Conidial Germination and Population of Aspergillus flavus in the Geocarposphere of Peanut

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ABSTRACT

Under greenhouse conditions, pegs of Virginia Bunch 46-2 peanut plants were introduced into small pots containing a nonsterile loamy fine sand artificially infested with washed conidia of a clone of *Aspergillus flavus* isolated from peanut fruit. Dilution plate analysis indicated the inner 0.5-mm layer of geocarposphere soil of pegs and mature fruits had *A. flavus* populations little different from nongeocarposphere soils. In contrast, the population of total fungi was greater, and the populations of bacteria and actinomycetes were much greater, in geocarposphere soils. Microscopic observation of the inner 0.5-mm soil layers indicated that no germination of conidia of *A. flavus* occurred in peg geocarposphere soil, and trace germination occurred in fruit geocarposphere soil for plants maintained in a growth chamber at 30 C. *A. flavus* conidia germinated readily in soil adjacent to pods after 16 hr at 30 C and 35 C when a 4- to 6-mm^2 area of pod surface was superficially injured and inoculated with infested soil. Dry conidia applied to aerial portions of pegs in the greenhouse germinated at a low percentage. These findings are discussed in relation to the exogenous carbon and nitrogen requirements for spore germination.

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Additional key words: fungistatic substances, rhizosphere.

Peanut (*Arachis hypogaea* L.) pegs (elongating tissue at base of developing fruit) or fruits are colonized before and after digging by *Aspergillus flavus* Link ex Fries, *A. parasiticus*, *Fusarium* spp., and certain other soil fungi (5, 6, 11, 13, 19). Invasion of peanut kernels by *A. flavus* and *A. parasiticus* is of prime concern because these fungi produce aflatoxins, and Garren et al. (7) reported that *Fusarium* spp. and certain other fruit-colonizing fungi have a potential for toxin production. Geocarposphere, a corollary to the term rhizosphere, was introduced by Garren (6) to indicate that portion of soil influenced by the developing peanut fruit. In a previous investigation, trace conidial germination by *A. flavus* was observed in the rhizosphere of peanut (8). It is not clear, however, under what conditions soil-borne propagules of *A. flavus* and other fungi are stimulated by peanut fruits. The influence of the peanut fruit on the population of *A. flavus* in nonsterile soil is also unclear (16). This investigation was undertaken to examine in nonsterile geocarposphere soil the conidial germination and population of an aflatoxin-producing clone of *A. flavus* isolated from peanut fruit, and the population of other soil microbes. A preliminary report has been given (10).

MATERIALS AND METHODS.—The clones of *A. flavus* and *F. oxysporum* Schlecht. emend. Snyd. & Hans. used were isolated from peanut by K. H. Garren at Holland, Va. (8), and *A. flavus* produced B_1 and G_1 aflatoxins (determined by T. C. Campbell, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University). Conidia of *A. flavus* were harvested from 2-week-old potato-dextrose agar (PDA) slants with 45 ml of 0.01 M potassium phosphate buffer (pH 6.5) containing 0.05% $MgCl_2$ and 0.1 ml of 0.25% Tween 20 [polyoxyethylene (20) sorbitan monolaurate, Atlas Chemical Industries, Inc., Wilmington, Del.]. The spore suspension (40-50 ml) was shaken on a wrist-action shaker in a 250-ml screw-cap Erlenmeyer flask (Burrell Corp., Pittsburgh, Pa.) at position 10 for 20 min. I followed this by washing the spores 3 times by centrifugation at 150 *g* for 20 min with 45-ml portions of the inorganic salt solution minus Tween 20. Conidia of *F. oxysporum* were harvested from 2-week-old cultures grown in 10-cm petri plates containing water agar (1.5%) and a 1-cm^2 PDA plug. Conidia were washed 3 times by centrifugation with the inorganic salt solution minus Tween 20 before use.

The soil used was a nonamended, nonsterile loamy fine sand collected in the vicinity of Holland, Va., and had a pH of 5.7 (water-saturation percentage method, 14), and at 0.1 atmosphere contained 12% water. The soil contained 6.2 μg NH_4^+-N and 7.1 μg NO_3^--N/g soil. This soil did not yield *A. flavus* colonies on dilution plates containing 0.01 g soil or less. The soil was air-dried to 4% moisture content and stored in large cans. For spore germination and geocarposphere population studies, the moisture content was raised to 12% at the time of infesting with conidia of either fungus. The *A. flavus* spore density used in population studies was 5 to 20 × 10^3 spores/g soil, and 5 to 10 × 10^5 spores/g soil were required for spore germination studies (8). The

infested soils were incubated for approximately 4 weeks at 30 C, when chlamydospores comprised the principal spore form in soil for *F. oxysporum* and the conidia of *A. flavus* remained ungerminated (8).

