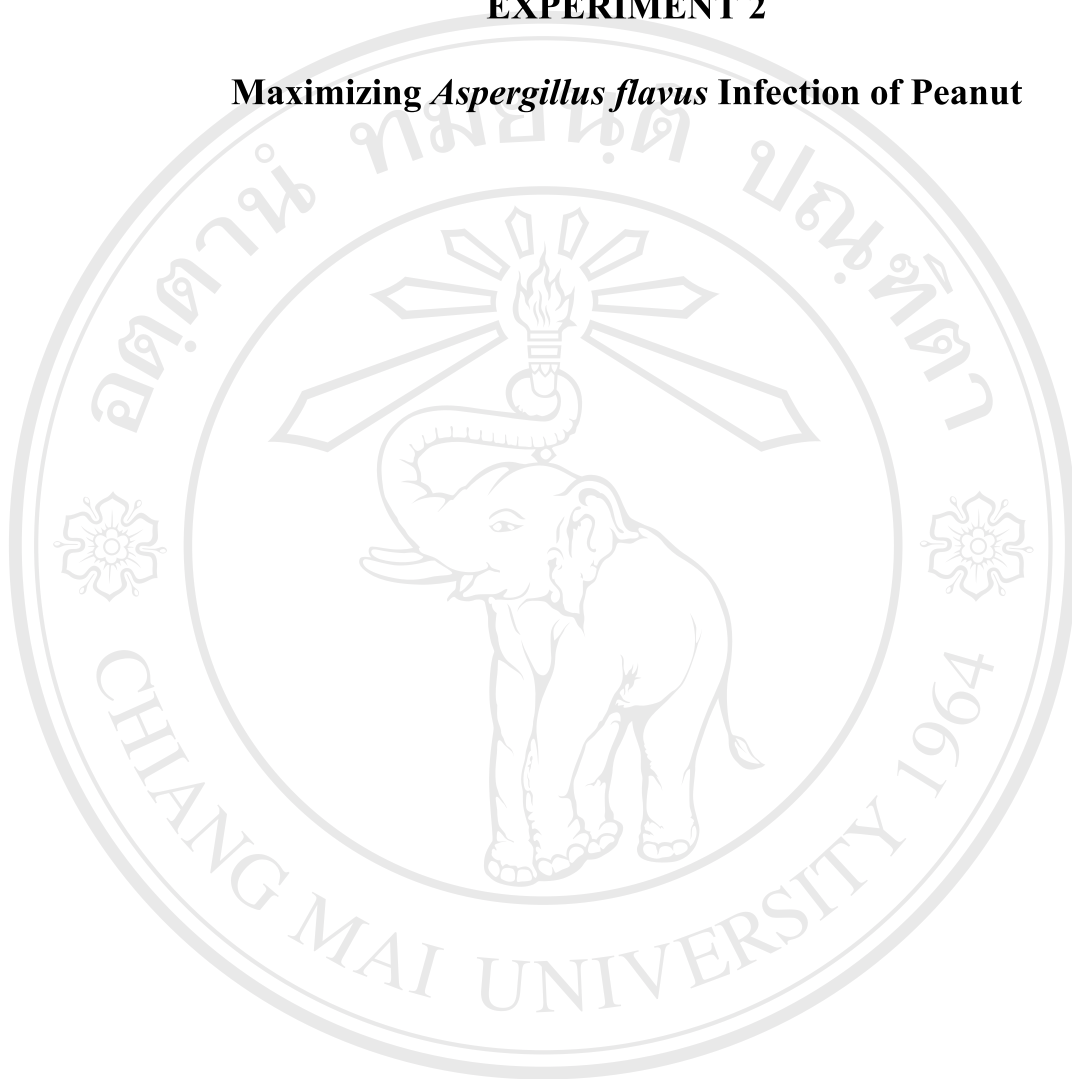
EXPERIMENT 2

Maximizing *Aspergillus flavus* Infection of Peanut



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INTRODUCTION

The saprophytic fungi, *Aspergillus* spp., are nearly ubiquitous on earth. Generally, these fungi do not infect healthy living tissues. Under drought conditions, however, *Aspergillus* spp. infect a large number of economically-important plants and may contaminate plant products with aflatoxin, one of the most carcinogenic toxins known. When peanut plants experience water deficit during pod filling, they are especially susceptible to aflatoxin contamination, which is largely produced by *Aspergillus flavus*.

