CHAPTER III

Materials and Methods

3.1 Materials

3.1.1 Laboratory Facilities and Equipment

1. Plastic Plates, American PrecisionPlastics (Northglenn, CO)

2. Edge GARD® Hood (Laminar flow hood), Baker Company Inc. (Sanford, ME)

3. Well Cell Culture Cluster and Netwell TM Mesh (74μm and 200 μm) and Plates System, Costar® Corning Incorporated (Corning, NY)

4. Summit HPLC system with a Photochemical Reactor for Enhanced Detection (PHRED, Aura Industries, NY), Dionex Corporation (Houston, TX)

5. Eppendorf tubes, Eppendorf (Germany)

6. Low Incubator, Lab-line Instruments Inc. (Melrose Park, IL)

7. Culture Plate Inserts (Pore Size: 0.4 μm), Millipore (Bedford, MA)

8. Horizontal Autoclave, Tuttnauer Corporation (Hauppauge, NY)

9. Low Temperature Incubator. VWR Company (VWR Scientific Model 2015)

3.1.2 List of Chemicals

Chemical	Manufacturer
2,6-Dichloro-4-nitroaniline	Aldrich
Acetonitrile	EMD Chemicals Inc.
Agar Bacteriological	Oxoid
Ammonium Iron (III) Citrate	Aldrich
Ammonium Molybdate	Fisher
Ammonium Molybdate	Mallinckrodt®
Ammonium Sulfate	J.T. Baker
Ammonium Sulfate	Fisher
Avid®	Novartis Crop Protection
Bacto Agar	BD
Bacto Malt Extract	BD
Bacto Peptone	BD
Bacto Yeast Extract	BD
Calcium Chloride	J.T. Baker
Chloramphenicol	EMD
Cupric Sulfate,	Pentahydrate Sigma
Dextrose, Anhydrous	J.T. Baker
Dextrose,Anhydrous (Glucose)	EMD
Difco Agar	BD
Ethyl Alcohol	USP Aaper

List of Chemicals (continue)

Chemical	Manufacturer
Ethanol (EtOH)	Aaper Alchohol and Chemical Co.
Ferric Sulfate, n-hydrate	J.T. Baker
Ferrous Sulfate, Heptahydrate	Sigma
Ferrous Sulfate 7-Hydrate	Mallinckrodt®
Glycerol	EMD
Manganous Sulfate, Monohydrate	Fisher
Methanol	EMD
Magnesium Sulfate 7-Hydrate	J.T. Baker
Manganese Chloride 4-Hydrate	Fisher
p-(Dimethylamino)benzaldehyde	J.T. Baker
pH 10.00 blue buffer	Fisher
pH 4.00 red buffer	VWR
pH 7.00 yellow buffer	VWR
Potassium Chlorate	EMD
Potassium Chloride	J.T. Baker
Potassium Phosphate, Dibasic	J.T. Baker
Potato Dextrose Broth	EM Science
Potato Dextrose Agar (PDA)	BD
Rose Bengal	Fisher
Sodium Chloride	J.T. Baker
Sodium Citrate Dihydrate	J.T. Baker

List of Chemicals (continue)

Chemical	Manufacturer
Sodium Hydroxide	J.T. Baker
Sodium Nitrate	J.T. Baker
Sodium Nitrite	J.T. Baker
Sodium Sulfate	EM Science
Sodium Sulfate, Anhydrous	EMD
Sodium Tetraborate Decahydrate	The Coleman & Bell Co.
Sucrose	J.T. Baker
Triton X-100	Sigma
Zinc Sulfate, Heptahydrate	Sigma
Zinc Sulfate 7-Hydrate	The Coleman & Bell Co.

3.1.3 Isolates

Isolates of *A. flavus* and some related species including *A. parasiticus*, *A. tamarii*, and *A. nomius* were obtained from the Fungi Section, BCU Herbarium (isolates 001 to 256), Department of Botany, Faculty of Science, Chulalongkorn University, Thailand.

Comparative standard *A. flavus* strains included NRRL 3357, NRRL 21882, TX 9-8, F3W4, Af 53, and Af 13 were obtained from Department of Plant Pathology & Crop Physiology, Louisiana State University, LA, USA.