Peanut (Virginia Bunch 46-2) plants were grown in the greenhouse in 26-cm-diam clay pots containing a 1:1 mixture of sand and nonsterile silt loam soil. When pegs formed at the margin of the pots, the plants were moved into a Percival 54B growth chamber with a 16-hr photoperiod at 30 C. The nonsterile loamy fine sand infested with conidia of *A. flavus* was placed in 50-ml beaker pegging chambers and covered with aluminum foil to retard water loss. A hole in the center of the foil cover allowed passage of the peg into the soil and gas exchange. After various periods, pegs or developing fruits were carefully removed from the beakers and geocarposphere soil examined for conidial germination. Soil samples were mechanically removed with dissecting needles and forceps from within 0.5 mm of the surface of pegs or fruits. Approximately 0.01 g soil was smeared on a glass slide, stained with 1.0% acid fuchsin in lactophenol, and air-dried. Microscopic observations of germination were made with the oil immersion objective at X1125. No spores resembling the echinulate *A. flavus* conidia were observed in noninfested soil samples.

Geocarposphere population studies were performed in the greenhouse with Virginia Bunch 46-2 peanut plants. When pegs were formed at the margin of 26-cm pots, they were directed into 8-cm-diam, clay-pot pegging chambers containing soil artificially infested with *A. flavus*. At zero time, two to six pegs of approximately equal size were introduced into an equal number of pegging chambers for each of 12 plants. The pots containing the plant roots were placed in depressions in a sand bench to insure that the soil surface levels in the pegging chambers and in the 26-cm pots were similar. Heating cables were used in the sand bench to maintain the sand temperature at 30 C or above. The plants were sprayed periodically with dicofol [4,4'-dichloro-α-(trichloromethyl)benzhdrol] and malathion (0,0-dimethyl dithiophosphate of diethyl mercaptosuccinate) to control mites and insects. The soil in the pegging chambers was watered with distilled water while the root system was watered with tap water. Soil in pegging chambers containing no pegs served as controls (nongeocarposphere soil) and was treated the same as soil in chambers containing developing pegs and fruits.

Microbial populations in nongeocarposphere soil (S) and geocarposphere soil (G) of pegs and fruits were determined by the dilution plate technique (3). Total fungal populations were determined on Martin's medium as used previously (8). For *A. flavus* population determinations, the medium was supplemented with 10 μg/ml 2,6-dichloro-4-nitroanaline (2). Bacterial and actinomycete populations were determined on yeast-extract agar (3) using the criteria of Clark (3, 4). Geocarposphere soil from pegs or fruits was obtained from within 0.5 mm of the plant surface. To obtain the samples, pegs or pegs and fruits were carefully removed from pegging chambers; with dissecting needles and forceps, soil was carefully removed until it was 0.5 mm or less in thickness. Pegs or fruits with geocarposphere soil, or nongeocarposphere soil samples (comparable in size to geocarposphere samples) were then placed in tared 250-ml screw-cap Erlenmeyer dilution flasks containing 95 ml water and shaken for 20 min on a Burrell wrist-action shaker at position 10 before dilutions were prepared. The amounts of geocarposphere and nongeocarposphere soils in flasks, and peg and fruit dry weights, were determined after drying at 105 C for 24 hr. Three replicate dilution series were prepared for geocarposphere and nongeocarposphere soils in each experiment. Twenty pegs or eight fruits were used per replicate. Soil dilutions and plates for bacteria were prepared immediately after shaking in order to minimize bacterial multiplication. Eight plates each of Martin's medium and *A. flavus* medium were used per replicate for counting total fungi and *A. flavus*. Colonies were counted after 7 days' incubation at 25-28 C. Bacteria and actinomycetes were counted on four suitable (3) plates per replicate after 13 days' incubation at 25-28 C. Separate sets of peanut plants and soils were used in experiments for pegs and fruits.