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 seed that are discolored are generally infected with aflatoxin and may be easily removed, so for our purposes, we use the term aflatoxin resistance to refer only to resistance of whole, sound peanut pods and seeds. Will *et al*. (1994) evaluated a mid-bloom organic matrix inoculation is an effective method for field screening for aflatoxin resistance. Holbrook *et al*. (1994) prepared *Aspergillus* inoculum using cracked corn at 20% moisture as an organic culture substrate in field screening trials. Mehan *et al*. (1988) 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* spores. Aqueous suspension of *A. flavus* or *A. paraciticus* conidia may be either mixed into the surface of the soil (Azaizeh *et al*., 1989) or sprinkled directly on the plant (Wilson and Stansell, 1983; Wilson *et al*., 1989). Unfortunately, none of these techniques gave consistent infection levels, which are needed to identify genotypes with aflatoxin resistance.

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

In addition to great variability in contamination, methods for the detection of aflatoxin are relatively expensive. The recent development of *A. flavus* strains that contain a gene that codes for production of a GFP (J. Carey, USDA-ARS, New Orleans, personal communication and G. Payne, NCSU, Raleigh, North Carolina, personal communication) offer great hope to our ability to detect *A. flavus* infection. When illuminated with UV light (350-380 nm) the GFP fluoresces green. Thus, the GFP-producing *A. flavus* strains may be easily and quickly detected with either a simple UV light source (Wangeli *et al*., 1999) or with an UV-illuminated microscope.

The development of *A. flavus* strains that produce GFP also offer the opportunity to track pathways of infection, which have not been clearly identified. For peanut, two principal pathways of have been proposed – through the pod wall and through floral organs. Because *A. flavus* is largely a soil-borne fungus, previous research has mostly studied possible infection through the pod walls. Clearly, as injury to pods underground generally leads to infection and contamination, this is an important pathway for infection. However, undamaged pods may also contain aflatoxin-contaminated seed. It is possible that spores, whether moved by wind or rain, may infect floral organs, including developing ovules, before the pegs elongate and thrust them into the soil.

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This research had two objectives: 1) to develop inoculation methods that would attain high levels of *A. flavus* infection that are needed in aflatoxin resistance breeding programs; and 2) to investigate the potential for *A. flavus* to infect peanut flowers and pegs.

MATERIALS AND METHODS

Research was conducted in growth chambers of the Georgia Envirotron, at the Griffin Campus of the University of Georgia, USA during 2000 and at the Faculty of Agriculture, Chiang Mai University, Thailand during 2001-2002.

Sub-experiment 1 – Inoculation methods for maximizing *Aspergillus flavus* infection

Seeds of peanut genotype 329CC, previously identified as aflatoxin resistant by C.C. Holbrook, were germinated in moist paper for 2 days. Three healthy seeds were planted in each of 20 plastic 20-L containers 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 hours, and CO_2 concentration near ambient at 400 μL L^{-1} (Appendix E). All containers were irrigated lightly by hand at 1- to 2-day intervals 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.

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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 flowers first appeared on the plants: 1) spray over top of plant with suspension of *A. flavus* spores in water at 10 and 20 days after first flowers appeared; and 2) no spray. A split plot design was used with two growth chambers having 8 containers each to isolate the plants of no spray treatment from those of the spray treatment in order to minimize likelihood of cross contamination.

Before the first spray, each container were attached with 4 cuvettes. 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 (Figure 2.1). Each cuvette on a container represented a sub-plot treatment with different inoculation treatment methods. The four sub-plot treatments were: 1) spore suspension was mixed into soil before filling cuvette; 2) cracked corn inoculum was mixed into soil before filling cuvette; 3) uninoculated soil was placed in cuvette and cracked corn inoculum was applied to the soil surface; and 4) cuvette soil was not inoculated. Thus there were 8 treatment combinations applied in a 2 × 4 split plot design with 4 replications. After treatments began, each cuvette were applied 60 ml water twice weekly.

