Colonization of Gnotobiotically Grown Peanuts by Aspergillus flavus and Selected Interacting Fungi

T. R. Wells, W. A. Kreutzer, and D. L. Lindsey

Junior Plant Pathologist and Professor of Botany and Plant Pathology, Colorado State University, Fort Collins 80521; and Assistant Plant Pathologist, New Mexico State University, Las Cruces 88001, respectively.

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ABSTRACT

Peanut plants (cultivar Tennessee Red) were grown under gnotobiotic conditions in the presence of *Trichoderma viride*, *Penicillium funiculosum*, and the toxin-forming *Aspergillus flavus*. These fungi singly or in combinations colonized aerial and subterranean parts of peanut plants; however, colonization by *T. viride* was restricted primarily to subterranean tissues. Immature pods, mature pericarps, and testae, to a lesser extent, were susceptible. Embryos showed limited invasion. Colonization of immature and mature pericarps by *A. flavus* was reduced in the presence of *T. viride*. *Penicillium funiculosum* not only nullified this antagonistic effect, but also appeared to stimulate colonization of mature peanut pericarps and testae by *A. flavus*.

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Additional key words: Bacillus subtilis, aerial peg colonization, plant isolator units.

In an earlier study (12), peanut seedlings (*Arachis hypogaea* L.) were grown to maturity in gnotobiotic environments and inoculated at 80-90 days of age with the aflatoxin-forming *Aspergillus flavus* (Lk.)Fr. Although the fungus penetrated pericarps, testae, and, to a limited extent, embryonic tissues, no evidence of pathogenicity was observed.

Research was expanded to include interactions of *A. flavus* with two additional dominant pod fungi of field-grown peanuts, *Trichoderma viride* (Pers.) Fr. and *Penicillium funiculosum* Thom (1, 2, 3, 5, 6, 7, 8) under gnotobiotic conditions. We believe that such studies on the peanut plant and its associated soil microorganisms offer the best approach to an eventual understanding of natural colonization of peanut tissues by the toxin-forming *A. flavus*.

MATERIALS AND METHODS.—Procedures were similar to those previously described (11, 12, 13) with some modifications.

Test organisms and their sources.—Mature peanut fruits (Tennessee Red cultivar) and isolates of *A. flavus*, *T. viride*, and *P. funiculosum* were supplied by K. H. Garren, ARS, USDA, Holland, Va.

Apparatus for growing axenic plants.—Plants were grown in pots within flexible polyvinyl isolators (16 ft^3). Isolators were equipped with overhead A-frame light banks (ca. 1,700 ft-c) operating on a diurnal 16-hr light and 8-hr dark cycle (11, 12, 15). Positive pressure was maintained by a filtered and temperature-controlled constant air flow (32-35 C, lights on; 22-24 C, lights off) which included a small amount of CO_2 (2.9 ml/hr). Relative humidities within isolators were 70-85%.

Sterilization of isolators, materials, and equipment.—Methods of cleaning and sterilizing isolator interiors and surfaces of introduced materials have been described (11, 13). All materials except rooting medium or test organisms were disinfested by autoclaving in sealed paper bags for 30 min at 121 C. Containers introduced into isolators were surface-disinfested by spraying in situ with 2% peracetic acid (PAA). The isolators were sealed for 24 hr and aerated for 120 hr prior to exposure of biological material. Rooting medium [50% washed river sand and 50% perlite mixture (v/v), in rigid polypropylene pots 16 cm deep × 28 cm in diam] was sealed within large paper bags and sterilized by autoclaving for 12 hr at 121 C. After exposure of biological material within isolators, additional materials to be introduced were surface-disinfested in a "transfer" isolator, aerated, and moved into the "growth" isolator through a sterilized polyvinyl lock.