Experiments were also undertaken in the greenhouse to determine whether dry conidia would germinate when in contact with aerial portions of pegs. Dry conidia of *A. flavus* were removed from 2-week-old slant cultures by tapping the culture over a glass petri dish. Conidia were picked up on the end of a washed camel's-hair brush and applied to pegs by gently brushing the lower 5- to 8-cm portion of a peg not in contact with soil. As conidia were applied at an unknown, and possibly high, conidial density, the same brush was used to apply conidia to a sequence of four pegs to obtain a decreasing number of conidia on each subsequent peg. Each inoculated peg was placed in a plastic bag moist chamber having two pin holes for gas exchange. No contact existed between pegs and 10 ml of water placed in the bottom of the bag. After 24 hr of incubation, pegs were detached from the plants and autoclaved. Smears were prepared from pegs, stained with acid fuchsin, and air-dried. Microscopic observations were made at X 1,125 with the oil immersion objective.

RESULTS.—*Microbial populations in geocarposphere and nongeocarposphere soils.*—Populations of fungi, bacteria, and actinomycetes were greater in the geocarposphere of peanut pegs and fruits. Two weeks after pegs entered the soil, dilution plate analysis indicated that the inner 0.5-mm layer of geocarposphere soil of pegs had a mean fungus population more than 4 times as great as nongeocarposphere soil, whereas the geocarposphere population of *A. flavus* was only slightly higher than the nongeocarposphere soil population (Table 1). Large increases in the populations of bacteria and actinomycetes were also observed in geocarposphere soil. Similar increases in

TABLE 1. Populations of *Aspergillus flavus*, fungi, actinomycetes, and bacteria in nongeocarposphere soil and in the inner 0.5-mm layer of geocarposphere soil of peanut pegs

	A. flavus	Fungi	Actinomycetes	Bacteria
	X 10³	*X 10⁴*	*X 10⁶*	*X 10⁷*
Geocarposphere soil (G)[a]	8.0±2.5[b]	74.8±8.3	35.6±9.4	181±48
Nongeocarposphere soil (S)	5.9±1.4	15.8±3.8	0.60±0.11	1.35±0.32
G/S	1.36	4.73	59.3	134

[a] Average g oven-dry soil/g of peg (dry wt) = 2.86; 2 weeks after Virginia Bunch 46-2 pegs made contact with the soil.
[b] Propagules per gram oven-dry soil. Variation expressed as standard error.

TABLE 2. Populations of *Aspergillus flavus*, fungi, actinomycetes, and bacteria in nongeocarposphere soil and the inner 0.5-mm layer of geocarposphere soil of mature peanut fruits

	A. flavus	Fungi	Actinomycetes	Bacteria
	X 10³	*X 10⁴*	*X 10⁶*	*X 10⁷*
Geocarposphere soil (G)[a]	17.1±2.0[b]	36.2±12.7	45.4±7.9	65.2±8.8
Nongeocarposphere soil (S)	18.0±2.9	6.2±0.7	1.02±0.24	1.15±0.21
G/S	0.95	5.84	44.5	56.7

[a] Average g oven-dry soil/g of fruit (dry wt) = 0.14; 10 weeks after Virginia Bunch 46-2 pegs made contact with the soil.
[b] Propagules/g oven-dry soil. Variation expressed as standard error.

microbial populations were observed in fruit geocarposphere soil. Ten weeks after pegs entered the soil, the mean population of fungi was more than 5 times as great in the inner 0.5-mm layer of geocarposphere soil as in nongeocarposphere soil (Table 2). No increase in the population of *A. flavus* was observed, however. The populations of bacteria and actinomycetes were much greater in geocarposphere soil than in nongeocarposphere soil. Microbial populations per g of fruit or peg may be calculated from these data, but comparisons may not be useful due to differences in plant part morphology. Similar results were observed in experiments conducted preliminary to those reported here, including high G/S values for bacteria and actinomycetes, for both fruits and pegs, after comparable periods of time.

Conidial germination in the geocarposphere.—After pegging, soil artificially infested with washed conidia of *A. flavus* was removed periodically from within 0.5 mm of the surface of pegs and fruits, and 100 conidia were counted per peg or fruit. In no instance was conidial germination observed in geocarposphere soil of 14 pegs examined 2-45 days after pegs first made contact with the soil surface. In only one instance was conidial germination observed in geocarposphere soil of 20 fruits examined 17-81 days after pegs contacted the soil; in this case, one conidium germinated in proximity to a mature peanut fruit after 78 days. In contrast, chlamydospores of *F. oxysporum* germinated readily in geocarposphere soil soon after pegs entered the soil in a similar experiment. Chlamydospore germination ranged from 31 to 56% for 5 pegs examined 5-7 days after pegging.