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In addition to the 8 treatment combinations described above, peanut plants were grown in 4 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.*

Figure 2.1 Photograph showing attachment of cuvettes to the sides of container. Cuvettes were covered with aluminum foil to exclude light so that pegs and pods would develop normally. One cuvette was attached to each of the four sides of the containers, with each cuvette on a container having a different inoculation treatment.

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Inoculum preparation and application

Two *A. flavus* strains, each modified to produce a green fluorescent protein (GFP), were used (Appendix F). One culture was obtained from Gary Payne (North Carolina State University, Raleigh, NC); the other was from Jeffery Carey (USDA-ARS, New Orleans, LA). While these two *A. flavus* strains may differ in relative

pathogenicity and location of the GFP gene (Carey, 1999 personal communication; Payne, 2000 personal communication), this study did not intend to study the difference between the strains. Rather, both strains were used 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/L) originally developed by Bell and Crawford (1967) and modified by Griffin *et al.* (1974). M3S1B medium had 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 ml acetone), and 1 L distilled water. When spores had been 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 up to 1000 ml (190 spores ml^{-1}) with sterile deionized water and then a hand pump spray bottle was used to spray the solution on the plant shoot of container treatments. For cuvette treatments, 2 ml of spore 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 each of four stoppered 250 ml flasks and autoclaved twice. After the cracked corn had cooled, a 50 ml aliquot from each spore 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.

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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 (Appendix G). Half of each sample was immediately observed with an ultraviolet illuminating fluorescence microscope (Model BX60F5, S/N: EXPO25754 Digital Output, Olympus, Melville, NY, USA). The other half was cultured on M3S1B medium in Petri dishes for 3 to 5 days. Cultured samples were observed with a hand held UV light and results were recorded as percent of samples infected with GFP *A. flavus*. 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 5-6 pegs that had reached the soil from each cuvette were excised. Pegs from each treatment combination were surface sterilized by dipping in 10% Clorox for 30 s, then rinsed twice in sterile water. Pegs 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 five 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

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fluorescence. Pegs from the absolute control plants were handled similarly to the sampled pegs from the two main growth chambers

Sub-experiment 2: Infection of peanut genotypes by *Aspergillus flavus*

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

A randomized complete block design with four peanut genotypes and four replications was used. Inoculum was prepared as in Sub-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 spore suspension of GFP *A. flavus* to the plant shoot and flowers; then four cuvettes were attached with cracked corn inoculum on applied to soil surface. Five days after inoculation, the wilted flowers and ovaries tagged with thread were excised. Flowers were cultured on M3S1B medium in 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.*

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Sub-experiment 3: *Aspergillus flavus* infection of peanut flowers under open-field conditions

This experiment was conducted at Faculty of Agriculture, Chiang Mai University, Thailand, to verify whether GFP *A. flavus* could infect developing flowers under field conditions. 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 (diameter 35.56 cm) with river sand soil that had been steam sterilized at 110-130 °C for 4 hr.

A completely randomized 4 × 2 factorial design with four replications was used. At flowering, half of the inoculated containers of each genotype were tagged 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. Ten ovaries were dissected longitudinally. Half was immediately placed on a microscope slide for observation with the microscope. The other half was placed on M3S1B medium in Petri dishes and cultured at room temperature for 5 days. After five 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*.

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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 package, 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 *Aspergillus flavus* infection of peanut flowers and pegs

Though under white light, flower tissues of inoculated treatments were not seen to have fungal mycelia, but by observation with a UV microscope at 5 days after inoculation GFP *A. flavus* was found on the surface of peanut flowers (Figure 2.2A-B) and hyphae of GFP *A. flavus* were observed to have penetrated into the flower tissues. On the other hand, I did not observe fluorescence on embryos of dissected ovaries at 5 days after inoculation under UV microscope observation. At 28 days after spraying with inoculum, embryos in dissected pegs fluoresced with GFP *A. flavus* (Figure 2.3A). Thus, either there was insufficient growth of the GFP *A. flavus* to observe fluorescence on embryos at 5 days after inoculation or the infection and growth of the *A. flavus* occurred between 5 and 28 days after inoculation.

