Chapter 3

Screening Procedures

3.1 Introduction

One of the first and most important phases of breeding for disease resistance is to identify a source of resistance. The efficient and accurate screening method is the one importance in breeding program. In the past, aflatoxins was considered predominantly only a post harvest problem and as such received little attention in crop improvement progress. The screening was done on rehydrated sound mature seed inoculated with conidia of *A. flavus* in an environment favorable to fungus development (Wynne *et al.,* 1991). Most researchers evaluated the levels of seed resistance in both laboratory and field conditions for identify resistant genotypes as the fungal infection levels and aflatoxins production of seed (Bartz *et al.,* 1978; Diener *et al.,* 1965; Fernandez *et al.,* 1997; Gembeh *et al.,* 2001; Ghewandee *et al.,* 1993; Mehan *et al.,* 1986; Mixon, 1980). Although, these methods are useful in screening, the data have been interpreted to indicate a possible resistance, or tolerance, to pre-harvest invasion.

Host-pathogen interaction begins at a very early stage in any encounter between a fungus and a plant. Disease resistance or susceptibility probably starts at or during the initial contact between the cell of host and the pathogen (Isaac, 1992). Aspergillus colonization can occur during flowering or during aerial peg formation (Griffin and Garren, 1974; Azaizeh *et al.,* 1989) when high temperature (28.0 - 30.5 °C) and water deficit conditions (Sander *et al.,* 1985). Information on

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the optimum procedure to surface sterilizing and the timing of groundnut peg infection *A. flavus* are needed to help researchers determine when to harvest groundnut genotypes to accurately determine levels of resistance. Therefore, the present investigation was designed to meet the following objectives:

- To find novel screening technique for assessing the resistant or susceptible groundnut genotypes using the staining *A. flavus* in groundnut tissues and identify infected position under UV microscope.
- To determine appropriate conditions for assessing pre and post harvest resistant genotypes to *A. flavus* infection.

3.2 Materials and methods

3.2.1 Aniline blue fluorescence and hematoxylin staining (AFHS) method

3.2.1.1 Preliminary finding

Before molecular individualization of the resistance response, there were interesting in defining the initial chronological divergence between infections in compatible versus incompatible interactions. To accomplish this it needed to determine the amount of fungal growth present and identify the type of host tissue infected. Use of hand-sectioned material was not practical because of the thick and complicates tissue made the unclear visibility (Figure 3.1). Thus, section by rotary microtrome at 13-15 μm thickness showed the single layer (Figure 3.2) but still not practical, so evaluation a whole mount staining technique that use KOH-aniline blue (Hood and Shew, 1996), safanin O and hematoxylin (Suriyong, 1997). This method gave very poor results; revealing only small fraction of the *A. flavus* hyphae present

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inside the tissue while autofluorescence of tissue was very brightness after staining safanin O or KOH-aniline blue (Figure 3.3A-C). However, hematoxylin stained tissues presented darkness (Figure 3.3D). Thus, to fix plant tissue from fluorescence, in next experiment, the staining technique using aniline blue fluorescence followed by hematoxylin was developed.

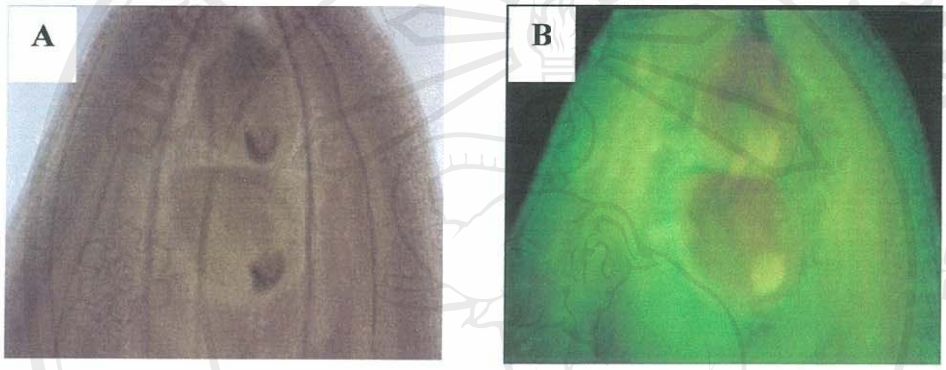

Figure 3. 1 Long-section by hand of groundnut embryos after flowering 3 days, A; under normal light and B; under UV light microscope

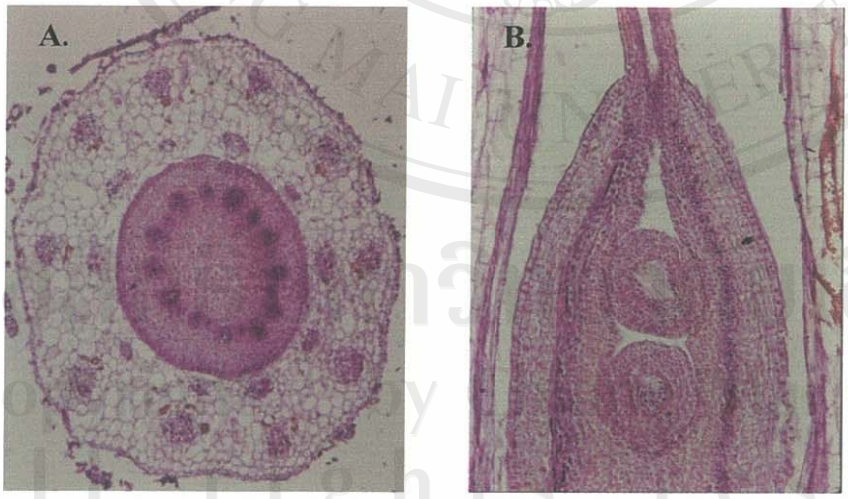

Figure 3. 2 Cross (A.) and long (B.) section by rotary microtrome and stained with hematoxylin of groundnut embryos under normal light microscope

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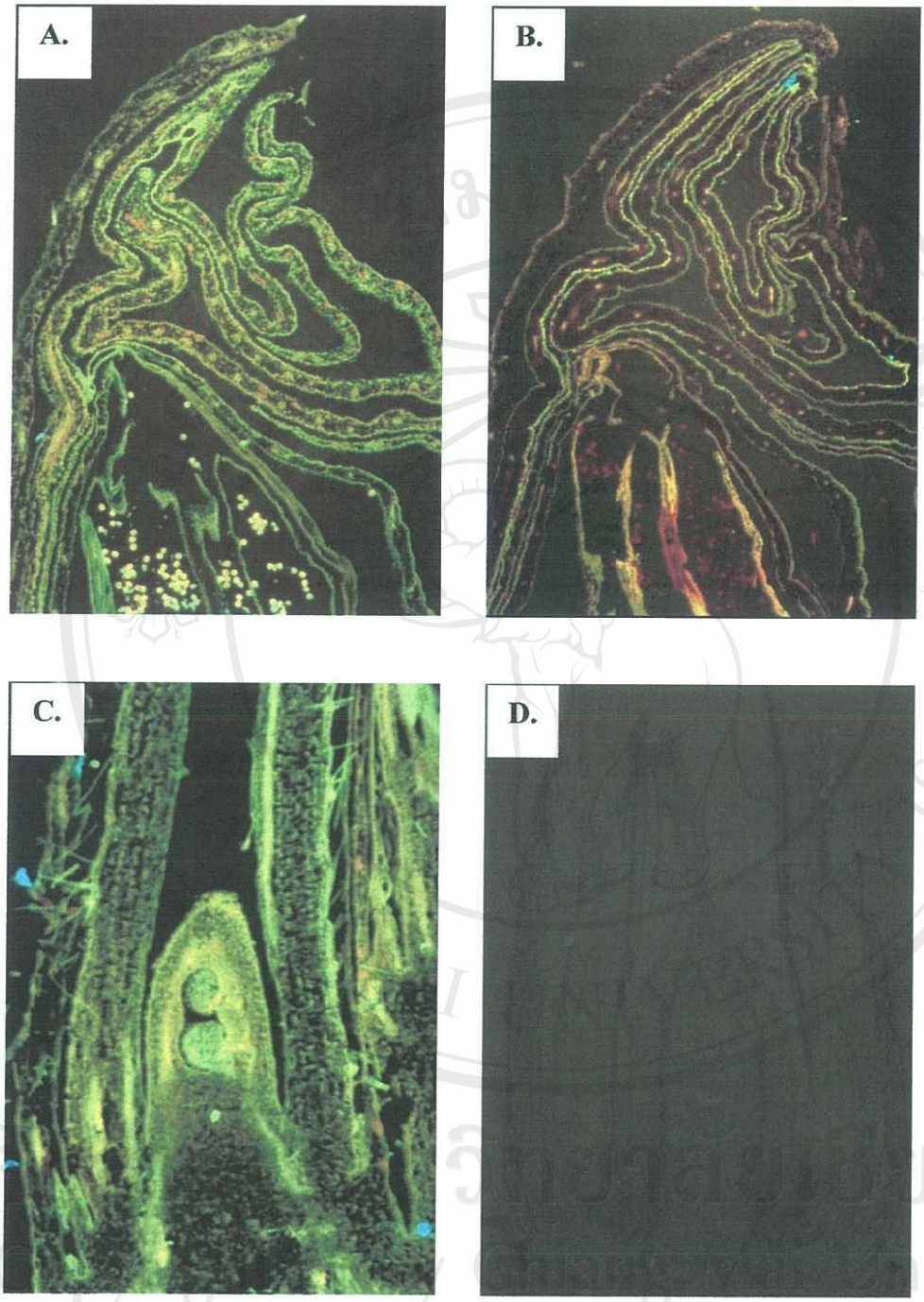

