by the substrate source and may not be involved in the differences in aflatoxin formation.

It was speculated that phenolic concentrations in the substrates may have been a factor in the observed variation in growth rates of the fungus and aflatoxin accumulation. However, there was no significant difference in total phenolics in the 2 substrates with a mean of 9.1 and 9.5 μg/mg for high- and low-yielding trees. Specific phenolic compounds were not determined, but 7 are known to occur in pecan kernels (19). The different growth rates of *A. parasiticus* may have been due to the concentration of one or more of these phenolic compounds rather than the total concentration of all phenolics present. Juglone, which occurs in pecan leaves and reported as a possible resistant factor to pecan scab (*Fusicladium effusum*) (20), did not occur in the kernels (19).

The data indicate that physiological differences, associated with low-yielding trees, resulted in improved tolerance to accumulation of aflatoxins by one isolate of *A. parasiticus*. Identification of the physiological differences may prove to be a new approach for developing the natural tolerance of pecan nutmeats to members of the *A. flavus* group. The relationship of yield with aflatoxin concentration indicates this variable should be considered in the field evaluation for infection by toxin-producing strains of the *A. flavus* group and the accumulation of aflatoxins.

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Aflatoxin Formation on Whole and Ground Cumin and Anise Seeds

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ABSTRACT

The purpose of this study was to evaluate the potential productivity and growth of *Aspergillus parasiticus* (NRRL 2999) and the resulting toxin production on natural and autoclaved (cooked) cumin and anise spice seed substrates. Both whole and ground seeds were used. Mycelia and sporulation were also noted in this 17-day experiment. Cumin and anise seeds are capable of supporting mycelial growth, sporulation and toxin production when the seeds are moist and maintained at room temperature. Toxin yields were higher on ground sterile seed substrates. Of the commercial samples tested, neither the resulting cultures of natural flora nor dry whole seeds were found to contain aflatoxin or aflatoxin-like producing organisms. The anise substrates were more conducive to mycelial growth, sporulation and aflatoxin production than the cumin. Toxin levels in the various anise substrates ranged from 0.83 to 6.5 μg/g total for the 4 aflatoxins, B_1, B_2, G_1 and G_2. Cumin seed substrates usually showed only B_1 and G_1 at total levels ranging from 0.23 to 0.63 μg/g. Both spice seeds had mycelial growth and sporulation to occur at some time during the experimental period. Both substrates could be considered as low-level-producer substrates for aflatoxins. Anise seeds should be monitored occasionally for aflatoxin contamination when the commodities are purchased and used in large quantities.

INTRODUCTION

Aflatoxins have been found in many agricultural products such as peanuts (1), red peppers (2) and cottonseed (3). Spices such as ginger, mace, cumin seed, dill seed, garlic powder, onion powder and the herbs, marjoram, rosemary, thyme and sage, were examined for aflatoxin with negligible levels reported (4). *Aspergillus flavus* has been isolated from mace, cumin seeds, coriander seeds and other spices (5,6). The potential for aflatoxin contamination of spices is quite evident. Spices are international commodities having widespread usage.

Anise (*Pimpinella anisum*) seeds are used to flavor pastry, cookies, candy and certain cheeses. They have a spicy taste and are used to flavor licorice. The oil is also used in medications, especially for treating children's stomach problems (i.e., flatulence). Oil of anise is volatile and contains anethole, parapropenyl phenyl methyl ether ($C_3H_5C_6H_4OCH_3$). Anethole and its derivatives such as anisole and anisaldehyde are used in perfumes and flavorings. Anise is imported into the U.S. from Europe and the Mediterranean countries (7).

Cumin (*Cuminum cyminum*) seeds are used in flavorings and resemble caraway seeds in odor. They are also known as comino seeds. Cumin is used commercially in the preparation of meats, pickles, cheeses, sausage, curry powder, chili powder and chutneys. The plants are natural to the Mediterranean and Middle East nations (7).

The purpose of this study was to evaluate the potential productivity and growth of *Aspergillus parasiticus* (NRRL 2999) and the resulting toxin production on natural and autoclaved (cooked) cumin and anise spice substrates.

