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# Occurrence of Zearalenols (Diastereomeric Mixture) in Corn Stalk Rot and Their Production by Associated *Fusarium* Species

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**Zearalenol was extracted from *Fusarium*-infected stems of corn from southern Italy. The toxin, which appeared as a single compound in various thin-layer chromatography systems, was resolved by high-pressure liquid chromatography into two components. A gas chromatography-mass spectrometry examination of a purified fraction confirmed the natural occurrence of zearalenol as a diastereomeric mixture and led to the identification of alpha (56 ng/g) and beta (27 ng/g) isomers. Among nine *Fusarium* species found associated with stalk rot in corn, only *Fusarium culmorum* (*F. roseum* 'Culmorum') and *F. equiseti* (*F. roseum* 'Gibbosum') produced zearalenol and always produced it in a diastereomeric mixture of alpha and beta isomers.**

Zearalenol, 2,4-dihydroxy-6-(6,10-dihydroxy-*trans*-1-undecenyl)-benzoic acid-*u*-lactone, is an estrogenic derivative of zearalenone produced by certain *Fusarium* species (7, 17). Of the two possible diastereomers of zearalenol, designated alpha-zearalenol (low melting point, 168 to 169°C) and beta-zearalenol or *epi*-zearalenol (high melting point, 174 to 176°C) (8, 14), only the former was reported as occurring naturally in *Fusarium* cultures (7) and in animal feed (11). In a rat uterus bioassay, Hagler et al. (7) found the alpha-zearalenol isomer to be three to four times more active than zearalenone, whereas the estrogenic activity of the beta-zearalenol isomer was equal to that of zearalenone.

Zearalenol, along with other *Fusarium* mycotoxins (zearalenone and deoxynivalenol), was found to be associated with foot and stalk rot in corn from southern Italy (Basilicata) during 1983 (3). The toxin was resolved by high-pressure liquid chromatography (HPLC) into two components, whereas it appeared as a single spot when tested by thin-layer chromatography (TLC).

The finding of zearalenol in corn stems infected by *Fusarium* species in the field led us to reinvestigate the natural diastereomeric form of this toxin and to study its production by *Fusarium* species associated with stalk rot.

## MATERIALS AND METHODS

**Corn samples.** Samples of *Fusarium*-infected corn stems were collected from fields in Basilicata, Italy. The lower internodes of the stems were cut open, and portions of the reddish or brown pith were separately selected. The resulting samples, after a subsampling for mycological purposes, were cut into short pieces and finely ground with a Buhler mill.

**Isolation and identification of *Fusarium* species.** Fragments of visibly infected corn stem tissues were plated out on *Fusarium*-selective peptone-PCNB medium (3, 13). Single-spore cultures of *Fusarium* spp., obtained on plates of potato sucrose agar, were identified in accordance with the nomenclature of Booth (1) and compared with the nomenclature of Snyder and Hansen (15) and Snyder et al. (16).

**Toxin production.** The *Fusarium* isolates were grown on 200 g of corn kernels brought to about 45% moisture in 500-ml Erlenmeyer flasks. The substrate was autoclaved for 20 min at 120°C and then inoculated with potato glucose agar cultures of freshly isolated *Fusarium* strains (single spore) from decayed corn stems. The cultures were incubated at either 27°C for 4 weeks or 27°C for 1 week plus 3 weeks at 12°C. After incubation, the cultures were dried at 60°C and finely ground.

