APPLIED MICROBIOLOGY, Apr. 1975, p. 522-526

Vol. 29, No. 4
Printed in U.S.A.

Mycotoxin-Producing Potential of Mold Flora of Dried Beans

P. B. MISLIVEC,* C. T. DIETER, AND V. R. BRUCE

Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204

Received for publication 31 December 1974

To evaluate the potential for mycotoxin production by molds in dried beans, the mold flora of 114 samples was determined both before and after surface disinfection of the beans with 5% NaOCl. Surface disinfection substantially reduced mold incidence, indicating that contamination was mainly on the surface. The flora, both before and after disinfection, was dominated by species of the *Aspergillus glaucus* group, the toxicogenic species *A. ochraceus*, *Penicillium cyclopium*, and *P. viridicatum*, and species of *Alternaria*, *Cladosporium*, and *Fusarium*. The toxicogenic species *Aspergillus flavis*, *A. versicolor*, *Penicillium citrinum*, *P. expansum*, *P. islandicum*, and *P. urticae* were encountered less frequently. Of 209 species of *Aspergillus* and *Penicillium* screened for mycotoxin production on sterile rice substrate, 114 produced one or more of the following mycotoxins: *A. flavus*, aflatoxins; *A. ochraceus*, ochratoxins; *A. nidulans*, *A. unguis*, and *A. versicolor*, sterigmatocystin; *P. cyclopium*, penicillic acid; *P. citrinum* and *P. viridicatum*, citrinin; *P. urticae*, patulin and griseofulvin. Sterigmatocystin production by *A. unguis* is reported for the first time.

The amount of dried beans produced yearly in the United States is sizable; in 1972, nearly 2 billion pounds were brought to market from U.S. farms (3). Because of their high protein content and relatively low cost, they are consumed in substantial amounts. Preparation of dried beans for the table usually is such that the beans as well as the liquid in which they were prepared, e.g., water, are eaten. Considering their method of preparation and the amount consumed, the question arose concerning the presence and relative prevalence of toxicogenic molds in or on these foodstuffs. The literature indicates that at present little is known about the mold flora of dried beans and its potential to produce mycotoxins. Lopez and Christensen (14) reported the presence of *Aspergillus glaucus* in 20% of pea beans in a surface-disinfected (SD) sample, but detected no molds in SD samples of pinto beans or black-eyed peas. Kurata et al. (12) isolated 1,300 fungal cultures from various Japanese foodstuffs, including soybean, red bean, and kidney bean flours. Of the 1,300 cultures, which included mycotoxic species of *Aspergillus* and *Penicillium*, 56 were isolated from the above flours; however, the authors did not delineate the species comprising these 56 cultures. Alpert et al. (2) examined 64 samples of Ugandan beans and detected aflatoxins in 46 samples. Types of beans examined were not designated. Beuchat and Lechowich (4) examined high-moisture kidney, pinto, and navy beans, inoculated with *Aspergillus parasiticus*, as suitable substrates for aflatoxin production. Aflatoxins were produced on all three bean types at 21, 28, and 35 C.

For these reasons, we conducted a study to determine the mold flora of a number of types of dried beans to establish whether a potential hazard might exist due to contamination of dried beans with mycotoxin-producing fungi.

MATERIALS AND METHODS

Samples. A total of 114 samples, including 12 different types of bean, were examined. All samples were obtained from warehouses and wholesale outlets by inspectors of the Food and Drug Administration (FDA); most beans had been grown and harvested from areas in California, Idaho, and Michigan. The FDA inspectors sent the samples to the FDA Mycotoxin Laboratory, New Orleans, La., where an aliquot of approximately 1 kg was taken from each sample and sent to our laboratory.

Mycoflora determination. Upon receipt, samples were held at 0 C for 72 h to kill any mites present, as mites are a primary source of cross-contamination in a mycology laboratory. From each sample, 100 beans were selected at random, avoiding visibly damaged or discolored beans, and five beans per plate were placed directly onto malt-salt agar plates containing 20 g of malt extract (Difco) per liter, 75 g of NaCl per liter, 40 μg of chlortetracycline per ml, and 15 g of agar (Difco) per liter. Malt-salt agar was utilized because its xerophytic nature prevents bean germination and, therefore, the subsequent rapid seedling development, which caused disorientation of petri plate lids

and stacks. Malt-salt agar inhibits growth of fast-growing species of *Mucorales* and slows down (but does not inhibit) growth of most other fungi, thus allowing detection of normally slow-growing species that otherwise might not be detected. The chlortetracycline, added just prior to the pouring of plates, adequately inhibits any bacterial growth.

