5000 ppm of Cl^-, Br^-, I^-, oxalate, citrate, sulphite, phosphate, nitrate, nitrite, tartrate, carbonate azide, acetate, bromate and 1000 ppm of urea, hydroxylamine, semicarbazide, thiourea, glucose, starch, 50 ppm of thioglycollic acid do not interfere, while even minute quantities of thiocyanate, perchlorate, periodate and iodate obscure the colour formation.

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1. Feigl, Fritz, *Spot Tests*, Elsevier Publishing Company, New York, 1954, p. 167.
2. —. *Spot Tests in Inorganic Analysis*, Elsevier Publishing Company, New York, 1958, p. 305.
3. —, *Ibid.*, 1958, p. 303.
4. Fujimoto and Masatoshi, *Bull. Chem. Soc. Japan*, 1956, 29, 567.
5. Gordon, J. Erdman, *Nature*, 1964, 203, 749.
6. Elbeih, I. I. M. and Gabra, G. G., *Chemist-Analyst*, 1963, 52, 36.
7. Korenman, I. M. and Belyakov, A. A., *Ser. Khim.*, 1958, 32, 93.
8. Cornea, F. and Cercasov, C., *Ser. Stiint Nature*, 1965, 14, 39.
9. Austerhof, H., *Dtsch-Apoth. Ztg.*, 1962, 102, 765; Cf. *Anal. Abstr.*, 1963, p. 318.
10. Feigl, Fritz, *Spot Tests in Organic Analysis*, 7th Edition, Elsevier Publishing Company, Amsterdam, 1966.
11. Jamber, B., *Agrokemea'es Talajtan*, 1956, 5, 89.
12. Weyg, F. and Frank, I., *Z. Naturforsch*, 1948, 36, 377.
13. Hill, W. H., *U.S.*, 2,848,307, August 19, 1958, Cf. *C.A.*, 53, 12098 *d*.
14. Amako, K., *Seito Gijutsu Kenkyukaishi*, 1958, 7, 65; Cf. *C.A.*, 54, 2002 f.

OCCURRENCE OF AFLATOXINS AND CITRININ IN GROUNDNUT (*ARACHIS HYPOGAEA* L.) AT HARVEST IN RELATION TO POD CONDITION AND KERNEL MOISTURE CONTENT

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ABSTRACT

Groundnut pods were collected from fields on the day of harvest in November, 1972, graded into undamaged and damaged pods and kernel moisture content was determined. The accumulation of a yellow pigment in some groundnut kernels, especially in damaged pods, was noticed and it was identified as citrinin. Only *Aspergillus flavus* isolates were found to produce aflatoxins while isolates of *Penicillium citrinum*, *P. jenseni* and *A. terreus* produced citrinin. High levels of aflatoxins and citrinin were associated with kernels having less than 30% of moisture, which occurred under rain-fed conditions. Where the moisture content of kernels is high (under irrigation) there was very little or no formation of the mycotoxins. In all the cases damaged kernels were found to contain the toxins. Kernel moisture content and pod damage appear to be the major governing factors for fungal infestation and toxin accumulation before harvest.

INTRODUCTION

PRESENCE of aflatoxins in groundnut kernels before harvest[8] and at the time of harvest[6 14] had been reported. Their presence was attributed to low kernel moisture content (30%), over maturity, unfavourable weather conditions and excessive pod damage. *Aspergillus flavus* Link. invades groundnut pods in the field before harvest, during storage or during handling[5·6·13 15]. Optimum kernel moisture content of 20–30%[2] and temperature of 25°–35° C[9] are favourable for aflatoxin production. Kernels are more susceptible to fungal infestation and toxin production when their moisture content is between 10 and 30%[8].

During our studies on fungal infection of groundnut and aflatoxin accumulation before harvest, we found an accumulation of a yellow pigment in groundnut kernels, especially in kernels from rotted pods (Fig. 1). Such kernels emit golden yellow fluorescence when exposed to UV light (Fig. 2). The chemical nature of this substance was determined and the conditions for its accumulation was also investigated.

MATERIALS AND METHODS

Groundnut samples were collected from the fields around Tirupati (A.P.), on the day of harvest in November, 1972 from three different localities. Two samples of 1 kg of pods for each were collected in polythene bags. The pods were graded into undamaged (sound mature) and damaged pods

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(rotted, insect bored, broken shells, etc.). The moisture content to kernels was determined by drying 20 g of sliced kernels for 5 hr at 102° C, cooled in a desiccator and weighed.

FIG. 1. Damaged pods of groundnut.