Strains of *Aspergillus* section *Flavi Aspergillus* section *Flavi* including *A. parasiticus* SRRC 75 and 143-A, *A. nomius* SRRC 375 and 362, *A. oryzae* SRRC 302, 2085, 480, 2079, and 2044, *A. tamarii* SRRC, and *A. pseudotamarii* SRRC SRRC 2420 and 2428 were obtained from the United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center (USDA-ARS, SRRC), New Orleans, LA, USA.

3.2 Methods

3.2.1 Collection of soil samples from corn fields in Thailand

Aspergillus section *Flavi* including *A. flavus* and *A. parasiticus* which produces aflatoxin, is widespread in the environment and have more ability to contaminate crops such as corn (Horn 2003). In the study, surveys were conducted in corn producing areas of three agro-ecological zones including Northern, Northeastern, and Central Thailand, to evaluate distribution of *Aspergillus* section *Flavi* in corn producing areas in Thailand. Soil samples (approximately 500 g) from the major corn producing areas in Thailand were collected for *Aspergillus* section *Flavi* isolation, identification and characterization. The samples were placed in an individual plastic bag. If condensation formed on the bag, the sample was dried in the convection air oven at 40 ^{O}C for 1 to 2 days.

3.2.2 *Aspergillus* section *Flavi* isolation

Aspergillus flavus and parasiticus agar (AFPA) was prepared and used in this study because both *A. flavus*, and *A. parasiticus* produced yellow orange when viewed from the reversed side of the plate (Pitt et al., 1983). Ten grams of soil were added into 250 ml Eelenmeyer flask containing 100 ml of 0.1% peptone saline solution (pH 5.5). The flasks were shaken at 150 rpm at 30^{O}C for 60 min. Soil suspension (10 µl) was poured on AFPA plates. The plates were then incubated at 30^{O}C for 3 to 5 days. *A. flavus* and *A. parasiticus* were identified by the orange color when viewed from the reverse side of the plate. Isolated fungi were re-inoculated into 5%V8 agar (pH 5.5) and incubated at 30^{O}C, 7 days for mycelium and spore formation respectively. Fungal spores were suspended in 1 ml sterile distilled water in 5 ml screw-capped tube or vial.

Spore suspension of each isolate of *Aspergillus* was inoculated on 5% V8 agar (pH 5.5) using cross-steak technique. The cultures were incubated overnight at 30 ^{O}C in the dark to obtain an isolated colony from a single spore. Colony of the fungus was observed, collected and re-inoculated into a new 5% V8 agar. The plates were incubated at 30 ^{O}C in the dark for mycelium and spore formation respectively (1 to 2 days). Fungal spores were collected and suspended in 1 ml of sterile distilled water in 5 ml screw-capped tube or vial.

3.2.3 *Aspergillus* section *Flavi* identification

Several media including Czapek Dox agar (Dox, 1910), Aspergillus flavus and parasiticus agar (AFPA), Potato dextrose agar (PDA), Corn meal agar (CMA), and 5% V8 agar were prepared and used to identify *Aspergillus* section *Flavi* based on morphological characteristics (Pitt et al., 1983 and Klich, 2002). Spore suspension of each isolated of *A. flavus* was inoculated on Czapek Dox agar, AFPA, PDA, CMA, and 5% V8 agar. The cultures were incubated at 30 ^{O}C in the dark, 7 days for colonization and sclerotium formation respectively. The morphological structures (conidium and sclerotium) of *Aspergillus* were studied and identified using the light microscope (Klich, 2002). For further characterization, after 5 days at 30°C in 5% V8 agar, isolates were classified on the basis of colony characteristics and conidial morphology (400X). Isolates with abundant small sclerotia (average diameter <400 μm) were initially classified as the S strain of *A. flavus* (Cotty et al., 1999). Isolates with smooth conidia and large sclerotia (average diameter over 400 μm) were classified as the L strain of *A. flavus* (Cotty, 1989). *A. tamarii, A. nomius* and *A. parasiticus* were initially identified by colony and spore

morphology (Klich et al., 1988) and identifications were confirmed by color reaction on AFPA (*A. flavus* and *A. parasiticus* agar, Pitt et al., 1983).