One hundred additional intact beans from each sample were surface disinfected for 1 min in 5% NaOCl, rinsed three times with sterile water, and plated on malt-salt agar. All plates were incubated at 23 to 26 C for 14 to 21 days prior to enumeration and identification of mold flora. Species of *Aspergillus* and *Penicillium* were identified according to Raper and Fennell (19) and Raper and Thom (20). Isolates of other genera were rarely speciated.

Mycotoxin determination. A total of 209 pure culture isolates of toxicogenic species of *Aspergillus* and *Penicillium*, subcultured from the beans, were screened qualitatively for production of their respective mycotoxins. Isolates were multispore inoculated into autoclaved (121 C, 30 min, 15 lb/in^2) 500-ml Erlenmeyer flasks containing 50 g of polished rice and 50 ml of tap water and were held for 10 days at 23 to 26 C for development of mold growth. The flask contents were then submerged under 250 ml of chloroform, the molded rice cake was broken up with a glass rod, and the mixture was heated on a steam bath until the chloroform had boiled rapidly for 5 min. The flask contents were then filtered, and the filter cake was discarded. Filtrates were evaporated to dryness in a rotary vacuum evaporator and then redissolved in 5 ml of chloroform. For qualitative mycotoxin determinations by thin-layer chromatography, 10-μl aliquots of the extracts were spotted on silica gel (Mallinckrodt 7-G) and the chromatograms were developed with a toluene/ethyl acetate/formic acid (50:40:10) solvent system in unlined, unequilibrated glass tanks. This system develops relatively rapidly and effectively separates those mycotoxins with which we were concerned from background material in the molded rice extracts.

Mycotoxins were identified by comparison with appropriate reference standards, including internal standards. All mycotoxin standards were provided by the Division of Chemistry and Physics, FDA, Washington, D.C. Developed plates were examined for fluorescing spots under long-wave ultraviolet light. Patulin and penicillic acid, neither of which fluoresce under long-wave ultraviolet light, were made to fluoresce by exposing developed plates to ammonia fumes for up to 5 min. Ciegler and Kurtzman (6) recommend this procedure for penicillic acid, and Norstadt and McCalla (18) recommend it for patulin. We found that ammonia fumes also enhance the normal long-wave ultraviolet fluorescence of the ochratoxins, as reported previously by Trenk and Chu (23). Visibility of sterigmatocystin was enhanced by spraying developed plates with $AlCl_3$ solution and heating for 10 min at 80 C; this procedure changes the normally dull, brick red sterigmatocystin to a bright-yellow fluorescing spot (21).

Following the above procedures, the lower limits of sensitivity for mycotoxin detection were estimated to be: aflatoxins, 5 μg/kg; ochratoxins, 50 μg/kg; sterigmatocystin, 2 mg/kg; and patulin and penicillin acid, 10 mg/kg. Lower limits of sensitivity for citrinin and griseofulvin were not estimated.

RESULTS

Mold flora of dried beans. In this study, occurrence of molds (genera and species) is defined as the percentage of dried beans examined that contained the respective molds. This method of quantitating seed mycoflora (and dried beans are seeds) is documented in the literature and is used widely by plant pathologists (5, 8, 16, 24).

Table 1 lists the 12 types of dried beans and the number of samples of each type that were examined. One hundred non-surface-disinfected (NSD) and 100 surface-disinfected (SD) beans from each sample were tested for the presence of mycoflora, resulting in a total of 11,400 beans for each treatment. The incidence of molds on NSD beans of each type approached or exceeded 90% of the beans examined, indicating a high level of surface contamination. Although surface disinfection substantially reduced the number of beans with viable mold, there was considerable mold invasion; the incidences, except for the one lentil sample, ranged from 16.7 to 58.2%.

