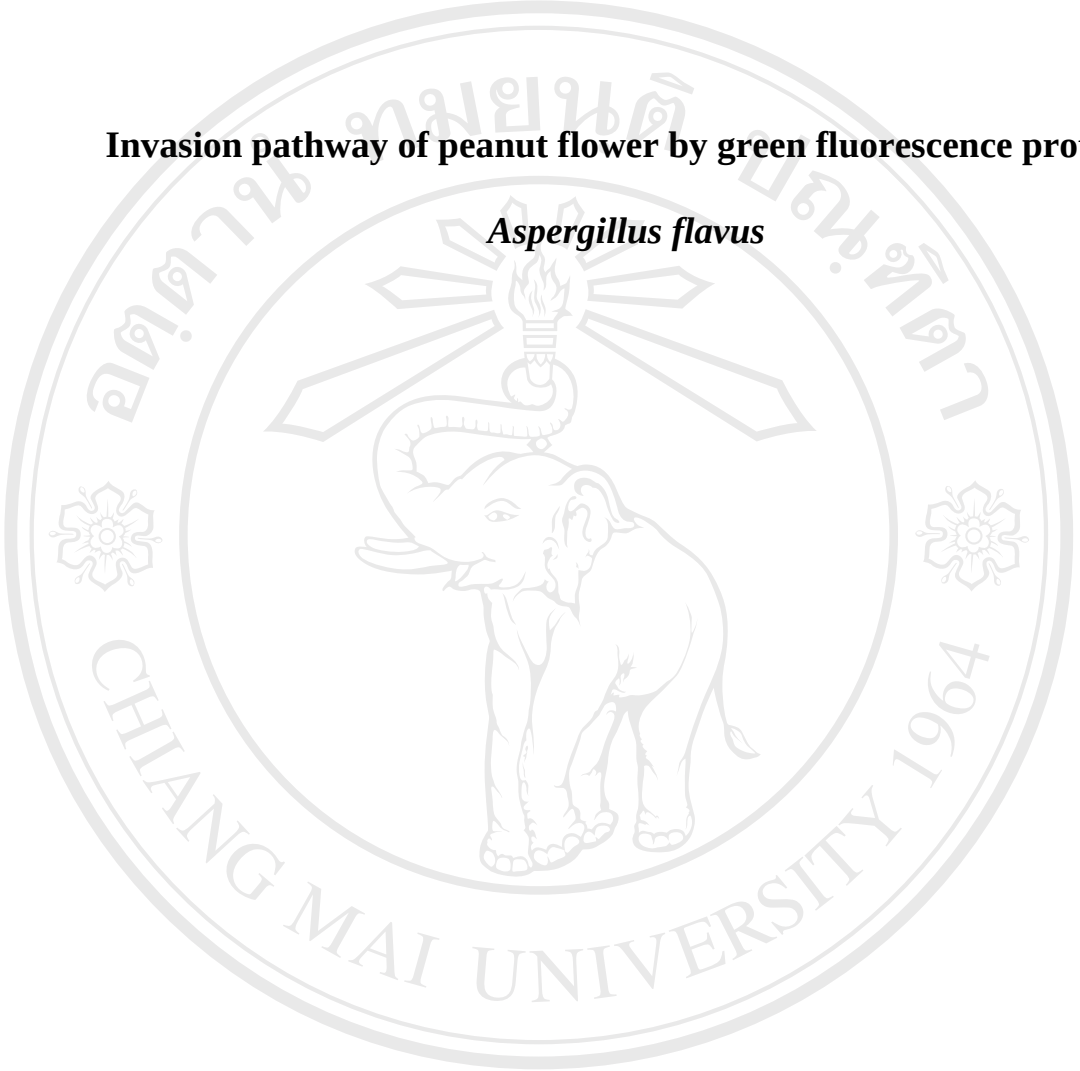
EXPERIMENT 3

Invasion pathway of peanut flower by green fluorescence protein *Aspergillus flavus*



INTRODUCTION

Seed infection is the establishment of pathogen within any part of a seed and many occur systemically either through the vascular system, through plasmodesmata between cells, or directly through floral infection or penetration of the ovary wall, the seed coat, or natural openings.

In peanut (*Arachis hypogaea* L.), colonization of shells and seeds by *Aspergillus flavus* and subsequent aflatoxin contamination is a serious problem in subtropical and tropical regions. Peanut could be invaded by *A. flavus* and subsequently become contaminated with aflatoxin before or after harvest (Sander *et al.*, 1985; Dorner *et al.*, 1989; Mehan and McDonald, 1991). The aflatoxigenic *A. flavus* is a soil-borne fungus and generally characterized as saprophytic fungus, which acts as weak pathogen and does not normally infect healthy tissues. *Aspergillus* fungi usually colonize dead or dying plant tissues that are on the soil surface or in the upper 5- to 10-cm soil layer (Blankenship *et al.*, 1984), which includes the peanut pod development zone.