Figure 3. 3 Long-section of groundnut flower after stained with aniline blue (A.) and safanin O (B.) and groundnut embryos after stained with aniline blue (C.) and Hematoxylin (D.) under UV light microscope

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3.2.1.2 Preparation of suspension of Aspergillus flavus

Aflatoxins-producing and Green Fluorescent Protein (GFP) strain of *A. flavus* were grow in 12 hr alternating daylight and darkness on M3S1B selective medium (Giffin and Garren, 1974) plates at 27 °C for 10 days (Figure 3.4). Conidia were washed from M3S1B plates using sterile deionized water containing 20 drops of Tween 20 L^{-1} and filtered through four layers of sterile cheesecloth. The concentration of conidia was determined with hemacytometer and adjusted with sterile deionized water to 4.0 x 10^6 conidia per ml. The prepared inoculum not immediately used was refrigerated at 4 °C.

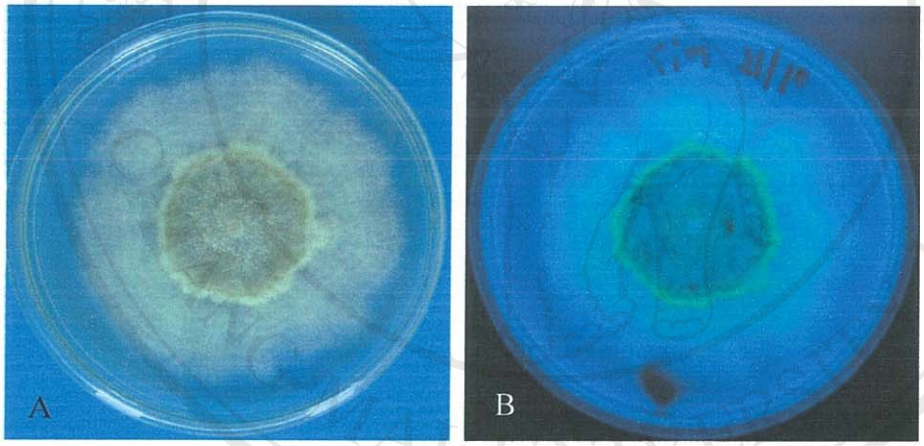

Figure 3.4 The 10 days *Aspergillus flavus* growth on M3S1B selective medium under normal light (A) and ultraviolet light (B).

3.2.1.3 Source of seeds

Groundnut seed samples were obtained from Department of Agronomy, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand. Among them, two varieties were a resistant J_{11} (Mixon, 1980) and a susceptible Tainan9 variety (Yingthongchai, 1994).

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3.2.1.3 Post-harvest determination

Seeds were inoculated with spore suspension *A. flavus* (prepared earlier as section 3.2.1.2) and placed in petri-dishes. The petri-dishes with seeds were maintained in 100% relative humidity (RH) at temperature favoring *A. flavus* growth, 30°C. After 7 days of incubation, the infected seedcoat were cut (Figure 3.5) and fixed in F.A.A. (50 % EtOH, 5 % glacial acetic acid, 10 % formalin and 35 % water (v/v)) at least 18 hr. Then followed by alcohol dehydration and paraffin/ TBA (tert-butyl alcohol) infiltration (Table 3.1).

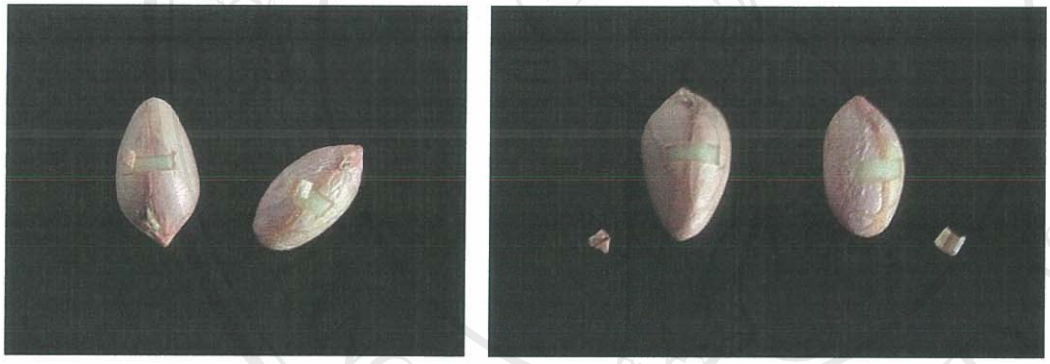

Figure 3.5 Infected seedcoat of groundnut by *Aspergillus flavus* were cut

The paraffin oil/TBA mixture was poured off all and added pure liquid paraffin to the bottle of tissues. Keep the bottle in hot oven at 58-60 °C about 7 days by changes the pure liquid 3-4 times. The tissue samples were embedded in paraffin and sectioned by rotary microtrome at 13-15 μm (Ruzin, 1999). The ribbons of tissues were pasted on glass microscope slides that smeared the adhesive suspension before.

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Table 3.1 Dehydrate and transfer to paraffin using TBA as the intermediate solvent, 1 night / step

Steps	95 % EtOH	100 % EtOH	Distilled water	TBA	Paraffin oil
a	40		50	10	
b	50		30	20	
c	50		15	35	
d	45			55	
e*		25		75	
f				100	
g				50	50

* Add 0.1% erythrosine in step "e"

Alternatively placed the slides overnight on hot plate at 40 °C. Deparaffinize in xylene followed by hydration to 30% EtOH. Stain 3-5 min hematoxylin ($NH_4Al_1(SO_4)_2 12H_2O$) and 8-10 min aniline blue fluorescence (0.05% Aniline blue dye CI # 42755 in 0.067 M K_2HPO_4 at pH 9.0). Dehydrate in EtOH, clear in xylene and mount cover slip with Canadabalsm. The specimens were identified under the UV microscope and separated *A. flavus* fluorescent positions by Guacos Quantitative Color System Version 2.5.2 program (Ingram *et. al*, 1998).