Pegs sampled after they entered the soil and cultured with M3S1B medium had the highest infection levels in treatments sprayed with spore suspension and differences were significant between spraying and no spraying treatments (Table 2.1).

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For the cuvette inoculations, treatments with cracked corn inoculum applied to the surface of soil had the greatest infection levels (Table 2.1). No spray and no inoculum cuvette treatments had 20.8% of pegs infected with *A. flavus* on pegs. Clearly, *A. flavus* spores are sufficiently mobile so that spores applied to or produced in other cuvettes could infect flowers or pegs growing into uninoculated cuvettes. On the other hand, no infection was observed in absolute control plants grown in a separate growth chamber. Spore mixed with soil and cracked corn mixed with soil in the inoculated cuvette were not different, moreover the percent infection of both treatment were lower than on the cracked corn applied to the surface of soil.

As observed under UV illumination, embryos in some dissected pegs fluoresced (Figure 2.3A), indicating internal colonization by GFP *A. flavus*. Figure 2.3 compares the appearance of an embryo having a network of fluorescing hyphae with an embryo sampled from the absolute control treatment that had no apparent fluorescence to indicate colonization GFP *A. flavus*.

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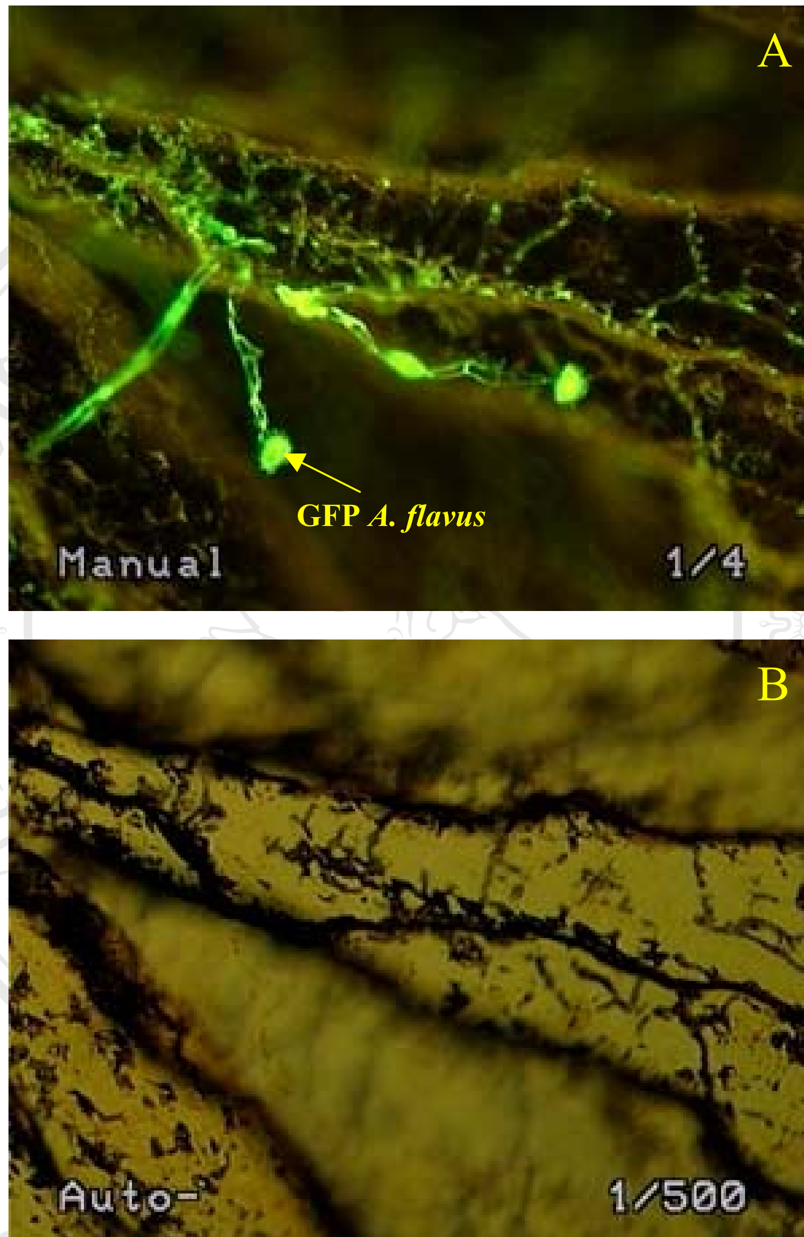