Because peanut pods and seeds develop underground, mechanically or biologically damaged pods in the soil are predisposed to invasion by *Aspergillus* fungi and frequently contain dangerous amounts of aflatoxins. Previous research has mostly studied underground infection through the peg and pod walls (Griffin and Garren, 1976; Cole *et al.*, 1986; Azaizeh *et al.*, 1989). Clearly, as injury to pods underground generally leads to infection and contamination, this is an important pathway for infection. However, healthy, undamaged peanut pods and seeds may also be infected by *A. flavus* and contaminated with aflatoxin. Norton *et al.* (1956) reported that 27.4%

of visibly unblemished Spanish peanut seeds were infected with fungi. In their study, the most common fungal species found in peanut seeds 6 weeks before harvest was *A. flavus*. Griffin (1972) observed that *A. flavus* conidia germinated on aerial pegs. These findings suggested that *Aspergillus* conlonization of pods might occur during flowering or during aerial peg formation from viable air-borne spores. Griffin and Garren (1976) demonstrated colonization of aerial peanut pegs and flowers by *A. flavus* and *A. niger* under field conditions. Isolation frequency from flowers was about 7% from flowers but only 1.5% from terminal aerial pegs and surface-sterilized aerial pegs. They concluded that low levels of colonization of peanut pods by *A. flavus* via flower and aerial peg colonization were possible under field conditions in Virginia.

Although colonization of peanut pods by *A. flavus* can occur either before or after harvest, and this fungus can infect pods in the soil during fruit development or earlier during the flower or aerial peg stage, little is known of the nature and mechanisms by which this fungus infects peanut flowers. To study the infection process, *A. flavus* strains that contain a gene that codes for production of a green fluorescent protein (GFP), which fluoresces green when illuminated with UV-light (350-380 nm), offers the great opportunity to track pathways of infection that have not previously been clearly identified. Moreover, there is very sparse data on aerial peanut peg colonization. This study was initiated to investigate the entry of *A. flavus* into the peanut flowers, ovary, aerial pegs, which relate to pre-harvest infection.

MATERIALS AND METHODS

Plant material and growth condition

Green house research was conducted at Lampang Agricultural Research and Training Center during 21 June to 3 August 2003. Seeds of three peanut genotypes: 511CC, drought tolerant and resistant to pre-harvest aflatoxin contamination (PAC); 419CC, drought intolerant and PAC susceptible; and Tainan 9, unknown drought and PAC resistant, were pre-germinated in moist germination paper and placed in growth chamber with 30^{o}C day/25^{o}C night temperature for five days. Then, four uniform seedlings of each genotype were transplanted into growing trays, covered with flexible opaque plastic film and placed over the foam box containers (Figure 4.1). This covering both supported the plants and prevented the entry of light into the box during pod development. A modified half-strength Hoagland's solution was supplied to the plants in each container (Hoagland and Arnon, 1950). Air pumps lines were used to aerate nutrient solution of all containers. All hydroponic containers (10 containers per genotype) were placed under green house conditions, which experienced 36.3^{o}C maximum and 27.4^{o}C minimum temperatures, 78.5% maximum and 39.3% minimum RH, and an average light intensity of 36.7 mol $m^{-2}day^{-1}$. The solution was replaced weekly. At flowering, perforations were made in the plastic film to allow the entry of developing pegs into the nutrient solution.

We used two strains of *Aspergillus flavus* that were genetically modified to produce green fluorescent protein (GFP) in this study (Appendix A), one strain was produced by Gary Payne (NCSU, Releigh, North Carolina), the other by Jeffrey Carey (USDA-ARS, New Orleans, Louisiana). Two *A. flavus* strains were maintained on

M3S1B medium and cultures were incubated at 30^{o}C for 7 days. Then conidia were washed from the Petri dishes using sterile distilled water containing 10 drops L^{-1} of Tween 20 and filtered through three layers of sterile cheesecloth. The concentration of conidia was measured with a hemacytometer and adjusted with sterile distilled water to $4x10^{6}$ conidia ml^{-1}. At 31 days after planting (DAP), 50 flowers of each genotype were tagged with colored thread around the hypanthia, and then each 0.5 ml of spore suspension was applied on those flowers by using a sterile micropipette. Sterile distilled water was sprayed over all peanut plants to generate high moisture condition, which promote spore germination. At 24 and 48 hours after inoculation, inoculated flowers were collected to examine *A. flavus* infection. Samples were kept in 250 ml beakers containing a formalin-acetic acid-alcohol (FAA) mixture (Appendix G) and stored in a refrigerator. This mixture was use to kill and fix infected plant tissues before making slides. After fixing, flowers were observed under stereoscopic microscope to examine the spores or sporulating fungal bodies on plant tissues. Then, flowers were separated into pistil (stigma, style, and ovary), stamen (anther, filament, and pollen grain), and petal (Figure 1.1 B). Ovaries were cut into small pieces using a razor blade. Then, each component was carefully removed and placed into a drop of sterile water on glass slide. A cover slip was placed over the mounting medium, carefully preventing entry of air bubbles and cover slip edges were sealed with nail varnish. Slides were examined under an ultraviolet illuminating fluorescence microscope to detect infection sites and the presence of fungal hyphae inside plant tissues. Some pistils, stamens, and petals from infected flowers were also stained with cotton blue lactophenol (Appendix G) and observed under white light microscope.