Table 2 lists the mold species and unspeciated genera encountered most often in the 114 bean samples both before and after surface disinfection. The data show the incidence as the number of samples in which these species and

TABLE 1. *Incidence of mold on various types of NSD and SD dried beans*

Type	No. of samples examined[a]	Incidence (%)[b]	
		NSD	SD
Navy	28	99.4	53.3
Red	6	97.7	33.7
Great Northern	20	93.2	24.7
Lima	10	98.2	51.0
White	9	89.8	43.3
Pinto	23	96.2	58.2
Kidney	7	100.0	53.4
Garbanzo	3	100.0	16.7
Black-eyed peas	2	66.0	38.0
Pink	3	100.0	56.7
Lentil	1	84.0	6.0
Black turtle	2	100.0	49.0

[a] For each sample lot, 100 NSD and 100 SD beans were examined for mold.

[b] Number of beans from which molds emerged expressed as percentage of total number of beans examined.

TABLE 2. *Number of NSD and SD bean samples from which mold species were isolated*[a]

Species	Incidence (no. of samples)	
	NSD	SD
Alternaria spp.	55	65
Aspergillus candidus	28	8
A. flavus	39	18
A. glaucus	106	77
A. nidulans	6	0
A. niger	20	8
A. ochraceus	85	42
A. restrictus	45	11
A. sydowi	18	5
A. tamarii	28	5
A. versicolor	59	20
Cladosporium spp.	80	64
Fusarium spp.	47	40
Penicillium brevi-compactum	31	6
P. citrinum	70	16
P. cyclopium	98	52
P. viridicatum	95	40

[a] A total of 114 samples were examined.

genera were encountered. The toxicogenic species *Aspergillus flavus*, *A. ochraceus*, *A. versicolor*, *Penicillium citrinum*, *P. cyclopium*, and *P. viridicatum* occurred at the sample level at a relatively high incidence rate.

Table 3 lists the incidence of the species and unspeciated genera detected most often; data are given as percentages of the 11,400 NSD and 11,400 SD beans examined. In both cases, the flora was dominated by the species of the *A. glaucus* group, primarily *A. repens*, *A. ruber*, and *A. chevalieri* (not listed separately in Table 3). Seven known toxicogenic species are listed; four of these, *Aspergillus ochraceus*, *A. versicolor*, *P. cyclopium*, and *P. viridicatum*, were frequently encountered in NSD beans, but their incidence was greatly reduced in SD beans. The other three listed toxicogenic species, *A. flavus*, *A. nidulans*, and *Penicillium citrinum*, occurred less frequently in both NSD and SD beans. Additional toxicogenic species, not listed in Table 3 because of sporadic occurrence (in less than 1% of NSD beans examined), were *Penicillium expansum*, *P. islandicum*, and *P. urticae*. Unidentified species of *Alternaria*, *Cladosporium*, and *Fusarium* were prominent in SD beans.

Additional species and genera encountered sporadically (in less than five NSD samples [Table 2] or in less than 1% of the 11,400 NSD beans examined [Table 3]) were: *A. fumigatus*, *A. unguis*, *A. wentii*, *Botryosporium* spp., *Cephalosporium acremonium*, *Chaetomium globosum*, *Cunninghamella* spp., *Helminthosporium* spp., *Mucor* spp., *Piricularia oryzae*, *Penicillium decumbens*, *P. expansum*, *P. islandicum*, *P. janthinellum*, *P. roqueforti*, *P. urticae*, *Pullularia pullans*, *Rhizopus nigricans*, and *Trichoderma viride*.

Although surface disinfection substantially reduced the incidence of toxicogenic molds in and on dried beans (Table 3), at least 10 toxicogenic species were encountered, and eight of these (including *P. expansum* and *P. urticae*, not listed in Table 3) were found both before and after surface disinfection. *A. nidulans* and *P. islandicum* (not listed in Table 3) were not encountered in SD beans. These data strengthen our initial concern that a potential hazard due to the presence of toxicogenic molds in dried beans may exist.