3.2.3.1 Effect of different surfactant types on fungal colonization

Several surfactants including Tween 40 and Triton X-100 were used to prepare fungal spore suspension. However, growth of *Aspergillus* was inhibited by some types or high concentration of surfactants. In the study, different surfactant types for spore suspension were used to observe *Aspergillus* colonization on the medium. Anionic surfactant (deoxycholic acid, and Niaproof), nonionic surfactants (digitonin, Triton X-100, and Tween 40), and cationic surfactant (CTAB) were used in the study. Spore suspension of each isolated of *A. parasiticus*, *A. nomius*, *A. tamarii*, and *A. flavus* were inoculated into 5% V8 agar (pH 5.5). The cultures were incubated at 30 ^{O}C, 7 days in the dark for colonization and sporulation. Fungal spores were separately suspended in 5 ml of (0.01% and 0.1% w/v) different surfactants in 20 ml screw-capped tube or vial contained. Spore suspension (5 µl) of each isolates in surfactants was inoculated in the Czapek Dox medium. The cultures were incubated at 30^{O}C, in the dark. Fungal colonization and sclerotium formation were observed.

3.2.3.2 Identification of *Aspergillus* by synnema formation on Czapek Dox agar containing Avid®

Czapek Dox agar was prepared and autoclaved at 121 ^{O}C for 15 min. After autoclaving, 40 µl of Avid® (0 .15 EC, Novartis Crop Protection, Greensboro, NC, USA) was added into 1,000 ml medium and stirred vigorously at 60 ^{O}C to clear the bubble

formation. Spore suspension of each isolated of *Aspergillus* was inoculated in the Czapek Dox agar containing Avid®. The cultures were incubated at 30 ^{O}C, 7 days in the dark for colonization and synnema formation respectively. *A. flavus* was identified from the related strain in *Aspergillus* section *Flavi* by synnema formation.

3.2.3.2.1 Effect of light and darkness on *Aspergillus* synnema formation

Spore suspension of each isolated of *Aspergillus* was inoculated on the Czapek Dox agar containing 40 µl/L of Avid®. The cultures were separately incubated in the dark and under the light at 30 ^{O}C for 7 days. *A. flavus* colonies and synnema formation were studied under the light microscope.

3.2.3.2.2 Effect of media types on *A. flavus* synnema formation

Spore suspension of each isolated of *A. flavus* were inoculated on different types of media including Czapek Dox agar, AFPA, PDA, and CMA containing 40 µl/L of Avid®. The cultures were incubated in the dark at 30 ^{O}C for 7 days. *A. flavus* colonies and synnema formation were observed.

3.2.3.2.3 Effect of nitrogen sources on *A. flavus* synnema formation

Czapek Dox agar was modified by replacing sodium nitrate with either 0.3% (w/v) ammonium sulphate, 0.3% (w/v) ammonium tartrate, or 0.3% (w/v) peptone. After autoclaving, 40 µl of Avid® was added into 1,000 ml media and stirred vigorously at 60 ^{O}C to clear the bubble formation. Spore suspensions of each isolated of *A. flavus* was

inoculated on these media. The cultures were incubated at 30 ^{O}C, 7 days in the dark for colonization. *A. flavus* colonies and synnema formation were observed.

3.2.3.2.4 Effect of Avid® concentration on *A. flavus* synnema formation

Spore suspension of each isolated of *A. flavus* was inoculated on the Czapek Dox agar containing different (0, 40, 80, 200, 500, and 1,000 µl/L) Avid® concentration. The cultures were incubated in the dark at 30 ^{O}C for 7 days. *A. flavus* colonies and synnema formation were observed.

3.2.4 The study of *A. flavus* distribution by vegetative compatibility groups (VCGs) technique

The laboratory analysis of VCGs with complementary *nit*rate-nonutilizing (*nit*) mutants is technically simple and requires little more than basic microbiological materials. Many filamentous fungi including *A. flavus*, physiologically distinct individuals of the same species can fuse asexually to form a heterokaryon. When the heterokaryon is stable, then the participants are said to be vegetatively compatible and to belong to the same vegetative compatibility group (VCG) (Leslie, 1996). In this study we use vegetative compatibility tests to demonstrate genetic diversity and on the relationship in the *A. flavus* population of corn fields in Thailand.

3.2.4.1 *nit* mutant selection

Spore suspensions of each isolated of *A. flavus* was inoculated on Rose Bengal-chlorate medium (Elias and Cotty, 1995). This medium, which containing $NaNO_3$ and

potassium chlorate, was used to produce auxotroph or nitrate non utilizing strains (*nit*) mutant of fungi. The plates were incubated at 30 ^{O}C in the dark approximately 2 weeks for nitrate-nonutilizing mutation (*nit* mutant). Morphological structures of the wild type with nitrate reductase, also showed very restricted dense and dark purple color mycelium with a radial less than 1 cm from the point of inoculation. The *nit* mutants of were observed and collected.

