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Studies on Mycoflora and Mycotoxins of Cowpea Cultivars

A. A. Zohri, M. and Khayria Abd. El. Gawad
Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

동부 콩열매에 있는 진균류와 균독소에 관한 연구

A. A. Zohri, M. Khayria, A. El. Gawad
이집트, Assiut 대학교, 식물학과

ABSTRACT: Thirty three species and two species varieties belonging to 14 genera of fungi were collected from 20 cowpea cultivars on glucose Czapek's agar (11 genera and 25 species+1 var.) and glucose-Czapek's agar supplemented with 10% NaCl (7 genera and 18 species+2 var.) at 28± 2°C. The total count of fungi were 6716 colonies/g in all cowpea cultivars. On glucose-Czapek's agar and identified; *Aspergillus flavus, A. niger, A. sydowii, A. flavus* var. *columnaris, A. terreus, Penicillium chrysogenum, Emericella nidulans* and *Rhizopus stolonifer.* The total count of halotolerant or halophilic fungi was 3515 colonies/g on 10% NaCl-glucose-Czapek's agar and identified; the most common species were: *A. flavus, A. sydowii, A. tamarii. A. flavipes, A. niger, A. flavus* var. *columnaris, A. ochraceus, A. oryzae* and *P. chrysogenusm.* Thin layer chromatographic analysis of chloroform extracts of the different seed samples revealed that four cultivars were naturally contaminated with aflatoxins B_1, B_2, G_1 and G_2 (45-112 μg/kg).

KEYWORDS: aflatoxin, Mycoflora, cowpea, mycotoxin

Cowpea (*Vigna unguiculata*) is a crop of worldwide distribution, especially in the arid and semiarid areas. Major use of this crop in Africa is in a form of dried seeds. Cowpea seeds are an important source of protein since it contains between 19-26% protein (Idusogie, 1971). In recent years international effort is being directed towards increase of the production of cowpea as an inexpensive source of protein and its ease of cultivation in low rainfall areas of the world (Summerfield *et al.*, 1974).

Mould contamination can occur on a crop during development, harvest, storing, or terminal shipment and processing. To ensure a mouldfree product at harvest, farmers must utilize proper agronomic technigues during plating and cultivation that will provide for the optimum crop development. From the continued high levels of contamination reported it would appear that farmers in the developing countries have not yet adopted recommended methods of growing, harvesting, drying and storing the crops designed to reduce mould invarion and mycotoxin production. These methods are expensive and impracticable for immediate application in these countries (Stoloff, 1977). The ideal solution to this problem would be the development of plant varieties which produce seeds that are resistant to the fungus or that inhibit development of mycotoxins though invasion by the fungus occurs (Detroy *et al.*, 1971).

Therefore, the present investigation was imdertalem tp evaluate 20 different genotypes of cowpea for: 1) the composition, density and frequency of occurrence of glucophilic and halotolerant or halophilic funigi, and 2) determination of the natural occurrence of mycotoxins.

Materials and Methods

Collection of cowpea samples: Twenty cultivars

of cowpea seeds were kindly provided by the Horticulture Department, Faculty of Agriculture, Assiut University, from the 1987/1988 crop. Sixteen cultivars were obtained from IITA (International Institute of Tropical Agriculture Ibadan, Nigeria) and the other four cultivars were obtained from EAO (Egyptian Agriculturai Organization, Egypt). Sources of these cultivars are indicated in Table 2. Moxst of these cultivars were developed at IITA which combine high yield, good seed quality and disease resistance.

Moisture content: The moisture content of cowpea seeds was determined by the oven method. Replicate of seed samples were ground in an electric mill and flour was dried at 105℃ in an electric oven for constant weight. The moisture content was then calculated as percentage on oven dry basis.

Isolation of fungi: The fungal flora of the samples was detected by using the dilution plate method (Johnson *et al.*, 1959). Two types of media were used: Glucose-Czapek's agar in which glucose (10 gm/*l*) replaced surcrose and Glucose-Czapek's agar medium fortified by 100 gm/*l* of sodium chloride. Streptomycin (20 μ/m*l*) and rosebengal (30 ppm) were applied to suppress bacterial growth (Smith & Dawson, 1944; Al-Doory, 1980). Ten plates were used for each sample (5 plates for each type of medium). The plates were incubated at 28± 2℃ for 1-2 weeks during which the developing colonies were counted, identified and the numbers were calculated per gm of each sample.

Identification: Fungal isolates were identified, whenever possible, in the original Petri-dish culture. When this was not possible, fungi were subcultured and stored for later identification, according to Raper & Thom, 1949; Gilman, 1957; Raper & Fennell, 1965; Ellis, 1971, 1976; Pitt, 1979, 1985; Samson, 1979; Domsch *et al.*, 1980; Onions *et al.*, 1981; Ramirez, 11982 and Sivanesan, 1984.