Figure 2.2 Infection of the external surface of peanut flower at 5 days after inoculation by GFP *Aspergillus flavus*. Hyphae of GFP *A. flavus* penetrated into the flower tissues as observed with (A) an UV-illuminated microscope or (B) with white light.

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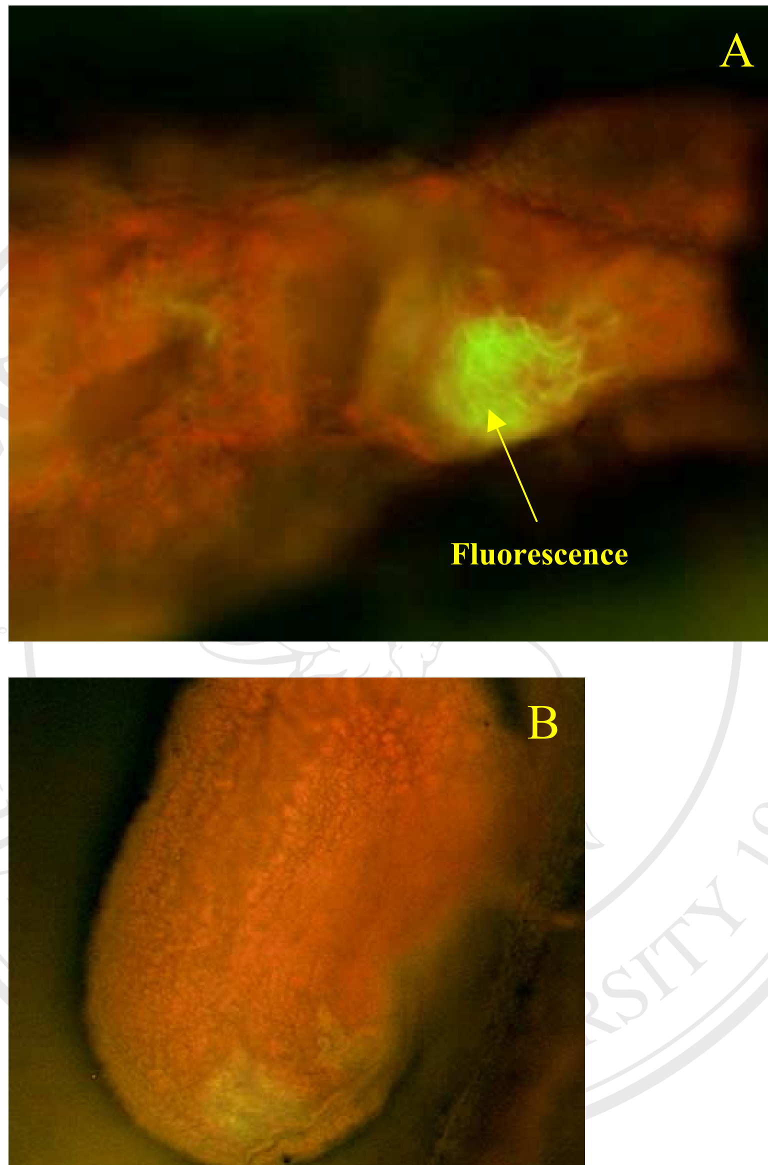

Figure 2.3 Dissected peanut peg at 28 days after inoculation as observed with a UV-illuminating microscope. (A) Network of GFP *Aspergillus flavus* hyphae colonizing the embryo inside a peg. (B) Embryo inside peg that is not colonized by GFP *A. flavus*.

