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Identification of Infection Pathways and Development of Inoculation Methods for Peanut Using Green-Fluorescence-Protein Aspergillus Flavus

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Abstract: Screening peanut (*Arachis hypogea* L.) germplasm for resistance to Aspergillus flavus was hampered by low and variable rates of infection. Three experiments were conducted to develop methods to inoculate Aspergillus flavus on peanut flowers, pegs and ovaries by strains of A. flavus, modified to produce a Green Fluorescence Protein (GFP). Maximum infection with GFP A. flavus was found by spraying an aqueous suspension of conidia over shoots and flowers of peanut which resulted in 100% infection of floral surfaces. Other methods tested included application of cracked corn inoculum to the surface of soil in cuvettes and mixing aqueous suspension or cracked corn inoculum with soil in cuvettes, designed to allow in situ observation of peg and pod development. Observations with an ultra violet-illuminated microscope showed fluorescence of GFP A. flavus on the surface of the peanut flowers and a fluorescing network of hyphae on ovules inside the peanut pegs before the pegs reached the soil surface. These experiments provide supporting evidence that A. flavus infection of peanut ovaries can occur during flowering or early peg formation.

Key words: Aspergillus flavus, floral infection, Green Fluorescence Protein (GFP), inoculation, peanut, peg

INTRODUCTION

The saprophytic fungi, Aspergillus sp., are nearly ubiquitous on earth. These fungi do not generally infect healthy tissues. Under drought conditions, however, Aspergillus sp. can infect a large number of economically-important plants and may contaminate plant products with aflatoxin, one of the most carcinogenic natural toxins known. When peanut (*Arachis hypogaea* L.) plants are subjected to water deficit during pod filling, they are especially susceptible to aflatoxin contamination which is largely produced by Aspergillus flavus (Link ex Fries)[1,2].

Efforts to develop aflatoxin-resistant peanut genotypes, that is, genotypes that have either resistance to A. flavus infection or prevention of aflatoxin production, or both, have made only modest progress. Peanut pods that are damaged or seeds that are discolored are generally contaminated with aflatoxin and can easily be removed, so for our purposes, we use the term aflatoxin resistance to refer only to resistance of whole, sound peanut pods and seeds to aflatoxin contamination. Will *et al.*[3] evaluated a mid-bloom organic matrix inoculation which used A. flavus cultured on cracked corn at 25% moisture. Organic-matrix inoculum was incorporated into the top 10 cm of the soil surface and an aqueous conidia suspension (1×10^6 conidia/mL) was sprinkled by hand on the foliage. This method was found to be effective for field screening for aflatoxin resistance. Holbrook *et al.*[2] prepared Aspergillus inoculum using cracked corn at 25% moisture as an organic culture substrate and each plot (1.5 m × 1.8 m) received 57 g inoculum in field screening trials. Mehan *et al.*[4] observed an increase in seed infection and aflatoxin contamination from using a labor-intensive method of inoculating developing pegs and pods with an aqueous suspension of A. flavus conidia, but did not assess the effects of this technique on soil A. flavus population levels or seed infection under field conditions. An aqueous suspension of Aspergillus sp. conidia may be either mixed into the surface of the soil[5] or sprinkled directly on the plant[6, 7]. Unfortunately, none of these techniques gave consistent correlations among aflatoxin concentration, percentage colonization of seed and shells and soil population densities of A. flavus infection levels which are needed to identify genotypes with aflatoxin resistance.

One of the greatest challenges to studies of aflatoxin resistance is the enormous variability in aflatoxin contamination among plants and among pods within plants, yet if as little as 0.1% of seed are contaminated, an entire lot may be condemned. Even in experiments where

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peanut plants are heavily inoculated with A. flavus and subjected to intense water deficit, typically less than 1 to 5% of seed are infected and contaminated.

Further, methods for aflatoxin detection are relatively expensive. The recent development of A. flavus strains that contain a gene that codes for production of a Green Fluorescence Protein (GFP) (J. Carrey, USDA-ARS, New Orleans, personal communication and G. Payne, NCSU, Raleigh, North Carolina, personal communication) offers great hope to our ability to detect A. flavus infection. When illuminated with ultra violet (UV) light (350-380 nm), the GFP fluoresces green. Wangeli *et al.*[8] suggested that GFP containing transformants could be useful in screening for the resistance of corn genotypes to aflatoxin accumulation and making screening faster. Thus, the GFP producing A. flavus strains may be easily and quickly detected with either a simple UV light sourceor with an UV-illuminated microscope.