Mycotoxinss analysis: Twenty gm of each sample were 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 m*l*. Chromatographic analysis of the chloroform extracts were achieved on precoated silica gel plate type 60 F 254 (Merck) for the presence of aflatoxins B_1, B_2, G_1 & G_2, citrinin, ochratoxin A, patulin, sterigmatocystin, T-2 toxin, diacetoxyscirpenol and zearalenone according to Scott *et al.* (1970) and Roberts & Patterson (1975).

Source of mycotoxin standard: All of mycotoxin standards used throughout this study were purchased from Makor Chemical Ltd. Jerusalem, Israel and kindly provided by Prof. Dr. I. A. El-Kady, Botany Dept., Faculty of Science, Assiut University, 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).

Results and Discussion

Mycoflora of cowpea cultivars: 33 species and two varieties belonging to 144 genera were isolated from 20 cowpea cultivars seed on glucose-Czapek's agar (11 genera and 25 species+1 var.) and on 10% NaCl glucose-Czapek's agar (7 genera and 18 species+2 var.) at 28± 2℃ (Table 1). The average total counts of glucophilic and halotolerant or halophilic fungi in all samples tested (20 cultivars) were 6716 and 3515 colonies/g dry seeds, respectively. All of these fungi were previously recovered from different beans, peas and other types of seeds in Egypt (Moubasher *et al.*, 1977, 1979; El-Kady *et al.*, 1986; Abdel-Hafez & Shoreit, 1986a, b; Mazen *et al.*, 1990) as well as from various types of seeds from different places of the world (Hitokoto *et al.*, 1981; Daclero *et al.*, 1983).

Aspergillus was the most common genus, recorded in 100% of the samples on the two isolation media used. It was represented by 13 species and 1 variety. *Aspergillus flavus* and *A. sydowii* were the most common species on the two types of media. *A. niger* and *A. flavus* var. *columnaris* were isolated with high occurrence on glucose-Czapek's agar medium. *A. tamarii, A. oryzae, A. flavipes, A. niger, A. flavus* var. *columnaris* and *A. ochraceus*

were isolated with high or moderate frequencies of occurrence on 10% NaCl glucose-Czapek's agar, while *A. terreus* was isolated with moderate occurrence on glucose-Czapek's agar. The remaining *Aspergillus* species were recorded with low or rare frequencres of occurrence and only on one of the two types of media used. The preceding *Aspergillus* species were isolated previously, but with variable densities and frequencies from different beans, peas and other types of seeds in Egypt (Moubasher *et al.*, 1977, 1979; El-Kady *et al.*, 1986; Mazen *et al.*, 1990). Abdel-Hafez and Shoreit (1986a, b) isolated 18 species and 3 species varieties of *Aspergillus* from bean (*Phaseolus vulgaris*) cultivated in upper Egypt on glucose- and cellulose-Czapek's agar at 28℃, and the most common species were *A. niger, A. flavus, A. flavus* var. *columnaris, A. ochraceus, A. terreus* and *A. fumigatus*. El-Khadem *et al.* (1983) isolated 10 *Aspergillus* species from Egyptian broad bean seeds on malt agar amended with 7.5% NaCl and the most prevalent species were *A. niger* and *A. flavus*. El-Kady *et al.* (1986) isolated 14 species and one variety of *Aspergillus* from soybean and chick-pea on 15% NaCl-water agar at 28℃, and the most common species were *A. sydowii, A. ochraceus* and *A. niger*. El-Maraghy (1989) isolated 5 species and one variety of *Aspergillus* from chick-pea and soybean varieties and hybrids seed on glucose-Czapek's agar at 28℃, and the most common species were *A. niger, A. flavus* and *A. fumigatus*. Hitokoto *et al.* (1981) isolated 11 species of *Aspergillus* from different kinds of beans and peas, and the most common species were members of *A. flavus* group.

Penicillium was isolated in high frequency of occurrence on the two isolation media used. It was recovered from 100% and 95% of the samples giving rise to 37.88% and 34.37% of total fungi on glucose-Czapek's and 10% NaCl glucose-Czapek's agar, respectively. It was represented by 6 species of which *P. chrysogenum* and *P. oxalicum* were the most prevalent. These two species were recovered, but with variable densities and frequencies, from bean seeds gathered from upper Egypt (Abdel-Hafez & Shoreit, 1986a, b). El-Kady *et al.* (1986) reported that *Penicillium* occupied the second place in soybean and chick-pea, and found that *P. chrysogenum* was the most common species on plates of 15% NaCl-water agar at 28℃. El-Maraghy (1989) isolated 4 species of *Penicillium* from chick-pea, and soybean varieties and hybrids seed on glucose-Czapek's agar at 28℃, and the most common species were *P. chrysogenum, P. viridicatum* and *P. lanosum*.