3.2.4.2 Characterization of the *nit* mutants

Nitrate-metabolism mutant selection media (Horn and Green, 1991 (modified from Cotty and Bayman, 1991)) were used to identify three different types of *nit* mutant (*niaD*, *nirA*, and *cnx*). The media were prepared by replacing sodium nitrate in Czpex Dox medium with the three different corresponding nitrogen sources including sodium nitrite, hypoxanthine, and ammonium tartrate. Each strain of the *Aspergillus nit* mutants was inoculated on these three types of Nitrate-metabolism mutant selection medium. The cultures were incubated at 30 ^{O}C in the dark for 7 days. All *niaD*, *nirA*, and *cnx* mutant strains of *A. flavus* were collected.

3.2.4.3 Complementation between different types (*niaD*, *nirA*, and *cnx*) of the *nit* mutants

All *niaD*, *nirA*, and *cnx* mutant strains of *A. flavus* were individually inoculated on PDA. The plates were incubated at 30 ^{O}C in the dark for 7 days. After sufficient conidiation, spores were dislodged from the colonies in sterile H_2O, to make spore suspensions.

Starch medium (Cotty and Tylor, 2003) was used in the study. Complementation between each *nit-* mutants was tested by seeding spore suspension of each *niaD* (mutant in the nitrate reductase structural locus) mutant at the middle of the plate and surround with four different strains of *cnx* (mutant in one of several genes for a molybdenum-containing cofactor necessary for nitrate reductase activity) or *nirA* (mutant in a regulatory locus governing nitrate and nitrite reduction) mutant. The cultures were incubated for 14 days at 30°C. Complementation was indicated by a zone of dense, wild-type growth, often accompanied by sclerotium formation. Isolates which complemented each other were assigned to the same VCG (also called heterokaryon incompatibility group) (Bayman and Cotty, 1991).

3.2.5 Determination of aflatoxins and Kojic acid producing ability of isolates of *Aspergillus* section *Flavi*

3.2.5.1 Qualitative determination of aflatoxin producing strains of *Aspergillus*

3.2.5.1.1 Determination of aflatoxins producing ability of isolates of *Aspergillus* section *Flavi* by growing in Citrate utilizing medium

Maggon et al (1977) reported atoxigenic strain showed higher tricarboxylic acid (TCA) cycle activity more than toxigenic strain. So atoxigenic strain more used of primary metabolism intermidate for TCA cycle, and did have no intermediate for aflatoxin production by toxigenic strain. Citrate utilizing medium (pH 7.0) (modified from Simmons' Citrate agar, Atlas and Snyder, 2006) complementary with bromothymol blue was used in the study. *Aspergillus* that ability to utilize citrate as sole carbon source turned the medium to blue color (alkali reaction) (Atlas and Snyder, 2006). Fungal spores

of each isolated of *Aspergillus* was suspended in 1 ml of citrate utilizing medium in 5 ml screw-capped cab tube or vial. The cultures were incubated at 30 ^{O}C and 7 days in dark condition for fungal colonization. Isolates *Aspergillus* had ability to utilize citrate, changed the medium to blue.

3.2.5.1.2 Determination of aflatoxins producing ability of isolates of *Aspergillus* section *Flavi* by growing on Czapek Dox agar

Aflatoxins production mechanism by fungi needs more enzymes and substances including xanthone and in anthraquinone (Payne and Brown, 1998). These substances showed yellow to brown color. At the hypothesis, aflatoxin producing strains of *Aspergillus* changed the medium color to brown faster than atoxigenic *Aspergillus*. Czapek Dox agar was used in the study because this media had colorless. The medium was prepared and autoclaved at 121 ^{O}C for 15 min. Spore suspension of each isolated of *Aspergillus* was inoculated on the medium. The cultures were incubated at 30 ^{O}C, 5-7 days in the dark for colonization.

3.2.5.2 Quantitative determination of aflatoxin producing strains of *Aspergillus*

Modified yeast extract sucrose (mYES) (Horn et al., 2000) was used to determine aflatoxin production by *Aspergillus*. Modified yeast extract sucrose (mYES) contained 5% sucrose, and 0.5% yeast extract in distilled water. The pH was adjusted to 6.5 with 1 N HCl. One ml of the medium was transferred to a five ml screw-capped vial. Spore suspension (15 µl of 10^5 spore/ml) of each isolated *Aspergillus* was suspended into mYES medium. The cultures were incubated at 30^{O}C, in darkcondition for 7 days.