**Toxin analysis.** Zearalenols were extracted by a multimycotoxin method previously described for zearalenone and trichothecenes (2, 19). Samples (50 g) of corn stems or samples (20 g) of dried *Fusarium* cultures were extracted with methanol-aqueous NaCl, defatted with hexane, and partitioned with dichloromethane (instead of chloroform). After evaporation of the solvent, the residue was brought up to 2 ml with methanol-water (40:60), passed through a Sep-Pak C-18 cartridge (Waters Associates, Inc., Milford, Mass.), and eluted with a new portion (2 ml) of the methanol-water mixture (fraction A). Further elution with two 2-ml portions of methanol yielded fraction B. The two fractions were separately evaporated to near dryness and reconstituted with 0.5 ml of methanol. Fraction A was examined for nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, and fusarenone; fraction B was examined for diacetoxyscirpenol, T-2 toxin, zearalenone, and zearalenols (alpha and beta). Analyses of zearalenone and trichothecenes were performed by TLC and gas chromatography (GC) (2). Deoxynivalenol was also confirmed by HPLC (18). The procedure for extraction and analysis of moniliformin has been reported elsewhere (5).

Total zearalenols were detected in stem samples by two-dimensional TLC with benzene-acetone (60:35) and toluene-ethyl acetate-formic acid (60:30:10) as the solvent systems and were quantitated as trimethylsilyl (TMS) derivatives by GC with a 3% OV-1-packed column (2, 12).

GC-mass spectrometry (GC/MS) was performed on a selected stem sample after TLC purification of its extract in the above-mentioned solvent systems. Low-resolution mass spectra were obtained at 70 eV in the electron impact mode on a Hewlett-Packard model 5987B GC/MS/DS (gas chromatograph-mass spectrometer-data system). The source tem-

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TABLE 1. Mycotoxins and *Fusarium* species associated with stalk rot in corn in southern Italy[a]

| Corn sample | Amt (ng/g) of: | | | *Fusarium* species[d] |
|---|---|---|---|---|
| | Zearalenol[b] | Zearalenone[b] | Deoxynivalenol[c] | |
| Reddish stem rot | | | | |
| 1 | 83[e] | 3,331 | 115 | 1, 2, 3, 4, 5, 6, and 7 |
| 2 | 51 | 7,433 | 668 | 1, 2, 3, 4, 6, 8, and 9 |
| 3 | 25 | 668 | 162 | 1, 2, 3, 4, 6, and 8 |
| Brown stem rot | | | | |
| 4 | 10 | 1,554 | 71 | 1, 2, 3, 4, 6, 7, and 8 |
| 5 | 86 | 883 | 81 | 1, 2, 3, 4, and 7 |
| 6 | 20 | 411 | 18 | 1, 2, 3, 4, 5, and 8 |

[a] No 3-acetyldeoxynivalenol, fusarenone, nivalenol, T-2 toxin, diacetoxyscirpenol, or moniliformin was detected.
[b] Determined by GC.
[c] Determined by HPLC.
[d] Classified in accordance with the nomenclature of Booth (1): 1, *F. moniliforme*; 2, *F. moniliforme* var. *subglutinans*; 3, *F. culmorum*; 4, *F. equiseti*; 5, *F. semitectum*; 6, *F. oxysporum*; 7, *F. acuminatum*; 8, *F. fusarioides*; and 9, *F. ventricosum*.
[e] Confirmed by GC/MS (full mass spectra) as a diastereomeric mixture of alpha-zearalenol (56 ng/g) and beta (*epi*)-zearalenol (27 ng/g).

perature was kept at 200°C, and the source pressure was kept at $5 \times 10^{-6}$ torr ($7 \times 10^{-7}$ kPa). Gas chromatographic separation of the zearalenol TMS derivatives was performed on a DB-5 glass capillary column (30 m in length and 0.25 mm in diameter) with a programmed temperature of 80 to 300°C at 20°C/min. The injector temperature was 250°C, and the carrier gas (helium) flows were 90 and 1.5 ml/min at the column inlet and outlet, respectively. TMS derivatives of zearalenols (alpha and beta) were obtained by reacting the extract (or the standard toxins) with Tri-Sil BT (Pierce Chemical Co., Rockford, Ill.)-chloroform (1:1) at room temperature for 30 min. The data relative to the GC/MS quantitative analysis were obtained as the mean value of individual quantitations performed on the same sample with the external standard method (a 1:1 mixture of alpha- and beta-zearalenols was used as the external standard) by operating the apparatus in the total-ion-chromatogram mode or in the selected-ion-monitoring mode at *m/z* 536 ($M^+$), 333, and 305. For individual quantitations in the different monitoring modes, the area counts (obtained by computer integration) of the chromatographic peaks having retention times identical to those of the reference standards were compared with the area counts obtained for the reference standards under the same experimental conditions. A baseline resolution of alpha- and beta-zearalenol signals was obtained with the DB-5 glass capillary column.