3.2.1.4 Preharvest determination

Growing aerial pegs (5-10 days old) were sprayed with spore suspension of *A. flavus* strain that prepared earlier as described in section 3.2.1.2. The inoculated pegs of plants were incubated in growth chamber at 30 °C and 100 % RH (Figure 3.6). After incubation at 7 days, the pegs were collected and cut into 1.5 - 2 cm long

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pieces from the end of peg. Then the pegs were fixed in F.A.A, dehydrated with alcohol, paraffin/TBA infiltrated, sectioned by rotary microtrome and stained hematoxylin and aniline blue fluorescence similarly with the seedcoat determination.

Figure 3.6 Inoculated pegs were incubated in growth chamber at 30 °C and 100 % relative humidity.

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3.2.2 Peg screening method

3.2.2.1 Optimum time for surface sterile

The aerial pegs (5-10 days old) of J11 and Tainan9 genotypes were collected at random from 50 plants of each genotype. The pegs were cut into 1.5 - 2.0 cm long pieces, rinsed for 2 min in running tap water. Half of amount pegs of each variety were used for control and remaining half was taken randomly and 10 pegs were placed in each petri-dishes. Five petri-dishes i.e. 10 pegs, were considered as one replication. Ten pegs were inoculated with 1 ml suspension of aflatoxin-producing *A. flavus* (prepared earlier as section 3.2.1.2) and incubated under 100 % relative humidity at 30 °C. Eight hours after incubation, sample pegs of each replication was soaked with 3 % sodium hypochlorite for 0, 1, 3, 5, 7, 9 and 11 minutes followed by rinsed in sterile distilled water for three times. The adhering water around the pegs was soaked by sterile blotter paper. Then the pegs were placed in sterilized 9 cm-diameter petridishes containing about 20 ml solidified M3S1B medium at the rate of 10 pegs per plate (Figure 3.7). The plates were kept under 12 hour alternating daylight and darkness at about 27 °C. After five days, the fungus-infected pegs were observed and recorded.

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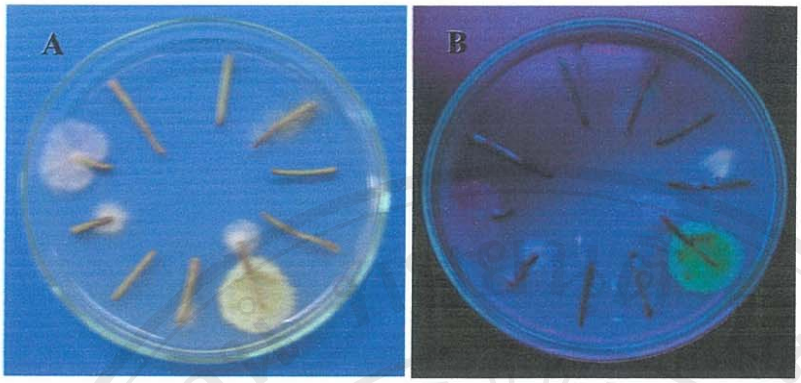

Figure 3.7 Inoculated pegs on M3S1B selective medium under normal light (A) and UV light (B).

3.2.2.2 Appropriate time after inoculation for assessing resistant genotypes by peg screening method

Seed of six-groundnut variety named as ICGX990090, ICGX990091, ICGX990092, ICGX990093, ICGX990094 and ACC232 were planted in 10-liter pots containing sterile soil. Aerial pegs (5-10 days old) were anointed with spore suspension (prepared earlier as Section 3.2.1.2). The inoculated pegs of plants were incubated in 30 °C and 100 % RH. After incubation at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days, the pegs were collected and cut into 1.5-2 cm long pieces. In each category, 5 replication, 10 pegs were considered as one replication. The pegs were surface sterilized with 3 % sodium hypochlorite for optimum time (as Section 3.2.2.1) followed by rinsed in sterile distilled water for three times. The adhering water around the pegs was soaked by sterile blotter paper. Then the pegs were placed in sterilized 9 cm-diameter petridishes containing about 20 ml solidified M3S1B selective at the rate of 5 pegs per plate. The plates were kept under 12 hour alternating daylight and darkness at about 27 °C. After five days, the fungus-infected pegs were observed and recorded.

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3.2.2.3 Appropriate time after inoculation for assessing resistant genotypes by AFHS technique

Six-groundnut variety in the same set as Section 3.2.2.2 were planted in 10-liter pots soil. Aerial pegs (5-10 days old) were anointed with spore suspension (prepared earlier as Section 3.2.1.2). The inoculated pegs of plants were incubated in 30 °C and 100 % RH. After incubation at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days, the pegs were collected and cut into 1.5-2 cm long pieces. Then the pegs were fixed in F.A.A, dehydrated with alcohol, paraffin/TBA infiltrated, sectioned by rotary microtrome and stained hematoxylin and aniline blue fluorescence. The specimens were identified under the UV microscope and separated *A. flavus* fluorescent positions by Guacos Quantitative Color System similarly in the section 3.2.1.

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3.2.3 Seed screening method

3.2.3.1 Optimum time for surface sterile

Sound mature seeds of uniform size and shape with intact seedcoat of J11 and Tainan9 genotypes are selected. Half amounts of seeds of each variety were used for uninoculation (control) and remaining half was taken randomly and 10 seeds were placed in each petri-dishes. Finding of three-petridishes i.e., each petri-dishes were considered as one replication. Each petridishes were inoculated with 1 ml suspension of aflatoxin-producing *A. flavus* and incubated under 100 % relative humidity at 30 °C. For uninoculation condition were put the sterilized distilled water 1 ml in each petridishes. After 8 hour of incubation, sample seeds of each replication was soaked with 3 % sodium hypochlorite for 0, 1, 3, 5, 7, 9 and 11 minutes followed by rinsed in sterile distilled water for three times. The adhering water around the seeds was soaked by sterile blotter paper. Then the seeds were placed in sterilized 9 cm-diameter petridishes containing about 20 ml solidified M3S1B medium at the rate of 10 seeds per plate (Figure 3.8). The plates were kept under 12 hour alternating daylight and darkness at about 27 °C. After five days, the fungus-infected seeds were observed and recorded

3.2.3.2 Appropriate time after inoculation for assessing resistant genotypes by seed screening method.

Seed of 11-groundnut genotypes named as J_{11}, Tainan9, VRR245, SK38, KK4, KK5, KK60-1, ICGV86325, ICGS110, PRO40-1, CM40-14-2 were assessment. In each category, 4 replication of each genotypes, 10 seeds were considered as one replication and put in each petridishes. Seed were inoculated with spore suspension of aflatoxins-producing *A. flavus*. The inoculated seeds were

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incubated in 30 °C and 100 % RH. After incubation at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days, the seeds were surface sterilized with 3 % sodium hypochlorite for optimum time (as Section 3.2.3.1) followed by rinsed in sterile distilled water for three times. The adhering water around the seeds was soaked by sterile blotter paper. Then the seeds were placed in sterilized 9 cm-diameter petridishes containing about 20 ml solidified M3S1B selective at the rate of 5-10 seeds per plate. The plates were kept under 12 hour alternating daylight and darkness at about 27 °C. After five days, the fungus-infected seeds were observed and recorded.

