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STUDIES ON SOME COWPEA CULTIVARS
II - SUITABILITY FOR ASPERGILLUS FLAVUS GROWTH AND AFLATOXIN PRODUCTION

By

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دراسات على بعض هجن اللوبيا
ملاءمة الهجن لنمو فطرة الأسبرجيلس فلافس
وإنتاج سموم الافلاتوكسين

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تم في هذا البحث حقن ١٦ هجين من بذور اللوبيا الخالية تماما من سموم الأفلاتوكسين بفطرة اسبرجيلس فلافس (IMI 89717) وذلك لدراسة مدى ملاءمتها لنمو الفطرة وإنتاج سموم الأفلاتوكسين . باستخدام طريقة التحليل الكروماتوجرافي على رقائق السليكا ، ثبت أن ثلاثة هجن عالية المقاومة لنمو الفطرة وانتاج سموم الافلاتوكسين وثمانية هجن لها مقاومة جزئية بينما الخمسة هجن الباقية كانت ذات قابلية عالية لنمو الفطرة وانتاج سموم الافلاتوكسين .

كما أثبتت النتائج عدم وجود أي علاقة في الهجن المختبرة بين الصفات المورفولوجية (مثل لون وشكل وحجم البذور) أو سمك القصره وكمية سموم الأفلاتوكسين المنتجة على البذور ، وكذلك لا توجد اختلافات واضحة في المحتوى النيتروجيني أو كمية الماغنسيوم بين الهجن المقاومة أو القابلة للأصابة وانتاج سموم الافلاتوكسين . أما كمية الكالسيوم والبوتاسيوم والفوسفات فكانت في البذور القابلة للإصابة بالفطرة وتراكم الافلاتوكسين قليلة بالمقارنة بتلك البذور التي تكون مقاومة للفطرة ، وكان عكس ذلك تماماً في حالة عنصري الصوديوم والخارصين .

Key Words: Cowpea, cultivars, resistance, mycotoxin, aflatoxin

ABSTRACT

Sixteen mycotoxin-free seed cultivar samples of cowpea (Vigna unguiculata (L.) Walp.) were inoculated with spores of *Aspergillus flavus* Link (IMI 89717) to determine varietal differences in terms of aflatoxin production. Thin-layer chromatographic analyses of chloroform extracts of the colonized seeds revealed that three cultivars were highly resistant and not shown aflatoxin accumulation while 8 cultivars showed partial resistance. The remaining 5 samples were highly susceptible to the establishment of *A. flavus* and aflatoxin accumulation.

The results did not show any relationship between morphological characters (seed colour, shape & size) or testa thickness and the amount of aflatoxin produced on the different cowpea cultivars. The results also revealed the absence of significant variation in either total nitrogen or magnesium contents of the susceptible, partially resistant and highly resistant cultivars. Calcium, potassium and total phosphate contents of both testas and kernels of the susceptible seeds were low as compared to those of partially and highly resistant seeds; and *vice versa* with sodium and zinc contents.

INTRODUCTION

The role of aflatoxins as highly toxic and carcinogenic substances is well documented (Esuruoso, 1975 and Bullerman, 1979). These substances are mainly produced by members of *Aspergillus flavus* group of fungi. The natural and artificial production of aflatoxin has been reported on various grains and seeds especially in tropical and subtropical countries where the storage conditions were sub-optimal.

The ideal way to prevent the formation of aflatoxins would be the development of plant varieties which produce seeds that resist fungal colonization or are not suitable for toxin formation. Therefore, the present investigation was undertaken to evaluate 16 different genotypes of cowpea for their ability to sustain *Aspergillus flavus* growth and aflatoxin production. Various constituents of the resistant and susceptible genotypes were analyzed to determine which factors effect aflatoxin formation.

MATERIALS AND METHODS

Collection of cowpea samples

Sixteen cowpea cultivars were kindly provided by the Horticulture Department, Faculty of Agriculture, Assiut University: from the 1987/1988 crop. Twelve cultivars were obtained from IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria) and the other four cultivars were obtained from EAO (Egyptian Agricultural Organization, Egypt). Sources of these cultivars are indicated in Table (1). Most of the cultivars combine high yield, good seed quality and a high level of disease resistance. All seed samples were tested for natural contamination with aflatoxins and proved to be naturally aflatoxin-free.