HPLC was used for individual determinations of both isomers in the fungal cultures after purification of the extracts by TLC in benzene-acetone (60:35). The HPLC apparatus was a Perkin-Elmer series 3B microcomputer-controlled pump module in connection with a Perkin-Elmer LC-75 UV variable-wavelength detector set at 236 nm. A Hibar prepacked column (250 mm by 4 mm [inner diameter]) of Li-Chrosorb RP-18 (7 μm) (E. Merck AG, Darmstadt, Federal Republic of Germany) was used. HPLC-grade methanol-water (65:35) was used as the mobile solvent at a flow



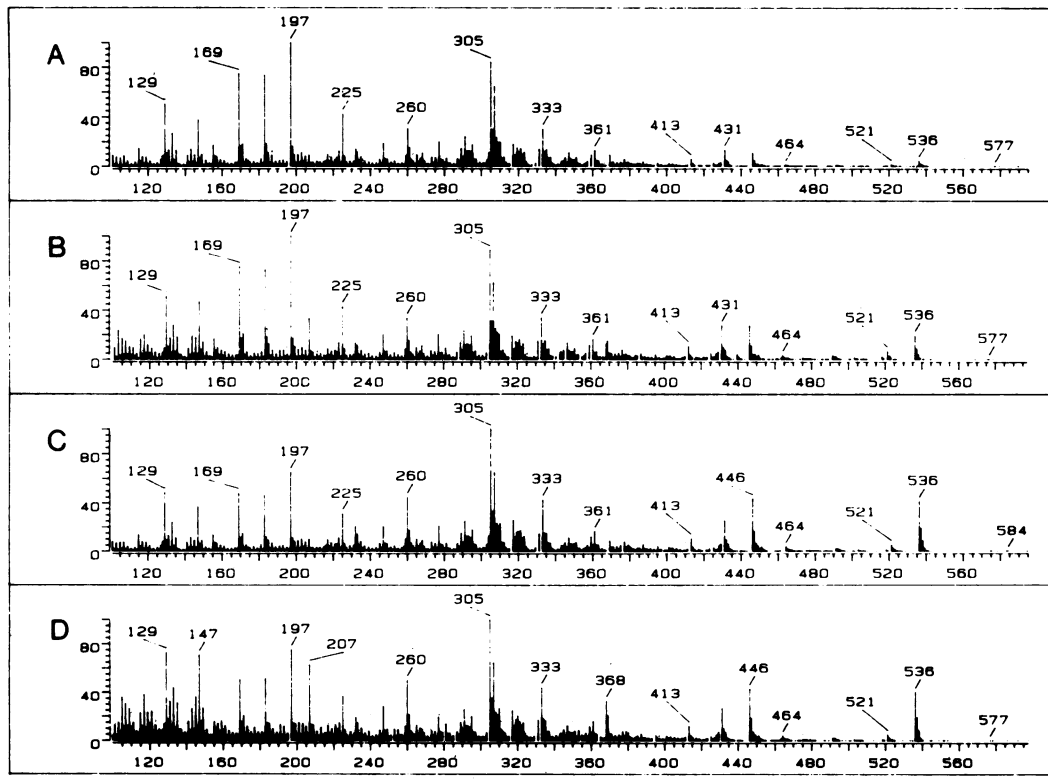


FIG. 1. Electron impact mass spectra (70 eV) of the TMS ethers of alpha- and beta-zearalenols present in sample (1) of decayed corn stalk (B and D, respectively) compared with the mass spectra of their reference standards (A and C, respectively). The source temperature was 200°C, and the source pressure was $5 \times 10^{-6}$ torr. The GC retention time (on a DB-5 glass capillary column) for A and B was 14.09 min, and that for C and D was 14.23 min.

rate of 1 ml/min (9). UV spectra from 200 to 400 nm were determined on the variable-wavelength recorder after the solvent flow was stopped at the appropriate retention times.