Sterilization of peanut embryos and the growing of seedlings.—Techniques used to obtain disinfested peanut embryos have been described (12). Briefly, hulled seeds were washed and surface-disinfested with acidified (pH 6.0) 0.5% NaOCl, followed by the removal of testae and all but 0.5 cotyledon/embryo, with a final disinfestation under vacuum (5 min in 1% NaOCl) and five sterile water rinses. Surface-disinfested embryos were transferred aseptically to sterile filter paper wicks in test tubes (25 × 200 mm) which contained 20 ml 50% Hoagland's solution with 1% glucose and were sealed with sterilized rubber stoppers or sterilized, stoppered, glass separatory funnel assemblies designed to permit introduction of liquid culture media into tubes. The sealed wick tubes were introduced into isolators and surface-sterilized, and the enclosed embryos were permitted to germinate. Twelve-day-old seedlings were flooded with sterile Bacto AC liquid medium and kept submerged for 96 hr to detect possible external bacterial contamina-

tion. Well-developed and apparently germfree seedlings were rinsed in sterile distilled water and transplanted (one seedling/pot; three pots/isolator). Plants were watered as needed with sterile 50% Hoagland's solution containing 5 μg Fe/ml (iron versenate).

Contamination monitoring and infestation of isolators.—The sterility of isolators and their contents was monitored each week by incubating plant tissues, rooting medium, and debris in fluid thioglycollate, peptone broth, Sabouraud-dextrose broth, and AC liquid medium for 21 days at 22-35 C and 14 additional days at 35 ± 1 C. We infested peanut plants (80-90 days old) with test fungi by pouring 330 ml of a conidial suspension from single-spored 14-day-old cultures (10^5 conidia/ml = ca. 10^4 conidia/g rooting medium) over uncovered immature pods and about the base of each plant.

Tissue colonization determinations.—After 120 days of plant growth, isolator interiors and contents were surface-disinfested with 150 ml of 2% PAA spray/isolator, sealed for 16 hr, and aerated 24 hr. Plant parts were then removed and treated in 0.5% NaOCl for 3 min, followed by three sterile water rinses. Isolations were made on 2% potato-dextrose agar (PDA) from leaves, stems, pegs, and roots of randomly selected plants, as well as from immature pods rated I-III, Kranz-Pucci scale (9), and shells, testae, and embryos of mature pods (IV to VII ratings) of all plants 30-40 days after inoculation. Culture plates were incubated for 7 days at 20-25 C, followed by 14 days at 35 ± 1 C.

Experimental design.—The results of four interactions, each utilizing five growth isolators (5 replications), were compared. All interactions were conducted under the same exacting physical conditions, but at different times. Each interaction, therefore, was considered to be a single treatment of an experiment extended in time. Interactions were as follows: I. *Arachis hypogaea* × *Aspergillus flavus*; II. *A. hypogaea* × *A. flavus* × *Trichoderma viride*; III. *A. hypogaea* × *A. flavus* × *Penicillium funiculosum;* and IV. *A. hypogaea* × *A. flavus* × *T. viride* × *P. funiculosum*. Summarized data from interaction I were used as a baseline for determining effects of added organisms in interactions II, III, and IV. All data were converted to percentages and analyzed following arcsine transformations (10).

RESULTS.—*Comparisons among interactions.*—1) *Effects of interacting fungi on the colonization of peanut fruit and seed tissues by A. flavus.*—Mean percentages of immature pods invaded by *A. flavus* in interactions I (*A. flavus* alone), III (*A. flavus* × *P. funiculosum*), and IV (*A. flavus* × *T. viride* × *P. funiculosum*) are not significantly different (Table 1). However, a significant reduction (P = .01) in immature pod colonization by *A. flavus* is shown in interaction II (*A. flavus* × *T. viride*).

Mean colonization percentages of mature shells (pericarps) by *A. flavus* in the four interactions are similar to those for immature pods. A comparison of means from interactions I (*A. flavus* alone) and II (*A. flavus* × *T. viride*) again shows that the presence of *T. viride* markedly reduced colonization by *A. flavus* (78.0 to 24.8%). Furthermore, *P. funiculosum* apparently nullified the antagonistic effect of *T. viride* on *A. flavus* as indicated by the nonsignificant difference between the means of interactions I and IV. The difference between colonization of mature shells by *A. flavus* where it was used alone (mean = 78.0%) and where it was used in combination with *P. funiculosum* (mean = 91.7%), although suggestive, is nonsignificant (P = .2-.4).