Mycotoxin production by dried bean mold isolates. Table 4 lists the toxicogenic species, the number of each examined, and the number from which at least one mycotoxin was detected. A total of 209 isolates were examined for production of their respective toxins on sterile rice substrate. Isolates of *P. islandicum* were not examined for production of luteoskyrin and islanditoxin because of the lack of reference standards. Three isolates of *P. expansum* examined for patulin production were negative. *A. unguis* (Table 4) had no history of toxicogenicity, but, because of its morphological similarity to *A. nidulans* and *A. versicolor* (19), we examined our only isolate and found that it produced

TABLE 3. *Incidence of mold species in NSD and SD dried beans*

Species	Incidence (%)[a]	
	NSD	SD
Alternaria spp.	6.3	5.0
Aspergillus candidus	1.6	0.1
A. flavus	2.9	0.9
A. glaucus	38.6	18.3
A. nidulans	0.3	0.0
A. niger	1.8	0.3
A. ochraceus	14.5	3.9
A. restrictus	3.3	1.3
A. sydowi	1.0	0.3
A. tamarii	1.3	0.2
A. versicolor	7.3	0.9
Cladosporium spp.	19.0	7.1
Fusarium spp.	5.8	4.9
Penicillium brevi-compactum	1.7	0.1
P. citrinum	3.5	0.4
P. cyclopium	17.3	3.3
P. viridicatum	18.0	2.4

[a] Percentage of the 11,400 NSD and 11,400 SD dried beans in which the species was detected.

TABLE 4. *Toxin production by toxicogenic molds isolated from dried beans*

Species	Mycotoxin	No. of isolates examined	No. of toxin-producing isolates
Aspergillus flavus[a]	Aflatoxin B_1	29	15
A. flavus	Aflatoxin B_2	29	13
A. flavus	Aflatoxin G_1	29	8
A. flavus	Aflatoxin G_2	29	7
A. flavus	Aflatoxin M_1	29	4
A. ochraceus[b]	Ochratoxin A	60	5
A. ochraceus	Ochratoxin B	60	3
A. nidulans	Sterigmatocystin	3	3
A. unguis	Sterigmatocystin	1	1
A. versicolor	Sterigmatocystin	31	30
Penicillium citrinum	Citrinin	10	10
P. cyclopium	Penicillic acid	51	38
P. urticae[c]	Patulin	9	9
P. urticae	Griseofulvin	9	9
P. viridicatum	Citrinin	15	3

[a] All five listings of *A. flavus* refer to the same 29 isolates.
[b] Both listings of *A. ochraceus* refer to the same 60 isolates.
[c] Both listings of *P. urticae* refer to the same nine isolates.

sterigmatocystin. At least one mycotoxin was produced by 114 of the 209 isolates of toxicogenic species. Some of the 95 negative isolates may have produced toxin(s), but at levels below the limits of sensitivity of our analytical procedure.

Fifteen of the 29 *A. flavus* isolates produced aflatoxins. Two produced B_1, B_2, G_1, G_2, and M_1, five produced B_1, B_2, G_1, and G_2, two produced B_1, B_2, and M_1, four produced B_1 and B_2, one produced B_1 and G_1, and one produced B_1 alone. Only five of 60 isolates of *A. ochraceus* produced detectable ochratoxin A, three of which also produced detectable ocharatoxin B. Some of these isolates were similar morphologically to *A. melleus*, a reported toxin producer (13), but the five positive isolates were *A. ochraceus*, based upon descriptions by Raper and Fennell (19).

Of the 35 isolates of *A. nidulans*, *A. versicolor*, and *A. unguis* examined for sterigmatocystin production, all were positive except for one isolate of *A. versicolor*. All nine isolates of *P. urticae* produced both patulin and griseofulvin, and the 10 isolates of *P. citrinum* all produced citrinin. Citrinin was also produced by three of the 15 isolates of *P. viridicatum*. Thirty-eight of the 51 isolates of *P. cyclopium* produced penicillic acid.