At 35 DAP, 50 flowers of each genotype were tagged and inoculated with same amount of GFP *A. flavus* spore suspension as described above, then aerial pegs were sampled after 10 days of inoculation to evaluate the presence of GFP *A. flavus* colonization. Aerial pegs that developed from tagged, inoculated flowers were sampled, totaling 20 pegs for 511CC, 18 pegs for Tainan 9, and 10 pegs for 419CC. All sampled pegs were cleaned with tap water, surface sterilized for 3 minutes in 10% Clorox, then rinsed twice with sterile distilled water. Apical portions of pegs about 1 cm long were dissected longitudinally and plated on M3S1B medium. Cultures were incubated at 30^{o}C for 5 days when the presence of GFP *A. flavus* colonization was tested for yellow-green fluorescence using a 365 nm-UV box. This observation was compared with original GFP *A. flavus* colonies. Results were recorded as percent of pegs that were infected with GFP *A. flavus*.

Statistical analysis

Data were analyzed by Chi-square test of SAS statistical package, Version 6.12 (SAS Institute, Cary, North Carolina, USA).

Figure 4.1 Three peanut genotypes growing in modified half-strength Hoagland's solution of a hydroponic system at Lampang Agricultural Research and Training Center.

RESULTS

Floral infection

Germination of *A. flavus* conidia on flowers organs were monitored by light microscopy after staining with cotton blue in lactophenol (Figure 4.2 A-D) and observing under UV-illuminating microscope (Figure 4.3-4.4). Conidia of *A. flavus* germinated within 24 hr after inoculation. Germination occurred first nearest the pollen grains and on the tip of stigma (Figure 4.2 B-C; Figure 4.3 A-C). Conidia also germinated on anthers and filaments (Figure 4.4 P-R) but not on styles or ovaries. Fungal hyphae spread across surfaces of stigmas and styles within 24 hr, producing extensive growth and lateral branching (Figure 4.2 A-D). Hyphae of *A. flavus* grew down the style, eventually reaching the top of the ovary (Figure 4.4 A-K), from which the fungus might enter following the path of the pollen tube through the stylar canal. In the absence of pollen grains, conidia germinated on the stigma and germ tubes grew through the stylar canal though no specialized infection structures, such as appressoria or penetration pegs were observed. No differences in spore germination or fungal growth were noted for different peanut genotypes during microscopic analysis. Similar conidia germinations and hyphal growth patterns of *A. flavus* were observed in all peanut genotypes. At 24 hr after inoculation, ovules of all genotypes were not visibly infected and no fluorescing hyphae were observed. There was, however, a green fluorescence visible occurred at the surface of ovary wall (Figure 4.4 L-M). By 48 hr, I observed fluorescing hyphae inside ovary tissues (Figure 4.4 N) and also at the base of ovule (Figure 4.4 O), such fluorescence was observed in only a very small

portion of ovaries (1/50). In some peanut flowers, *A. flavus* also found to colonize the stamens in as little as 24 hr. Sporulation was also visible on the anthers and distal portions of the filaments (data not shown). Heavy colonization of pollen grains was accompanied by varying amounts of *A. flavus* mycelium on pollen-sac surfaces (Figure 4.4 P-R). Hypanthia were also infected by GFP *A. flavus*. I could observe fungus colonization and sporulation on the hypanthia when cultured on selective medium (Figure 4.5 A). Conidiophores and conidia formed over the entire surface of peanut flowers within 48 hr after inoculation (Figure 4.5 B).