TABLE 1. Effects of *Trichoderma viride* and *Penicillium funiculosum* on colonization of peanut fruit and seed tissues by *Aspergillus flavus*

Interactions[a]	Mean percentage colonization by *A. flavus*[b]			
	Immature pods	Mature pods		
		Shells	Testae	Embryos
I	100.0	78.0	14.7	17.2
II	25.6*[c]	24.8*	20.7	10.7
III	100.0	91.7	70.3*	12.6
IV	87.2	79.9	67.0*	18.6

[a] I = *A. hypogaea* × *A. flavus*; II = *A. hypogaea* × *A. flavus* × *T. viride*; III = *A. hypogaea* × *A. flavus* × *P. funiculosum;* and IV = *A. hypogaea* × *A. flavus* × *T. viride* × *P. funiculosum*.

[b] Analysis performed on arcsine transformations (10). Percentage data based on a total of 4,003 isolations.

[c] * = Transformed mean significantly different from that of interaction I (P = .01-.05).

Testa invasions by *A. flavus* in two of the four interactions show a departure from pod-shell patterns in that there is no significant difference between percentage colonizations by this fungus in interactions I (*A. flavus* alone = 14.7%) and II (*A. flavus* × *T. viride* = 20.7%). The presence of *P. funiculosum*, however, in interactions III and IV significantly increased mean colonizations of testae by *A. flavus* to 70.3 and 67.0%, respectively.

Percentage colonization of embryos by *A. flavus* (principally cotyledons) was low, differences between interaction I and interactions II, III, or IV being nonsignificant.

2) *Colonization of peanut fruits and seeds by T. viride and P. funiculosum in the presence of A. flavus.*— Peanut fruit and seed colonizations by all fungi of the four interactions are summarized in Fig. 1. Effects of *T. viride* and *P. funiculosum* on *A. flavus* have been described (Table 1). It next is pertinent to consider responses of *T. viride* and *P. funiculosum* from interactions II, III, and IV.

Trichoderma viride in the presence of *A. flavus* (interaction II) invaded 94.3% of the immature pods, 88.0% of the mature shells, 8.6% of the testae, and 6.2% of the embryos (Fig. 1-B). In the presence of both *A. flavus* and *P. funiculosum* (interaction IV), *T. viride* colonized 55.5% of the immature pods, 71.5% of the mature shells, 4.1% of the testae, and 2.4% of the embryos (Fig. 1-D). Mean percentage colonization differences between homologous tissues in the two interactions are significant (P = .05-.01).