The foregoing results suggested that the nutritional stimulus in the geocarposphere was not sufficient for *A. flavus* conidial germination. Experiments were conducted to determine whether mechanical injury of the peanut shell would stimulate conidial germination in soil. Mature or nearly mature peanut fruits, grown under greenhouse conditions, were detached from plants and brought to the laboratory. With a dissecting needle, a 4- to 6-mm^2 area of pod surface was scored superficially several times. Nonsterile soil (12% water) artificially infested with *A. flavus* conidia was applied with a small spatula to cover just the injured area. For some fruits, I added 0.06 ml of glass-distilled water after applying the soil to reduce drying of the soil during incubation, and to simulate wetting of freshly dug fruits by rainfall. Fruits were incubated in glass moist chambers at 30 C for 16 hr. After this time, drying of the soil applied to injured areas was noticeable for fruits receiving no added water, but not for fruits receiving supplemental water. Based on a count of 100 conidia/fruit, microscopic observation of soil smears indicated that *A. flavus* conidia germinated at high percentages adjacent to injured fruits receiving water (55.9% = mean and 29-76% = range for 14 fruits), and adjacent to injured fruits receiving no added water (63.4% = mean and 56-74% = range for five fruits). No germination was observed for soil not in contact with fruits or in contact with nonscored fruits. In other tests, similar high degrees of conidial germination were observed at 30 C for injured,

TABLE 3. Germination of dry conidia of *Aspergillus flavus* applied to aerial Virginia Bunch 46-2 peanut pegs

Peg sequence[a]	% Germination[b]	
	Thin germ tubes	Typical germ tubes
First	0.4±0.4	0.0
Second	1.8±0.8	0.5±0.3
Third	4.5±1.2	2.7±1.3
Fourth	4.3±0.8	2.7±0.7

[a] Sequence in which conidia were applied to a series of four pegs. Dry conidia applied to first peg with washed camel's-hair brush and then directly to subsequent pegs, by which decreasing conidial densities on the pegs were obtained.

[b] Based on a count of 200 conidia/peg for each of five pegs/peg sequence number. After 24 hr. Variation expressed as standard error.

immature fruits with added water. In an experiment in which the influence of temperature was examined, conidial germination (7.3% = mean of four fruits) was observed at 20 C when injured, mature fruits with supplemental water were incubated for 16 hr, whereas highest germination was observed at 35 C (55.0% = mean of four fruits).

Germination of dry conidia applied to aerial portions of pegs.—Low percentages of typical germ tube formation by *A. flavus* conidia were observed for dry conidia applied to pegs (Table 3). However, this was not observed for the first pegs in the peg sequence. Microscopic observation of peg smears indicated that the frequency of conidia on slides for the first peg was much greater than for the other pegs, or for the soil smears of geocarposphere soil described above. The lack of typical germination on the first pegs in the series may have been due to this high conidial density. Thin germ tube formation was also observed on pegs. The frequency of both germ tube types was generally greater on pegs having fewer conidia (Table 3). In contrast to typical germ tube formation, thin germ tube formation occurs without conidium swelling. Thin germ tube formation has also been observed in the laboratory in axenic culture studies of conidial germination by *A. flavus*, and at X 1,125 appears as a line emerging from the conidium rather than as a stout, two-dimensional structure characteristic of typical germ tube formation (20). Low degrees of typical and atypical (thin germ tube) germination were also observed in two other similar experiments performed. It is not known whether thin germ tubes are capable of continued elongation.

DISCUSSION.—Methods for obtaining rhizosphere or geocarposphere soil samples have not been satisfactory. As acknowledged by Rovira (21), there are serious limitations in shaking and washing procedures. Mechanical removal of rhizosphere (or geocarposphere) soil with forceps, as recommended by Tesic (23), appears to be a better method, but is very tedious. The technique used here and in a previous rhizosphere investigation (8) is a modification of the latter, and is an attempt to define more clearly the volume of soil actually sampled. The geocarposphere-geocarpoplane (or rhizosphere-rhizoplane) is a continuum, and the samples collected here were obtained from the inner 0.5-mm portion of this continuum for either pegs or fruits.