Moisture content

The moisture content of cowpea seeds was determined by the oven drying. Replicate seed samples were ground in a mill and flour dried at 105°C in an electric oven to constant weight. The moisture content was then calculated on an oven dry basis.

Artificial infection of cowpea seeds by *Aspergillus flavus*

25 g of healthy seeds representing each of the 16 aflatoxin-free cultivars were surface disinfected with 2% aqueous solution of calcium hypochloride as described by Seenappa *et al.* (1981). The disinfected seeds were then transferred to 250 ml sterile Erlenmyer flasks and moisted to different moisture contents (15%, 20% and 30%) by adding sterile distilled water according to the method described by Lutey and Christensen (1963). Each flask was inoculated with 2 ml of concentrated spore suspension from 10 day old culture on PDA of *Aspergillus flavus* IMI 89717, Obtained from the International Mycological Institute (Kew, Surrey, England). After inoculation, the flasks were gently swirled to spread the inoculum evenly. The inoculated samples incubated for 10 days at 28±2° under relative humidity of 90±2%.

Table 1

Sample number (S. No.), cultivars, source, thickness of testa (mm), testa colour, moisture content (M.C.%) of different cowpea cultivars and visual growth (VG) & aflatoxin production (µg/kg) by *Aspergillus flavus* IMI89717 on the different cultivars.

S. No.	Cultivars	Source	Thickness of testa	Testa Colour	M. C. %	Infected by A. flavus	
						VG***	Aflatoxin detected (µg/kg)
1	IT82D-79	IITA*	0.011	White with red eye	8.97	+++	60
2	Pusa Ph. St.	IITA	0.009	White with red eye	9.60	+++	125
3	Sabaheia	IITA	0.007	White with black eye	11.11	+++	55
4	Azmerly	EAO**	0.008	White with black eye	9.09	+++	65
5	Fetriyat	EAO	0.009	Cream	8.89	+++	90
6	IT82C-32	IITA	0.014	Light Brown	10.96	++	25
7	IT82D-812	IITA	0.012	Light Brown	8.72	++	25
8	IT82D-889	IITA	0.012	Brown	9.30	++	25
9	IT82D-716	IITA	0.012	White with red eye	9.32	+++	25
10	Assan	IITA	0.010	Cream	9.09	++	25
11	Barasadi	IITA	0.009	White with black eye	9.68	++	25
12	Cream 7	EAO	0.007	Cream	10.70	+++	25
13	Pusa Phalyngi	IITA	0.008	White with red eye	10.27	+++	25
14	IT82C-16	IITA	0.010	Brown	10.20	+	0
15	IT81D-1032	IITA	0.011	Brown	9.69	0	0
16	Balady	EAO	0.008	White with red eye	8.95	0	0

* IITA : International Institute of Tropical Agriculture, Ibadan, Nigeria.
** EAO : Egyptian Agricultural Organization, Egypt.

*** VG : Visual growth rated on a scale; +++ = Good ++ = Moderate += Poor 0 = No growth

Aflatoxin analysis

At the end of the incubation period, the growth of the fungus was visually assessed on each seed sample which was then defatted by extraction with cyclohexane for 10 h using a Soxhlet-type extractor. The defatted residue was extracted for another 10 h with chloroform. The chloroform extract was dried over anhydrous sodium sulphate, filtered and then distilled under vacuum to near dryness. The residue was diluted with chloroform to one ml. Chromatographic analysis of the chloroform extracts were achieved on precoated silica gel plate type 60 F254 (MERCK) for the presence of aflatoxin B1, B2, G1 & G2 according to Scott *et al.* (1970) and Roberts & Patterson (1975).

Source of aflatoxin standards

Aflatoxin standards used throughout this study were kindly provided by prof. Dr. I. A. El-Kady, Botany Dept. Fac. of Sci. Assiut Univ., Assiut, Egypt.

The presence of aflatoxins in the chloroform extracts were confirmed by derivative methods of Przybylski (1975) and quantitatively determined according to the methods of Jones (1972).

