Trichothecenes and Other Mycotoxins
Edited by J. Lacey

CHAPTER 7

Zearalenone production in groundnut*

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INTRODUCTION

Fusarium spp. are important components of the seed mycoflora of healthy groundnut pods (Jackson, 1965; Joffe, 1969; McDonald, 1969), and are also implicated in pod rots (Mercer, 1977; Mehan *et al.*, 1981). Some species of this genus are well-known producers of mycotoxins such as zearalenone, diacetoxyscirpenol and neosolaniol (Mirocha *et al.*, 1976; Ueno, 1977). Zearalenone, an oestrogenic mycotoxin, has mainly been found in cereal grains and their products (Mirocha and Christensen, 1974). Zearalenone has been reported to occur naturally in sesame (Mirocha *et al.*, 1976) and in groundnuts (Mehan and McDonald, 1982). This chapter reports the natural occurrence of zearalenone and the effects of cultural conditions on its production in groundnut seeds by a toxigenic strain of *F. oxysporum*.

MATERIALS AND METHODS

Partially rotted pods were collected from the 1981 and 1982 rainy season and the 1981/82 and 1982/83 post-rainy season groundnut crops at ICRISAT Center farm at Patancheru near Hyderabad, India. The pods were hand-shelled and seeds with pink to purple-stained testae typical of *Fusarium* infection were selected for testing. A total of 204 samples from four seasons were tested for the presence of zearalenone.

Zearalenone extraction

Seed samples of 25 g each were finely ground in a Waring blender at full speed for 5 min. Zearalenone was extracted with dichloromethane and purified using acetonitrile : petroleum ether partition as described by Mirocha *et al.* (1967).

* Submitted as ICRISAT Conference Paper No. 163 by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P.O. 502 324, A.P., India.

Detection, confirmation and quantitation zearalenone

Purified extracts were spotted on activated silica gel G (250 μm thickness) coated thin-layer plates. A known quantity of a standard solution of zearalenone in chloroform was also applied to the plates for comparison. The plates were developed with toluene : ethyl acetate : chloroform : 90% formic acid (7 : 5 : 5 : 2) in an unlined, unequilibrated tank (Gimeno, 1980). Zearalenone spots in sample extracts were identified by their correspondence with the standard toxin spots in R_f value and fluorescence under UV light: bluish-green at 365 nm and greenish at 254 nm.

Zearalenone spots of sample extracts were confirmed after additional chemical tests. The toxin spots turned yellowish-brown when the developed thin-layer chromatoplates were sprayed with freshly prepared 50% H_2SO_4 in methanol and then heated in a drying oven at 100 °C for 10 min (Mirocha *et al.*, 1967). The fluorescence of the toxin spots was enhanced when the thin-layer plates were sprayed with freshly prepared 15% aluminium chloride in methanol (Eppley *et al.*, 1974). Spots eluted in methanol gave a positive reaction with an aqueous solution of ferric chloride. When sample extracts eluted in absolute ethanol were scanned for absorption maxima between 220 and 330 nm in a Beckman spectrophotometer, the toxin exhibited characteristic peaks at 236, 274 and 314 nm, corresponding to that of standard zearalenone. The amount of zearalenone was calculated by determining absorbance at 236 nm after eluting the zearalenone spots from the plates with absolute ethanol. The amount of zearalenone was computed from a standard curve prepared by determining the absorbance at 236 nm of different concentrations of standard zearalenone.

Testing *Fusarium* species for production of zearalenone

The *Fusarium* cultures used in this investigation were obtained from the seeds and shells of rotted groundnut pods. Thirteen *Fusarium* isolates belonging to five different species were obtained from rotted pods from the 1978/79 post-rainy season groundnut crop at the ICRISAT Center. Single-spore isolates of different *Fusarium* species were maintained on Czapek–Dox agar slants at 4 °C. The following isolates were tested for their ability to produce zearalenone: five each of *F. solani* and *F. oxysporum*, and one each of *F. moniliforme*, *F. nivale* and *F. fusarioides*. In another experiment, 54 isolates belonging to *F. solani*, *F. semitectum*, *F. oxysporum* and *F. moniliforme*, obtained from groundnut shells and seeds from the 1982 rainy season and the 1982/83 post-rainy season crops, were also screened for their capacity to produce zearalenone.

Inoculum of different isolates of *Fusarium* species was raised in shake cultures in 50 ml Czapek–Dox broth in 250 ml conical flasks for 10 days at 25 °C. One millilitre of inoculum (6.1×10^4 conidia/ml) of each isolate was used

to separately inoculate 25 g samples of autoclaved polished rice (with 45% moisture content before autoclaving) in 250 ml Erlenmeyer flasks. Inoculated samples were incubated at 25 °C for 2 weeks, then at 15 °C for 8 weeks. After incubation the mouldy rice samples in the flasks were dried overnight at 50 °C. The dried material was then ground in a blender and zearalenone was extracted and analysed as described earlier.