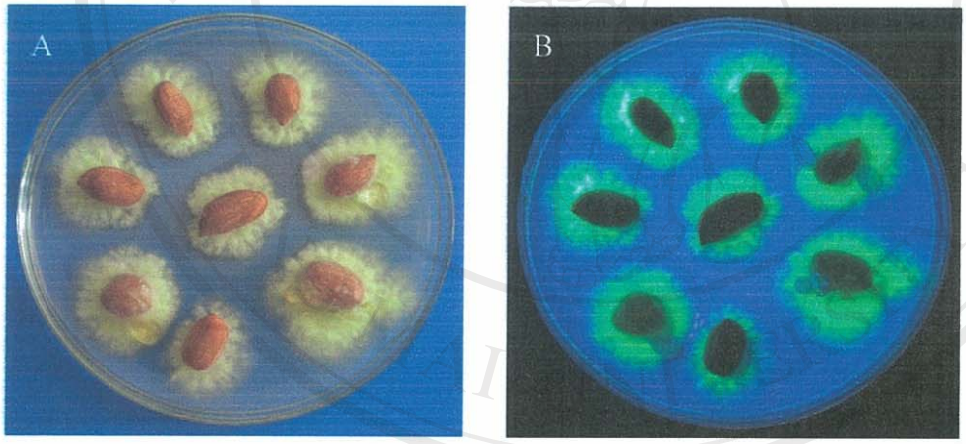

Figure 3.8 Inoculated seeds on M3S1B selective medium under normal light (A) and UV light (B)

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3.3 Results

3.3.1 Aniline blue fluorescence and hematoxylin staining (AFHS) method

Figure 3.9 shows the fluorescence of hyphae, conidia and conidiophores under UV light (B) more clearly than under normal light (A) after staining with aniline blue fluorescence. Similarly, under UV light, the hematoxylin and aniline blue stained specimens, the fungus also fluoresced brightly while the fixed plant tissues were dark. Figure 3.10C illustrates the position of the fluorescent fungus (AF), which distinguished clearly from the dark area of plant tissue (DP). However, differences in the fluorescent areas could be observed among the two groundnut varieties. For the J_{11}, the fluorescent area was detected in outer epidermis, whereas in Tainan9, detecting can be seen further into parenchyma and inner epidermis (Figure 3.10).

Distinguishing of *A. flavus* fluorescent position of seed coat tissue by Quacos program indicated the interaction between treatments and varieties was ecisted (Table 3.2). In inoculation condition, Tainan9 showed significantly higher fluorescent area (14.63 %) than the J_{11} variety (3.11 %). However, under uninoculation condition, percent of fungal infected area did not differed between varieties.

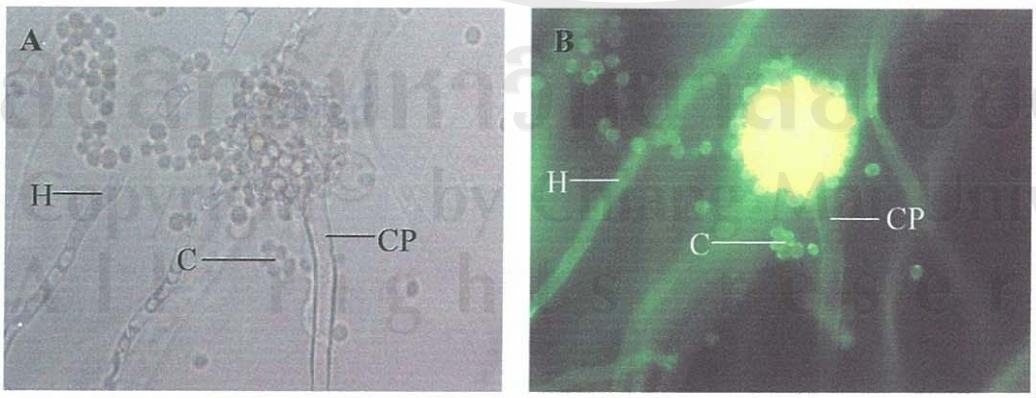

Figure 3.9 The hyphae (H), conidia (C) and conidiophores (CP) of *Aspergillus flavus* under normal light (A) and UV light (B) microscope

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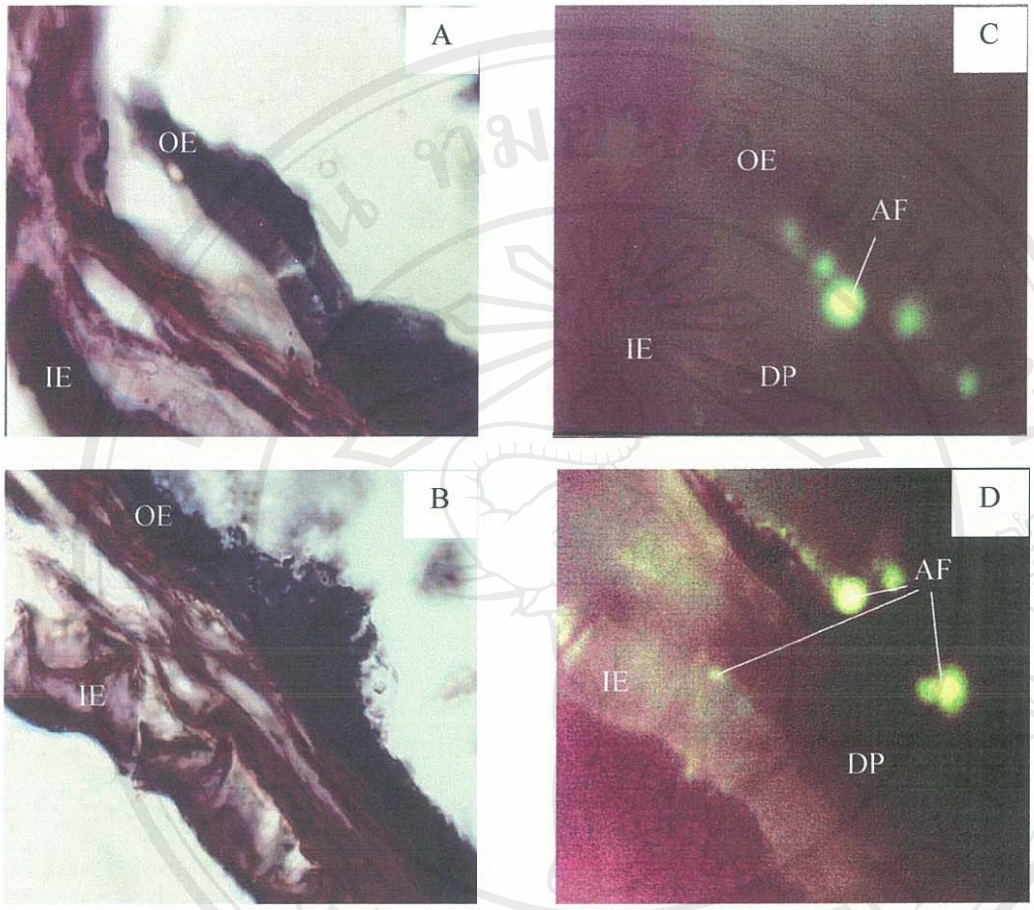

Figure 3.10 Infected seedcoats after staining with aniline blue fluorescence and hematoxylin of J_{11} (A) and Tainan9 (B) under normal light and under UV light of J_{11} (C) and Tainan9 (D), outer epidermis (OE), inner epidermis (IE), *Aspergillus flavus* fluorescence (AF) and dark plant tissue position (DP).

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Table 3.2 Percentage of *Aspergillus flavus* fluorescence in seed coat tissues after staining with aniline blue fluorescence and hematoxylin and separated the fluorescent position by Quacos program

Treatment	*A. flavus* fluorescence area (%)		Average
	Varieties		
	J_{11}	Tainan9	
Uninoculation	0.05	0.02	0.03
Inoculation	3.11	14.63	8.87
Average	1.58	7.32	4.45

LSD (0.05) Varieties = 4.28; Treatments = 4.28;

Varieties x Treatments = 6.06, SE = 1.01

Preharvest determination in pegs found that the fluorescence of infected area was likewise fluoresced in the seedcoat (Figure 3.11). Figure 3.11A shown spotless fungal fluorescence in the cross section peg, the center was embryo and the green fluorescence was vascular bundles. While, Figure 3.10B exposed greatly blight yellow fluorescence of the *A. flavus* infected positions (AI) that can be separated the difference of fluorescence color from the color of vascular bundles (VB). In addition, the position fungus in peg was clearer than in the seedcoat. Figure 3.11D was enlarged from the upper of Figure 3.11C on which showed fungus might be presented mostly between peg cells.