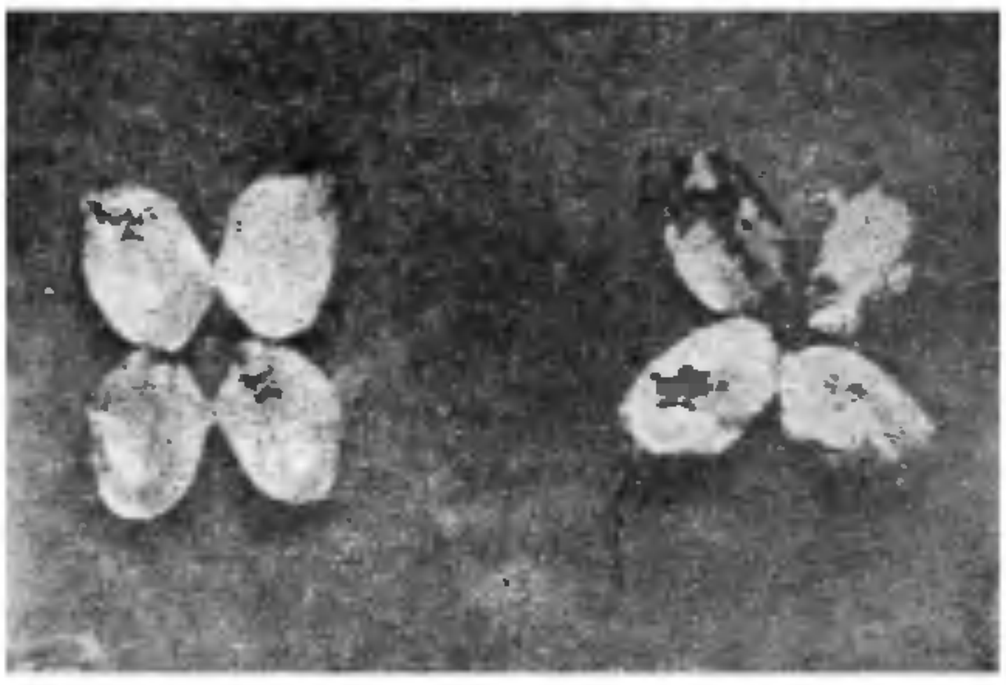

FIG. 2. Undamaged and damaged groundnut kernels as seen under Ultraviolet light.

From each sample 100 kernels were surface sterilized by immersing for two minutes in 0·1% $HgCl_2$, rinsed repeatedly in sterilized distilled water and plated out on Czapek-Dox rose bengal streptomycin agar (CDRSA) in petridishes (5 seeds/9 cm plate). The plates were incubated at 28 ± 2° C and examined for fungi after 4 and 8 days. Fungi coming out from the kernels were counted and isolated on potato dextrose agar (PDA) slants. The isolates were purified by single spore isolation in case of sporulating fungi or by cutting and subculturing the hyphal tips in the case of non-sporulating fungi.

Groundunt samples were analysed for mycotoxins. 50 g of each sample was extracted with 250 ml of methanol-water (55 ; 45, V/V) *plus* 100 ml of *n*-Hexane in a waring blender. After centrifugation of the mixture, 50 ml of the aqueous methanol layer was shaken with two 50 ml portions of chloroform. The combined chloroform extracts were evaporated on water bath, residue was redissolved in 1 ml of chloroform and analysed for aflatoxins by thin layer chromatography (TLC). Another 50 ml of the aqueous methanol layer was acidified to 1·5 to 2·0 pH with conc. sulphuric acid and extracted as above and was analysed by TLC[11]. The following solvent systems were used : Toluene ; Ethyl acetate ; 90% Formic acid (60 ; 30 ; 10, V/V/V) and Ether ; Methanol ; Water ; 90% Fomic acid (95 ; 4 ; 1 ; 1, V/V/V/V) in a tank lined with filter paper. The plates were viewed under UV light and estimated quantitatively by comparing the intensity of fluorescence of spots form extracts with that of standards. The kernels which showed the yellow pigment were found to contain citrinin by this method.

The fungal isolates were screened for aflatoxin and citrinin production by the method of van Walbeed *et al.* (1968). They were grown on PDA slants enriched with 0·2% yeast extract. Conidial or mycelial suspension was made with sterile water containing Tween-80 (0·05%) and 0·1 ml of this suspension was added to a test-tube containing 5 ml of yeast extract sucrose (YES) medium (15 g of sucrose, 2 g of yeast extract and 100 ml of distilled water). The test-tubes were incubated in a slanting position for one week at 28 ± 2° C. For each isolate 5 replicates were maintained. The culture filtrate of all the replicates pooled and extracted by shaking vigorously for two minutes with 50 ml of hot (60° C) chloroform in a separating funnel. The lower chloroform layer was passed through anhydrous sodium sulphate in a column. The column was washed with another 10 ml of hot chloroform and the washings were pooled, evaporated on water bath to dryness and kept in deep freeze (0° C) for TLC analysis.