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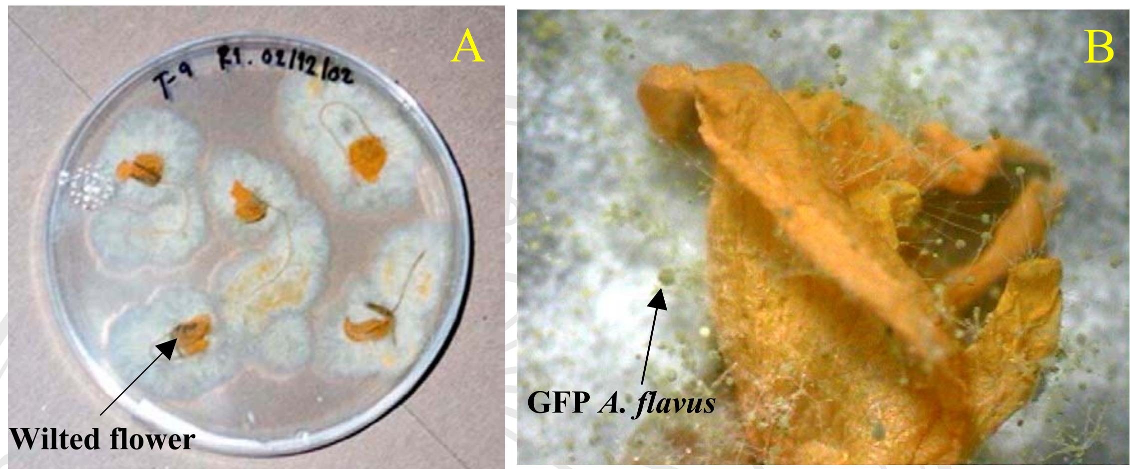

Figure 2.4 Wilted peanut flowers at 5 days after inoculation infected with GFP *Aspergillus flavus* that cultured on the M3S1B medium. (A) Whole flower (keel, standard, wing, calyx, hypanthium, stigma and anther) infected with GFP *A. flavus* and (B) close up as observed with an UV-illuminating microscope.

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Table 2.1 Percent of peanut peg infection by GFP *Aspergillus flavus* at 28 days after inoculation by eight combinations of inoculation methods. Main plot treatments were with and without spore suspension inoculation. Subplot treatments were four soil inoculation methods. The absolute control treatment was not infected by GFP *A. flavus*.

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

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Infection of peanut flowers and ovary by *Aspergillus flavus* under growth chamber condition

The investigation of infection by GFP *A. flavus* on peanut flowers and embryos of pegs confirmed floral infection for four peanut genotypes. Floral tissues of the four peanut genotypes were infected at moderate to high frequencies by *A. flavus* hyphae when observed after culturing on M3S1B medium (Figure 2.4A-B). Ovary infection of each genotype was not statistically related with flower infection (Table 2.2). Infection rates of dissected ovaries from 329CC genotype were significantly lower than for Tainan 9 variety, yet both genotypes had high infection on the flower tissues.

Findings of both experiments indicated that *A. flavus* could infect peanut during flowering and fertilization of peanut flowers. A comparison of pollen grains, spore of GFP *A. flavus* is shown in Figure 2.5A. *A. flavus* spores had covered the pollen grains and hyphae of fungus rapidly colonized the outside of pollen grains (Figure 2.5B), so it is probable that colonized pollen grains provided substrates for fungal growth.

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Table 2.2 Percent of peanut flower infection by GFP *Aspergillus flavus* at 5 days after inoculation with a spore 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	36.8 a*
511CC	100	23.5 ab
419CC	100	11.1 ab
329CC	100	5.0 b

* Means followed by the same letter are not significantly different by χ^2 at P = 0.05 level.