Development of A. flavus strains that produce GFP offers the opportunity to track pathways of infection which have not been clearly identified. For peanut, two principal pathways have been proposed:

- Underground infection by growth of hyphae through the epidermis of pegs or pod walls; and
- Above ground infection through germination of conidia on floral organs followed by growth of hyphae through the calyx and into the ovary.

Because A. flavus is largely a soil-borne fungus, previous research has mostly studied underground infection through the pod walls. Clearly, as injury to pods underground generally leads to infection and contamination, this is an important pathway for infection. It is possible that conidia, whether moved by wind, rain, or some other factor may infect floral organs before the pegs elongate and thrust them into the soil.

This research had two objectives:

- To develop an inoculation methods that would attain high and consistent levels of A. flavus infection that are needed in aflatoxin-resistance breeding programs; and
- To investigate the potential for A. flavus to infect peanut flowers and pegs.

MATERIALS AND METHODS

Study was conducted in growth chambers of the Georgia Envirotron, at the Griffin Campus of the University of Georgia, USA. and at the Faculty of Agriculture, Chiang Mai University, Thailand.

Experiment 1-Inoculation methods for maximizing A. flavus infection: Seeds of peanut genotype 329CC, previously identified as aflatoxin-resistant by Holbrook *et al.*[9], were germinated on moist paper for 2 days. Three healthy seeds were planted in each of 20 plastic containers of 20-L capacity filled with commercial potting medium (Metro-Mix 360, Scotts, Marysville, OH, USA.). Containers were placed in growth chambers (PG72, Conviron, Winnipeg, Manitoba, Canada) set to 33/25°C day/night, 75/95% RH day/night, light intensity level 5, about 1400 μmol PAR $m^{-2}s^{-1}$, photoperiod of 16 h and CO_2 concentration near ambient at 400 μl L^{-1}. All containers were irrigated lightly by hand at 1- to 2-day interval until seedlings established. After establishment, containers were watered twice weekly with half-strength Hoagland's solution, using an automatic irrigation system to apply solution until drainage appeared from the bottom of each container.

Inoculation treatments were selected to identify differences in location and pathway of infection and were imposed in a split plot design. Two main plot treatments were imposed beginning 10 days after first flowers appeared on the plants:

- Spray over top of plant with aqueous suspension of A. flavus conidia in water at 10 and 20 days after first flowers appeared; and
- No spray. A split plot design was used with two growth chambers having eight containers each to isolate the non sprayed plants from those of that were sprayed in order to minimize likelihood of cross contamination between the treatments.

Before the first spray, we attached four cuvettes to each container. Cuvettes were filled with Tifton loamy sand soil from the Blackshank Farm, Tifton, Georgia. Soil composition was 86% sand, 8% clay and 6% silt. Cuvettes were 10 cm high×20 cm long×1 cm thick, made of clear acrylic and covered with removable opaque shields to prevent light from affecting peg and pod development. Cuvettes were attached to the sides of the containers such that the tops of the cuvettes were level with the container so that pegs could grow naturally into the soil of the cuvettes. Each cuvette on a container represented a sub-plot treatment with different inoculation treatment methods. The four sub-plot treatments were:

- Aqueous suspension of conidia was mixed into soil before filling the cuvette;
- Cracked corn inoculum was mixed into soil before filling the cuvette;

- Uninoculated soil was placed in the cuvette and cracked corn inoculum was applied to the soil surface; and
- The soil in the cuvette was not inoculated. Thus there were eight treatment combinations applied in a 2×4 split plot design with four replications. After treatments began, we applied 60 mL water to each cuvette twice weekly.

In addition to the eight treatment combinations described above, we grew plants in four containers in a third growth chamber without inoculation to serve as an absolute control. Four cuvettes were attached to each of the absolute control containers, but soil in these cuvettes was not inoculated with A. flavus.