The extracts were redissolved in 0·5 ml of chloroform and appropriate amounts were spotted on TLC plates along with authentic samples of aflatoxins and citrinin. The following solvent systems were employed to detect the toxins in the extracts. Chloroform ; Acetone (9 ; 1, V/V). Chloroform ; Methanol (93 ; 7, V/V), Toluene ; Ethyl acetate ; 90% Formic acid (60 ; 30 ; 10, V/V/V) and Benzene; Methanol ; Acetic acid (24 ; 2 ; 1, V/V/V). Extracts containing fluorescent materials having the colour and R_f values similar to those of authentic aflatoxins and citrinin were co-chromatographed.

RESULTS AND DISCUSSION

The infestation of kernels and the accumulation of aflatoxins and citrinin in kernels obtained from undamaged and damaged pods were estimated in 1972 *Kharif* crop (August–November) under rainfed conditions and under supplement irrigation

TABLE I

*Fungal infestation and toxin content in groundnut kernels in 1972-*Kharif *Crop*

Water source	Pod condition	% of Kernels infested with			% of clean Kernels	Kernel moisture content %	Toxin content (μg/kg)	
		A. flavus	*Penicillium* sps.	Other fungi			Aflatoxins	Citrinin
Rain-fed	Undamaged	32	76	82	18	29·2	940	10
Irrigated	,,	25	50	55	45	46·0	68	0
Rain-fed	Damaged	85	100	100	0	35·0	4980	950
Irrigated	,,	100	100	100	0	50·1	2100	86

TABLE II

Toxin content and kernel moisture content in groundnut at harvest in relation to pod condition and nature of water source

Crop season	Water source	Pod condition	No. of samples tested	Range of moisture content (%)	No. of samples containing				Range of aflatoxins content (μg/kg)	Range of citrinin content (μg/kg)
					Only aflatoxins	Only citrinin	Aflatoxins + citrinin	No. toxins		
1972 *Kharif*	RF	UDP	25	28–31	12	0	5	8	360–1200	0–60
	I	UDP	20	38–46	6	0	0	14	Tr–130	0
	RF	DP	25	25–31	20	0	5	0	2500–5850	200–140
	I	DP	20	45–40	17	0	3	0	1500–2250	Tr–1200
1973 *Rabi*	I	UDP	10	42–45	0	0	0	10	0	0
	I	DP	10	41–43	8	0	0	2	Tr–210	0
1973 *Kharif*	RF	UDP	15	28–32	6	0	1	8	176–950	0–Tr
	I	UDP	10	40–42	1	0	0	9	0–Tr	0
	RF	DP	15	30–33	13	0	2	0	750–2800	70–150
	I	DP	10	40–44	10	0	0	0	420–1850	0

RF=Rain fed; I=Irrigated; UDP=Un damaged pods; DP=Damaged pods.

separately (Table I). Kernel moisture content was lower while the percentage of kernels infested with fungi was much higher under rain-fed conditions than under irrigation. All the damaged kernels were infested with fungi.

Aflatoxin and citrinin content of kernels of undamaged and damaged pods collected from fields in 1973-*Rabi* (February–May) and 1973-*Kharif* were also estimated (Table II). No toxins were found in 1973-*Rabi* samples and only a low per cent of samples containing toxins were found in 1972 and 1973 *Kharif* under irrigated conditions. The range of aflatoxins and per cent of samples containing toxins was high in samples of rain-fed plots in 1972 and 1973 *Kharif*. In all the cases damaged kernels were found to contain the toxins but the toxin content was much higher under rain-fed conditions.

In all the cases the presence of aflatoxins and citrinin were confirmed by spraying *p*-anisaldehyde[10]. The presence of aflatoxins and citrinin was further confirmed by bioassay using *Bacillus mageterium* (NRRL. 1368), *B. brevis* (NRRL. 1874) and *B. subtilis*. The spots on TLC plates were eluted and dissolved in chloroform, centrifuged and the chloroform layer was evaporated to dryness. They were redissolved in chloroform. These eluates were transferred to Whatman No. 1 filter paper discs (3 cm), dried and placed on seeded agar plates. The inhibition zones were noticed after 24 hr.