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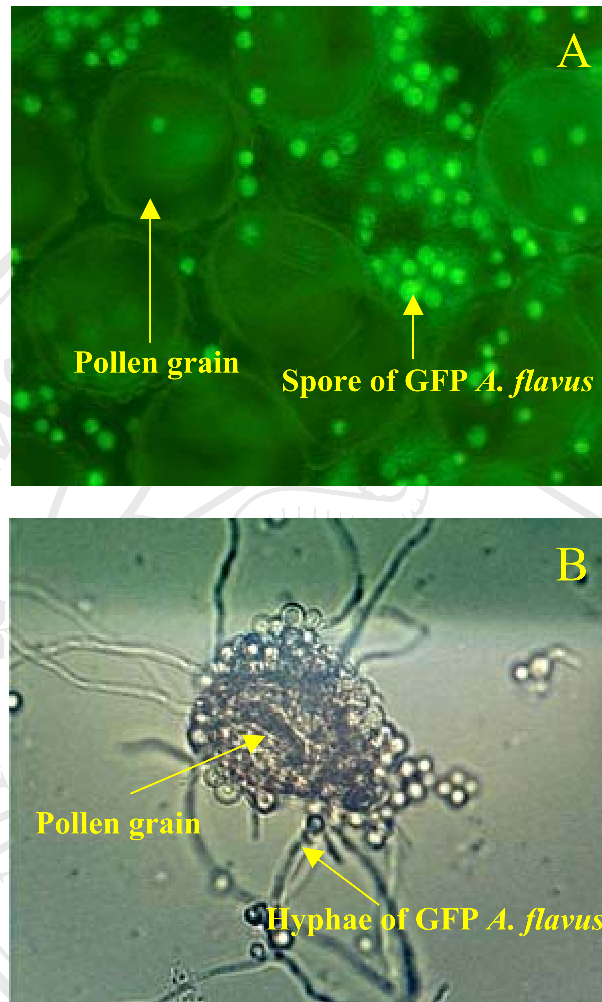

Figure 2.5 Comparison of pollen grains and spores of GFP *Aspergillus flavus*. Pollen grains of peanut flower have diameters more than 10 times larger than spores of GFP *A. flavus*. (A) The green fluorescent dots were the spore of fungi observed with an UV-illuminating microscope. (B) Spores and hyphae of GFP *A. flavus* covering the outside of a pollen grain observed under white light at 3 days after inoculation.

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Infection of peanut flowers under open-field conditions

Under open-field conditions, the highest infection of floral tissues was found on the treatment with cracked corn inoculum applied to the soil surface (Table 2.3). Inoculation and no inoculation treatments differed significantly, with nearly all inoculated flowers showing infection and slightly more than half of uninoculated flowers were infected. Wilted and fresh unwilted flowers of four peanut genotypes from inoculated containers had higher frequencies of infection by *A. flavus* than uninoculated plants (Table 2.3). However, uninoculated plants also had *A. flavus* colonization of peanut flowers. Ovary infection in the inoculated treatment was high, but there was no infection by *A. flavus* in the control treatments. Fresh unwilted flowers also had higher rates of infection in the inoculated treatment than in the control.

Under open-field condition, the soil was likely already colonized by *A. flavus*, which could produce spores capable of infecting peanut flowers. At 5 days after inoculation, varieties were already infected with the fungus. These data confirm that peanut infection by GFP *A. flavus* occurs during flowering and may result in infection of ovaries and seeds. All of these infections may be associated with wind or rain, which are vectors that can move spores from the soil to the flowers.

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Table 2.3 Percent of infection for peanut flowers and ovaries by GFP *Aspergillus flavus* in open-field conditions at 5 days after application of cracked corn inoculum.