Mineral analysis

Total nitrogen contents were determined by the use of Nessler reagent (Vogel, 1968). Sodium and potassium were determined by the flame photometer method (Williams & Tuine, 1960). Calcium and magnesium were determined by the versene titration method (Schwarzenbach & Biedermann, 1948). Total phosphorus was determined colorimetrically according to Woods & Mellon (1941). Atomic absorption spectrophotometry (Zeiss FMD3) was used for determination of zinc.

Statistical analysis

Differences in the concentrations of the different elements content were tested for statistical significance between groups, using one-way analysis if variance of means (PC-State Computer Program).

RESULTS AND DISCUSSION

Production of aflatoxin on cowpea cultivars seed

The results presented in Table 1, show that the amount of fungal growth and aflatoxin produced varied among the different cultivars tested. Three cultivars (IT82C-16, IT81D-1032 and Balady) were found to be highly resistant to seed invasion and aflatoxin production by strain of *Aspergillus flavus.* Five cultivars (IT82D-79, Pusa Ph. St., Sabaheia, Azmerly and Fetriyat) were highly susceptible to the establishment of *A. flavus* and aflatoxin accumulation (the amount of aflatoxin formed ranged from 55 µg/kg to 125 µg/kg seeds). The remaining 8 cultivars showed partial resistance (aflatoxin formed was about 25 µg/kg seeds).

Similar observations were reported by Shotwell *et al.* (1978) on soybeans where aflatoxin accumulation was dependent on the variety of soybean. Priyadarshini & Tulpule (1978), while working with six varieties of groundnuts and a toxigenic isolate of *Aspergillus parasiticus* showed that the amount of aflatoxin produced was ranged between 57.8-206.8 mg/kg seeds.

In our study on cowpea cultivars, the range of aflatoxin produced on different cultivars varied between 25 and 125 µg/kg seeds. Similar low levels of aflatoxin were also obtained when other leguminous crops were artificially infected by toxigenic fungi. Seenappa *et al.* (1983) observed that all 22 cowpea cultivars seed tested were susceptible to *Aspergillus parasiticus* NRRL 3145 infection and subsequent aflatoxin production. They reported that the amount of aflatoxin produced on different cowpea cultivars ranged between 500 and 1800 µg/kg of seeds. They also reported that there is, at least, partial resistance to aflatoxin production in cowpea cultivars on the basis of the amount of aflatoxin produced. Hitokoto *et al.* (1981) examined 604 samples of six different types of beans to determine their suitability for use as solid substrates for mycotoxin production. They found that aflatoxin levels produced were 0.25, 0.5, 1.0., 2.0, 2.0 and 4.0 mg/kg seeds of pea beans, red beans, lima beans, kidney beans, green peas and cowpeas, respectively. Recently, El-Kady *et al.* (1991) examined 100 different cultivars and lines of broad bean seeds to determine varietal differences which may support or resist aflatoxin production and found that 11 cultivars/lines were highly resistant to seed invasion and aflatoxin production while 9 cultivars/lines showed partial resistance.

Varietal differences of cowpea related to aflatoxin accumulation

The elucidation of precise chemical nature of factors responsible for varietal differences in susceptibility to aflatoxin production will help plant geneticists to breed strains with such desirable characteristics. A systematic examination of the different constituents (total nitrogen, total phosphate, Ca^{++}, Mg^{++}, Zn^{++}, K^{+} and Na^{+} contents) of both the testas and kernels of the susceptible, partially resistant and highly resistant cowpea seed cultivars was undertaken. Testa thickness and some morphological characters (seed colour, shape and size) were also investigated.

The results obtained in Table 1, show no differences in aflatoxin incidence could be attributed to colour, shape and size of the seeds or testa thickness of different cowpea cultivars. These results agree with the findings of Seenappa *et al.* (1981, 1983) who found no relation between the seed colour, size or shape and fungal infection or aflatoxin production in seeds of cowpeas and beans in Tanzania. They also presumed that variation in testa thickness of different cultivars might in part be responsible for differences in quantities of aflatoxins accumulated. However, Calvert *et al.* (1978) found that the production of aflatoxins B1 and G1 was significantly greater in *Zea mays* hybrids with thin rather than thick-pericarps.