Although the composition of the fungal population of geocarposphere samples has been studied (13, 16, 18), the general magnitude of the geocarposphere effect on major soil microbe populations (the relative populations of total fungi, actinomycetes, and bacteria in geocarposphere soil and nongeocarposphere soil samples) has not been examined. The results reported here indicate that the increase of microbe populations in geocarposphere soil samples is comparable to or greater than that found for the rhizosphere effect of many plants (17). However, there is little evidence that the *A. flavus* population is increased. Joffe (15), working with sterilized soil infested with *A. flavus*, reported a higher *A. flavus* population in geocarposphere samples than in nongeocarposphere samples at the end of the growing period. This finding is difficult to evaluate, however, due to the elimination of the natural population of soil microbes, and the increase in carbon and nitrogen substrates accompanying soil sterilization. In field studies, Joffe (16) found higher populations of *A. flavus* in nongeocarposphere samples (soil between the rows) than in geocarposphere samples for two of three soils studied. No differences were observed in the other soil. Joffe indicated, however, that only very wide differences in numbers could be compared because of differences in collection procedures for geocarposphere and nongeocarposphere soils. *A. niger* populations were higher in geocarposphere soil samples than in nongeocarposphere samples for all three soil types. No indication was given of the size of the geocarposphere soil samples obtained per fruit or per gram of fruit. McDonald (18) reported the populations of *A. flavus* and other fungi in geocarposphere and nongeocarposphere (field) samples, but these samples were collected in different years. Fruit soil samples (soil lost during vigorous shaking of fruits in a polythene bag) had a higher total fungus population than field soil samples of the same year for most of the collection periods tested, but the population of *A. flavus* in these soils was variable.

Trace germination of *A. flavus* occurred in the geocarposphere. Mechanical injury of the shell, however, resulted in high conidial germination of *A. flavus* (in the presence or absence of supplemental water); conidia germinated readily at 30 and 35 C. Injury of peanut pods due to growth cracks, mechanical, or biological agents has been associated with colonization of peanut kernels by *A. flavus* or the development of aflatoxins in kernels (1, 19, 22). Low degrees of conidial germination were observed when dry conidia of *A. flavus* were applied to pegs. Air-borne conidia of the fungus may come in contact with pegs under field conditions and germinate. Colonization by *A. flavus* of aerial pegs may occur, as

has been reported by Hanlin (11).

Pass & Griffin (20) reported that germination of washed conidia of *A. flavus* in axenic culture is nearly fully dependent on exogenous carbon over a range of conidial densities. Full dependence on exogenous carbon was observed for settling tower-deposited dry conidia of *A. flavus*. Glucose plus L-proline or certain amino acid mixtures were particularly effective in supporting germination (20). In rewetted, nonamended, nonsterile peanut-field soil, no *A. flavus* conidial germination was observed by Griffin (8) whereas glucose plus an amino acid source amendment favored high germination. In addition, Hora & Baker (12) have reported that volatile substances from soil inhibited conidial germination by *A. flavus* on agar discs. These results suggest that a capability for exogenous carbon-independent germination by conidia on aerial plant surfaces or in soil is unlikely for *A. flavus*, and that exudation of carbon and nitrogen substrates by plant parts (together, potentially, with soil NH_4^+ and NO_3^-) accounts for most of the conidial germination observed. Possible volatile and nonvolatile fungistatic substances and increased competition from other soil microbes may account, in part, for the nearly nil and lower conidial germination observed in the peanut geocarposphere and rhizosphere (8) than on aerial peg surfaces. An increase in the amounts and types of carbon and nitrogen substrate exudation, particularly amino acids, may be responsible for the high conidial germination observed following pod injury.

The exogenous carbon and nitrogen requirements for spore germination may be related further to the ecology of spore germination and to peg-fruit colonization. Hanlin (11) reported a much higher frequency of peg colonization, both in air and soil, by *Fusarium* spp. than by *A. flavus* group and other *Aspergillus* spp., and Garren (6) found *Fusarium* spp. to be dominant early colonizers of peanut fruits in Holland, Virginia field soils. In contrast to *A. flavus* conidia, *F. oxysporum* chlamydospores germinate readily in the peanut rhizosphere and (8) in the geocarposphere soon after pegs enter the soil, and are less exacting (qualitatively and quantitatively) in their exogenous carbon and nitrogen requirements for germination in peanut-field soil (8). The exogenous carbon and nitrogen requirements in axenic culture for chlamydospore germination by a peanut-fruit-colonizing clone of *F. solani* (9) are also less exacting than for conidial germination by *A. flavus* (20).

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