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

† 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

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DISCUSSION

Field inoculation studies have been used to screen for resistance or susceptibility to *A. flavus* infection with results showing great variability and inconsistency. There have also been attempts to develop laboratory screening methods to identify germplasm resistant to colonization by *A. flavus* and preharvest aflatoxin contamination (Blankenship *et al*., 1985). The spraying of spore suspension of GFP *A. flavus* to the plant shoot, especially on flowers resulted in high levels of infection on the flower stage and pegging stage. Inoculum of *A. flavus* applied to the surface of soil also led to high levels of peg infection (Table 2.1). These inoculating methods were artificial treatments that had certainly infected the peanut by *A. flavus* fungi. Colonization of the peanut fruit by soil-borne fungi may occur through the flowers (Griffin *et al*., 1976; Mehan *et al*., 1980) (Table 2.3) in a process similar to infection of corn through the silks (Payne, 1983). Griffin *et al*. (1976) suggested that *A. flavus* does not always establish a successful systemic infection following flower infection. They found that conidia of *A. flavus* in soil may germinate adjacent to developing peanut fruit and infect pods, particularly following injury of the fruit. Griffin (1972) also observed much higher levels of *A. flavus* conidial germination in soil adjacent to injured peanut fruits than on aerial peanut pegs.

Fungal ingress was observed through flower and peg tissues. By spraying plants with a spore suspension, infection percent was higher than without spraying spores. Fungal spores attached to the tips of stigma with pollen grain (Figure 2.5A-B). Certain pathogens follow the path of pollen. Fungal spores lodge on the stigma and germinate, following the pollen tube as it enters the style, then infects the ovary. As gynophore elongates, the fungus remains with the ovary, becoming established in

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developing seeds. Therefore, *Aspergillus* colonization can occur during flowering or during aerial peg formation (Griffin and Garren, 1974) from viable air- borne propagules originating from peanut soil. Soil-borne fungi, such as *A. flavus*, usually colonize on fruiting zone, which had promoted the substrates for *A. flavus* growth under soil. Griffin *et al.* (1976) and Pitt *et al*. (1991) had reported that direct invasion of seeds only occurs after pod injury. In this study, *A. flavus* infected peanut flowers and pegs, which explains how *A. flavus* can infect seeds within undamaged pods.

Peanut pegs had the greatest infection under *A. flavus* inoculum applied on the soil surface. Almost all subterranean tissues (pegs, pods, fibrous and tap roots) were infected with *A. paraciticus* by the end of the growing season (Kisyombe *et al*., 1985), and the fungus was present within vegetative tissue but was restricted in its ability to infect the seeds. Haixin *et al*. (2000) observed one case where the fungus had penetrated through the outer cell wall layers of the pericarp, numerous pods had extensive colonization of pericarp tissue. Alternatively, initial infection may occur through the peg or in very young stages. Griffin and Garren (1976) showed that even when aerial pegs were surface-sterilized with 0.5 % NaClO for 3 minutes and then cultured on a selective medium for *A. flavus*, a small portion (0.3%) of pegs were colonized by *A. flavus*. Although the NaClO may have penetrated several cell layers of the peg epidermis, it is clear that *A. flavus* hyphae had penetrated deeper into the peg than the NaClO. Sander *et al*. (1981) found that infection occurred in young fruit at high levels and that maturity was delayed by drought. Pegs are in direct contact with soil fungal populations, pegs are most susceptible to invasion by *A. flavus*. Hence, Payne (1983) concluded that water stress had no effect on the percentage of infected seeds, where inoculum was applied. In this study, it was found that the

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infection of flower and pollen grains by GFP *A. flavus* may be related to seed infection. If fungal spores and mycelia are present on the surface of ovaries or pegs, they may invade and injure these parts. Percent of peanut flower infection by GFP *A. flavus* at 5 days after inoculation with a spore suspension spray and cracked corn inoculum applied to the soil surface is shown in Table 2.2.

This experiment suggested that peanut infection by GFP *A. flavus* could occur during flowering or peg formation. *A. flavus* can become associated with the peanut fruit early in its development (Griffin and Garren, 1976). Seed infection can occur by fertilization of pollen grains that were contaminated with *A. flavus*. Production field are usually contaminated with numerous spores of *A. flavus*, so it can infect the flowers or spores can be contaminated on the soil surface. Therefore a high soil-surface population of applied toxigenic strains had the effect to the flower infection with subsequent aflatoxin contamination.

The combined applications of a spore suspension over shoots with flowers and a cracked corn inoculum applied to the soil should result in the greatest *A. flavus* infection levels, thereby facilitate efforts to screen peanut germplasm for aflatoxin resistance that is for sound and undamaged pods.

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