EXPERIMENTAL PROCEDURES

Whole, commercial spice seeds and their ground powders were the sole source of seeds used as substrates. All seeds were from individually canned containers. Anise seeds and ground cumin were processed by C.F. Sauer Co., Richmond, VA, and cumin seeds were processed by Twin Tree Gardens Flavor Delight, Inc., Medford, NJ. Ground anise was prepared in a chemically sterile blender from whole anise seed and was ground to a consistency similar to that of ground cumin.

Sterile glass prescription bottles (30-mL) with cotton stoppers served as culture flasks for all the seeds tested. Four g of whole or ground seed was placed in the bottles. Preliminary results indicated that 8 mL of tap water was needed to adequately moisten the substrates. Triplicate sets were made for each of the 8 treatment sets used. The first treatment sets were the controls. Here, the whole and ground cumin and anise substrates were autoclaved at 15 psi for 15 min. They were not inoculated with the fungal spores. The experimental substrate treatment groups included triplicate sets of whole and ground anise and cumin seed that were not sterilized but cultured in sterile bottles with sterile tap water. They were the noninoculated natural flora cultures. Additional substrate treatment groups were all inoculated with equal aliquots of spores from *Aspergillus parasiticus* NRRL 2999. This is an aflatoxigenic strain known to produce B_1, B_2, G_1 and G_2 on favorable substrates under favorable conditions. Spores used herein came from a single slant. Slants of this isolate have been maintained in the laboratory on potato-dextrose agar medium. Triplicate sets of natural flora cultures and sterilized cultures were inoculated. Such sets were prepared for both the whole and ground seeds of anise and cumin. This provided a total of 8 different substrate treatment groups for each spice: sterilized whole seeds, sterilized ground seeds, natural flora whole seeds, natural flora ground seeds, inoculated natural flora whole seeds and inoculated natural flora ground seeds.

The mold was allowed to grow for 17 days in darkness at 21 ± 3 C. Bottles were cultured on their flat side to provide maximal surface area for the mold growth. After the first 60 hr, each culture bottle was observed daily for mycelial growth and sporulation. After the 17th day, 10 mL of $CHCl_3$ was added to each bottle. The bottles were capped and placed in a shaker for 20 min. The $CHCl_3$ served to attenuate the cultures. Cultures were allowed to air-dry and then an additional 20 mL of $CHCl_3$ was added for extraction purposes. After shaking for 20 min, the solids were allowed to settle for 2 hr, and then μL quantities of the liquid phase were removed and spotted on silica gel thin layer chromatography (TLC) plates. Triplicate spots were made and mean values read by visual examination and then subsequent dilutions were made of spotted reference samples containing aflatoxins B_1, B_2, G_1 and G_2. A standard solvent system approved for use with foods and feeds by the Association of Official Analytical Chemists (8) was followed. These tests were made at the Mycotoxin Laboratory, Division of Consolidated Laboratory Services, Department of General Services, Commonwealth of Virginia, Richmond.

RESULTS

No aflatoxins were detected in the 24 samples of the whole and ground cumin and anise seeds that were used as controls. Some of the natural flora cultures, however, had a heavy growth of *Rhizopus* sp. No *A. parasiticus*-like organisms were observed in the natural flora or sterilized controls in either the ground or the whole seed cultures. Also, tests to determine aflatoxin content were negative for both types of cultures. However, large concentrations of the aflatoxins were detected in the cultures inoculated with *A. parasiticus* NRRL 2999 (Table I).

The amount of growth of *A. parasiticus* was slightly

TABLE I

Formation of Aflatoxins on Various Anise and Cumin Seed Substrates[a]