DISCUSSION

The predominance of species of the *A. glaucus* group in the dried bean samples was not unexpected since members of this group can grow on substrates of relatively low moisture, moistures in equilibrium with a relative humidity as low as 70% (6). The predominance of *P. cyclopium* and *P. viridicatum*, among the *Penicillum* species encountered, also may be due to their relatively low moisture requirements. Mislivec and Tuite (15) found that both species could grow at 81% relative humidity, which is low for most species of *Penicillium*. The relatively high incidence of *A. versicolor* and *A. ochraceus* also may be due to their relatively low moisture requirements, since their conidia can germinate at a relative humidity of 81% (P. B. Mislivec, C. T. Dieter, and V. R. Bruce, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, G56, p. 29).

Production of sterigmatocystin by *A. unguis* is reported for the first time. The close morphological and possible genetic relationship of *A. unguis* to the toxin-producing species *A. nidulans* and *A. versicolor* may explain, in part, this ability. However, five isolates of *A. sydowi*, which is closely related morphologically to *A. versicolor*, were screened in this study for sterigmatocystin production with negative results.

The ratios between the number of isolates of mycotoxicogenic species screened for toxin production versus the number found to be positive (Table 4) are of interest. Approximately one-half of the *A. flavus* isolates produced aflatoxins. The ratio is consistent with the results of others as reviewed by Detroy et al. (9). The finding that five of 60 *A. ochraceus* isolates produced ochratoxins agrees partially with earlier results. Lai et al. (13) screened 34 isolates of the *A. ochraceus* group and found only four ochratoxin producers: one *A. ochraceus*, one *A. sulphureus*, and two *A. melleus*. Nesheim (17) found that only four of 14 isolates of *A. ochraceus* tested were capable of producing ochratoxins. Hesseltine et al. (11), however, screened 44 strains of nine species of the *A. ochraceus* group and found that polished rice, the substrate used in this study, was a poor substrate for ochratoxin production as compared with pearled wheat and cracked corn; 21 of the 44 strains they screened, including six of 11 strains of *A. ochraceus*, produced ochratoxin A. Thus, some of our negative isolates may have produced ochratoxin on a substrate other than polished rice. Of the 31 isolates of *A. versicolor* we screened for sterigmatocystin production, 30 were positive. Halls and Ayres (10) found that ten of 16 cultures of *A. versicolor* isolated from cured ham produced sterigmatocystin. Thirteen of 15 cultures of *A. versicolor* isolated from smoked meats by Alperden et al. (1) produced sterigmatocystin. The production of citrinin by

all 10 *P. citrinum* isolates and of patulin by all nine *P. urticae* isolates agrees with Raper and Thom's taxonomic description of the two species (20). No reports were found in the literature with which the 38:51 ratio of penicillic acid production by *P. cyclopium* isolates could be compared.

All 114 bean samples were screened by the FDA Mycotoxin Analytical Laboratory in New Orleans for the natural presence of aflatoxins, with negative results; 40 of these samples with a high incidence of *A. ochraceus* (more than 20% of the beans containing the species) were also examined for the natural presence of ochratoxins, with negative results. However, Thorpe and Johnson (22) of the Division of Chemistry and Physics, FDA, detected penicillic acid at levels ranging from 11 to 179 μg/kg in five of 20 samples with a high incidence of *P. cyclopium*.

The finding that intact beans with no visible evidence of mold growth harbored viable mold when plated on agar media indicates that visual observation may not be sufficient when evaluating moldiness of a foodstuff. Isolates included a number of toxicogenic species, many of which produced their respective mycotoxins on sterile rice substrate, although in different ratios (number of isolates of each species screened versus the number positive). Data in Table 4 suggest that sterigmatocystin, patulin, griseofulvin, citrinin, and penicillic acid may be of more concern than considered previously because of the abilities of the respective species to regularly produce these toxins in detectable amounts. Although naturally occurring aflatoxins and ochratoxins were not detected in the beans themselves, penicillic acid was detected (22), indicating that dried beans are substrates suitable for mycotoxin production. The possibility of the natural occurrence of sterigmatocystin, patulin, griseofulvin, and citrinin in beans has not been determined because of the lack of appropriate analytical methods for these mycotoxins in beans.

