Effect of Aspergillus flavus on Peanuts Grown under Gnotobiotic Conditions

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ABSTRACT

Two varieties of peanuts (Tennessee Red and Virginia Bunch 46-2) were grown under gnotobiotic conditions, and the pods were inoculated with a conidial suspension of the toxin-producing fungus *Aspergillus flavus*. No evidence of pathogenicity to peanut plants or pod rot symptoms was observed. *A. flavus* penetrated the shell tissue consistently, but was limited in its seed invasion to the testa. Invasion and colonization of the embryos by *A. flavus* appeared to be limited. Phytopathology 60:208-211.

There has been considerable interest and research in recent years on peanut fruit mycoflora, and especially on the toxin-producing fungus, *Aspergillus flavus* Link. Under field conditions, *A. flavus* is a minor but persistent constituent of pod-surface (8) and shell mycoflora (4, 8) of sound pods. As a rule, the seeds are free of *A. flavus* at maturity (1, 2, 6, 7, 8, 11); however, invasion and colonization of seeds before removal of pods from the soil have been reported (13, 14). Invasion and colonization of peanut seeds by *A. flavus* in one instance increased markedly in the soil as the pods matured (14), and in several instances this colonization of seeds by *A. flavus* increased after maturity and during the period between removal from soil and drying (1, 2, 7, 11).

My purpose was to determine the capacity of *A. flavus* to penetrate and colonize seeds of living, attached peanut pods. The complex and abundant mycoflora associated with peanut fruit under natural conditions make it difficult to study the penetration of pod tissue by individual microorganisms. For this reason, peanut plants (*Arachis hypogaea* L.) were grown in a gnotobiotic environment where the mycoflora could be eliminated or controlled.

MATERIALS AND METHODS.—Flexible film isolators described by Trexler & Reynolds (15) provided gnotobiotic conditions. An A-frame light bank with 12 General Electric F48PG17 cool-white power groove fluorescent tubes was suspended over each isolator. Light intensity at plant height ranged from 1,700 ft-c at the center of the isolator to 1,000 ft-c at the corners of the isolator. Air temperatures in the isolators were 29-31°C with lights on, and 22-24°C with lights off. A diurnal cycle of 16-hr light and 8-hr dark was maintained. Relative humidity in the isolators ranged from 85 to 95%.

The method of sterilization of isolators and equipment was described in an earlier paper (10).

The rooting medium consisted of a 50% washed river sand and 50% perlite mixture (v/v). Sterilization of the rooting medium was accomplished by autoclaving the medium for 12 hr at 121°C.

Germ-free peanut plants were obtained by the following procedure. Seeds were surface-sterilized in 0.5% NaOCl for 3 min, then the testae were removed. The axis, with one-half a cotyledon attached, was aseptically excised from these seeds and surface-sterilized in 1% NaOCl for 5 min under vacuum, then rinsed three times in sterile distilled water. These were aseptically transferred onto a filter paper wick in sterile test tubes (25 × 100 mm) containing 15 ml of 50% Hoagland's solution with 3% glucose, and stoppered. After 14 days, the best-developed seedlings were transplanted into plastic pots (16 cm in depth × 28 cm in diam) containing the rooting medium. Only one plant was grown in each pot; there were three plants per isolator. Two varieties of peanuts (Virginia Bunch 46-2 supplied by K. H. Garren, Agricultural Research Service, Holland, Va., and Tennessee Red supplied by K. H. Garren and R. S. Matlock, Department of Agronomy, Oklahoma State Univ., Stillwater, Okla.) were used in this study. Plants were watered with sterile 50% Hoagland's solution according to their needs. Iron was added in the form of iron versenate at the rate of 5 ppm iron/liter. The pH of the nutrient solution was 5.2 after autoclaving.

Sterility of the plants and isolators was confirmed every week. Samples of plant tissue, rooting medium, and debris from the isolator floor were placed in the following culture media: fluid thioglycollate, nutrient broth, AC medium, and Sabouraud dextrose broth. The culture tubes were incubated in the isolator from 7 to 21 days, and outside the isolator at 23-25°C for 7 days. At the termination of each experiment, pieces of the root, stem, and leaves were dissected from the plant and surface-sterilized in 0.5% NaOCl for 3 min, then plated out on potato-dextrose agar (PDA) and NIH agar.