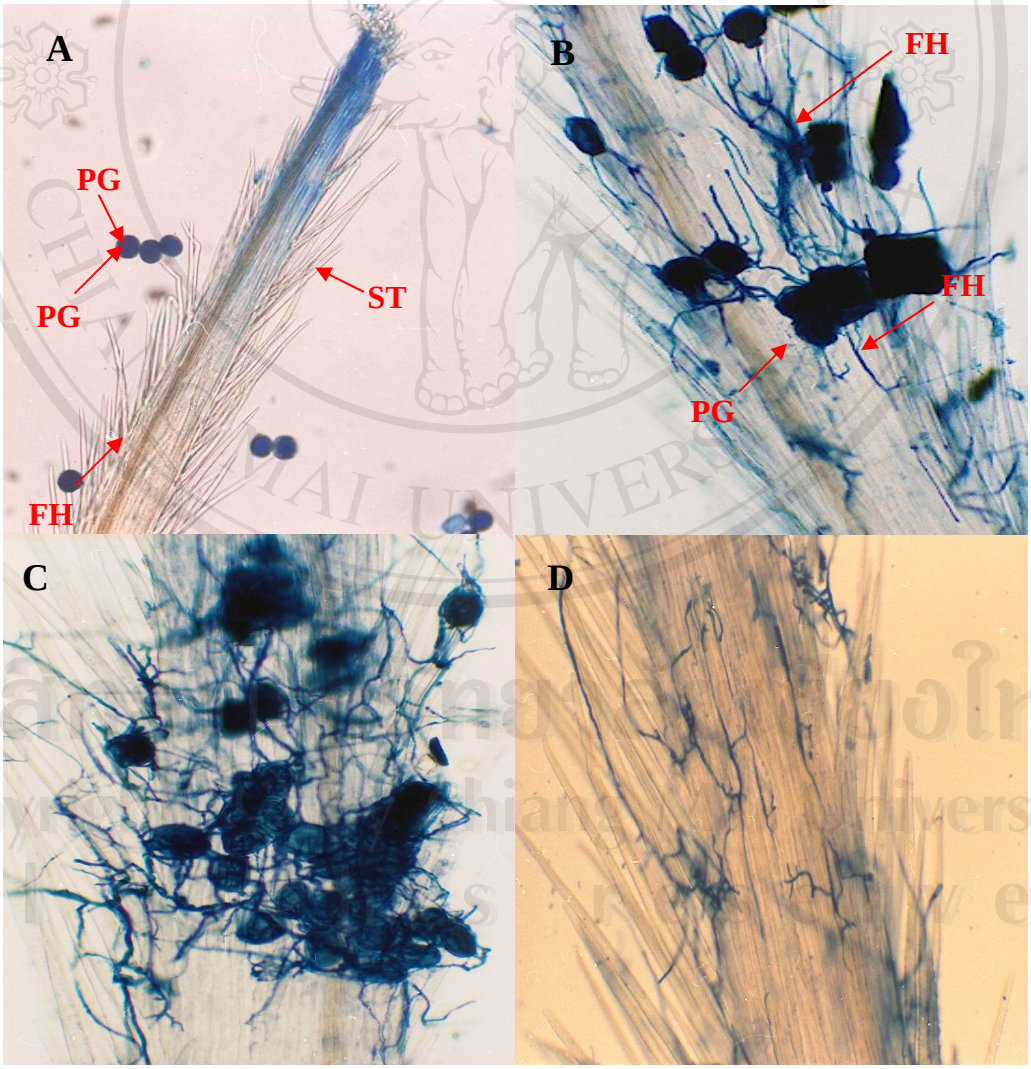


Figure 4.2 Photomicrograph of peanut flower infection by GFP *Aspergillus flavus* after staining with cotton blue in lactophenol and observed under light microscopy. **A,** Stigma (ST) and Pollen grains (PG) of noninoculated flower. **B-D,** Fungal hyphae (FH) growing on surface of stigma and colonized pollen grains at 24 hr after inoculation. Magnifications: A, ×100; B-D, ×200.