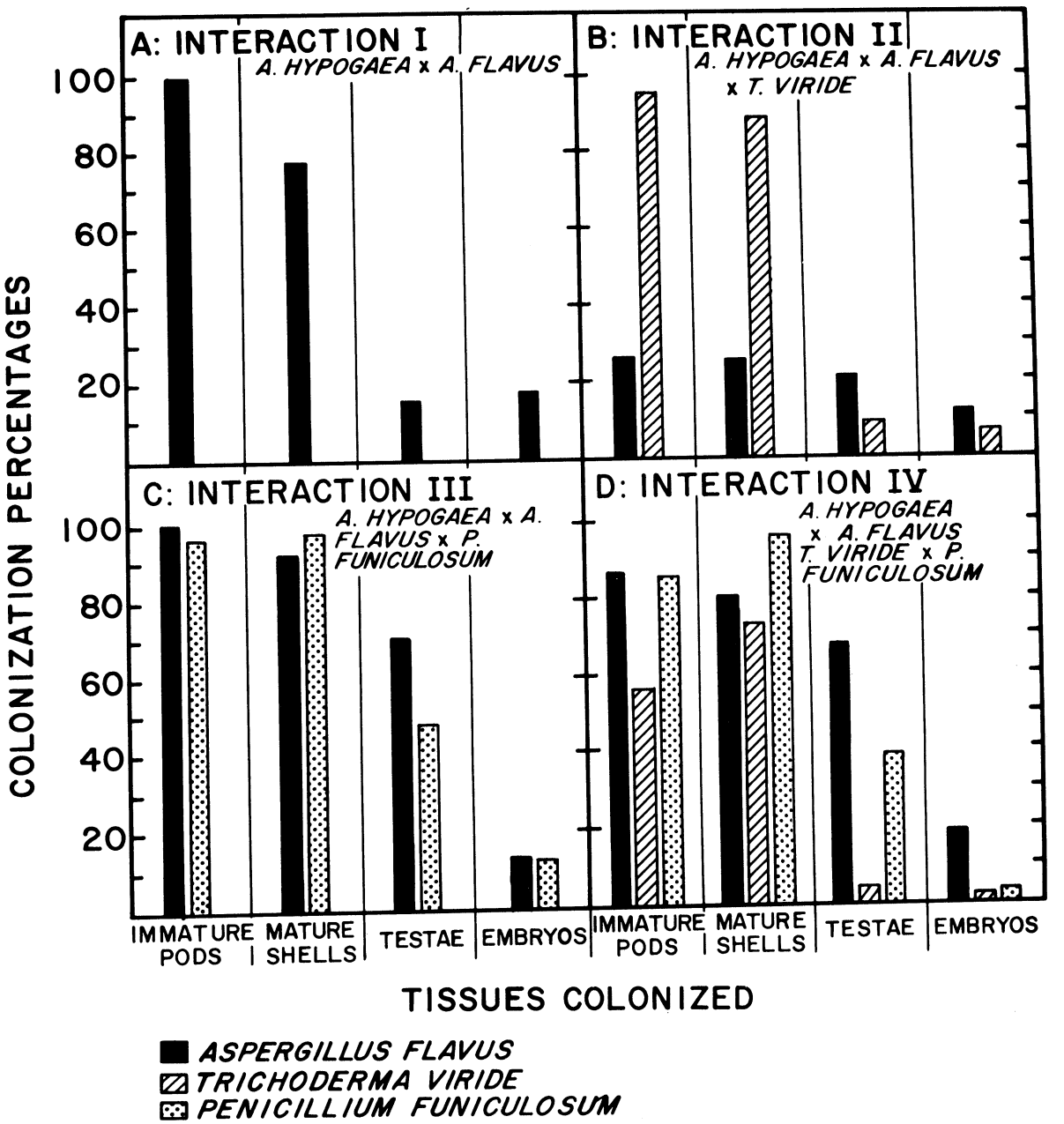

Fig. 1. Mean percentage colonizations of peanut fruit and seed tissues by the component fungi of four gnotobiotic interactions. **A)** Interaction I, colonization by *Aspergillus flavus*; **B)** Interaction II, colonizations by *A. flavus* and *Trichoderma viride*; **C)** Interaction III, colonizations by *A. flavus* and *Penicillium funiculosum*; and **D)** Interaction IV, colonizations by *A. flavus, T. viride*, and *P. funiculosum*.

Penicillium funiculosum in the presence of *A. flavus* (interaction III) colonized 96.5% of the immature pods, 96.6% of the mature shells, 46.6% of the testae, and 11.6% of the embryos (Fig. 1-C). In the presence of both *A. flavus* and *T. viride* (interaction IV), *P. funiculosum* invaded 86.5% of the immature pods, 95.1% of the mature shells, 39.0% of the testae, and 2.9% of the embryos (Fig. 1-D). Mean percentage colonization differences between homologous tissues in the two interactions are nonsignificant (P = .2-.5).

3) *Comparisons within interaction IV. Colonizations of aerial and subterranean peanut tissues by interacting fungi.*—In interaction IV (*A. flavus* X *T. viride* X *P. funiculosum*), one plant 80 days old in each isolator was covered with a large clear plastic bag for 48 hr immediately after inoculation, and similarly covered on the 8th to 10th days of three following 10-day intervals. At 120 days, isolations were made

TABLE 2. Colonizations of aerial and subterranean peanut tissues by the fungal components of interaction IV (*Aspergillus flavus* X *Trichoderma viride* X *Penicillium funiculosum*)

Colonizing fungus	Mean percentage colonization[a]				
	Aerial tissues	Subterreanean tissues			
	Pegs	Immature pods	Mature shells	Testae	Embryos
A. flavus	72.7	87.2	79.9	67.0	18.6
P. funiculosum	46.7*[b]	86.5	95.1	39.0*	2.9*
T. viride	3.6*	55.5*	71.5	4.1*	2.4*

[a]Data based on a total of 1,538 isolations. Analysis performed on arcsine transformations (10).
[b] * =Transformed mean significantly different (P = .01-.05) from that of base line (*Aspergillus flavus*) colonization.

from aerial tissues (pegs, leaves, and stems) of bagged plants, and from subterranean tissues (immature and mature pods) of all plants.