Inoculum preparation and application: Two A. flavus strains, each modified to produce a Green Fluorescence Protein (GFP), were used. One culture was obtained from Gary Payne (North Carolina State University, Raleigh, NC); the other was from Jeffery Carrey (USDA-ARS, New Orleans, LA). While these two A. flavus strains may differ in relative pathogenicity and location of the GFP gene (J. Carrey, 1999 personal communication; G. Payne, 2000 personal communication), this study did not intend to study the difference between the strains. Instead, we applied both strains to increase the overall probability of infection. Strains were cultured separately on Petri dishes containing M3S1B medium, an A. flavus-A. niger group selective medium. The medium was a 2,6-dichloro-4-nitroaniline-amended medium (10 mg/liter) originally developed by Bell and Crawford[10] and modified by Griffin and Garren[11]. M3S1B medium has following composition: 5.0 g peptone, 10.0 g glucose, 1.0 g KH_2PO_4 0.5 g $MgSO_4.7H_2O$, 30.0 g NaCl, 20.0 g agar, 50.0 mg streptomycin sulfate, 50.0 g chlorotetracycline, 1.0 mg 2,6-dichloro-4-nitroaniline (added in 3 mLacetone) and 1 liter distilled water. When conidia had formed, they were washed from the mycelia with 50 mL sterile deionized water and stored in a refrigerator at 3 to 5°C.

Before spraying, stock suspension was diluted to 1000 mL with sterile deionized water and then a hand pump spray bottle was used to spray the suspension on the plant shoot of container treatments. For cuvette treatments, 2 mL of aqueous conidia suspension were mixed into the soil of each cuvette.

Corn (Zea mays L.) seeds were coarsely ground in a blender to make cracked corn. Then 200 g of cracked corn was placed in stoppered 250 mL flasks and autoclaved twice. After the cracked corn cooled, a 50 mL aliquot from each conidia strain was added to each flask and incubated at 30°C for 5 days. After 5 days, this cracked corn inoculum was either used immediately or stored in a freezer for later use. For cuvette treatments receiving cracked corn inoculum, either 2 g were mixed into the soil for a single cuvette or 2 g of inoculum were spread evenly over the soil surface of a cuvette.

First flowering was defined as the date when there was at least one flower visible in each container. At 10 days after first flowering, 10 fresh flowers on each container were tagged by tying pieces of cotton thread around the stem adjacent to the hypanthia. Then all inoculation treatments were applied.

Five days after inoculation, 10 tagged flowers and ovaries from each treatment were excised. Flowers and ovaries were dissected longitudinally. Half of each sample was immediately observed with an ultraviolet-illuminating fluorescence microscope (Model BX60F5, S/N: EXPO25754 Digital Output, Olympus, Melville, NY, USA.). Flowers and ovaries of the absolute control plants were Observed similarly to the samples from the two main growth chambers.

By 28 days after spraying, pegs began to reach the soil surface and we excised 5 to 6 pegs that had reached the soil from each cuvette. Pegs from each treatment combination were surface-sterilized by dipping in 10% Clorox for 30 s, then rinsed twice in sterile water. Apical portions of pegs (about 1 cm) were dissected longitudinally. Half of each peg was placed on a microscope slide for immediate observation with the fluorescence microscope. The other half was placed on M3S1B medium in Petri dishes and cultured in an incubator at 27°C for 5 days. After 5 days, cultured pegs were observed under a hand-held UV light to detect infection by GFP A. flavus. Results of pegs observed immediately under the microscope were recorded as percent of pegs infected with A. flavus whereas those observed after culturing were recorded as percent with green fluorescence. Pegs from the uninoculated control plants were handled similarly to the sampled pegs from the two main growth chambers.

Experiment 2: Infection of peanut genotypes by A. flavus: The experiment was conducted at Georgia Envirotron, University of Georgia, USA. In this study, we observed A. flavus infection of peanut flowers and ovaries in the soil cuvette system described above. Four peanut genotypes (511CC: drought-and aflatoxin-resistant; 419CC: drought-and aflatoxin-susceptible; 329CC: aflatoxin-resistant[9]; Tainan 9: commercial variety in Thailand) were grown in 20-liters plastic containers with metro media, a commercial mix and four cuvettes attached to each container. We planted four pre-germinated seeds in each container. All containers were irrigated lightly at 1- to 2-day interval until seedlings established.

A randomized complete block design with four peanut genotypes and four replications was used. Inoculum was prepared as in Experiment 1. At 30 days after planting, 10 fresh flowers of all varieties were tagged with thread. All inoculated containers of four genotypes were sprayed with aqueous conidia suspension of GFP A. flavus to the plant shoot and flowers; then we attached four cuvettes with cracked corn inoculum applied on soil surface. Five days after inoculation, the wilted flowers and ovaries tagged with thread were excised. Flowers were cultured on M3S1B medium on Petri dishes. Ovaries were dissected longitudinally; half of each was observed with the fluorescence microscope and the other half was placed on M3S1B medium and cultured at 27°C for 5 days. Results were recorded as percent of flowers and ovaries that either fluoresced or were infected with A. flavus.

