum ‘Graminearum’, produced detectable amounts of this mycotoxin on soybeans. Fusarium sporotrichioides NRRL 3299, the T-2 toxin-producing isolate tested, produced T-2 toxin as well as T-2 tetraol, HT-2 toxin and neosolaniol on soybeans. HT-2 toxin production was greatly enhanced on soybean meal in comparison to rice cultures. These findings plus previous field observations suggest that soybean products present a mycotoxic hazard which warrants attention.

Recently, investigation of two North Carolina cases of apparent mycotoxicosis in swine revealed that the animals were consuming soybean meal containing T-2 toxin at concentrations ranging from 0.3 to 10 µg/g soybean meal (Stanislaw, Hagler and Hamilton, unpublished data). These cases prompted investigation of the production of zearalenone and selected trichothecenes on whole and cracked soybeans and soybean meal inoculated with Fusarium strains known to be toxigenic.

MATERIALS AND METHODS

Mycotoxins

Crystalline T-2 toxin was produced by the method of Hagler et al. (13). Authentic zearalenone was obtained from IMC Inc., Terre Haute, IN. T-2 tetraol, HT-2 toxin and neosolaniol were produced by selective hydrolysis of T-2 toxin (3); identities of these trichothecenes were confirmed by gas liquid chromatography/mass spectrometry (GC/MS). Solvents and other chemicals and supplies were reagent-grade (Fisher Scientific, Raleigh, NC) unless otherwise noted.

Cultures

Fusarium roseum ‘Gibbosum’, F. roseum ‘Graminearum’ and F. oxysporum, producers of zearalenone (15), and Fusarium sporotrichioides NRRL 3299, a producer of T-2 toxin (5,13), were maintained at 5°C on moist autoclaved soil (15). Inoculum for the experiments was produced by sprinkling small portions of soil culture on potato dextrose agar plates which were then incubated at 25°C for 5 d (15) before blocks of agar (0.5 cm²) covered with mycelium were transferred to the substrates being evaluated for their ability to support toxin production.

Medium preparation

The solid rice medium contained 4 g of rice (Uncle Ben’s, Inc., Houston, TX) per screw-cap vial (20 ml). The influence of moisture content on toxin production was studied by adding 2, 4 or 6 ml of deionized water to the vials containing rice which were then autoclaved for 30 min on two consecutive days before inoculation. The soybean media were prepared by placing 4 g of whole soybeans, cracked soybeans or soybean meal plus 2, 4 or 6 ml of deionized water into 20-ml screw-cap vials that were autoclaved for 30 min on two consecutive days before inoculation.
Extraction of toxins

After incubation of the rice and soybean cultures for 28 d at 25°C, 10 ml of aqueous acetonitrile (50%) were added to the cultures which were broken into small fragments with a clean spatula, shaken and allowed to stand tightly capped overnight before decantation. The extraction was repeated once. The initial extract was filtered through Whatman No. 1 paper and evaporated to dryness in vacuo at 50°C. The second extract was combined with it the next day. The combined extracts were partitioned in a separatory funnel twice against 10-ml portions of hexane to remove interfering lipids. The defatted extracts were evaporated to dryness and redissolved in 1 ml of acetone for thin-layer chromatography.

Thin-layer chromatography

A 0.25-mm layer of silica gel 60 on 20 × 20-cm glass plates was used for TLC (E. Merck, Darmstadt, Germany). Portions of culture extracts and toxin standards were spotted on plates using 2-μl capillary pipettes (Drummond Scientific Co., Broomall, PA). Plates were developed in unlined, unequilibrated in a separatory funnel twice against 10-ml portions of portions (500 μl) of each extract was transferred to a 2-ml equilibrium in 100 ml of chloroform:methanol (97:3, vol/vol).

For detection of zearalenone, plates were examined under longwave (365 nm) and shortwave (254 nm) UV light. Confirmation was by the colorimetric spray reagent Fast Violet B [Sigma Chemical Co., St. Louis, MO; (23)] or 4-methoxybenzene-diazonium-fluoroborate [Reanal, Budapest, Hungary; (23)].

T-2 toxin, HT-2 toxin, neosolaniol and T-2 tetraol were tentatively identified on TLC plates by using the p-anisaldehyde-based spray reagent of Scott et al. (27) or 25% H2SO4 in methanol before charring at 100°C for 5 to 10 min.