Out of 233 isolates belonging to 19 species of fungi tested for aflatoxins and citrinin production, only *A. flavus* isolates (38 out of 52) produced aflatoxins. *A terreus* (5 out of 14) and all the isolates of *Penicillium citrinum* and *P. jenseni*

produced citrinin (Table III). Out of 38 aflatoxin producing *A. flavus* isolates, 29 produced $B_1 + G_1$ and the others produced $B_1 + G_1 + B_2 + G_2$.

Kernel moisture content and pod damage appear to be the major governing factors for fungal infestation and toxin accumulation before harvest. Hundred per cent kernel infestation was observed in damaged pods and this is said to be due to the exposure of kernel surface to the soil[1,3,4,7,12]. Even in undamaged pods high per cent of fungal infestation was observed in rain-fed plots. Here the kernel moisture content seems to exert much influence on fungal infestation because kernel moisture was around the maximum limit of 30% for aflatoxin accumulation. Though the per cent of kernels infested by fungi in damaged pods in both irrigated and rain-fed plots is more or less same, higher levels of toxin accumulation were observed under rain-fed conditions. This is most probably due to low kernel moisture content in rain-fed plots.

TABLE III

Aflatoxin and citrinin production by fungi isolated from groundnut kernels in 1972 Kharif *crop*

Fungus	No. of isolates screened	No. of isolates producing aflatoxins	No. of isolates producing citrinin
Alternaria tenuis	2	0	0
Aspergillus flavus	52	38	0
A. fumigatus	12	0	0
A. niger	28	0	0
A. terreus	14	0	0
A. ustus	5	0	0
Cunninghamella sp.	1	0	0
Fusarium equiseti	2	0	0
F. oxysporum	12	0	0
F. solani	15	0	0
Macrophomina phaseoli	12	0	0
Neochosmospora vasinfecta	2	0	0
Penicillium citrinum	26	0	26
P. funiculosum	5	0	0
P. jenseni	20	0	20
Penicillium sp.	18	0	0
Rhizoctonia solani	1	0	0
Rhizopus sp.	5	0	0
Sclerotium rolfsii	1	0	0

This study indicates that fungal infestation as well as the kernel moisture content are important in determining the accumulation of toxins in the field. Even in the damaged pods toxin accumulation seems to be governed to a greater extent by kernel moisture content. From this point of view supplement irrigation of fields during *Kharif* season is beneficial. Another interesting aspect brought out by this study is the finding that citrinin also accumulates in groundnut kernels in the field, which has not been so far reported.

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1. Ashworth, L. J. Jr., and Langley, B. C., *Pl. Dis. Reptr.*, 1964, 48, 875.
2. —, Schroeder, H. W. and Langley, B. C., *Science*, 1965, 148, 1228.
3. Aucamp, J. L., "The role of mites in the development of aflatoxins in groundnuts," *Paper Read at First International Congress on Plant Pathology*, London, 14–26, July, 1968.
4. McDonald, D. and Harkness, C., *Trop. Sci.*, 1963, 5, 143.
5. — and —, *Ibid.*, 1964, 6, 12.
6. — and —, *Ibid.*, 1967, 9, 148.
7. Minton, N. A. and Jackson, C. R., *Oleagineux*, 1967, 22, 543.
8. Pettit, R. E., Taber, R. A., Schroeder, H. W. and Harrison, A. L., *Appl. Microbiol.*, 1971, 22, 629.
9. Schroeder, H. W. and Hein, H., Jr., *Ibid.*, 1967, 15, 441.
10. Scott, P. M., Lawrence, J. W. and van Walbeek, W., *Ibid.*, 1970, 20, 839.
11. —, van Walbeek, W., Harvig, J. and Fennell, D. J., *Can. J. Plant Sci.*, 1970, 50, 580.
12. Sellschop, J. P. E., "Field observations on conditions condusive to the contamination of Groundnuts with the mould *Aspergillus flavus*. Symposium on mycotoxins in food stuffs (Agricultural aspects) Pretoria, February, 1965, 25, 47.
13. Subrahmanyam, P., *Ph.D. Thesis Entitled*, "A study of mycotoxin producing fungi associated with groundnut (*Arachis hypogaea*, L.) in relation to agricultural practices," S.V. University, Tirupati (A.P.), India (in preparation).
14. — and Rao, A. S., *Curr. Sci.* (India) (Accepted for publication), 1974.
15. Taber, R. A. and Schroeder, H. W., *Appl. Microbiol.*, 1967, 15, 140.
16. van Walbeek, W., Scott, P. M. and Thatcher, F. S., *Can. J. Microbiol.*, 1968, 14, 131.