Experiment 3: A. flavus infection of peanut flowers by soil surface inoculation: This experiment was conducted at Faculty of Agriculture, Chiang Mai University, Thailand, to verify whether GFP A. flavus could infect developing flowers by soil surface inoculation. Four peanut genotypes (511CC: drought-and aflatoxin-resistant; 419CC: drought-and aflatoxin-susceptible; Tainan 9: commercial variety in Thailand; and Luhua 11: an aflatoxin-resistant variety from China) were grown in plastic containers (35.6 cm diameter) with river sand soil that had been steam-sterilized at 110 to 130°C for 4 h.

A 4 × 2 factorial design with four replications was used. At flowering, half of the inoculated containers of each genotype were tagged for 10 samples of fresh flower with thread and then inoculated with 10 g of cracked corn inoculum (prepared as described above) on the soil surface.

At 5 days after inoculation, wilted flowers that had contacted the soil were carefully excised and taken to the laboratory to be observed with a UV-illuminated microscope (Model CX41-32L02-SET, Olympus optical, Shibuya-ka, Tokyo, Japan) or cultured on M3S1B medium on Petri dishes. Ovaries were dissected longitudinally. Half was placed on M3S1B medium on Petri dishes and cultured at room temperature for 5 days. After 5 days, cultured samples were observed for fluorescence of GFP A. flavus under a hand-held UV light. Results were recorded as percent of peanut flowers and ovaries that were infected with GFP A. flavus.

Fresh unwilted flowers were also observed in a similar manner at 5 days after inoculation.

Statistical analysis: Data were analyzed by the general linear model procedure of SXW (Statistix for Windows; Analytical Software, Tallahassee, FL) and SAS (Statistical Analysis System Version 7, SAS Institute, Cary, North Carolina, USA.). Means were compared by Least Significant Difference (LSD). Unless otherwise stated, all differences referred to in the text were significant at $p \leq 0.05$.

RESULTS

Maximizing A. flavus infection of peanut flowers and pegs Although under white light, we could not see fungal mycelia on flower tissues of inoculated treatments, observation with an UV-illuminated microscope at 5 days after inoculation, permitted observation of GFP A. flavus on the surface of peanut flowers (Fig. 1) and hyphae of GFP A. flavus were observed to have penetrated into the flower tissues. We did not observe fluorescence on ovules of dissected ovaries at 5 days after inoculation either, but by 28 days after spraying with inoculum, ovules in dissected pegs fluoresced with GFP A. flavus (Fig. 2A), indicating internal colonization by GFP A. flavus. Figure 2 compares the appearance of an ovule having a network of fluorescing hyphae with an ovule sampled from the absolute control treatment that had no apparent fluorescence to indicate colonization GFP A. flavus.

Pegs sampled after they entered the soil and cultured on M3S1B medium had the highest infection levels in treatments sprayed with aqueous conidia suspension and differences were significant between spraying and no spraying treatments (Table 1). For the cuvette inoculations, treatments with cracked corn inoculum applied to the surface of soil had the greatest infection

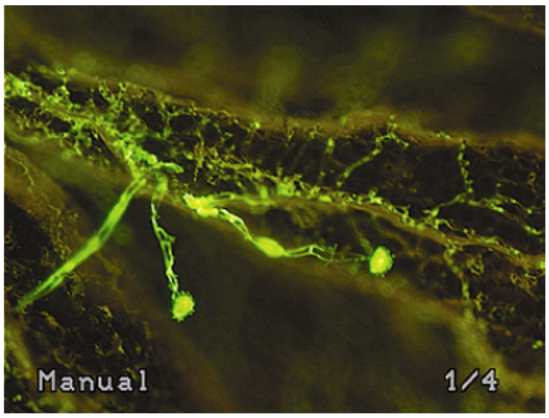
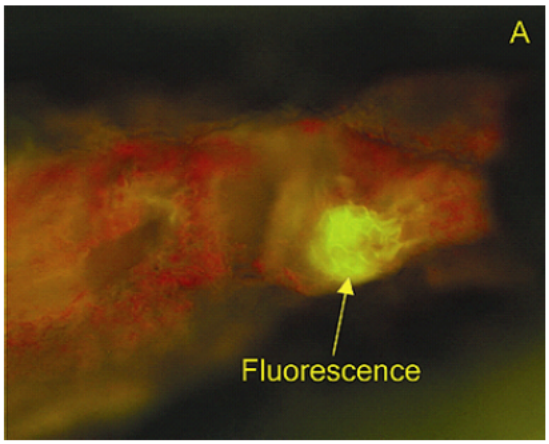