Examination of the different seed constituents revealed an absence of significant variation in total nitrogen and magnesium in susceptible, partially resistant and highly resistant cowpea seed cultivars (Table 2). The susceptible seeds contained the lower calcium concentration followed by the partially resistant and the highest calcium level was recorded in the highly resistant seeds. This agrees with the finding of Howell (1970) who examined 3114 different seed and grain samples and recorded that soybeans did not constitute a good substrate for aflatoxin production. He speculated that the levels of calcium in soybeans, which are higher than those of cereals, may be inimical to toxin production. Mashaly & El-Deeb (1983) and Zeinab El-Bazza *et al.* (1983) found that calcium stimulated aflatoxin production and enhanced the growth rate of all seven *Aspergillus flavus* and two *A. parasiticus* strains tested in synthetic media. The effect gradually increased with increasing concentrations of calcium in the culture medium.

Table 2

The mean values (MV) ± standard deviation (SD) of calcium, magnesium, total nitrogen, zinc, potassium, sodium and total phosphate contents (mg/g of dry seed) in different groups of cowpea cultivars which are susceptible (A: No. 1-5), partially resistant (B: No. 6-13) and highly resistant (C: No. 14-16) with respect to aflatoxin production.

Elements	Groups	Kernel MV±SD	testa MV±SD	Total MV±SD
	A	4.180 ± 0.95 (a*)	7.600 ± 1.22 (a)	11.780 ± 1.34 (a)
Calcium	B	4.650 ± 0.89 (a)	9.180 ± 1.44 (ac)	13.830 ± 1.25 (b)
	C	6.820 ± 0.79 (c)	10.260 ± 1.42 (c)	17.080 ± 1.08 (c)
	A	1.320 ± 0.14 (a)	2.550 ± 0.49 (a)	3.870 ± 0.39 (a)
Magnesium	B	1.230 ± 0.20 (a)	2.630 ± 0.35 (a)	3.860 ± 0.42 (a)
	C	1.130 ± 0.12 (a)	2.600 ± 0.58 (a)	3.730 ± 0.59 (a)
	A	2.850 ± 0.31 (a)	3.370 ± 0.25 (a)	6.220 ± 0.40 (a)
Total Nitrogen	B	3.090 ± 0.53 (a)	3.470 ± 0.81 (a)	6.560 ± 0.61 (a)
	C	3.110 ± 0.27 (a)	3.400 ± 0.35 (a)	6.510 ± 0.50 (a)
	A	0.320 ± 0.02 (a)	0.395 ± 0.02 (a)	0.715 ± 0.03 (a)
Zinc	B	0.226 ± 0.01 (c)	0.220 ± 0.01 (c)	0.446 ± 0.02 (c)
	C	0.205 ± 0.01 (c)	0.225 ± 0.01 (c)	0.430 ± 0.01 (c)
	A	11.120 ± 0.40 (a)	6.800 ± 1.04 (a)	17.920 ± 1.00 (a)
Potassium	B	11.990 ± 0.61 (c)	6.510 ± 0.59 (a)	18.500 ± 0.96 (a)
	C	12.070 ± 0.82 (c)	6.680 ± 0.45 (a)	18.750 ± 1.24 (a)
	A	0.870 ± 0.08 (a)	1.560 ± 0.18 (a)	2.430 ± 0.22 (a)
Sodium	B	0.520 ± 0.15 (c)	1.370 ± 0.19 (a)	1.890 ± 0.23 (b)
	C	0.440 ± 0.07 (c)	1.000 ± 0.19 (c)	1.440 ± 0.26 (c)
	A	3.590 ± 0.45 (a)	1.380 ± 0.34 (a)	4.970 ± 0.49 (a)
Total phosphate	B	5.080 ± 0.71 (c)	1.670 ± 0.41 (a)	6.750 ± 0.93 (c)
	C	5.330 ± 0.71 (c)	1.720 ± 0.71 (a)	7.050 ± 0.85 (c)

* Values in the same column followed by the same letter are not significantly different at 5% level by Duncans multiple range test.