Emericella (2 species) and *Rhizopus* (one species) were recovered in high frequencies of occurrence on glucose-Czapek's agar medium. *Eurotium* (2 species) and *Emericella* (one species) were isolated with moderate and low frequencies of occurrence, respectively on 10% NaCl glucose-Czapek's plates. The reaining genera and species (*Fusarium oxysporum, Alternaria alternata, Cephaliophora tropica, Chaetomium globosum, Scopulariopsis brumptii, S. halophilica, Syncephalastrum globosum, Scopulariopsis brumptii, S. halophilica, Syncephalastrum racemosum, trichoderma hamatum, Cladosporium sphaerospermum, Giberella fujikuroi* and sterile mycelia) were isolated in less frequency on one or the two types of media used as shown in Table 1. The preceding genera and species were previously isolated, but with variable densities and frequencies, from different seeds (Moubasher *et al.*, 1977, 1979; Hitokoto *et al.*, 1981; El-Khadem *et al.*, 1983; El-Kady *et al.*, 1986; Abdel-Hafez & Shoreit, 1986a, b; El-Maraghy, 1989; Mazen *et al.*, 1990).

In conclusion, it could be said that there were no specific fungal flora for cowpea seeds, since these mycoflora were recovered with variable densities and frequencies from different types of seeds and grains either in Egypt or abroad. Also, there was a high existence of well known pathogenic fungal species such as: *Aspergillus flavus* and these fungi in addition to other species cause serious seed deteriorations and could mycotoxins.

Natural occurrence of mycotoxin on cowpea cultivars: Thin-layer chromatographic analysis of the chloroform extracts of 20 cowpea cultivars showed that four cultivars seed samples were naturally contaminated with aflatoxins B_1, B_2, G_1 and G_2 (Table 2). Aflatoxin was detected in the four contaminated samples at concentrations ranged between

Table 1. Average total counts (ATC, calculated per g dry weight in all cultivars sample), number of cases of isolation (NCI, out of 20 cultivars sample) and occurrence remarks (OR) of fungal genera and species isolated from cowpea cultivars sample on plates of glucose- and 10% NaCl glucose-Czapek's agar media at 28± 2℃.

Genera & species	Glucose-Czapek's agar		10% NaCl glucose-Czapek's agar	
	ATC	NCI & OR*	ATC	NCI & OR*
Aspergillus	3798	20H	2121	20H
A. flavus Link	1994	20H	858	18H
A. niger van Tiegh.	948	19H	43	7M
A. sydowii (Bain. & Sart.) Thom & Church	156	12H	391	17H
A. flavus var. *columnaris Raper & Fennell*	136	11H	62	6M
A. terreus Thom	64	7M	32	4L
A. carbonarius (Bain.) Thom	60	5L	0	0
A. fumigatus Fresenius	168	5L	0	0
A. tamarii Kita	204	5L	468	15H
A. ochraceus Wilhelm	20	4L	62	6M
A. awameri Nakazawa	32	2R	0	0
A. flavipes (Bain. & Sart.) Thom & Church	16	2R	100	7M
A. oryzae (Ahlb.) Cohn	0	0	69	8M
A. janus Raper & Thom	0	0	24	2R
A. ustus (Bain.) Thom & Church	0	0	12	2R
Penicillium	2544	20H	1208	19H
P. chrysogenum Thom	2308	20H	976	17H
P. oxalicum Currie & Thom	204	5L	116	4L
P. aurantiogriseum Dierckx	4	1R	0	0
P. funiculosum Thom	24	1R	0	0
P. purpurogenum Stoll	4	1R	0	0
P. citrinum Thom	0	0	116	1R
Emericella	116	14H	40	4L
E. nidulans (Eidam) Vuillemin	154	12H	40	4L
E. quadrilineata (Thom & Raper) Benjamin	12	2R	0	0
Rhizopus stolonifer (Ehrenb.) Lindt	120	11H	0	0
Fusarium oxysporum Schlecht.	20	3L	0	0
Alternaria alternata (Fries) Keissler	4	1R	0	0
Cephaliophora tropica Thaxter	20	1R	0	0
Chaetomium globosum Kunze	4	1R	0	0
Scopulariopsis	24	1R	4	1R
S. brumptii Salvanet-Duval	24	1R	0	0
S. halophilica Tubaki	0	0	4	1R
Syncephalastrum racemosum (Cohn) Schroeter	4	1R	0	0
Trichoderma hamatum (Bon.) Bain.	4	1R	0	0
Cladosporium sphaerospermum Penzig	0	0	24	2R
Giberella fujikuroi (Sawada) Wollenw.	0	0	4	1R
Eurotium	0	0	114	7M
E. chevalieri Mangin	0	0	64	4L
E. chevalieri var. *intermedium* (Thom & Raper) Malloch & Cain	0	0	50	3L
Sterile mycelia (White * dark colour)	8	2R	0	0
Gross total count	6716		3515	
Number of genera	11		7	
Number of species+varieties	25± 1 var.		18+2 var.	