	Anise seeds							
	Ground & sterilized seeds		Whole sterile seeds		Ground seeds with natural flora		Whole seeds with natural flora	
	Aflatoxin (μg/g substrate)	%	Aflatoxin (μg/g substrate)	%	Aflatoxin (μg/g substrate)	%	Aflatoxin (μg/g substrate)	%
B_1	2.80 ± 3.00	43	2.19 ± 1.62	45	1.77 ± 1.03	43	0.44 ± 0.10	53
B_2	0.04 ± 0.00	1	0.12 ± 0.54	3	0.10 ± 0.00	2	ND[b]	–
G_1	3.65 ± 3.35	56	2.28 ± 0.92	49	1.98 ± 1.18	48	0.39 ± 0.12	47
G_2	0.05 ± 0.00	1	0.07 ± 0.02	2	0.28 ± 0.27	7	ND[b]	–
Total[c]	6.54	–	4.66	–	4.13	–	0.83	–
	Cumin seeds							
B_1	0.32 ± 0.14	51	0.13 ± 0.03	40	0.12 ± 0.04	40	0.23 ± 0.05	100
B_2	ND[b]	–	ND[b]	–	ND[b]	–	ND[b]	–
G_1	0.31 ± 0.11	49	0.19 ± 0	60	0.18 ± 0.04	60	ND[b]	–
G_2	ND[b]	–	ND[b]	–	ND[b]	–	ND[b]	–
Total	0.63	–	0.31	–	0.30	–	0.23	–

[a]Mean and SD from 3 replicates.

[b]ND = none detected.

[c]Overall total: means of individual toxins may not total exactly to this value.

TABLE II

Coverage of Cultures by Mycelium as a Percentage of Total Surface Area[a]

	Anise seed			
Time (hr)	Surface area covered by mycelia in ground and sterilized seed (%)	Surface area covered by mycelia in whole and sterilized seed (%)	Surface area covered by mycelia in ground seeds with natural flora (%)	Surface area covered by mycelia in whole seeds with natural flora (%)
60	53 ± 15	120 ± 8	28 ± 22	70 ± 10
72	90 ± 10[b]	30 ± 20[b]	77 ± 15[b]	97 ± 3[b]
84	97 ± 6	33 ± 19	82 ± 14	100 ± 0
96	98 ± 3	52 ± 11	88 ± 9	
108	100 ± 0	65 ± 6	93 ± 5	
120		89 ± 1	99 ± 1	
132		100 ± 0	100 ± 0	
144				
252				

[a]Mean % and standard deviation for 3 sets.
[b]The first observable occurrence of sporulation.

greater on the ground seeds than on the whole seed, as evaluated by mycelial area (Tables II and III). The mean yield of aflatoxins/g of culture of *A. parasiticus* is given in Table I. The yields on the ground seed cultures were greater than those in the whole seed cultures. The sterilized cultures also showed a greater yield of aflatoxin than the natural flora cultures. The sterilized ground anise seed had a total mean value of 6.54 μg aflatoxin/g of substrate compared to 4.13 μg aflatoxin/g of substrate in the ground natural flora culture.

The cumin seed followed the same pattern, though did not produce as much aflatoxin as the anise cultures. The sterile ground cumin seed had a mean value of 0.63 μg of aflatoxin/g of substrate compared to 0.30 μg of aflatoxin/g of substrate in the ground seed with natural flora.

The whole seed sterilized cultures showed a greater yield than the natural flora seeds in both the cumin and anise seed, though neither produced as much aflatoxin as the ground substrate (Table I). The sterilized whole anise seed had a total mean value of 4.66 μg aflatoxin/g of substrate compared to 0.83 μg of aflatoxin/g of substrate in the whole natural flora seed. In the cumin, the whole sterilized seed had a total mean value of 0.31 μg aflatoxin/g of substrate compared to 0.23 μg aflatoxin/g of substrate in the whole natural flora seeds.

In Tables II and III, the coverage of cultures by mycelium as a percentage of total mycelial surface area available and the amount of time it took to attain maximal growth are shown. *A. parasiticus* grew better on the anise seeds than on the cumin seeds and a larger amount of aflatoxin was produced on the anise cultures than on the cumin cultures (Table I).