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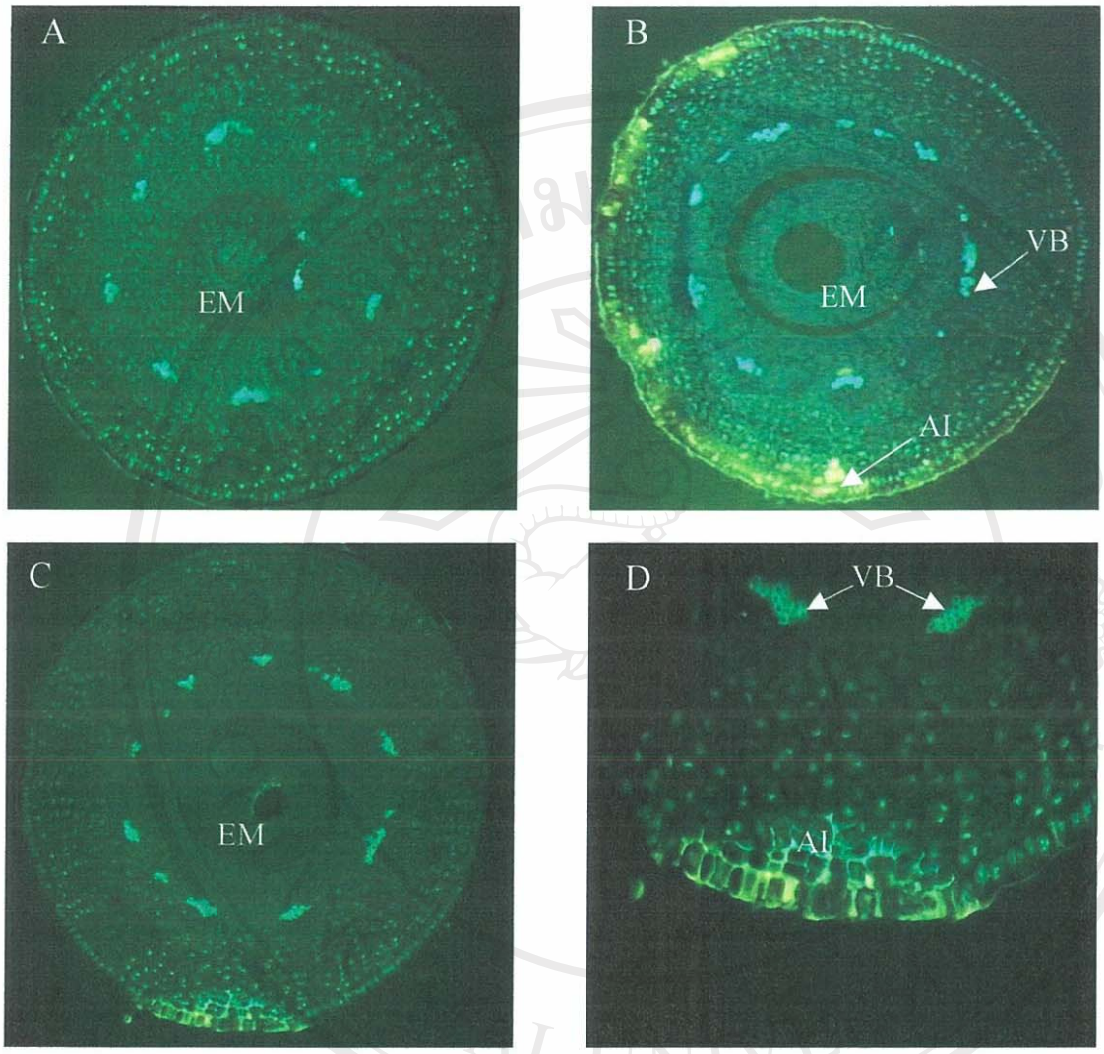

Figure 3.11 Cross section of groundnut pegs and stained with aniline blue fluorescence and hematoxylin under UV light microscope, *Aspergillus flavus* infected positions: AI, vascular bundles: VB and embryo: EM.

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3.3.2 Peg screening method

3.3.2.1 Optimum time for surface sterile

The percentages of *A. flavus* infection were decreased when increasing time for surface sterile pegs by 3 % sodium hypochlorite both in J_{11} and Tainan9 groundnut genotypes (Figure 3.12). In Table 3.3, groundnut pegs were infected 100 percent by *A. flavus* in inoculation treatment after surface sterile by sterilized distilled water but only 50 and 30 % of J_{11} and Tainan9 were found respectively in uninoculation treatment. After surface sterilized for 1 min, percent-infected pegs were declined to zero under uninoculation condition but was 100 % in J_{11} and slightly decreased in Tainan 9 genotype (85 %) under inoculation treatment. The percent-infection were appeared significantly difference between genotypes under inoculation condition after sterile by 3 % sodium hypochlorite for 5 min.

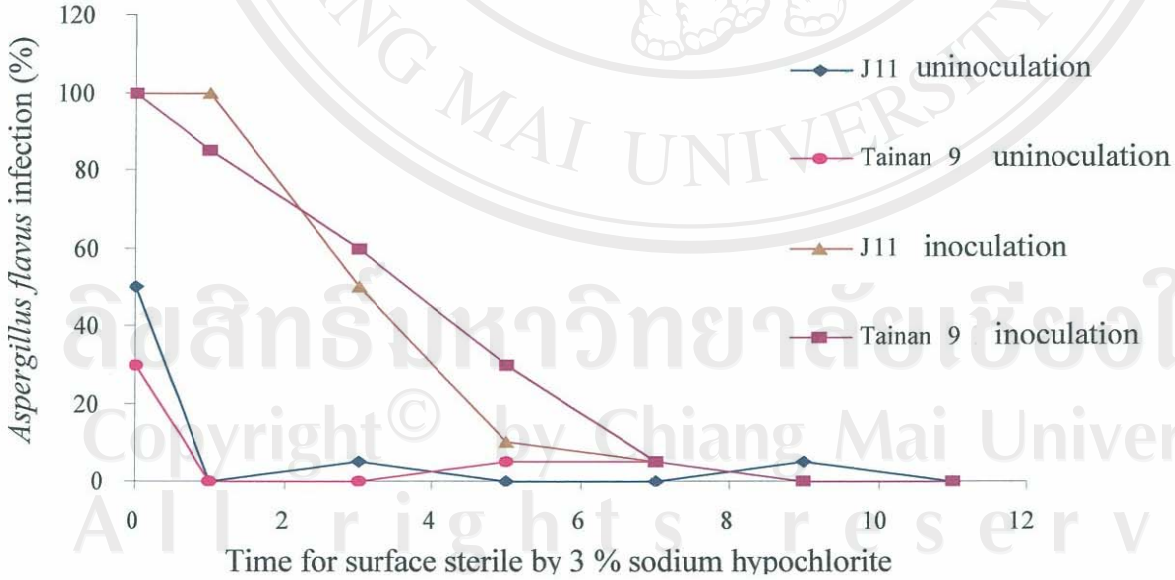

Figure 3.12 Percent infection by *Aspergillus flavus* of J_{11} and Tainan9 groundnut genotypes under inoculation and uninoculation condition.

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Table 3.3 The percentage of *A. flavus* infection after varies time for surface sterile pegs by 3 % sodium hypochlorite in J_{11} and Tainan9 groundnut genotypes.

Time for clean by Clorox 3 %	*A. flavus* contamination (%)				Mean
	Un-inoculation		Inoculation		
	J_{11}	TN9	J_{11}	TN9	
0	50	30	100	100	70.0
1	0	0	100	85	46.3
3	5	0	50	60	28.8
5	0	5	10	30	11.3
7	0	5	5	5	3.8
9	5	0	0	0	1.3
11	0	0	0	0	0.0
Mean	8.6	5.7	37.9	40.0	23.0

$LSD_{0.05}$ Treatment (A) = 5.4 Time (B) = 7.2 Interaction (A x B) = 15.1 CV (%) = 44.6

3.3.2.2 Appropriate time after inoculation for assessing resistant genotypes by peg screening method

The effects of time periods after inoculation by *A. flavus* caused percentage-infected pegs are shown in Figure 3.13. It showed that the percent-infected pegs increased when the incubation time increased after inoculation.