To determine the ability of *A. flavus* to colonize peanut pod tissue, peanut plants were grown under

gnotobiotic conditions until pods had formed (80 to 90 days), then inoculated by uncovering the pods and pouring 330 ml of a conidial suspension of *A. flavus* (conidia washed from two 14-day-old cultures on PDA slant tubes suspended in 1,000 ml of 50% Hoagland's solution) over them. Two strains of *A. flavus,* 6-911 and 6-885, supplied by K. H. Garren, Agricultural Research Service, Holland, Va., were used in this study. After a given incubation period, the plants were harvested and the pods rated on their stage of development according to the Kranz & Pucci system (9), which ranges from Stage 1 (immature pod 2-3 mm in diam) to Stage 7 (fully mature pod). The pods then were dissected into three parts: shell, testa, and embryo; surface-sterilized in 0.5% NaOCl for 3 min; and plated out on PDA and NIH agar. The petri plates were incubated at 23-25°C for 5 to 7 days.

To determine the effect of *A. flavus* on peanut pod development, pegs of germ-free peanuts were guided into glass cylinders (60 × 105 mm) containing 360 g of *A. flavus*-infested sand (conidia of *A. flavus* suspended in 60 ml of 50% Hoagland's solution + 0.5% glucose were added to the sand), and the pods allowed to develop. Inoculum density was 10,000 conidia/g of sand.

RESULTS.—Growth of peanuts to maturity under gnotobiotic conditions was difficult, as a bacterial contaminant (gram variable rod) was detected in most experiments after 2 months. The source of the contamination was not determined. The contaminant had no visual effect on plant growth or pod development. Peanut plants grown to maturity under germ-free conditions developed normally, and no stimulatory or detrimental effects were observed.

Tennessee Red peanuts grown under gnotobiotic conditions for an average of 120 days (germ-free for 56 days, then plants + bacterial contaminant for final 64 days) had the following average characteristics: flowered after 56 days; 19 pegs/plant; 7 pods/plant; shoot wt 7.16 g/plant; root wt 0.94 g/plant; pod wt 2.67 g/plant; and total dry wt of 10.77 g/plant. The above averages are from a total of 15 plants. The one experiment in which the plants were germ-free for 136 days had the following average characteristics: flowered after 61 days; 17 pegs/plant; 8 pods/plant; shoot wt 4.93 g/plant; root wt 0.53 g/plant; pod wt 1.48 g/plant; and total dry wt of 6.94 g/plant. The above averages are from a total of 3 plants.

Virginia Bunch 46-2 peanuts grown under gnotobiotic conditions for an average of 108 days (germ-free 63 days, then plants + bacterial contaminant for final 45 days) had the following average characteristics: flowered after 54 days; 41.4 pegs/plant; shoot wt 18.15 g/plant; root wt 1.21 g/plant; and a total dry wt of 19.36 g/plant. The above averages are from a total of 5 plants.

The bacterial contaminant was isolated consistently from the roots and stems of both varieties, but only from a small percentage of the pods (0-15%).

Plant appearance and growth were good in all experiments. No harmful effects were observed on peanut plants grown in the germ-free environment or gnotobiotic environment (peanut plant + bacterial contaminant).

Colonization of Tennessee Red pod tissue by A. flavus.—Peanut plants (Tennessee Red) were grown under gnotobiotic conditions for 84 days (plants germ-free the first 64 days and plants + bacterial contaminant the next 20 days), then inoculated with *A. flavus* 6-885 and harvested 21 days later. The pods were separated into three groups. The 0 days group was immediately plated out after harvest, while the other two groups were incubated in sterile petri dishes for 7 and 18 days, respectively.

At 0 days' incubation, *A. flavus* had colonized a high percentage of whole immature pods and shell pieces of the more mature pods (Fig. 1). *A. flavus* was able to penetrate the shell tissue and colonize the testae of living attached pods; however, its ability to penetrate the testa and colonize the embryo appeared to be limited.

At 7 days, there was an increase in the percentage of whole immature pods and shell pieces colonized by *A. flavus,* while there was a decrease in *A. flavus* isolated from the testae and embryos.