Gas liquid chromatography

After identification of the toxins by TLC analysis, GLC was performed for quantification and for additional confirmation. A portion (500 μl) of each extract was transferred to a 2-ml screw-cap vial fitted with a Teflon-lined cap and evaporated to dryness under a gentle stream of N2. Trimethylsilyl (TMS) derivatives of standards and extracts were made by reacting the subsample with 50 or 100 μl of Tri-Sil TBT (Pierce, Rockford, IL) for 0.5 h at 25°C. Injection volumes of 1 to 2 μl were applied to a 3% OV-17 column in an instrument (Model GC-6AM, Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a flame ionization detector. Either N2 or He was the carrier gas at 40 ml/min. Injector and detector were operated at 300°C. Air and H2 were supplied to the detector at 110 ml/min. The oven was programmed from 150 to 260°C at 6°C/min for zearalenone and 150 to 290°C at 6°C/min for trichothecenes. Final temperatures were held for an additional 5 min. Data were collected on a recorder-integrator (Model CR-1A, Shimadzu Scientific Instruments, Columbia, MD).

Mass spectrometry

Mass spectra of the TMS-derivatives of zearalenone, T-2 toxin, HT-2 toxin, T-2 tetraol and neosolaniol in culture extracts were obtained by GC/MS (HP 5985 mass spectrometer and data system, Hewlett Packard, Palo Alto, CA) at 70 eV. The temperature of the injection port and detector were 275 and 200°C, respectively, and the column temperature was programmed from 120 to 250°C at 10°C/min, with an initial hold time of 5 min and a final hold time of 25 min. The carrier gas was He at 40 to 600 amu. The spectra and chromatograms were reproduced by the hard-copy unit of the computer system.

RESULTS

The four Fusarium isolates used in this study produced detectable amounts of zearalenone or T-2 toxin on the rice medium (data not shown for isolates that did not produce toxin on soy substrates). However, only two of these isolates were toxigenic on whole and cracked soybeans and soybean meal. Of the three cultures known to produce zearalenone, only F. roseum ‘Graminearum’ produced the toxin in soybeans (Table 1), whereas F. sporotrichioides NRRL 3299 produced several trichothecenes on soybeans (Table 2) that were identified and confirmed by GC/MS (Figs. 1 through 4). Spectra obtained from culture extracts were the same as spectra of standards. No peaks above m/e 350 were observed with neosolaniol in culture extracts due to the low concentration present (<0.1 μg/g).

Zearalenone production by F. roseum ‘Graminearum’ (Table 1) was associated with the particle size of the soy substrate and dependent on the moisture level. In general, soybean meal yielded significantly (P<0.05) higher concentrations of zearalenone than did whole soybeans or cracked soybeans. The effect of moisture level was more complex. High moisture resulted in higher zearalenone production in cracked soybeans, but lower production in whole soybeans (P<0.05). The concentration of zearalenone in soybean meal was not significantly (P>0.05) different than in rice.

Fusarium sporotrichioides NRRL 3299 produced T-2 toxin and HT-2 toxin on the three forms of soy substrates at all moisture levels (Table 2). T-2 tetraol was produced in detectable amounts on soybean meal and cracked soybeans only at the lowest moisture level (Table 2). Neosolaniol production was not detected by TLC or GLC, but it was observed in low concentrations in mass spectra of culture extracts (Fig. 4).

T-2 toxin production was generally much higher in soybean meal than in cracked or whole soybeans (Table 2). Its production in cracked and whole soybeans was enhanced by low moisture levels rather than by high levels. However, moisture levels appeared to have no significant (P>0.05) effect on T-2 toxin production on soybean meal. HT-2 toxin production in the soy substrates was always higher than T-2 toxin production, particularly in soybean meal which supported higher production of HT-2 toxin than did cracked or whole soybeans. The response of HT-2 production to moisture level differed somewhat from the response of T-2 production, with HT-2 toxin production being enhanced by low moisture in cracked soybeans, whereas high moisture enhanced production in whole soybeans and soybean meal. Higher concentrations of T-2 toxin and T-2 tetraol were produced on rice than on soybeans. However, HT-2 toxin production was greater in soy products (particularly soybean meal) than on rice. Production of all three toxins on rice was generally enhanced by increasing the moisture content.
TABLE 1. Zearalenone production by F. roseum 'Gramininearum' on soybean and rice media.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Moisture level (% dry wt)</th>
<th>33</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole soybeans</td>
<td></td>
<td>219 ± 26a</td>
<td>99 ± 8</td>
<td>106 ± 25</td>
</tr>
<tr>
<td>Cracked soybeans</td>
<td></td>
<td>90 ± 15</td>
<td>145 ± 17</td>
<td>201 ± 11</td>
</tr>
<tr>
<td>Soybean meal</td>
<td></td>
<td>265 ± 25</td>
<td>271 ± 38</td>
<td>318 ± 10</td>
</tr>
<tr>
<td>Rice</td>
<td></td>
<td>298 ± 12</td>
<td>233 ± 5</td>
<td>227 ± 31</td>
</tr>
</tbody>
</table>

Tabular values are means ± S.E. of zearalenone (µg/g of soybeans or rice) in four replicates.