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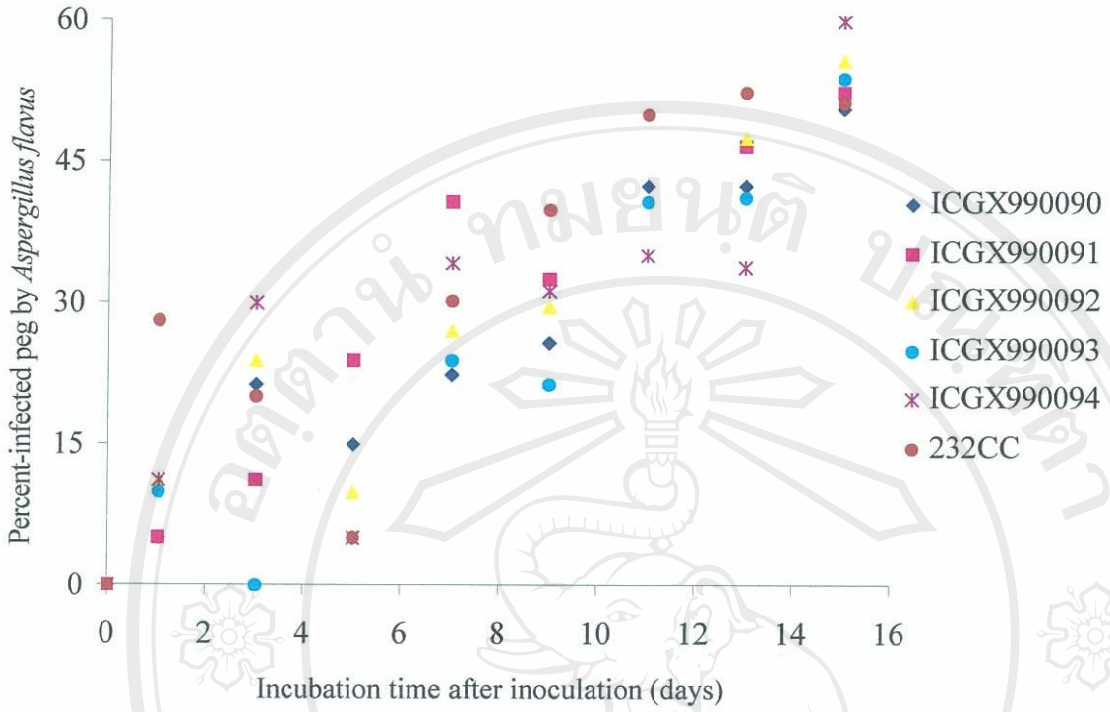

Figure 3.13 Relationship of incubation time to percent-infected pegs by *Aspergillus flavus* in six groundnut genotypes.

Percentage of peg infection did not significantly differed among these six groundnut genotypes (Table 3.4), but differently significant was found between the day periods after inoculation. At 15 days of incubation showed the highest of pegs contamination but not different with 11 and 13 day of incubation. However, the percentages of pegs contamination after incubation for 1-5 days were lower than the incubation time for 7 days until 15 days.

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Table 3.4 Percentage of infected peg after varies the incubation times (days) from 0 to 15 days of six groundnut genotypes.

	Aspergillus flavus infection of groundnut genotypes (%)						
Days	ICGX990090	ICGX990091	ICGX990092	ICGX990093	ICGX990094	232CC	Days
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	5.0	5.0	11.3	10.0	11.3	28.0	11.8
3	21.3	11.3	23.8	0.0	30.0	20.0	17.7
5	15.0	23.8	10.0	5.0	5.0	5.0	10.6
7	22.5	40.8	27.0	23.8	34.3	30.3	29.8
9	25.8	32.5	29.6	21.3	31.3	40.0	30.1
11	42.5	63.8	62.5	40.8	35.0	50.0	49.1
13	42.5	46.6	47.5	41.3	33.8	52.5	44.0
15	50.8	52.5	55.8	53.8	60.0	51.3	54.0
G^+	25.1	30.7	29.7	21.8	26.7	30.8	27.5

$LSD_{0.05}$, Days (A) = 12.52 genotypes = ns Interaction (A x B) = ns CV (%) = 60.53

$^+$ Genotypes

3.3.2.3 Appropriate time after inoculation for assessing by AFHS technique

The percent-infected peg area fluorescence by *A. flavus* in six groundnut genotypes was increased after prolong the time after inoculation or the incubation time from 1 to 15 days (Figure 3.14).

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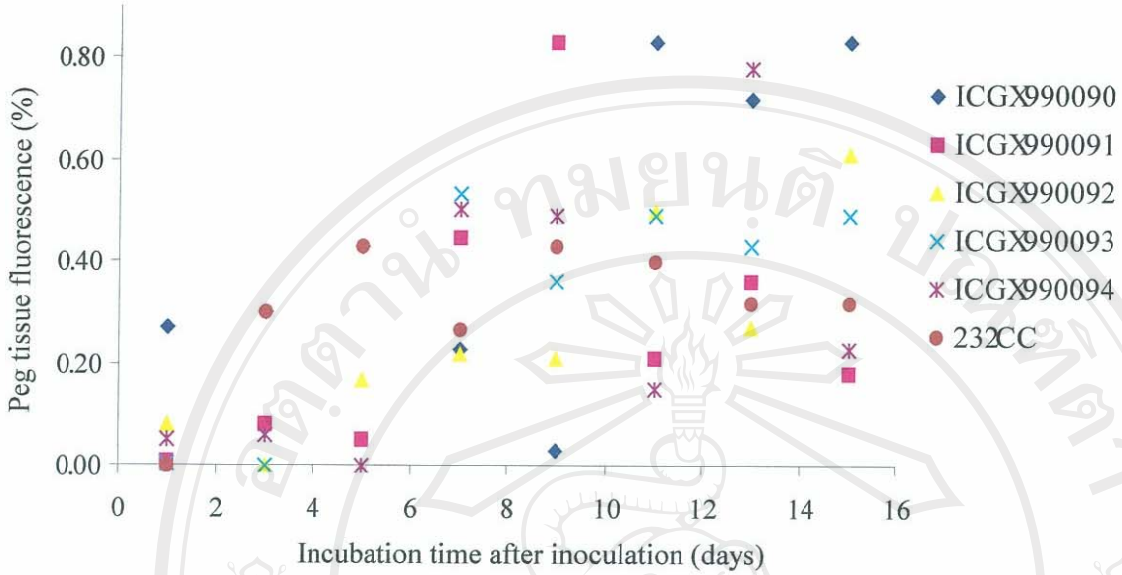

Figure 3.14 The effects of incubation time after inoculation to percent-infected peg area fluorescence by *Aspergillus flavus* in six groundnut genotypes.

Percent-infected peg area fluorescence showed significantly difference ($p \leq 0.05$) after delay incubation time (Table 3.5). This result showed that the percent infected peg area fluorescence were slowly increasing from 0.07 % at 1 day after inoculation to the highest at 13 days (0.48 %). Significant difference was not identified between groundnut genotypes.

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Table 3.5 Percentage of infected peg area fluorescence after vary the incubation times (days) of six groundnut genotypes.