## RESULTS

**Incidence of zearalenols.** Zearalenol (diastereomeric mixture), along with other *Fusarium* mycotoxins (zearalenone and deoxynivalenol), was present in all six samples assayed at levels ranging from 10 to 86 ng/g (Table 1). The values reported in Table 1 represent the total amount of zearalenols derived from the GC analysis after comparison with a reference standard (a 1:1 mixture of both isomers). Sample 1, analyzed by GC/MS, contained 56 ng of alpha-zearalenol and 27 ng of beta-zearalenol per g.

**Identities of the naturally occurring zearalenols.** Zearalenol isomers had the same $R_f$ values in both TLC solvent systems used in this study. Only one spot was observed when two-dimensional TLC was used, corresponding to the total amount of zearalenols. The two isomers were not readily resolved by GC with a 3% OV-1-packed column, whereas they produced distinct signals when analyzed by GC/MS with a DB-5 glass capillary column. The retention times of alpha- and beta-zearalenol TMS ethers under our GC/MS experimental conditions were 14.09 and 14.23 min, respectively. The indentities of the naturally occurring zearalenols in sample 1 of decayed corn stalk were determined by comparing their retention times and mass spectra with those of the reference standards. The retention times (on a DB-5 glass capillary column) relative to those of the reference toxins were also 14.09 and 14.23 min, respectively, and their mass spectra matched those of the corresponding reference toxins well (Fig. 1). No significant differences were observed between the mass spectra of both isomers TMS derivatives, although the relative intensity of the molecular ion (536) for the beta isomer appeared to be higher than that for the alpha isomer.

Good resolution was obtained by HPLC when the two isomers were eluted after 8.20 min (beta) and 11.82 min (alpha). The high-performance liquid chromatogram of an extract from a culture of *Fusarium equiseti* on corn after TLC purification in benzene-acetone (60:35) is shown in Fig. 2 (upper curve). Zearalenone, which has a longer retention time than alpha-zearalenol, is not shown in the figure because of its exclusion from the sample during the TLC purification step. A further confirmation of the identities of both isomers in the *Fusarium* cultures was obtained by comparison of the UV spectra of the extracted alpha- and beta-zearalenols eluted from the HPLC apparatus with the UV spectra of reference toxins eluted at the same retention times.

***Fusarium* species associated with stalk rot in corn.** The *Fusarium* species isolated from the lower internodes of several samples of infected corn stems are shown in Table 2. Nine different species were identified in accordance with the nomenclature of Booth (1), i.e., *F. moniliforme* Sheldon, *F. moniliforme* var. *subglutinans* Wollenweber et Reinking, *F. culmorum* (W. G. Smith) Saccardo, and *F. equiseti* (Corda) Saccardo, which were isolated from all samples, and *F. oxysporum* Schlechtendahl, *F. fusarioides* (Fragoso et Ciferri) Booth, *F. acuminatum* Ellis et Everhart, *F. ventricosum* Appel et Wollenweber, and *F. semitectum* Berkeley et Ravenel, which were isolated much less frequently and not from all samples. Identified in accordance with the nomenclature of Snyder and Hansen (15) and Snyder et al. (16) were *F. culmorum*, *F. equiseti*, and *F. acuminatum*, which corresponded to *F. roseum* 'Culmorum', *F. roseum* 'Equiseti' (*F. roseum* 'Gibbosum'), and *F. roseum* '*Acuminatum*', respectively.