Based on the new Duncan's multiple range test while accepting a 5% error, the *A. parasiticus*-inoculated whole seeds with natural flora in the substrate produced significantly lower levels of total aflatoxin than the other 3 substrates. All cumin substrates were statistically similar and all cumin substrate treatments were significantly lower than all the anise cultures. In our study, the mean yield of all 4 types of aflatoxins showed an increase for the sterilized

TABLE III

Coverage of Cultures by Mycelium as a Percentage of Total Surface Area[a]

	Cumin seed			
Time (hr)	Surface area covered by mycelia in ground and sterilized seed (%)	Surface area covered by mycelia in whole and sterilized seed (%)	Surface area covered by mycelia in ground seeds with natural flora (%)	Surface area covered by mycelia in whole seeds with natural flora (%)
60	ND[b]	ND[b]	7 ± 3	37 ± 15
72	ND	ND	63 ± 6	70 ± 26[c]
84	ND	ND	67 ± 5	83 ± 21
96	13 ± 6	ND	75 ± 1	93 ± 12
108	32 ± 12	ND	83 ± 2[c]	100 ± 0
120	72 ± 4[c]	6 ± 4	94 ± 1	–
132	85 ± 5	11 ± 1	100 ± 0	–
144	100 ± 0	11 ± 8[c]	–	–
156	–	11 ± 8	–	–
168	–	11 ± 9	–	–
180	–	12 ± 10	–	–
192	–	13 ± 13	–	–
204	–	15 ± 15	–	–
216	–	16 ± 17	–	–
228	–	17 ± 20	–	–
240	–	17 ± 20	–	–
252	–	27 ± 21	–	–

[a]Mean % and standard deviation from 3 sets.
[b]ND = no detectable growth.
[c]First observable occurrence of sporulation.

cultures (Table I). Most of the total increase in aflatoxins may be attributed to the production of G_1 and B_1 in the sterile cultures of the anise. Aflatoxin B_2 and G_2 were not produced on the cultures with natural flora and whole seeds and B_1 and G_1 production was lower in these cultures also.

DISCUSSION

In recent years, mycotoxin contamination of food products, both in the drying and storage process and in the home, have been studied. Many different substrates are susceptible to aflatoxin production under various conditions, both natural and experimental (9-11). It has been demonstrated in the laboratory that fungi of *Aspergillus* sp. can grow and produce aflatoxins on a very wide variety of commodities. Toxin production under laboratory conditions are not directly comparable to natural conditions, but at least may be indicative in some measure to the problem and potential in natural environments. Fungal growth or mold contamination per se is not necessarily an indication of toxin production in a particular commodity, as toxin production seems to occur only in a narrower range of conditions than those which permit growth (12,13).

A number of parameters affect aflatoxin production. The influence of temperature, pH, carbon source, available zinc, relative humidity, oxygen and carbon dioxide, among others, are all important in aflatoxin production (14).

In this study, the texture of the substrate, the barrier provided by the seed coats present on whole seeds, the influence and/or competition of the naturally occurring microorganisms, and the effect of cooking (autoclaving) on the substrates may have been significant parameters. For example, ground seeds having the seed coat broken and the contents pulverized proved to be a better substrate for mold growth, sporulation and toxin production for both spices. Toxin production was significantly greater for anise. Sterilization and accompanying cooking via autoclaving also provided similar results for the parameters measured. Apparently, cooking (autoclaving) helped make nutrients more available and reduced microflora competion. Other studies have reported greater aflatoxin production on autoclaved substrates (15).

When cumin and anise substrate yields are compared to aflatoxin levels produced by this fungal strain on other products tested in laboratory studies, the following results are evident. Soybeans produced from 22 to several thousand $\mu g/g$ of the total aflatoxin produced (15). Cocoa beans that were cooked produced nearly 400 $\mu g/g$ total aflatoxin but uncooked beans were not producers (16). Apricots and figs were low-level producers when this strain was used with total aflatoxin levels up to 5 $\mu g/g$ found in figs and less than 0.1 $\mu g/g$ found in apricot media (17). Lettuce, cauliflower, celery and taro root failed to produce detectable levels of aflatoxins when inoculated with *A. parasiticus* NRRL 2999 (18). Pineapples have produced 13-15 $\mu g/g$ total aflatoxins (17). It is apparent that cumin seeds could be ranked as a negligible producing substrate, whereas anise seeds would rank as a low-level producing substrate.

In the cumin cultures, B_2 and G_2 were not formed. In the sterilized cultures, B_1 and G_1 were produced, but in the cultures with natural flora, less B_1 was produced and in the whole seed with natural flora only B_1 was produced.

Cumin appears to be a harsh substrate and could contain some inhibitory agents against aflatoxin production. We doubt that cumin seeds would require monitoring, as based on our laboratory culture tests.

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