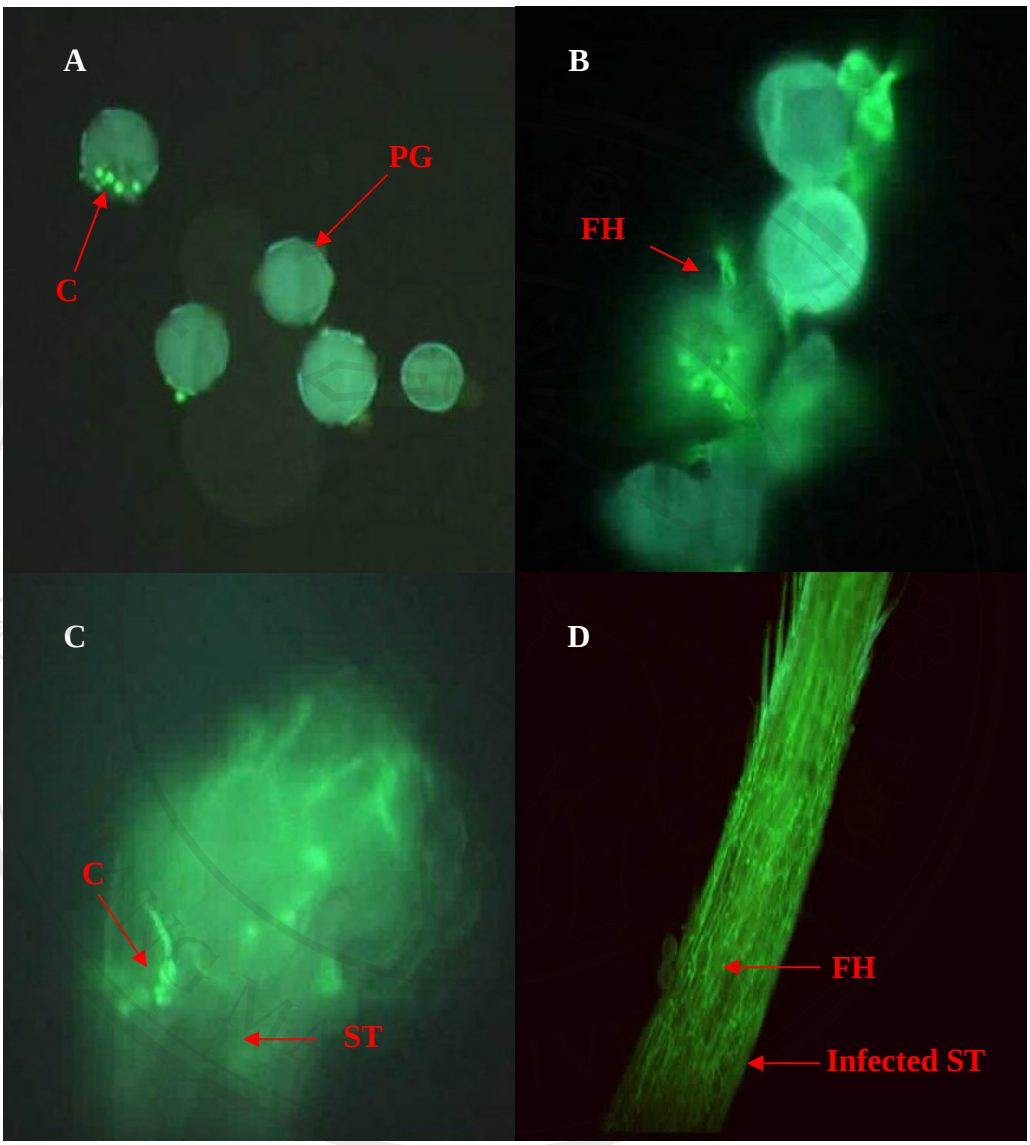


Figure 4.3 Bright green fluorescent conidia (C) and fungal hyphae (FH) of GFP *Aspergillus flavus* observed with a UV-illuminating microscope. **A-B,** Conidia germinated and colonized pollen grains (PG) at 24 hr after inoculation. **C,** Conidia germinated on the tip of stigma (ST). **D,** Fungal hyphae growing on surface and colonizing surface of stigma at 48 hr after inoculation. Magnifications: A, ×200; B, ×400; C, ×400; D, ×100.