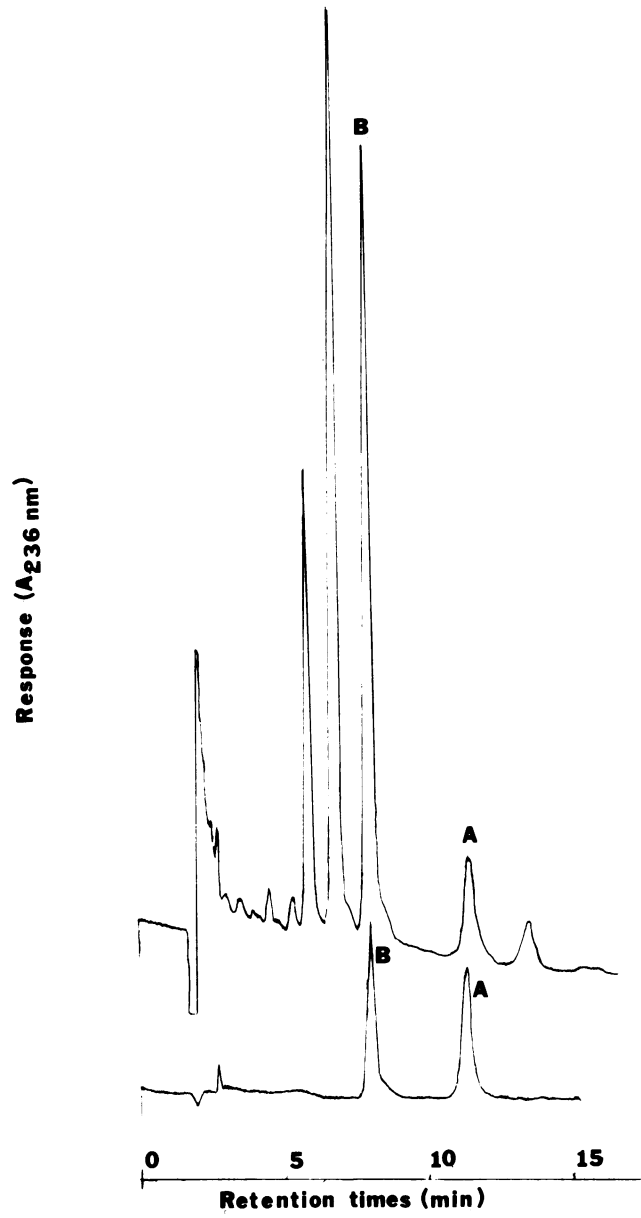


FIG. 2. High-pressure liquid chromatograms of an extract from a culture of *F. equiseti* on corn (upper curve) and a standard solution containing 25 ng each of alpha- and beta-zearalenols (lower curve). (A) Alpha-zearalenol; (B) beta-zearalenol. HPLC was done with reverse-phase Li-Chrosorb RP-18 with methanol-water (65:35) as the mobile phase (the culture extract was previously purified by TLC in benzene-acetone [60:35]). $A_{236}$nm, Absorbance at 236 nm.

**Production of zearalenol and zearalenone.** Of the nine *Fusarium* species found associated with stalk rot in corn, only *F. culmorum* and *F. equiseti* produced zearalenol in cultures (Table 2). The toxin was produced by all isolates of *F. culmorum*, but only by two of five isolates of *F. equiseti*. However, the toxigenic *F. equiseti* isolates produced higher amounts of the toxin than did the *F. culmorum* isolates. Zearalenol was always produced as a diastereomeric mixture, and *F. culmorum* isolates produced more alpha isomer than beta isomer, whereas *F. equiseti* isolates produced