Fig. 1: Infection of the external surface of peanut flower at 5 days after inoculation by GFP Aspergillus flavus. Hyphae of GFP A. flavus penetrated into the flower tissues as observed with an UV-illuminated microscope



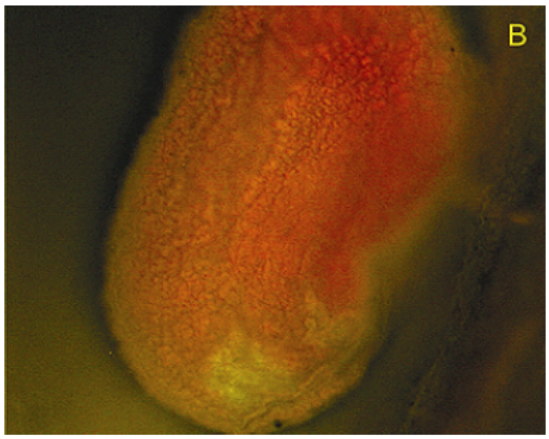


Fig. 2: Dissected peanut peg 28 days after inoculation either Aspergillus flavus as observed with a UV-illuminating microscope. A, Network of GFP A. flavus hyphae colonizing the ovule inside a peg; B, Ovule inside peg that was neither inoculated nor colonized by GFP A. flavus

levels (Table 1). The no-spray and no-inoculum cuvette treatments had 20.8% of pegs infected with A. flavus on pegs while no infections were observed in absolute control plants grown in a separate growth chamber. Infection levels of treatments with conidia or cracked corn mixed into soil in the cuvette did not differ significantly and were lower than for the treatment with cracked corn applied to the surface.

Infection of peanut flowers and ovary by A. flavus: The investigation of infection by GFP A. flavus on peanut flowers and ovules in ovaries confirmed floral infection for all four peanut genotypes after inoculation by spraying plants with a suspension of conidia. Floral tissues of all

Table 1: Peanut peg infection by GFP Aspergillus flavus at 28 days after inoculation for eight combinations of inoculation methods. Main plot treatments were with and without aqueous conidia suspension inoculation. Subplot treatments were four soil inoculation methods. The absolute control treatment was neither inoculated with nor infected by GFP A. flavus

Cuvette / Pot	Conidia mixed with soil	Cracked corn applied to the surface of soil	Cracked corn mixed with soil	No inoculum	Mean
	%				
No spraying	68.8c*	94.2a	76.2b	20.8d	65.0B
Spraying	77.5b	98.5a	83.9b	68.9c	82.2A
Mean	73.1B	96.3A	80.1B	44.8C	73.6

*Means followed by the same letter are not significantly different at p = 0.05 by LSD. Lower case letters are used for simple means and upper case letters for the main and sub plot means. LSD (0.05) main plot = 8.11. LSD (0.05) sub plot = 10.53. LSD (0.05) main x sub = 7.09

Table 2: Peanut flower infection by GFP Aspergillus flavus at 5 days after inoculation with an aqueous conidia suspension spray and cracked corn inoculum applied to the soil surface. No infection by GFP A. flavus was observed in the absolute control growth chamber

Genotype	Flower	Ovary
	%	
Tainan 9	100	36.8a*
511CC	100	23.5ab
419CC	100	11.1ab
329CC	100	5.0b

* Means followed by the same letter are not significantly different by $\div^2$ at p = 0.05 level

four peanut genotypes were colonized by A. flavus hyphae in 100% of flowers observed after culturing on M3S1B medium (Table 2). Infection rates of dissected ovaries from genotype 329CC were significantly lower than for Tainan 9, yet both genotypes had equally high infection of flowers.