Aflatoxins (AF) in the mYes medium were extracted by adding 1 ml of chloroform to each vial, mix well for 30 s. of chloroform extract (95 µl) was transferred to a new vial, and was evaporated to dryness under nitrogen gas, then it was re-dissolved in 1.9 ml of HLPC injection solvent (H_2O:Acetronitrile:MeOH, 6:2:3 (10-fold dilution)) (modified from Horn et al., 1996). Each extracts from mYES was passed through an alumina column (Sobolev & Dorner 2002) to HPLC autosampler vial (2 ml, 8-425). The vial was closed with cap containing a PTFE/silicone slit septa and aflatoxins were determined.

Aflatoxin Analysis Using TLC

Silica gel plate (silica gel 60 F254, layer thickness 250 µm, size 20 X 20 cm) were prepared by spraying with 2% oxalic acid solution. The plates were dried under air dry condition. Draw straight line across the bottom of the plate with a soft pencil. Samples were spotted on line. Pencil graphite did not seem to interfere with rising solvents.

Place sample (25 µl) and standards (AF) on pencil line with a clean Hamilton syringe. Samples spaced 1 cm apart worked well. Labeling samples in graphite under the pencil line did not seem to interfere with chromatography results. Holding the needle close to the plate were produced smaller sized sample spots and cleaner chromatography results.

Place 150 ml of chloroform:methyl-isobutylketone (120:30) solvent into a chromatography chamber. Place spotted plate in chamber making certain that samples were above solvent line. Allow approximately 1 hour for solvent and to travel up plate. After one hour, plate was removed from chromatography chamber and mark edge of

solvent front with a soft pencil. Allow plate to air dry or use a blower. Dried plate was observed aflatoxins under longwave UV (366 nm). Aflatoxin showed blue spot under longwave UV light.

Aflatoxin analysis using HPLC

Aflatoxins were determined by a Dionex Summit HPLC system. These system was composed of P 580 Pump, RF 2000 Fluorescence Detector, ASI-100 Automated Sample Injector (20 µl sample) and a Aura Industries post column Photochemical Reactor for Enhanced Detection. The whole system was controlled using Dionex Chromeleon software (Version 6.20). An Acclaim 120 column (C18, 5 µm, 120 Å, 4.6 X 250 mm) was used at 1 ml per min flow rate of H_2O: acetonitrile: methanol (6:2:3 v/v). The fluorescence detector was set at an excitation wavelength of 365 nm and detected emission of 440nm. Each sample was run for 20 min with the aflatoxin standards peak emerging at approximately at 16.9 min. The amounts of aflatoxins were calculated by comparison with previously run standards using Chromeleon software.

3.2.5.3 Determination of Kojic acid producing strains of *Aspergillus*

Kojic acid is the common secondary metabolite, produced by *Aspergillus* section *Favi*. The other sections of *Aspergillus* in cluding *Nigri* and *Circumdati* difficultly produced this compound (Samson et al., 2007). In the study, spore suspension (15 µl of 10^8 spore/ml) of each isolated of *Aspergillus* was inoculated in Rice medium (modified from Shotwell et al, 1966) containing of 5 g of long grain rice, and 15 ml distilled water. The media were incubated at 30 ^{O}C for 7 days in the dark. After incubation, one ml of 1%

(w/v) $FeCl_3$ was added in each culture to detect kojic acid in the medium (Murakami, 1971). Isolates *Aspergillus* with ability to produce kojic acid were determined by the presence of red color in the medium solution.

3.2.6 Determination of intraspecific aflatoxins inhibition of *A. flavus* by atoxigenic isolates

3.2.6.1 Determination of aflatoxins inhibition of *A. flavus* by atoxigenic isolates

A. flavus NRRL 3357 was used as the standard toxigenic strain of *A. flavus* in this study. Modified yeast extract sucrose (mYES) broth (Horn et al., 2000) consisted of 5% sucrose, 0.5% yeast extract in distilled water. The medium was prepared, and pH was adjusted to 6.5 with HCl. One ml of the medium was transferred to five ml vials. Spore suspension (15 µl of 10^8 spore/ml) of toxigenic *A. flavus* NRRL 3357, and atoxigenic *A. flvus* were co-inoculated into mYES medium (30 µl of 10^8 spore/ml). The cultures were incubated at 30^{O}C, in the dark for 7 days at stationary condition. After incubation, aflatoxins were extracted and determined. Atoxigenic *A. flavus* with highest ability to inhibit aflatoxin production by toxigenic *A. flavus* NRRL 3357 were selected and used to study on aflatoxin inhibition of *A. flavus* by this isolate within 7 days.