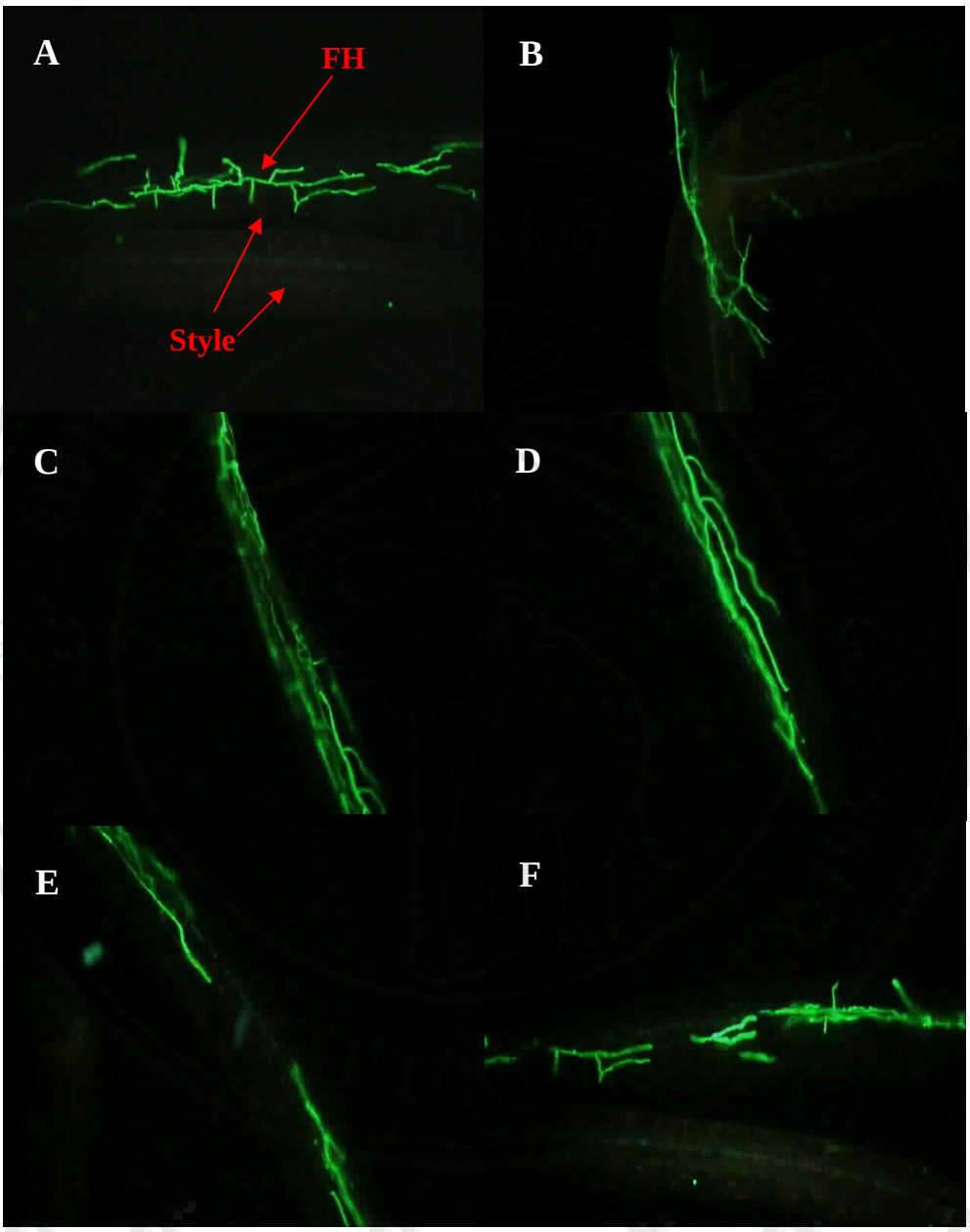
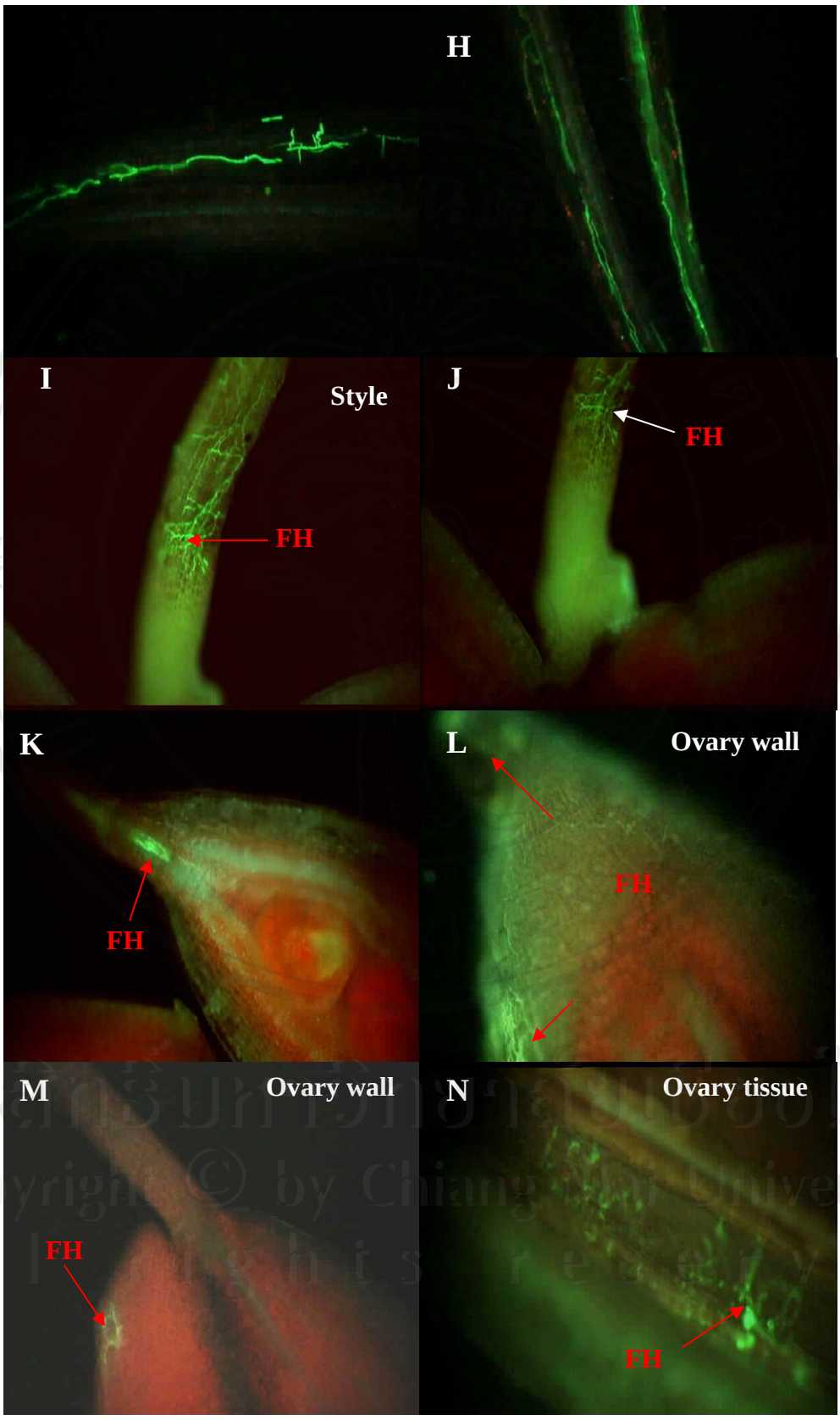


Figure 4.4 Fluorescence micrographs of GFP *Aspergillus flavus* growth on inoculated peanut flowers. **A-J,** Fungal hyphae penetrated through the style of peanut (the same style but different portions). **K-M,** Fluorescing hyphae occurred in the top of the ovary and outside ovary surface 24 hr after inoculation. **N,** Colonizing of fungal hyphae inside ovary tissue 48 hr after inoculation. **O,** Network of fungal hyphae colonized the ovule 48 hr after inoculation. **P-R,** Conidia germinated on anther and filament of peanut. Magnifications: A-M and P-Q, ×100; N-O, ×200.