Days	*Aspergillus flavus* infection of groundnut genotypes (%)						
	ICGX990090	ICGX990091	ICGX990092	ICGX990093	ICGX990094	232CC	Days
1	0.27	0.01	0.00	0.00	0.05	0.00	0.07
3	0.18	0.08	0.00	0.00	0.06	0.30	0.12
5	0.43	0.05	0.17	0.00	0.56	0.08	0.18
7	0.23	0.45	0.22	0.00	0.02	0.29	0.37
9	0.03	0.22	0.53	0.43	0.07	0.40	0.39
11	0.83	0.21	0.50	0.49	0.15	0.40	0.43
13	0.72	0.36	0.27	0.43	0.78	0.32	0.48
15	0.83	0.18	0.61	0.49	0.23	0.32	0.44
G^{+}	0.46	0.27	0.26	0.29	0.28	0.31	*0.31*

$LSD_{0.05}$, Days (A) = 0.2471 genotypes = ns Interaction (A x B) = ns

$^{+}$ Genotypes

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3.3.3 Seed screening method

3.3.3.1 Optimum time for surface sterile

After increasing time from 0 to 11 min for surface sterile groundnut seeds by 3 % sodium hypochlorite, the percent-contaminated seed by *A. flavus* under inoculation and uninoculation were decreased from 100 % to zero (Figure 3.15). Table 3.6 shows the percent-contaminated seed after surface sterile by sterile distilled water were 100 % under inoculation condition but only 20 and 30 % (J_{11} and Tainan9 respectively) in uninoculation treatment. After surface sterile by 3 % sodium hypochlorite for 1 min, the percent-infected seed were decreased to zero under uninoculation condition, while maintain 40 and 30 % under inoculation treatment of J_{11} and Tainan9 respectively. However, the significant difference between these two genotypes was noticed under inoculation condition after surface sterile seed by 3 % sodium hypochlorite from 1 to 9 min.

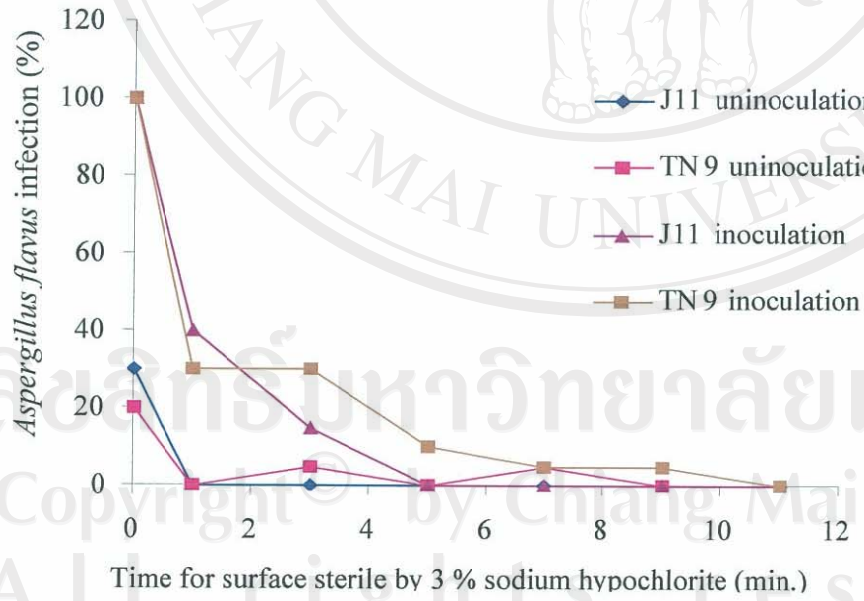

Figure 3.15 Percentage of groundnut seed contaminated by *Aspergillus flavus* after varies the time for surface sterile by 3 % sodium hypochlorite under uninoculation and inoculation treatments.

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Table 3.6 Percentage of contaminated seed after surface sterilized by 3 % sodium hypochlorite of J_{11} and Tainan9 groundnut genotypes.

Time for clean by Clorox 3 % (min.)	Contaminated seed (%)				
	Uninoculation *A. flavus*		Inoculation *A. flavus*		mean
	J_{11}	Tainan9	J_{11}	Tainan9	
0	20	30	100	100	62.5
1	0	0	40	30	17.5
3	5	0	15	30	12.5
5	0	0	0	10	2.5
7	5	0	0	5	2.5
9	0	0	0	5	1.3
11	0	0	0	0	0.0
Mean	4.3	4.3	22.1	25.7	14.1
$LSD_{0.05}$	Treatment (A) = 1.5		Time (B) = 1.9	A x B	= 3.9
$LSD_{0.01}$	= 2.0		= 2.6		= 5.2

CV (%) = 16.90

3.3.3.2 Appropriate time after inoculation for assessing resistant genotypes by seed screening method

The effects of time periods after inoculation by *A. flavus* to percent infected seed of eleven groundnut genotypes shows in Figure 3.16. It showed that the increasing of incubation time after inoculation will increase the percent-infected seed by *A. flavus.*

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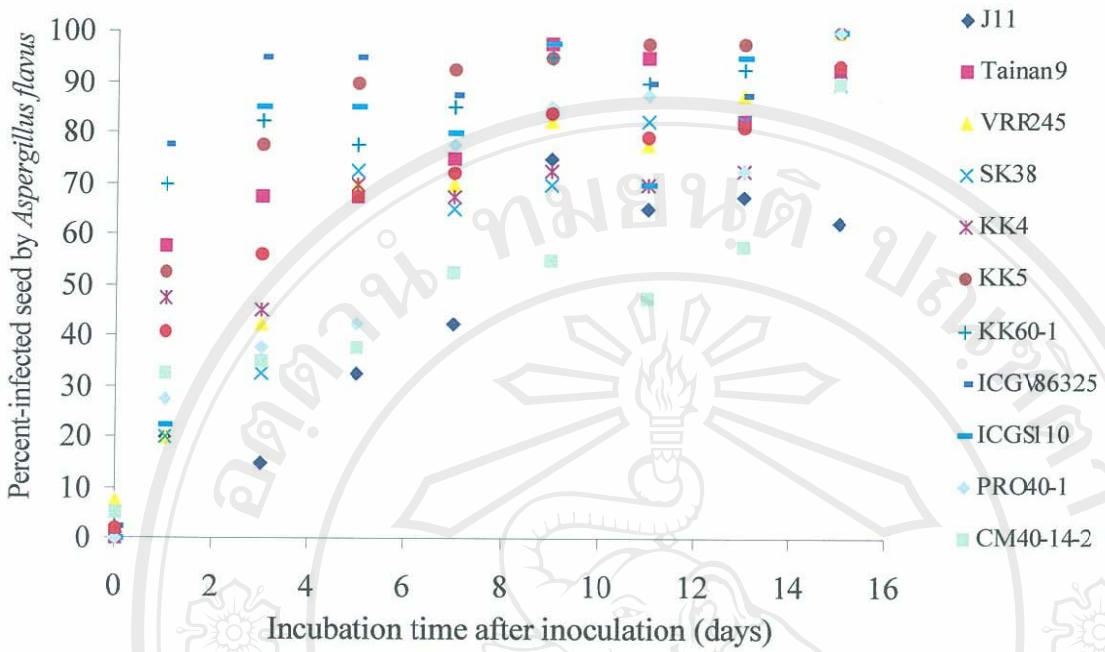

Figure 3.16 The effects of incubation time after inoculation caused percent-infected seeds by *Aspergillus flavus* in eleven groundnut genotypes.

Table 3.7, shows percent-seed contamination which were significant difference among the time of incubation, genotypes and their interaction. It varied between genotypes from 42.22 % (J_{11}) to 81.39 % (ICGV86325). After increasing incubation time the percent seed infection increased from 2.05 % (0 day, not incubation) to 93.41 % (15 days of incubation). When comparing the percent infected seed of each genotypes after delayed incubation times found that only one day of incubation time shows the difference between genotypes, but some of susceptible genotypes (ICGS110, SK38) were not differed. However, after 3 days of incubation could be separated all genotypes from the resistance J_{11}, excepted CM40-14-2 at 5 day of incubation.