The mean values of zinc in both the testa and kernel of the partially and highly resistant seeds were significantly low as compared to those obtained from susceptible cowpea seed cultivars (Table 2). The stimulatory effect of zinc on aflatoxin formation is well documented (Mateles & Adye, 1965; Lee *et al.*, 1966; Davis *et al.*, 1967; Lillehoj *et al.*, 1974; Marsh *et al.*, 1975). Of all the trace elements previously investigated, zinc seems to play a key role in the biosynthesis of secondary fungal metabolites including aflatoxins. At least twenty enzymes have been found to be zinc dependent (Parisi & Valee, 1969) which may partly account for its key role. Studies by Adye & Mateles (1964) and Mateles & Adye (1965) of glucose-ammonia-salts synthetic medium showed that deletion of zinc (2 μg/g) reduced aflatoxin yield by 88% without restricting the growth of the fungus. However, Obidoa & Ndubuisi (1981) reported that absence of zinc completely blocks fungal growth and aflatoxin production.

Phytate phosphorus comprised about 80% of the total phosphates (O'Dell *et al*, 1972). The results obtained in the present study clearly show significant differences in the total phosphate concentrations of the different cultivars (Table 2). The total phosphate content of the susceptible, partially resistant and highly resistant seeds were 4.97, 6.75 and 7.05 mg/g of seeds, respectively. In terms of phosphate content, the phytic acid present (80% of total phosphate) constitutes 3.18, 5.40 and 5.64 mg/g of susceptible, partially resistant and highly resistant seeds, respectively. This result is similar to that recorded by Gopalan *et al.* (1971) and Gupta & Venkitasubramanian (1975) who recorded that the phytic acid concentrations in soybeans, groundnut, wheat, peas and rice were 6.90, 3.90, 3.06, 2.98 and 1.90 mg/g, respectively. Gupta & Venkitasubramanian (1975) attributed the negligible amount of aflatoxin obtained with non autoclaved soybeans (0.335 mg/100 g of seeds) to binding of zinc with phytic acid which then rendered it unavailable. In case of autoclaved soybeans a large amount of aflatoxin was obtained (6.85 mg/100 g of seeds). They concluded that at high temperatures the phytic acid is broken down and the zinc would be released.

The seeds of resistant cowpea cultivars contain higher phytate concentrations (5.64 mg/g) and lower zinc contents (0.43 mg/g) as compared to the high susceptible seeds (Table 2). Since phytate binds zinc in ratio of 6 zinc : 1 phytic acid (Hensarling *et al.*, 1983) more of zinc in these seeds should be complexed and rendered biologically unavailable. There is also evidence that phytic acid decrease the availability of calcium (Harrison & Mellanby, 1939), iron (Sharpe *et al.*, 1950) and magnesium (Roberts & Yudkin, 1960).

The results in Table 2 reveal a significant variation in potassium concentration in seed kernels of the different cultivars. The mean potassium content was low in the susceptible compared to the partially and highly resistant seed kernels. These results agree with the findings by Davis & Diener (1967) who showed that increasing the amount of potassium sulphite (K_2SO_5) inhibited production of aflatoxin without a corresponding decrease in growth. They also reported that potassium fluoride somewhat inhibited aflatoxin synthesis.

Significant differences in sodium concentration were recorded between the different seed cultivars. The susceptible seeds contained the higher sodium concentration, followed by the partially resistant and the lowest sodium level was recorded in the highly resistant seeds (Table 2). This results agrees with that recorded in several previous reports (Shih & Marth, 1972; Buchanan & Ayres, 1976; Uraih & Chipley, 1976; Mashaly & El-Deeb, 1983) which showed significant increases in both mycelial growth and aflatoxin production by *Aspergillus flavus* and/or *A. parasiticus* when culture media were supplemented by 1-4% sodium chloride.

Evidence, from previous results (Shih & Marth, 1972; Uraih & Chipley, 1976; Mashaly & El-Deeb, 1983) and that obtained during this investigation suggest that low concentrations of sodium may stimulate some enzymes responsible for aflatoxin synthesis by toxigenic fungi.

In conclusion, it appears that susceptibility or resistance of cowpea cultivars for *A. flavus* colonization and aflatoxin formation is influenced by the complex interaction of several factors. Calcium, potassium, sodium, total phosphate and zinc are essential trace elements for aflatoxin synthesis. Zinc and sodium levels were markedly increased in susceptible cultivars as compared to those of resistant seeds. On the contrary, the highest levels of calcium, potassium and total phosphate were recorded in the highly resistant seed cultivars.

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