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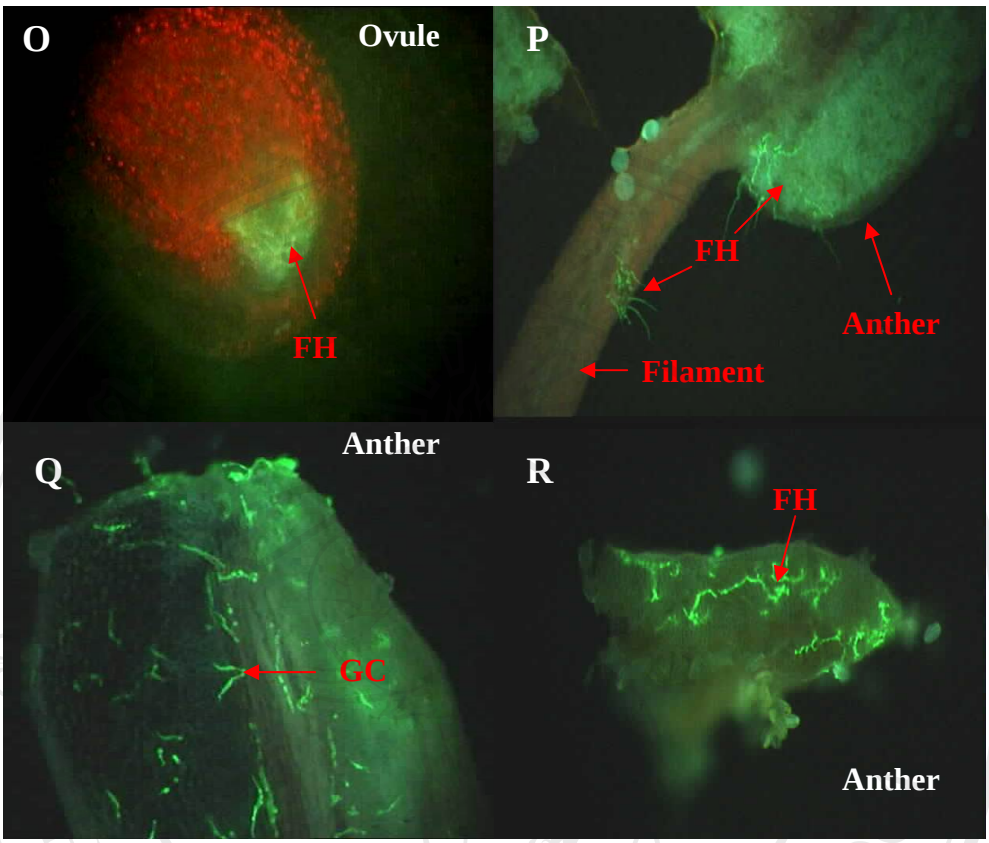

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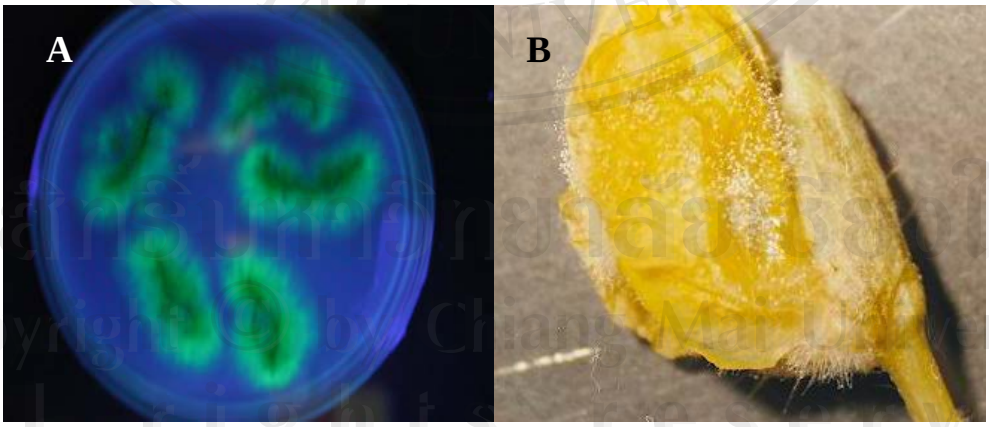

Figure 4.5 Floral infection by GFP *Aspergillus flavus* observed at 48 hr after inoculation. **A,** Sporulation of GFP *A. flavus* on hypanthia cultured on selective medium. **B,** Conidiophores and conidia on a peanut flower.

Aerial peg infection

Sporulation of *A. flavus* from plated, surface-sterilized aerial pegs sampled 10 days after inoculation was observed within 3 days after incubation. There were statistically significant differences in overall susceptibility among the three peanut genotypes, with the greatest incidence of fungal colonization found in Tainan 9 (80%) followed by 419CC (71%) and 511CC (40%) (Table 4.1). There was no *A. flavus* colonization on aerial peg developing from non-inoculated flowers.

Table 4.1 *Aspergillus flavus* colonization of aerial pegs for three peanut genotypes at 10 days after inoculation.

Genotype	% infected*	No. assessed
511CC	40.0	20
419CC	70.8	18
Tainan 9	80.0	10

* Significant difference in overall susceptibility between peanut genotypes (χ^2= 7.42, P<0.05).