TABLE 2. Production of zearalenol (diastereomeric mixture) and zearalenone by *Fusarium* species associated with stalk rot in corn in southern Italy[a]

| *Fusarium* species[b] | No. of isolates tested | No. of isolates producing toxin | Amt (ng/g) of zearalenol isomers produced[c] | | Amt (μg/g) of zearalenone produced[d] |
|---|---|---|---|---|---|
| | | | Alpha | Beta | |
| *F. acuminatum* | 1 | 0 | | | |
| *F. culmorum* | 4 | 4 | 238 | 157 | 36.5 |
| | | | 442 | 290 | 115 |
| | | | 612 | 338 | 375 |
| | | | 577 | 434 | 1,865 |
| *F. culmorum*[e] | 1 | 1 | 373 | 290 | 550 |
| *F. equiseti* | 5 | 3 | 271 | 1,108 | 8.3 |
| | | | 816 | 4,927 | 17 |
| | | | | | 10 |
| *F. fusarioides* | 2 | 0 | | | |
| *F. fusarioides*[e] | 1 | 0 | | | |
| *F. moniliforme* | 2 | 0 | | | |
| *F. moniliforme* var. *subglutinans* | 2 | 0 | | | |
| *F. oxysporum* | 2 | 0 | | | |
| *F. semitectum* | 1 | 0 | | | |
| *F. ventricosum* | 1 | 0 | | | |

[a] *Fusarium* species were grown on autoclaved corn kernels at 27°C for 4 weeks.
[b] In accordance with the nomenclature of Booth (1).
[c] Determined by HPLC.
[d] Determined by GC.
[e] Incubated at 27°C for 1 week plus 3 weeks at 12°C.

more beta isomer than alpha isomer. The production of zearalenol was always associated with that of zearalenone, but one isolate of *F. equiseti* produced only zearalenone (10 mg/kg of dry culture). Zearalenol and zearalenone were normally produced by both of the toxigenic species under a constant temperature of 27°C, as well as by an *F. culmorum* isolate under temperature stress conditions of 12°C.

## DISCUSSION

Low levels of zearalenol have previously been reported in cultures of zearalenone-producing isolates of *F. culmorum* (6), but no confirmation with analytical techniques other than TLC or mention of the diastereomeric composition of zearalenol was made. The other *Fusarium* species found in this study to be a zearalenol producer (*F. equiseti*) is probably identical to *F. roseum* 'Gibbosum' (7), Zearalenol has also been produced by *F. semitectum* (17). Zearalenol production seems more constant in *F. culmorum* than in *F. equiseti*, which produces the largest amount of the beta isomer; however, this isomer is three times less estrogenic than the alpha isomer (7). The production of both zearalenol isomers, together with zearalenone and zearalanol, by *Fusarium* spp. in rice cultures has been recently observed by K. E. Richardson, W. M. Hagler, and C. J. Mirocha (personal communication).

The occurrence of zearalenol in animal feed (oats and corn) has previously been reported by Mirocha et al. (11), but only the alpha isomer was found in that circumstance as a naturally occurring diastereomer. We emphasize the ability of *Fusarium* species to produce both diastereomers of zearalenol not only under laboratory conditions but also in naturally infected field samples. *Fusarium* toxins previously encountered in decayed corn stalks were zearalenone and deoxynivalenol (also found in the samples studied here ) and T-2 toxin (10).

We found a very high incidence of zearalenol in both reddish pith and brown pith of *Fusarium*-infected corn stalks standing in the field. Moreover, its production seems to be widespread in *F. culmorum* and *F. equiseti* isolates on cereals. This leads us to assume that zearalenol, along with zearalenone, may contribute to outbreaks of hyperestrogenism in swine, and it might be more significant thatn currently realized (11). Moreover, it is noteworthy that, as with zearalenone (4), there are toxigenic isolates of *F. culmorum* and *F. equiseti* which do not necessarily require a low temperature for zearalenol synthesis.