Infection of peanut flowers by soil surface inoculation with GFP A. flavus: The highest infection of floral tissues was found in the treatment with cracked corn inoculum applied to the soil surface (Table 3). Inoculation and no-inoculation treatments differed significantly, with nearly all flowers being infected in the inoculated treatments and slightly more than half flowers infected in uninoculated treatments. Wilted and fresh unwilted flowers of four peanut genotypes from inoculated containers had higher frequencies of infection than uninoculated plants (Table 3). However, uninoculated plants also had A. flavus infection of peanut flowers. Ovary infection in the inoculated treatment was high (89%), but no ovaries were observed to be infected by A. flavus in the control treatments. Fresh unwilted flowers also had higher rates of infection in the inoculated treatment than in the control.

In production fields, soils already infested by A. flavus which can produce conidia capable of infecting peanut flowers. At 5 days after inoculation, varieties were

Table 3: Infection of peanut flowers and ovaries by GFP Aspergillus flavus at 5 days after application of cracked corn inoculum

	Wilted flower †		Ovary ‡		Unwilted flower Γ	
	control	inoculated	control	inoculated	control	inoculated
Genotype	%		%		%	
511CC	46.2	98.0	0.0	86.7	56.7b	98.0a
419CC	51.2	97.0	0.0	87.8	28.3c	98.0a
Tainan 9	66.2	97.2	0.0	83.3	66.7b	97.7a
Luhua 11	58.3	97.0	0.0	98.3	16.7d	96.7a
Mean	55.2B*	97.3A	0.0B	89.0A	42.1B	97.6A

† Wilted flowers that had touched the soil surface. ‡ Ovaries dissected from wilted flowers, then cultured on M3S1B medium. Γ Unwilted fresh flowers that had not touched the soil surface. * Means followed by the same letter are not significantly different at p = 0.05 by LSD

already infected with the fungus. These data confirm that peanut infection by GFP A. flavus occurs during flowering and results in infection of ovaries.

DISCUSSION

Floral infection pathway by GFP A. flavus: Soil-borne fungi, such as A. flavus, usually colonize dead or dying plant tissues that are on the soil surface or in the upper 5- to 10-cm soil layer which includes the peanut pod development zone. It is therefore not surprising that most previous research on A. flavus infection of peanut have emphasized on infection of pods and seeds in the soil. Sampling 100-day-old pods from soil into which they had incorporated cracked corn inoculum, Haixin *et al.*[12] observed only one case where the fungus had fully penetrated through the outer cell wall layers of the pod pericarp, though numerous pods had extensive colonization of pericarp tissue. Based on observations of larger populations of A. flavus in soil adjacent to injured pods than on aerial peanut pegs, Griffin[13] suggested that the primary infection pathway was through germination of A. flavus conidia in soil adjacent to developing peanut pods, particularly for injured pods.

If direct infection of seeds occurs only after pod injury, the question then, is how infection occurs in seed of whole, undamaged peanut pods, which is the most challenging problem for development of aflatoxin resistance in peanut[2]. Three possibilities exist:

- A. flavus may penetrate pegs in the soil and infect ovules before pods form;
- A. flavus may infect aerial pegs before they reach the soil; or
- A. flavus may penetrate ovaries of the flower and infect ovules before pegs form.

A. flavus does not infect only soil-borne fruits such as peanut. It commonly infects fruits of Zea mays, Gossipium hirsutum, tree nuts and others. Several researchers have hypothesized that A. flavus conidia may infect flowers and Griffin and Garren[14] found some colonization of uninoculated flowers when cultured on a selective medium. They showed that even when aerial pegs were surface-sterilized with 0.5 % NaClO for 3 min and then cultured on a selective medium for A. flavus, a small portion (0.3%) of pegs were still colonized by A. flavus. It is likely that A. flavus recovered from these pegs derived from a floral infection rather than infection through the epidermis of the peg itself.

The findings of this experiment strongly support the hypothesis that A. flavus infection of peanut ovaries results from infection of floral tissues. Even before culturing, we observed a network of fluorescing GFP A. flavus hyphae at the base of some ovules in dissected pegs at 28 days after inoculation. Although we did not observe fluorescing GFP A. flavus on ovules at 5 days after inoculation, from 5 to 37% of ovules sampled at 5 days after inoculation were found to be infected when cultured on selective medium. It is likely that the hyphae were present in the ovary but had not yet reached the ovule, or that the mass of hyphae was insufficient for direct detection at that time.