Effects of cultural conditions on *in vitro* production of zearalenone in groundnuts

Using a toxigenic isolate of *F. oxysporum* (F6), the effects of moisture, temperature and incubation period on *in vitro* production of zearalenone in groundnut seeds were investigated. Mature groundnut seeds (cv. Robut 33–1), free of internally borne fungi, were used. Seed samples were surface-sterilized by soaking them in an aqueous solution of 1% sodium hypochlorite for 3 min and then rinsing thrice in sterile distilled water. Seed moisture content was adjusted by soaking the seeds in sterile distilled water for different periods. Seed lots (25 g each) were adjusted to three levels of moisture content, i.e., 20, 35 and 50%. The seed testae were scarified using a sterile needle. Each seed lot was then transferred to a sterile 250 ml conical flask and inoculated with 2 ml of the spore suspension (6.1×10^4 conidia/ml) of a 10-day-old culture of the toxigenic strain of *F. oxysporum*. After inoculation, seeds in the flasks were subjected to eight different incubation temperatures: (i) 18 °C for 3 weeks, (ii) 18 °C for 6 weeks, (iii) 27 °C for 3 weeks, (iv) 27 °C for 6 weeks, (v) 25 °C for 1 week then 15 °C for 2 weeks, (vi) 25 °C for 2 weeks then 15 °C for 4 weeks, (vii) a diurnal temperature cycle of 30 °C by day (6 a.m.–6 p.m.) and 18 °C by night (6 p.m.–6 a.m.) for 3 weeks and (viii) a diurnal temperature cycle of 30 °C by day and 18 °C by night (6 p.m.–6 a.m.) for 3 weeks and (viii) a diurnal temperature cycle of 30°C by day and 18°C by night for 6 weeks. Each treatment was replicated thrice.

After incubation, samples were dried at 50 °C for 24 h and ground finely in a Waring blender at full speed for 5 min. Zearalenone was extracted and analysed as described earlier.

RESULTS AND DISCUSSION

the 204 seed samples tested, one sample each from the 1981 rainy season and the 1981/82 post-rainy season and two samples from the 1982/83 post-rainy season groundnut crops were contaminated with zearalenone ranging from 0.72 to 1.84 mg/kg (Table 1). None of the 94 samples from the 1982 rainy season crop contained zearalenone. Of 13 strains tested in 1981, only one isolate of *F. oxysporum* (F6) produced zearalenone (at a level of 148 mg/kg) in rice culture. No isolate of any other species of *Fusarium* tested (*F.*

Table 1 Natural occurrence of zearalenone in *Fusarium*-infected groundnut seeds

Crop/year	No. of samples tested	No. of samples containing zearalenone	Level of zearalenone (mg/kg)
Rain-fed, 1981	12	1	1.5
Irrigated, 1981/82	60	1	1.8
Rain-fed, 1982	94	0	—
Irrigated, 1982/83	38	2	0.7, 1.1

solani, *F. moniliforme*, *F. fusarioides*, and *F. nivale*), nor any of 54 isolates tested in 1983, including *F. solani*, *F. semitectum*, *F. oxysporum* and *F. moniliforme*, produced zearalenone. Eugenio *et al.* (1970) failed to find zearalenone production by *F. solani*, *F. nivale*, *F. moniliforme* and *F. oxysporum*, but Mirocha *et al.* (1969, 1976) reported its production by some isolates of *F. oxysporum* and *F. moniliforme*. The small number of isolates tested in all these studies precludes any generalization as to the likelihood of occurrence of isolates able to produce zearalenone.

Zearalenone was produced in groundnut seeds at all moisture contents, incubation temperatures and incubation periods tested (Table 2). Significant differences were found in the quantities of zearalenone produced with different treatments. The production of zearalenone increased significantly with increasing seed moisture content and period of incubation. Of the three moisture contents tested, fungal growth was more vigorous at 50% seed moisture content that at either 35% or 20% which did not differ markedly. Similar effects of moisture content on fungal growth were noted by Sherwood and Peberdy (1972) while investigating zearalenone production in wheat, barley and oats by a toxigenic isolate of *F. graminearum*. Fungal growth was not affected by temperature differences. Toxin production was highest at 50% seed moisture content irrespective of temperature treatment (Table 2).

At constant temperatures, toxin production was significantly greater at 18 °C than at 27 °C. Treatments in which temperature was altered, either after one-third of the incubation period from 25 °C to 15 °C, or diurnally from 30 °C by day to 18 °C at night, gave higher levels of zearalenone than did the constant-temperature treatments. Incubation at 15 °C, after a period at 25 °C, enhanced zearalenone production but the lower temperature did not appear to be essential as considerable amounts of the toxin were produced during constant incubation at 27 °C. Similar observations have been reported by Sherwood and Peberdy (1972) and Naik *et al.* (1978) for zearalenone production in cereals by toxigenic isolates of *F. graminearum*. Schroeder and

Table 2 Effects of temperature, moisture content and incubation period on zearalenone production in groundnut seeds by *Fusarium oxysporum*