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## LITERATURE CITED

1. **Booth, C.** 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, England.
2. **Bottalico, A., P. Lerario, and A. Visconti.** 1983. Mycotoxins occurring in *Fusarium*-infected maize ears in the field, in some European countries, p. 375–382. *In* Proceedings of the International Symposium on Mycotoxins. Publishing Science Department, NIDOC, Dokki, Cairo, Egypt.
3. **Bottalico, A., and A. Logrieco.** 1984. Fusariotoxins associated with stalk rot in Maize, in southern Italy, p. 209–211. *In* Proceedings of the Sixth Congress of the Mediterranean Phytopathology Union. Egyptian Phytopathological Society, Cairo, Egypt.
4. **Bottalico, A., A. Visconti, and P. Lerario.** 1982. Influenza della temperatura sulla produzione di zearalenone da parte di isolati di *Fusarium* da cereali, in Italia. Phytopathol. Mediterr. **21**: 79–82.
5. **Bottalico, A., A. Visconti, and M. Solfrizzo.** 1982 Production of moniliformin by *Fusarium* species, in Italy. Phytopathol. Mediterr. **21**:105–106.
6. **Chelkowski, J., A. Visconti, and M. Manka** 1984. Production of trichothecenes and zearalenone by *Fusarium* species isolated from wheat. Nahrung **28**:493–496.
7. **Hagler, W. M., C. J. Mirocha, S. V. Pathre, and J. C. Behrens.** 1979. Identification of the naturally occurring isomer of zearalenol produced by *Fusarium roseum* 'Gibbosum' in rice culture. Appl. Environ. Microbiol. **37**:849–853.
8. **Hidy, P. H., R. S. Baldwin, R. L. Greasham, C. L. Keith, and J. R. McMullen.** 1977. Zearalenone and some derivatives: production and biological activities. Adv. Appl. Microbiol. **22**:59–82.
9. **James, L. J., L. G. McGirr, and T. K. Smith.** 1982. High pressure liquid chromatography of zearalenone and zearalenols in rat urine and liver. J. Assoc. Off. Anal. Chem. **65**:8–13.
10. **Mirocha, C. J., B. Schauerhamer, C. M. Christensen, and T. Kommedahl.** 1979. Zearalenone, deoxynivalenol, and T-2 toxin associated with stalk rot in corn. Appl. Environ. Microbiol. **38**:557–558.
11. **Mirocha, C. J., B. Schauerhamer, C. M. Christensen, M. L. Niku-Paavola, and M. Nummi.** 1979. Incidence of zearalenol (*Fusarium* mycotoxin) in animal feed. Appl. Environ. Microbiol. **38**:749–750.
12. **Mirocha, C. J., B. Schauerhamer, and S. V. Pathre.** 1974. Isolation, detection, and quantitation of zearalenone in maize

and barley. J. Assoc. Off. Anal. Chem. **57**:1104–1110.
13. **Nash, S. M., and W. C. Snyder.** 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soil. Phytopathology **52**:567–572.
14. **Shipchandler, M. T.** 1975. Chemistry of zearalenone and some of its derivatives. Heterocycles (Sendai) **3**:471–520.
15. **Snyder, W. C., and H. N. Hansen.** 1945. The species concept in *Fusarium* with reference to discolor and other sections. Am. J. Bot. **32**:657–666.
16. **Snyder, W. C., H. N. Hansen, and J. W. Oswald.** 1957. Cultivars of the fungus *Fusarium*. J. Madras Univ. Sect. B **27**:185–192.
17. **Stipanovic, R. D., and H. W. Schroeder.** 1975. Zearalenol and 8′-hydroxyzearalenone from *Fusarium roseum*. Mycopathologia **57**:77–78.
18. **Visconti, A., and A. Bottalico.** 1983. Detection of *Fusarium* trichothecenes (nivalenol, deoxynivalenol, fusarenone and 3-acetyldeoxynivalenol) by high-performance liquid chromatography. Chromatographia **17**:97–100.
19. **Visconti, A., A. Bottalico, F. Palmisano, and P. G. Zambonin.** 1984. Differential-pulse polarography of trichothecene mycotoxins. Determination of deoxynivalenol, nivalenol and fusarenone-X in Maize. Anal. Chim. Acta **159**:111–118.