DISCUSSION

Floral infection

This experiment provides compelling evidence that preharvest peanut seed infection by *A. flavus* may occur systemically directly through floral infection. Initial infections may take place from different part of peanut flower organs as follows:

Infected pollen grain: Dense growth of mycelia of *A. flavus* in infected pollen grains observed in the present study suggests that pollen plays a critical role in the establishment of *A. flavus* growth on stigma and style, presumably because it is a rich source of carbohydrates, amino acids, and minerals (Pfahler and Linskens, 1971; 1974) and is therefore an excellent substrate for growth of this fungus. Pollen grains could be important in natural infection because anthers form a relatively large area for fungal colonization and conidia produced on the anthers are in a perfect position to act as secondary source of inoculum to infect other flowers

Penetration through the stigma: As a saprophyte, *A. flavus* generally infects only injured tissues. I observed fungal conidia attached to the tips of stigma with pollen grain. Thus, when conidia germinated on the stigma surface and fungal hyphae could penetrate into the style following the pollen tube, which effectively causes a channel of injury to allow infection of the ovary and establishment of *A. flavus* colonies in developing pegs. Such a path has been proposed for *Monilinia oxycocci* in cranberry flowers by McManus *et al.* (1999) and for *M. vaccinii-corymbosi* on blueberry flower by Ngugi *et al.* (2002).

Penetration directly through the hypanthium, style, and ovary wall: In peanut flower, the style is contained within a hypanthium, which can elongate up to 5 cm during 24 hr prior to anthesis (Moss and Ramanatha Rao, 1995). After anthesis peanut flowers wither within 24 hr, however the withered hypanthia with styles still attached to an ovary during peanut peg development until reaching the soil surface. This study, I observed sporulation of *A. flavus* on hypanthium tissues within 48 hr after inoculation (Figure 4.5 A) as well as fluorescent hyphae on the surface of ovary tissues at 24 hr after inoculation (Figure 4.4 L-M) and inside ovary tissues within 48 hr after inoculation (Figure 4.4 N-O).

A. flavus conidia may germinate on hypanthia and penetrate directly through these tissues, passing through the style until hyphae reach an ovary wall. However this pathway could not be confirmed and there is no documented report that saprophytic *Aspergillus* fungi can infect directly through the ovary wall, though other fungi are known to penetrate through ovary walls, including *Ustilago nuda* on barley (Kozera, 1968) and *Alternaria alternata* in wheat (Bhowmik, 1969).

Incidence of aerial peg infection by *A. flavus*

Observations of *A. flavus* colonization on plated, surface-sterilized terminal portions of aerial pegs developing from inoculated flowers after 10 days of inoculation confirm that *A. flavus* infection of peanut ovaries results from infection of floral tissues. On the other hand, this fungus may colonize peanut ovaries non-pathogenically and remain associated with the apparently sound developing peanut peg tissues until stress occurs, such as drought, or some other injury weakens tissues and allows *A. flavus* to infect additional tissues. Although, I did not observe

fluorescing of GFP *A. flavus* on ovule of aerial peg at 10 days after inoculation, I found fluorescence on ovary walls and also inside ovary tissues. It is likely that the fungal hyphae were presented in the ovary but had not yet reaching ovule, that the mass of hyphae was insufficient for direct detection at that time, or that dissected tissues were not thin enough to see the fluorescing hyphae in peg tissues. Griffin and Garren (1976) 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%) for pegs were infected and the source of infections for these pegs was more likely a floral infection than infection through the epidermis of the peg itself. Puntase *et al.* (2006) observed that the maximum infection of GFP *A. flavus* was produced by spraying an aqueous suspension of conidia over shoots and flowers of peanut, which resulted in 100% infection of floral surfaces. They also found the fluorescence hyphae of this fungus on the surface of flowers and on ovules inside the peanut pegs before the pegs reached the soil surface.

The incidence of *A. flavus* colonization on aerial pegs differed among peanut genotypes. In this study, aerial peg infection was observed most thoroughly on Tainan 9 peanut genotype, although there was no obvious microscopic difference in floral infection. Results suggest that each peanut genotype may have different structural barriers or defense chemical compounds to fight fungal colonization of peg tissues. Tannins extracted from peanut seed coat have been reported to inhibit the growth of aflatoxigenic *Aspergillus spp. in vitro* and the inhibition rate depended on concentration and peanut cultivar (Sanders *et al.*, 1981). In Experiment 2, I have found that genotype 511CC, which was classified as drought and aflatoxin resistant

(Holbrook *et al.*, 1993), contains greater amounts of tannins in seed coat than genotype 419CC and Tainan 9. This compound and its antifungal activities may also exist in peanut ovaries. Genotypes with high tannin contents may be considered to be at least partly-resistant to *A. flavus* infection.

In conclusion, initial infection occurred anywhere on the surface of peanut flowers (stigma, style, pollen grain, hypanthium, standard, wing, or ovary). Pollen grain, stigma, and style appeared to be particularly susceptible to colonization as fungal hyphae were often found in these tissues. Floral infection is likely to be the most important pathway of infection, particularly infection of whole, sound pods and seeds. Thus knowledge of the floral infection could be a key to optimizing control of preharvest *A. flavus* infection and subsequent aflatoxin contamination. Future research would be warranted to identify irrigation, row orientation, and other crop management factors that would prevent the movement of conidia form the soil surface to the flowers.