Additional evidence that floral infection is the dominant pathway for infection of peanut ovaries is that peg and ovary infection levels were much smaller when either cracked corn inoculum or conidia were incorporated into the soil than when the conidia suspension was sprayed directly over the flowers. Infection levels were also high when cracked corn inoculum was applied to the soil surface, which may be explained by the large number of conidia produced by this inoculum that could move to flowers with air currents. The direction of airflow in the growth chambers was upward, which would facilitate movement of conidia to the flowers. Moreover, because the air was recirculated, any conidia suspended in the air would remain in the chamber, giving multiple opportunities for infection and may explain high infection levels of uninoculated pegs and flowers.

As a saprophyte, A. flavus generally infects only injured tissues. We observed fungal conidia attached to the tips of stigma with pollen grain. We hypothesize that the germinating A. flavus hyphae follow the germinating pollen tube, which effectively causes a channel of injury through the style and stigma to the ovaries. As gynophore elongates, the fungus remains with the ovary, which could be established in developing fruits. This mechanism would explain the association of A. flavus with peanut during early fruit development, as was observed by Sander *et al.*[15]

An important question is the level of infection that occurs in these immature ovules. We observed fluorescence only at the surface of the ovule, where the

pollen tube would have penetrated during fertilization. We did not observe systemic infection of either the ovule or ovary tissues. It is likely that A. flavus cannot infect these healthy tissues and remains quiescent, in a sort of latent infection, until drought or some other injury weakens the tissues and allows A. flavus to infect additional tissues. Such a latent infection would explain observations by Payne[16] that water stress did not affect the percentage of infected seeds when inoculum was applied. Furthermore, the same drought and high temperature conditions that promote the advance from latent to active infection are likely to promote the production of aflatoxin by A. flavus.

In recognition that floral infection is likely to be the most important pathway of infection, then research would be warranted to identify irrigation, row orientation and other factors that would promote the movement of conidia from the soil surface to the flowers.

Inoculation method for research on A. flavus resistance of peanut: Selection of an inoculation method will depend on the purpose and location of the experiment. We would recommend different methods for field and greenhouse experiments. The advantages of cracked corn inoculum are:

- It may be prepared and applied in large quantities; and
- Because the fungus continues to grow on the cracked corn medium, it will produce conidia capable of infection over a period of time, though it has not been established how long this inoculum will produce conidia. The use of cracked corn inoculum also has some disadvantages, most notable are that it does not allow direct control of conidia that reach flowers and that it exposes researchers to high populations of conidia which are associated with allergies and may affect people with immunity deficiency system.

The use of aqueous suspension of conidia has the advantages that it uses a relatively simple laboratory preparation, requires less material to be handled and applies a large concentration of conidia to flowers, thereby increasing the probability of infection. While multiple applications may be required to increase the period over which peanut flowers are inoculated, exposure of researchers to potentially-unhealthy A. flavus conidia is minimized. While it may be possible to develop larger-scale methods for producing A. flavus conidia, present methods for the use of an aqueous suspension for inoculation are suited only to relatively small-scale study.

Our results would support the continued use of cracked corn inoculum for field screening trials. This inoculum should be applied only to the surface of the soil and not incorporated as has been done by Holbrook *et al.*[2] and Will *et al.*[3].

Spraying of aqueous conidia suspension and application of cracked corn inoculum applied on the soil surface did not differ in peanut peg infection by A. flavus (Table 1), which suggests that both would be suitable inoculation methods under greenhouse conditions. Two applications of aqueous suspension of A. flavus conidia alone also consistently increased A. flavus population densities in the soil, but not as much as cracked corn inoculum. The choice of inoculation method for greenhouse research would depend on the purpose of the experiment and the design of the greenhouse. For greenhouses with sufficient airflow and turbulence to circulate conidia, cracked corn applied to the soil would be easier. If airflow and turbulence are not likely to move conidia to flowers, then an aqueous suspension of conidia would be preferred. In hydroponic culture systems, cracked corn inoculum would not be possible but it would be possible to spray an aqueous suspension of conidia.

This experiment using GFP A. flavus clearly demonstrated that the fungus can infect peanut ovaries during flowering. Peanut production fields are usually already contaminated with A. flavus which can infect flowers if wind, water or insect vectors move conidia produced at soil surface to the flowers. Thus, the flowers and ovaries from uninoculated soil can be infected through mechanisms similar to the inoculated soil with conidia of A. flavus.

Future research on aflatoxin resistance of peanut that focuses on floral resistance to A. flavus infection would be merited. Such research should attempt to identify morphological and biochemical traits of flowers and floral organs that may confer resistance to A. flavus infection.

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