After 18 days, *A. flavus* was able to penetrate the testae as indicated by the large increase in percentage of embryos colonized, but only a slight increase in the percentage of testae colonized. *A. flavus* was still iso-

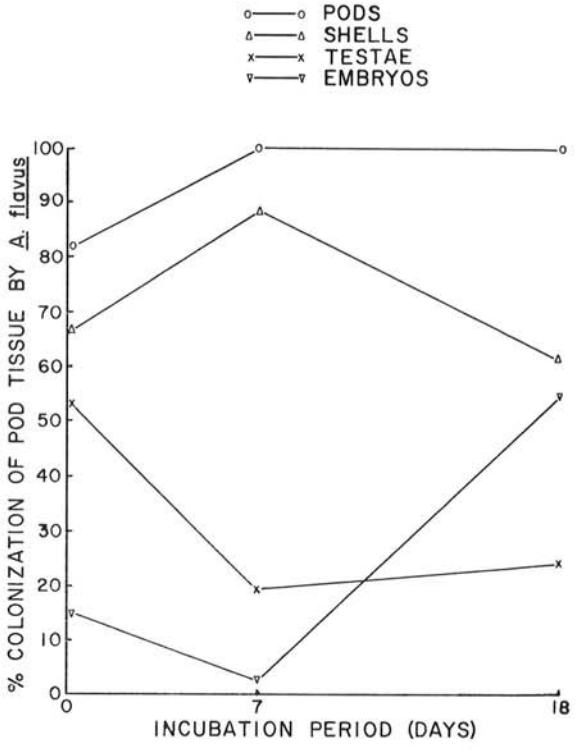

Fig. 1. Colonization of peanut pod tissue (Tennessee Red) by *Aspergillus flavus* isolate 6-885 at different incubation periods after harvest. Peanut plants were grown under gnotobiotic conditions for 84 days (plants germ-free the first 64 days and plant + bacterial contaminant the next 20 days), then inoculated with *A. flavus* and harvested 21 days later.

lated from all the whole immature pods while the percentage of shell pieces colonized decreased.

The bacterial contaminant was isolated from whole immature pods, shell pieces, and testae, but not from the embryos.

To determine the effect of a longer incubation period on the colonization of living attached pods by *A. flavus*, peanut plants (Tennessee Red) were grown under gnotobiotic conditions for 82 days (plants germ-free the first 64 days and plants + bacterial contaminant the next 18 days). Then the plants were inoculated with *A. flavus* 6-911, and harvested 46 days later instead of 21 days later, as in the previous experiment.

The increased exposure time of pods to *A. flavus* under gnotobiotic conditions resulted in a slightly higher percentage colonization of the whole immature pods and shell pieces of more mature pods, but no increase in percentage of testae and embryos colonized. All of the immature pods and a high percentage of the shell pieces were colonized by *A. flavus* (Table 1). *A. flavus* was able to penetrate the shell tissue and colonize 44% of the seeds; however, when the seeds were separated into 2 parts, testa and embryo, it was isolated from only 14% of the testae and 17% of the embryos. Again, the bacterial contaminant was isolated from shell pieces and testae, but not from immature pods or embryos.

Colonization of Virginia Bunch 46-2 pod tissue by A. flavus.—Peanut plants (Virginia Bunch 46-2) were grown under gnotobiotic conditions for 91 days (plants germ-free the first 50 days and plants + bacterial contaminant for the next 41 days), then inoculated with *A. flavus* 6-911 and harvested 21 days later.

The percentage colonization of pod tissue of the variety Virginia Bunch 46-2 by *A. flavus* was similar to that observed with the variety Tennessee Red. *A. flavus* colonized a high percentage of the whole immature pods and shell pieces; however, it was isolated from only a small percentage of the testae and embryos

TABLE 1. Colonization of peanut pod tissue (Tennessee Red and Virginia Bunch 46-2) by *Aspergillus flavus* isolate 6-911 in a gnotobiotic environment

	Pod colonization by *A. flavus*			
	Tennessee Red[b]		Virginia Bunch[c]	
Stage of pod development[a] & pod tissue	No. samples	% Colonized	No. samples	% Colonized
No. 1-3 pods	56	100	66	81
No. 4-6 pods				
shells	179	78	105	82
seeds	25	44		
testae	86	14	46	9
embryos	94	17	53	3

[a] Kranz & Pucci (9) classification of pod development.
[b] Gnotobiotic environment—first 64 days, plants germ-free; the next 18 days peanut plants + bacterial contaminant; and final 46 days peanut plants + bacterial contaminant + *A. flavus*.
[c] Gnotobiotic environment—first 50 days, plants germ-free; the next 41 days peanut plants + bacterial contaminant; and final 21 days peanut plants + bacterial contaminant + *A. flavus*.