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Table 3.7 Percentage of contaminated seed after vary the incubation times (days) of 11 groundnut genotypes.

Genotypes	Percent infected seed by *Aspergillus flavus* after vary time of incubation (Days)									Genotypes (B)
	0	1	3	5	7	9	11	13	15	
J11	0.0	20.0	15.0	**32.5**	**42.5**	75.0	65.0	67.5	62.5	**42.22**
Tainan9	0.0	57.5	67.5	67.5	75.0	97.5	95.0	82.5	92.5	**70.56**
VRR245	7.5	20.0	42.5	70.0	70.0	82.5	77.5	87.5	100.0	**61.94**
SK38	0.0	20.0	32.5	72.5	65.0	70.0	82.5	82.5	90.0	**57.22**
KK4	0.0	47.5	45.0	70.0	67.5	72.5	70.0	72.5	92.5	**59.72**
KK5	5.0	52.5	77.5	90.0	92.5	95.0	97.5	97.5	100.0	**78.61**
KK60-1	2.5	70.0	82.5	77.5	85.0	95.0	90.0	92.5	100.0	**77.22**
ICGV86325	2.5	77.5	95.0	95.0	87.5	97.5	90.0	87.5	100.0	**81.39**
ICGS110	0.0	22.5	85.0	85.0	80.0	97.5	70.0	95.0	100.0	**70.56**
PRO40-1	0.0	27.5	37.5	42.5	77.5	85.0	87.5	72.5	100.0	**58.89**
CM40-14-2	5.0	32.5	35.0	**37.5**	**52.5**	55.0	47.5	57.5	90.0	**45.83**
Days (A)	**2.05**	**40.68**	**55.91**	**67.27**	**72.27**	**83.86**	**79.32**	**81.36**	**93.41**	**64.02**

$LSD_{0.05}$ Days (A) = 4.87 Genotypes (B) = 5.38 A x B = 5.36

$LSD_{0.01}$ = 6.38 = 7.05 = 7.05

CV (%) = 18.13

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3.4 Discussion

The appropriate method in controlling *A. flavus* infection is the abilities of variety to resist the penetration of hyphae in mesophyll tissue (Hilu and Hooker, 1964). Plant health can be greatly influenced by interaction with pathogenic or mutualistic fungi, and microscopic examination of these interactions provides information about biology of host-parasite relationships and serves as a useful tool for diagnosticians. Hood and Shew (1996) developed KOH-aniline blue fluorescence staining technique to study of plant-fungal interaction. Therefore, this technique produced a high degree of resolution and contrast between hyphae and host-plant tissues. Trese and Loschke (1990) improved on a novel staining technique to monitor fungal growth in corn inoculated with *Exerohilum turcicum, Bipolaris maydis or Bipolaris zeicola.* They also found the high contrast resolution of the mycelia of pathogenic fungi in corn leaf tissue after staining with calcofluor and destaining with cellulose. However, determining for *A. flavus* infected position in groundnut tissues (*Arachis hypogae* L.) by using fluorescent-dyes and brighteners, as the technique has been problematic. Both the plant tissues and the fungus are automatically fluorescent under that technique thereby masking the position.

Aniline blue fluorescent has been a good general stain for botanical and histochemistry, particularly to stain β-1,3-glucans such as callose in phloem and composition of fungal cell wall. This callose will be fluorescing yellow to green at near UV excitation wavelength (Hood and Shew, 1999; Kang and Buchenauer, 2000; Du *et al.*, 1999; Ruzin, 1999). Callose depositions were also found in host structural responses for plant resistant mechanisms. The accumulation of these polymers in cell walls of resistant seedlings was rapid and localized around the pathogen, apparently to

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restrict pathogen entry (Mert Türk, 2002; Trillas *et al.,* 2000). While, host-produced callose deposits often were not observed around penetration structure (Hood and Shew, 1999). However, in present research, the fluorescence may be both callose from *A. flavus* cell wall and its accumulation in plant cells. Especially, in infected peg fluorescent should be both mechanisms clause this fluorescence. To be a result of the groundnut still growing during the fungus infection and plant may have abilities to produce chemicals or mechanisms for affected the invasion of the pathogen higher than the seed period.

As the *A. flavus* is fluorescent clearly under the UV light, determining for the fungus infection would be more effective. Separation of the plant tissue from the fungus infected position could be made using the hematoxylin and aniline blue staining process, in which the plant tissues were fixed as dark areas in contrast with the fluorescent area of the infected fungus. The higher fluorescent area observed in inoculated Tainan9 would proof the response of susceptibility of the variety referring back to Tainan9 susceptible to *A. flavus* infection (Yingthongchai, 1994) in consequence, indicate the effective of the staining procedure. Excellent resolution and contrast visualization of fungal taxa were provided by the aniline blue fluorescence and Hematoxylin technique compared to other fluorescent methods of staining hyphae in plant tissue, KOH-aniline blue (Hood and Shew, 1996) and safanin O and hematoxylin (Suriyong, 1997).

Resistance in groundnuts to infestation of peg and seeds by *Aspergillus flavus* has been reported (Bartz *et al.,* 1978, Ghewande *et al.,* 1993, Nayak and Khatua, 1992, Upadhyaya *et al.,* 2001). This resistance has been based on a dried seed screening and peg screening assays. While, sodium hypochlorite is the one essential

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chemical to surface sterilized that the fungal contamination was decreased after longer rinsed by sodium hypochlorite or the higher concentration. In this experiment, 3 % sodium hypochlorite for 5 min was the optimum for surface sterilized groundnut pegs and 3 min for seed. It is to be result of the difference between resistant genotype (J_{11}) and susceptible genotype (Tainan9) could be distinguished. Not only the condition, temperature and moisture (Holbrook *et al.,* 2000), for screening was important to determine the resistant genotype but the optimum method to evaluation also critical (Wynne *et al.,* 1991). While, the frequency of epidermal hair or papillae and the differently rough surface of plant structures support to differed efficient surface sterilization and obstruct the pathogen penetration (Huang, 2001; Comménil *et al.,* 1997).

Under suitable conditions, *A. flavus* invade the susceptible cultivar faster, a short time to infection and high rate of infection, than the resistant cultivar (Vazquez-Barrios *et al.,* 2001). Due to this reason, the critical time after inoculation or invasion of the fungus is the significant period for assesses the resistant genotypes. From the results could be suggested that should be screened the resistant pegs and seed by peg and seed screening methods and AFHS technique as previously to infection by *A. flavus* after 7 days of inoculation under 100 % RH and 30 °C. Vazquez- Barrios *et al.* (2001) reported that the time of infection to symptom development of *A. flavus* at 0.97 A_w levels was 6.4 days in pecan kernels. The corresponding values for *A. parasiticus* were 5.2, 10.2 and 22.9 days, respectively at 0.97, 0.90 and 0.86 A_w levels. Complete colonization was not reached when kernels were kept at 0.86 A_w. Means of rate of infection for 0.90 and 0.97 A_w were 0.20 and 0.73 for *A. parasiticus* and 0.13 and 0.53 for *A. flavus*, respectively. However, not different between

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groundnut genotypes in peg screening method may because these groundnut genotypes has been evaluated as the resistant genotypes to *A. flavus* infection at ICRISAT, ICGX990090, ICGX990091, ICGX990092, ICGX990093 and ICGX990094 (ICRISAT, 2002) and aflatoxin production (232CC) at Georgia university. Thus six genotypes may contain some characters resistance to the infection of *A. flavus*.

In conclusion, this research indicated that Aniline blue fluorescence and hematoxylin staining (AFHS) technique was the efficient method to evaluate mechanism of groundnut resistance to infection by *A. flavus*, especially in active mechanism of groundnut peg. The appropriate procedures, optimum surface sterilization by 3 % sodium hypochlorite for 5 min was the optimum for groundnut pegs and 3 min for seed and time after inoculation for assessment was more than 7 days at 100 % RH 30 °C.

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