3.2.6.2 Determination of aflatoxins inhibition of *A. flavus* by toxigenic isolate

This method was modified to determine aflatoxins inhibition of *A. flavus* by toxigenic isolate. mYES broth (Horn et al., 2000) was prepared. Spore suspension (15 µl of 10^8 spore/ml) of *A. flavus* NRRL 3357, and toxigenic *A. flvus* were co-inoculated into mYES medium (30 µl of 10^8 spore/ml). The cultures were incubated at 30^{O}C, in the dark

for 7 days at stationary condition. After incubation, aflatoxins were extracted and determined.

3.2.6.3 Determination of aflatoxins inhibition of *A. flavus* by atoxigenic isolate at agitation and stationary condition

Yu et al (2002) reported fungi are deficient in sporulation were unable to produce aflatoxins. So, this method was modified to compare aflatoxin production by *A. flavus* between agitation and stationary condition. Ten ml mYES broth (Horn et al., 2000) was prepared and then was transferred to a 250 ml flask. Spore suspension (150 µl of 10^8 spore/ml) of toxigenic *A. flavus* NRRL 3357, and a selected atoxigenic *A. flavus* were co-inoculated into mYES medium (300 µl of 10^8 spore/ml final concentration). The cultures were separately incubated at stationary and agitation (150 rpm) condition, at 30^{O}C, in the dark for 7 days. After incubation, aflatoxins were extracted and determined.

3.2.6.4 Determination of aflatoxins inhibition of *A. flavus* by atoxigenic isolate in the medium containing gallic acid

Some phenolic compound including gallic acid was determined as an indicator of potential for inhibition of aflatoxigenesis (Mahoney and Molyneux (2004)). In the hypothesis, Aflatoxin production activities were inhibited when adding gallic acid in the medium. Ten ml mYES broth (Horn et al., 2000) was prepared and then was transferred to a 250 ml flask. After autoclaving, 100 and 1,000 µl of 10 mM gallic acid was added into the medium. Spore suspensions (150 µl of 10^8 spore/ml) of toxigenic *A. flavus* NRRL 3357, and a selected atoxigenic *A. flavus* were co-inoculated into mYES medium

(300 µl of 10^8 spore/ml final concentration). The cultures were incubated at 30^{O}C, in the dark. After incubation, aflatoxins were extracted and determined.

3.2.6.5 Determination of aflatoxins inhibition of *A. flavus* by the addition time of atoxigenic culture.

Ten ml of the mYES medium was transferred to a 250 ml flask. Spore suspension (150 µl of 10^8 spore/ml) of each *A. flavus* NRRL 3357 and selected atoxigenic *A. flavus*, was separately inoculated into mYES medium. The cultures were incubated at 30^{O}C, in the dark. In order to see whether the time after germination of atoxigenic culture affected the inhibition, *A. flavus* NRRL 3357 culture was mixed with atoxigenic *A. flavus* culture within 0, 1, 2, 3, 4, 5, and 6 days. After 7 days incubation, aflatoxins were extracted and determined.

3.2.6.7 Determination of intraspecific aflatoxins inhibition of *A. flavus* by atoxigenic isolates using well plate technique

Cylinder with membrane attached at bottom is inserted into a well culture plates containing fungal spores. Spacers on the cylinder bottom allow free movement of medium underneath to the membrane and diffusion of medium through the membrane into the cylinder. A suspension of the *Aspergillus* conidia is put inside the cylinder and kept separate from the medium in the well (modified from Janisiewicz *et al.* 2000).

Two ml of the medium was transferred to the sterilized plastic well plate. Each spore suspension of (15 µl/ml of 10^8 spore/ml) atoxigenic and (15 µl/ml of 10^8 spore/ml) toxigenic strains of *A. flavus* was separated or added together into insert well plate (An

insert well is a polystyrene cylinder with a membrane (Polycarbonate) with pore sizes (12 μm) attached to the bottom of the cylinder) (figure 3). The cultures were incubated at stationary, at 30^{O}C, in the dark for 7 days. After incubation, aflatoxins were extracted and determined.