Incubation period (weeks)	Seed moisture content (%)	Zearalenone production (mg/kg)			
		Incubation temperatures			
		Constant at		At 25 °C for first third of period, then at 15 °C	At 30 °C during day and 18 °C at night[b]
		27 °C	18 °C		
3 weeks	20	3.1	6.2	10.0	8.0
	35	4.9	10.1	16.1	14.1
	50	6.0	11.9	20.1	17.6
6 weeks	20	5.1	9.8	11.9	10.9
	35	7.8	13.1	20.6	18.8
	50	9.8	16.2	26.6	21.3
SE ±		0.50			
CV (%)		4.10			

[a] Mean of three replications.
[b] Day period = 6 a.m. to 6 p.m.; night period = 6 p.m. to 6 a.m.

Hein (1975) also reported that the toxigenic isolate (S-74-lc) of *F. graminearum* produced considerably more zearalenone in cracked corn or grain sorghum when incubated at a constant 25 °C than when changed to 10 °C after growth was established at 25 °C. These results suggest that varied temperature stress and relatively low temperatures are not essential for production of zearalenone, and that wide variations in optimum temperatures for zearalenone production exist among the isolates of *Fusarium* species. There is a need to assess the toxin-producing potential of many more isolates of *F. oxysporum*, *F. moniliforme*, *F. solani* and *F. roseum* from groundnuts.

REFERENCES

Eppley, R. M., Stoloff, L., Trucksess, M., and Chung, M. W. (1974). 'Survey of corn for *Fusarium* toxins', *J. Assoc. Off. Anal. Chem.* **57**, 632–635.

Eugenio, C. P., Christensen, C. M., and Mirocha, C. J. (1970). 'Factors affecting production of the mycotoxin F-2 by *Fusarium roseum*', *Phytopathology*, **60**, 1055–1057.

Gimeno, A. (1980). 'Improved method for thin-layer chromatographic analysis of mycotoxins', *J. Assoc Off. Anal. Chem.*, **63**, 182–186.

Jackson, C. R. (1965). 'A list of fungi reported in peanut pods and kernels', *Univ. Georgia College Agric. Mimeo. Series*. N.S. 234, 6 pp.

Joffe, A. Z. (1969). 'The mycoflora of fresh and stored groundnut kernels in Israel', *Mycopathol. Mycol. Appl.*, **39**, 255–264.

McDonald, D. (1969). 'The influence of the developing groundnut fruit on soil mycoflora in Nigeria', *Trans. Brit. Mycol. Soc.*, **53**, 393–406.

Mehan, V. K., and McDonald, D. (1982). 'Mycotoxin-producing fungi in groundnuts—potential for mycotoxin contamination', In *Proc. International Symposium on Mycotoxins and Phycotoxins*, pp. 98–101, 1–3 September 1982, Vienna, Austria.

Mehan, V. K., McDonald, D., and Rao, V. R. (1981). 'Pod rot disease of peanut at ICRISAT, India', *Proc. Am. Peanut Res. Educ. Soc.*, **13**, 91.

Mercer, P. C. (1977). 'A pod rot of peanut in Malawi', *Plant Dis. Rep.*, **61**, 51–55.

Mirocha, C. J., and Christensen, C. M. (1974). 'Oestrogenic mycotoxins synthesized by *Fusarium*', in *Mycotoxins* (ed. I. F. H. Purchase), pp. 129–148. Elsevier, Amsterdam.

Mirocha, C. J., Christensen, C. M., and Nelson, G. H. (1967). 'Estrogenic metabolite produced by *Fusarium graminearum* in stored corn', *Appl. Microbiol.*, **15**, 497–503.

Mirocha, C. J., Christensen, C. M., and Nelson, G. H. (1969). 'Biosynthesis of the fungal estrogen F-2 and a naturally occurring derivative by *Fusarium moniliforme*', *Appl. Microbiol.* **17**, 482–483.

Mirocha, C. J., Pathre, S. V., Schauerhamer, B., and Christensen, C. M. (1976). 'Natural occurrence of *Fusarium* toxins in feed stuffs', *Appl. Environ. Microbiol.*, **32**, 553–556.

Naik, D. M., Busch, L. V., and Barron, G. L. (1978). 'Influence of temperature on the strain of *Fusarium graminearum* Schwabe in zearalenone production', *Can. J. Plant Sci.*, **58**, 1095–1097.

Schroeder, H. W., and Hein, H. (1975). 'A note on zearalenone in grain sorghum', *Cereal Chem.*, **52**, 751–752.

Sherwood, R. F., and Peberdy, J. F. (1972). 'Factors affecting the production of zearalenone by *Fusarium graminearum* in grain', *J. Stored Prod. Res.*, **8**, 71–75.

Ueno, Y. (1977). 'Trichothecenes: overview address', in *Mycotoxins in Human and Animal Health* (eds J. Rodricks, C. W. Hesseltine and M. A. Mehlman), pp. 189–207. Pathotox Publishers Inc., Illinois, U.S.A.