TABLE 2. Colonization of peanut pod tissue (Virginia Bunch 46-2) by *Aspergillus flavus* 6-911 in infested sand in a gnotobiotic environment[a]

	Pod colonization by *A. flavus*	
Stage of pod development[b] & pod tissue	No. samples	% Colonized
No. 1-3 pods	19	32
No. 4-5 pods		
shells	60	75
seeds		
testae	31	77
embryos	40	0

[a] Gnotobiotic environment—First 89 days, plants germ-free; the final 51 days peanut plants + *A. flavus* + bacterial contaminant (pegs were guided into *A. flavus*-infested sand).
[b] Kranz & Pucci (9) classification of pod development.

(Table 1). The bacterial contaminant was isolated from the whole immature pods, shell pieces, and testae, but not from the embryos.

To determine the effect of *A. flavus* on peanut pod development and its ability to penetrate pod tissue, peanut plants (Virginia Bunch 46-2) were grown under gnotobiotic conditions for 89 days (plants were germ-free the first 89 days; a bacterial contaminant was detected on the 89th day). Then pod chambers containing sand infested with *A. flavus* 6-911 were placed in the isolator. Pegs were guided into the pod chambers, and the pods allowed to develop in the presence of *A. flavus* for 51 days.

A. flavus did not affect the development of peanut pods under gnotobiotic conditions, and produced no peg or pod rot symptoms. When pods developed in the presence of *A. flavus*, the fungus colonized a high percentage of the shell pieces and testae; however, it was not able to colonize the embryos (Table 2). The bacterial contaminant was isolated from all pod tissue except the embryos.

No harmful effects on peanut plants were observed in any experiment where the plants were inoculated with *A. flavus*.

DISCUSSION.—When peanut plants were grown under gnotobiotic conditions and the pods allowed to develop in the presence of *A. flavus*, or when the root and pod systems were inoculated with *A. flavus*, no evidence of pathogenicity to peanut plants or pod rot symptoms was observed, although the fungus was isolated from the base of the stem, roots, and pods. Bell (3) also found no root necrosis when seedlings grown in tests under "known fungal" conditions were inoculated with *A. flavus;* however, he did observe necrosis of the plumule and cotyledonary laterals.

Under gnotobiotic conditions (peanut plant + bacterial contaminant), *A. flavus* penetrated consistently and colonized a high percentage of shell tissues of living attached immature and mature pods, while under natural conditions, Garren (4) and Jackson (8) found *A. flavus* in only a small percentage of the shells, and Porter & Garren (14) found less *A. flavus* in the shell portion of freshly dug fruits than in the seeds contained in those shells. Either the normal endogeocarpic mycoflora associated with shells is antagonistic to *A. flavus*

(which limits its colonization of shell tissue), or the faster-growing components of the mycoflora masks *A. flavus* when shell tissue is placed on agar media.

In this study, a high percentage of the seeds (9-77%) from immature and mature pods was colonized by *A. flavus;* however, under natural conditions the seeds were generally free from *A. flavus* (1, 2, 7, 8, 11, 12), although invasion of seeds before harvest has been reported (13, 14). There was no evidence of natural resistance to invasion of seeds in living attached pods as suggested by Austwick & Ayerst (2). These results suggest that the presence of normal endogeocarpic mycoflora provides a barrier to the invasion of seeds by *A. flavus.*

A. flavus was found primarily in the testae of colonized seeds under gnotobiotic conditions, but only a small percentage of the embryos were colonized. In the only experiment in which this was not the case, *A. flavus* was isolated from 44% of the seeds; however, when seeds were dissected into their component parts, *A. flavus* was isolated from 14% of the testae and 17% of the embryos. The lower percentage of *A. flavus* isolated from the testae as compared to seeds and embryos indicated that the disinfectant used may have penetrated and sterilized the inner cell layers of the testa. Similar observations have been reported by Jackson (5) in a study to determine the location of fungi in infected seeds; he found all fungi to be more abundant in the testae than in the embryos.

Invasion and colonization of the embryos by *A. flavus* increased in pods harvested from gnotobiotically grown peanut plants after 7 days. There was no increase in the percentage of *A. flavus* isolated from embryos before the 7th day.

During the period of time before harvest and approximately 7 days after harvest, the embryo appears to be resistant to the invasion and colonization by *A. flavus.* Whether the testa acted as a barrier to the invasion of the embryo tissue by *A. flavus* and other fungi, as suggested by Jackson (8), or whether the embryo was resistant to attack by *A. flavus,* was not determined.

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