TABLE 2. Trichothecene production by F. sporotrichioides NRRL 3299 on soybean and rice media.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Moisture level (% dry wt)</th>
<th>T-2 Toxin</th>
<th>HT-2 Toxin</th>
<th>T-2 Tetraol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole soybeans</td>
<td>33</td>
<td>32 ± 4a</td>
<td>49 ± 5</td>
<td>0b</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>37 ± 4</td>
<td>80 ± 9</td>
<td>0</td>
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<tr>
<td></td>
<td>60</td>
<td>17 ± 3</td>
<td>109 ± 10</td>
<td>0</td>
</tr>
<tr>
<td>Cracked soybeans</td>
<td>33</td>
<td>71 ± 4</td>
<td>111 ± 4</td>
<td>27 ± 1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>17 ± 2</td>
<td>71 ± 1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>15 ± 3</td>
<td>46 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>33</td>
<td>103 ± 19</td>
<td>285 ± 43</td>
<td>52 ± 4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>110 ± 15</td>
<td>284 ± 33</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>92 ± 12</td>
<td>439 ± 96</td>
<td>0</td>
</tr>
<tr>
<td>Rice</td>
<td>33</td>
<td>54 ± 6</td>
<td>8 ± 2</td>
<td>33 ± 6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>187 ± 12</td>
<td>41 ± 8</td>
<td>141 ± 9</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>214 ± 23</td>
<td>58 ± 7</td>
<td>105 ± 11</td>
</tr>
</tbody>
</table>

Tabular values are means ± S.E. of the trichothecenes (µg/g of rice or soybeans) in four replicates.

DISCUSSION

The results indicated clearly that heat-sterilized soybeans and soybean products can support the production of zearalenone, T-2 toxin, HT-2 toxin, T-2 tetraol and neosolaniol by certain strains of Fusarium. Two of the three Fusarium isolates tested failed to produce zearalenone on soybeans and soybean products, whereas they did so in rice cultures. Soybeans have been previously investigated for zearalenone production by isolates of F. roseum and none was observed to be produced (11). The isolate that produced zearalenone on soy substrates, produced greater concentrations of zearalenone on soybean meal and rice than on whole or cracked soy-
beans. These results suggest that soybeans (cracked or whole) possess some heat stable factor(s) that limit production of toxins by Fusarium isolates which may be removed in the processing of soybeans into soybean meal.

HT-2 toxin was the predominant trichothecene produced on soy substrates by F. sporotrichioides (Table 2). This result suggests that trichothecene problems may be overlooked when suspect samples are only analyzed for the presence of T-2 toxin. Previous animal studies with ducklings and rodents have indicated that HT-2 toxin (equivocal to T-2 toxin), suggests that soybeans possess a factor that contributes in the processing of soybeans into soybean meal.

Fusarium isolates which may be re-produced in the processing of soybeans into soybean meal. HT-2 toxin exhibits similar toxicity to that of T-2 toxin, but only limited research has been done on the effects of neosolanoil and T-2 tetraol (24). The data and observations reported here suggest that the so-called “minor” trichothecenes should be more thoroughly investigated for their effects in animals. The limited ability of F. sporotrichioides to produce T-2 tetraol (a completely deacylated product of T-2 toxin) in soybeans in comparison to rice (Table 2) plus the enhanced ability of the fungus to produce HT-2 toxin (a partially deacylated product of T-2 toxin), suggests that soybeans possess a factor that controls acylation reactions by the fungus.

Data reported here indicate that certain Fusarium isolates are able to produce trichothecenes on soybeans and soybean meal. These findings support field observations of toxicity in animals consuming Fusarium-infested soybean products containing Fusarium toxins (Stanislav, Hagler, and Hamilton, unpublished data).

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REFERENCES


JOURNAL OF FOOD PROTECTION. VOL. 48, MARCH 1985

con't. p. 245
TESTING OYSTERS FOR *E. coli*

| Table 1. Results of the analysis of 25 oyster samples for *E. coli* using EC-MUG. |
|---------------------------------|------------------|
| Total positive EC tubes         | 127              |
| Total fluorescent tubes         | 103              |
| Fluorescent +, *E. coli* +      | 102              |
| Fluorescent -, *E. coli* -      | 24               |
| Fluorescent +, *E. coli* -      | 1                |
| Fluorescent -, *E. coli* +      | 0                |

the fluorescence was missed when making the isolation from the EMB plates since the *Klebsiella* isolates were checked for fluorescence in MUG-EC and found to be negative.

While it would be ideal to be able to monitor the presence of *E. coli* during the first stage of the MPN procedure, incorporating the MUG into the EC broth was a workable alternative for oyster samples. Fluorescent reactions were strong even at the elevated temperature of incubation, and the EMB plates contained fewer contaminating bacteria than when streaked from the LST tubes. A saving of 3-4 d in identification time is still gained by the use of EC-MUG since biochemical confirmation of *E. coli* from positive EC tubes would not be necessary.

REFERENCES


Richardson, et al., con't. from p. 243