Elongating aerial pegs showed a colonization mean of 72.7% for *A. flavus* (Table 2) with significant reductions in means for *P. funiculosum* (46.7%) and *T. viride* (3.6%). Immature (subterranean) pods showed colonization means of 87.2% and 86.5% for *A. flavus* and *P. funiculosum*, respectively, with a significant reduction for *T. viride* (55.5%). Mature shell colonization means were 79.9% for *A. flavus*, 95.1% for *P. funiculosum*, and 71.5% for *T. viride* (differences nonsignificant). Significant differences were shown between the mean testa colonizations for *A. flavus* (67.0%) and *P. funiculosum* (39.0%) or *T. viride* (4.1%). The mean colonization of embryos by *A. flavus* (18.6%) was significantly higher than that of *P. funiculosum* (2.9%) or *T. viride* (2.4%)

At high relative humidities (85-100%), other aerial tissues such as leaves, petioles, and stems were invaded in varying degrees by both *P. funiculosum* and *A. flavus* (ca. 40-90%). Very little aerial colonization, however, was found for *T. viride* (ca. 0-5%).

DISCUSSION.—Under the conditions of these experiments, subterranean and aerial peanut tissues were readily invaded by *A. flavus* and *P. funiculosum*. Colonization by *T. viride* was primarily restricted to subterranean tissues. Mature or moribund tissues were heavily colonized, whereas immature tissues were apparently invaded nonpathologically. Colonization of mature peanut shells by *A. flavus* was markedly reduced in the presence of *T. viride*. This antagonistic effect was nullified by *P. funiculosum*, which also appeared to have a direct stimulating effect on tissue colonization by *A. flavus*.

Garren et al. (4) theorized that aerial tissues (i.e., pegs) could be invaded by fungi prior to pod formation in the soil. Corroborative field evidence was supplied by Hanlin (6). Our work supports these conclusions. We concur that colonization of mature tissues may arise from a quiescent fungal state in immature tissues (4).

The antagonistic effect of *T. viride* on *A. flavus* appears to be related directly to the capacity of the former to colonize peanut fruit and seed tissues (interactions II and IV). *T. viride* readily invaded immature pods or mature shells, but seldom became established in testae or embryos. We would, therefore, expect the major antagonistic effect of *T. viride* on *A. flavus* to be in the zone of shell colonization. *Aspergillus flavus* colonized 78-100% of peanut pericarps in the absence of *T. viride*, and only 24.8% of the mature pericarps in the presence of *T. viride*. Effects of *T. viride* on *A. flavus* colonizations of testae and embryos were neglible.

The antagonistic effect of *T. viride* on *A. flavus* apparently was nullified by the presence of *P. funiculosum* (interaction IV), since shell invasion by *A. flavus* rose to a level paralleling that in interaction I. Also, colonization of immature pods by *T. viride* was reduced from 94.3% in the absence of *P. funiculosum* to 55.5% in its presence. Evidence of a stimulatory effect of *P. funiculosum* on *A. flavus* was shown by the relatively high testa invasion by *A. flavus* in the presence of *P. funiculosum*, irrespective of the presence or absence of *T. viride* (67-70%). Testa colonization by *A. flavus* in the absence of *P. funiculosum* ranged from 15 to 21%.

A confounding factor, first noted in interaction I (12), was the appearance of a light bacterial "contamination" in 20-40% of the plants in all interactions, usually at the end of 70 days' growth. These organisms were identified by F. E. Clark as strains of *Bacillus subtilis*. Present evidence indicates that the bacteria are carried internally by the peanut plant (Wells & Kreutzer, *unpublished data*). Such an observation is not new; Pettit et al. (14) reported the presence of *B. subtilis* within the subepidermal tissues of healthy peanut cotyledons. The sources and effects of these bacteria are presently being investigated. Their influence on fungal interactions appears to be minor.

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