OR*: Occurrence of remarks:
H: High occurrence, between 11-20 cases (out of 20).
M: Moderate occurrence, between 6-10 cases.
L: Low occurrence, between 305 cases.
R: Rare occurrence, less than 3 cases.

Table 2. Sample number, cultivars, source, moisture content (M.C.%) and mycotoxin detected (μg/kg) of the different cowpea cultivars

Sample No.	Cultivars	Source	M.C.%	Toxins detected	μg/kg
1	IT82C-9	IITA*	11.70	Aflatoxins B_1, B_2, G_1 & G_2	112
2	IT82C-16	IITA	10.20	− ve	−
3	IT82C-32	IITA	10.96	− ve	−
4	IT82D-812	IITA	8.72	− ve	−
5	IT82D-889	IITA	9.30	− ve	−
6	IT82D-79	IITA	8.97	− ve	−
7	IT82D-716	IITA	9.32	− ve	−
8	IT81D-1032	IITA	9.69	− ve	−
9	IT81D-1137	IITA	9.55	Aflatoxins B_1, B_2, G_1 & G_2	86
10	TVU21	IITA	9.88	Aflatoxins B_1, B_2, G_1 & G_2	60
11	Assan	IITA	9.09	− ve	−
12	Pusa Ph. St.	IITA	9.60	− ve	−
13	Black eye 9	IITA	9.52	Aflatoxins B_1, B_2, G_1 & G_2	45
14	Sabaheia	IITA	11.11	− ve	−
15	Barasadi	IITA	9.68	− ve	−
16	Cream 7	EA**	10.70	− ve	−
17	Pusa Phalyngi	IITA	10.27	− ve	−
18	Balady	EAO	8.95	− ve	−
19	Azmerly	EAO	9.09	− ve	−
20	Fetriyat	EAO	8.89	− ve	−

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

45 and 112 μg/kg seeds. Aflatoxin has been previously reproted to be the main mycotoxin contaminated of different pulses (Wogan, 1968; Campbell, 1969; Shank *et al.*, 1972). In Thailand, 322 of different types of beans (e.g. soya, red, black, brown, yellow, white, horse and chick-pea) were analyzed and showed a contamination rate of 3%. The main concentration of total aflatoxins of the contaminated samples was 213 μg/kg (FAO, 1971, 1976). Habish (1972) found aflatoxin in 41 out of 74 samples of various pulses. Mislivec *et al.* (1975) examined a total of 114 samples including 12 different types of beans for the natural presence of aflatoxins and ochratoxins with negative results. Saleha *et al.* (1982) reproted that chick-pea samples were contaminated with aflatoxin B_1 only during storage. Recently, El-Maraghy (1989) reproted the presence of aflatoxins B_1, B_2, G_1 and G_2 in the extract of one variety of chick-pea out of five varieties tested.

Citrinin, ocharatoxin A, patulin, sterigmatocystin, T-2 toxin, diacetoxyscirpenol and zearalenone were not detected in any sample of cultivar seeds tested. This finding agrees with the results of several surveys which indicated that aflatoxin shown to be the main mycotoxin contamination of various pulses (Habish, 1972; Shank *et al.*, 1972; FAO, 1979).

摘 要

Czpaek agar을 이용하여, 동부품종의 콩열매에서, 11속 25종 1품종의 진균과 10% NaCl을 첨가한 배지에서 7속 18종 2품종의 진균을 분리하였다. 이때

분리된 진균의 코로니는 6716/g이였다. Czpaek 배지를 통하여 분리 동정된 균은 대부분이 *Aspergillus, Pericillium, Emericella* 및 *Rhizopus*속에 속하는 균들이였다. 위에서 소금에 내성이 강한 균으로 분리 동정된 균들은 *A. favus, A. sydowii, A. tomari, A. flavipes, A. niger, A. flavar* var. *columnaris, A. ochraceus, A. oryzae* 및 *P. chrysogerism* 이였다. 각각의 시료를 사용하여 Aflacoxin B_1, B_2, G_1 및 G_2을 분석한 결과, 잔존된 것을 관찰하였다.

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