3.2.7 Statistical Analysis

Statistical analyses were performed using one way analysis of variance (ANOVA) withTukey's Studentized Range (HSD) Test at significance level $p < 0.05$

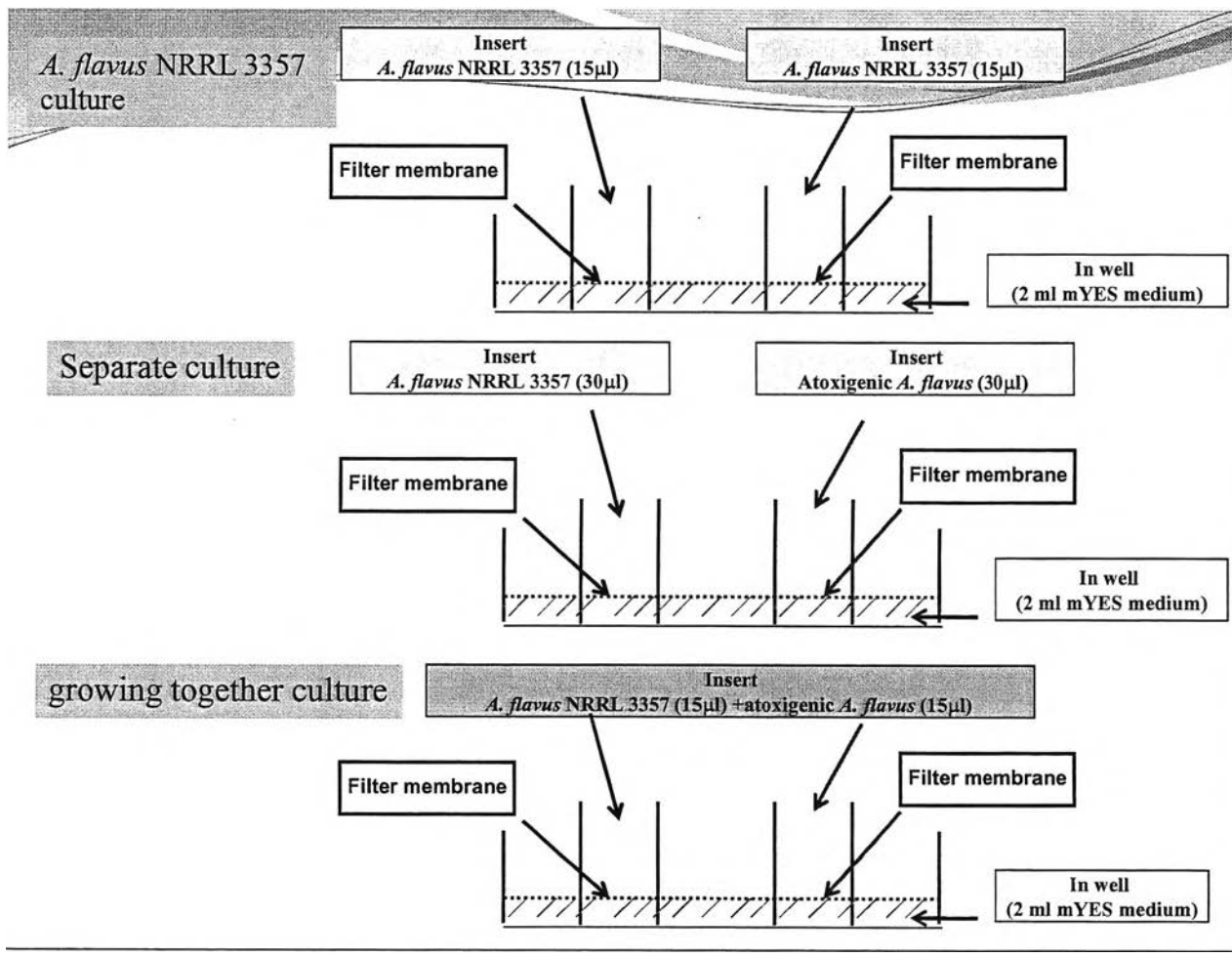

Figure 3.1 diagram of intraspecific inhibition method using filter well plate system