LITERATURE CITED

1. Alperden, I., H. J. Mintzlaff, F. Tauchmann, and L. Leistner. 1973. Bildung von Sterigmatocystin in mikrobiologischen Nahrmedien und in Rohwurst durch *Aspergillus versicolor*. Fleischwirtschaft **53**:707-709.
2. Alpert, M. E., M. S. R. Hutt, G. Wogan, and C. S. Davidson. 1971. Association between aflatoxin content of food and hepatoma frequency in Uganda. Cancer **28**:253-260.
3. Anonymous. 1973. Agricultural statistics, p. 283. U.S. Government Printing Office, Washington, D.C.
4. Beuchat, L. R., and R. V. Lechowich. 1970. Aflotoxins: production on beans as affected by temperature and moisture content. J. Milk Food Technol. **33**:373-376.
5. Christensen, C. M., H. A. Fanse, G. H. Nelson, F. Bates, and C. J. Mirocha. 1967. Microflora of black and red pepper. Appl. Microbiol. **15**:622-626.
6. Christensen, C. M., and H. H. Kaufman. 1965. Deterioration of stored grains by fungi. Annu. Rev. Phytopathol. **3**:69-84.
7. Ciegler, A., and C. D. Kurtzman. 1970. Penicillic acid production by blue-eye fungi on various agricultural commodities. Appl. Microbiol. **20**:761-764.
8. de Temple, J. 1961. Routine methods for determining the health condition of seeds in the seed testing stations. Proc. Int. Seed Test. Assoc. **26**:27-60.
9. Detroy, R. W., E. B. Lillihoj, and A. Ciegler. 1971. Aflatoxins and related compounds, p. 16-18. *In* A. Ciegler, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. VI. Academic Press Inc., New York.
10. Halls, N. A., and J. C. Ayres. 1973. Potential production of sterigmatocystin on country-cured ham. Appl. Microbiol. **26**:636-637.
11. Hesseltine, C. W., E. E. Vandegraft, D. I. Fennell, M. L. Smith, and O. L. Shotwell. 1972. Aspergilli as ochratoxin producers. Mycologia **64**:539-550.
12. Kurata, H., H. Tanebe, K. Kanota, S. Udagawa, and M. Ichinoe. 1968. Studies on the population of toxigenic fungi in foodstuffs. IV. Aflatoxin-producing fungi isolated from foodstuffs in Japan. J. Food Hyg. Soc. Jpn. **9**:29-34.
13. Lai, M., G. Semeniuk, and C. W. Hesseltine. 1968. Nutrients affecting ochratoxin A production by *Aspergillus* spp. Phytopathology **58**:1056.
14. Lopez, L. C., and C. M. Christensen. 1962. Invasion of and damage of bean seed by storage fungi. Plant Dis. Rep. **46**:785-789.
15. Mislivec, P. B., and J. Tuite. 1970. Temperature and relative humidity requirements of species of *Penicillium* isolated from dent corn kernels. Mycologia **62**:75-88.
16. Naumova, N. A. 1970. Testing of seeds for fungous and bacterial infections. Israel Program for Scientific Translations, Jerusalem.
17. Nesheim, S. 1967. Note on ochratoxin. J. Assoc. Offic. Anal. Chem. **50**:370.
18. Norstadt, F. A., and T. M. McCalla. 1969. *Penicillium urticae* Bainier enumeration in soils. Plant Soil **30**:129-133.
19. Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus*. The Williams and Wilkins Co., Baltimore.
20. Raper, K. B., and C. Thom. 1949. A manual of the penicillia. The Williams and Wilkins Co., Baltimore.
21. Stack, M., and J. V. Rodricks. 1971. Method for analysis and chemical confirmation of sterigmatocystin. J. Assoc. Offic. Anal. Chem. **54**:86-90.
22. Thorpe, C. W., and R. L. Johnson. 1974. Analysis of penicillic acid by gas-liquid chromatography. J. Assoc. Offic. Anal. Chem. **57**:861-865.
23. Trenk, H. L., and F. S. Chu. 1971. Improved detection of ochratoxin A on thin layer plates. J. Assoc. Offic. Anal. Chem. **54**:1307-1309.
24. Tuite, J. 1961. Fungi isolated from unstored corn seed in Indiana in 1956-1958. Plant Dis. Rep. **45**:212-215.