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Mechanism of intraspecific toxin inhibition in Aspergillus flavus

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MECHANISM OF INTRASPECIFIC TOXIN INHIBITION IN
ASPERGILLUS FLAVUS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Atoxigenic *Aspergillus flavus* was demonstrated by others as a promising biocontrol agent to minimize preharvest aflatoxins in susceptible crops. But the mechanism was unclear. A filter insert-well plate system was used to study the mechanism in lab. There was no inhibition when toxigenic *A. flavus* isolate 53 and inhibitory atoxigenic isolates were separated by 0.4 µm membrane, approximately 50% inhibition occurred when separated by 12 µm membrane, and complete inhibition occurred when a 74 µm membrane was used. This result suggested that touching or close physical interaction is needed for toxin inhibition and the nutrient competition hypothesis was not supported.

Isolate 53 and inhibitory atoxigenic isolate 51 were used to study the timing of intraspecific toxin inhibition. The result showed that inhibition occurred when 0 - 4 day old isolate 51 was added within the first 16-hour growth of isolate 53. However, two-day old isolate 51 inhibited toxin production by two-day old isolate 53 and twenty-four hour old isolate 51 inhibited toxin production by 48-hour old isolate 53. These results suggested that there is a 16-hour "window" for the conidial inhibition ability of atoxigenic isolate but for mycelia, the "window" is expanded to 48 hours.

Isolate Af70-GFP was acquired to microscopically examine the touch inhibition interaction. Surprisingly, none of the completely inhibitory atoxigenic isolates from our collection or NRRL 21882 inhibited toxin production by Af70-GFP. Isolate K49 and two Australian isolates were shown to be able to inhibit toxin production by Af70-GFP. The inhibitory abilities of additional atoxigenic isolates were tested with toxigenic isolates 53, Af70s-GFP and NRRL 3357. Different patterns were obtained among those three isolates. These results showed that there was specificity in the touch inhibition interaction. Af70-GFP and isolate K49 were used to continue microscopy work.

The growth of Af70s-GFP appeared to be inhibited and vacuoles present in Af70s-GFP were absent when it was paired with K49.

Biocontrol once thought to be due to competitive exclusion probably requires close physical growth or touching and displays specificity. Multiple atoxigenic isolates each specific to a subset of the toxigenic isolate population may be needed for an effective biocontrol application.

CHAPTER 1. LITERATURE REVIEW

1.1 *Aspergillus flavus* Problem and Biocontrol

Aspergillus flavus is an asexual filamentous fungus of agronomic and health importance. Under favorable environmental conditions, drought stress and high temperature, it can infect multiple crops, such as peanut, tree nut, corn and cotton (Payne 1998). It can contaminate the crops with aflatoxin which is carcinogenic and toxic both to humans and animals (Bennett & Klich 2003).

Research on aflatoxin-related problems began with the turkey X disease in Great Britain in the early 1960s (Sargeant *et al*. 1961, Goldblatt 1969, 1971; Pons & Goldblat 1969; Pons 1976). Surveillance and/or control of aflatoxin contamination are increasingly important (Arim 1995). This problem has been reported throughout the tropical and subtropical regions of the world (Arim 1995; Kaaya & Warren 2005; Njapau *et al*. 1998; Pitt 1998). Aflatoxin research in the Philippines began with an aflatoxin survey of various foods in 1967. Uganda is an Eastern African country with tropical climate and was one of the countries where aflatoxin studies first started (Kaaya & Warren 2005).

Suitable measures to combat this problem are very important. Among them, biological control by introducing atoxigenic strains of *A. flavus* to reduce toxin contamination in the field is one strategy that has recently gained prominence. Biological control has been used to reduce aflatoxin contamination in various crops such as cotton (Cotty 1994), peanut (Dorner *et al*. 1998) and corn (Brown *et al*. 1991). Utilization of atoxigenic isolates to control aflatoxin is an important project of the US Department of Agriculture (http://www.ars.usda.gov/research/ projects/projects.htm?ACCN_NO=406618). This approach was adopted in Africa and became a major component of the project "Aflatoxin risk assessment, biological control options and intervention" funded by the German Development Agency (BMZ) (http://www.gtz.de/de/

dokumente/en-beaf-bmz-list-of-funded-projects-2006.pdf).

There are a lot of difficulties for the application of this strategy. First, it is not easy to identify a reliable biocontrol isolate. Some strains which show good inhibitory ability in laboratory testing may not be good for field use (Cotty & Bhatnagar 1994). Second, it is hard to determine doses. When crops are exposed to conditions highly conducive to aflatoxin contamination, unacceptable toxin levels may occur even when doses of atoxigenic strains are applied that were effective under less conducive conditions (Cotty & Bhatnagar 1994). Third, time of application is critical. Some effective atoxigenic strains will lose their ability to reduce aflatoxin contamination when application timing is not proper. Fourth, a good biocontrol isolate is region restricted, which means that some isolates only function in particular geographic regions (Bandyopadhyay & Cardwell 2004). Therefore, determining the mechanism is very important for proper application of this biocontrol method.

1.2 Biocontrol Mechanism

This mechanism has been studied following the application of atoxigenic *A. flavus* (Cotty 1990; Brown 1991; Dorner 2002). Though the mechanism is not well understood, several possible hypotheses have been suggested.

- **Competitive Exclusion**

One hypothesis concerning the biocontrol mechanism is that "competitive exclusion" of toxigenic isolates by atoxigenic isolates occurs thereby lowering the toxigenic inoculum pressure in soil, which is an epidemiologically based mechanism. Cotty & Bayman (1993) tested the competitive ability of atoxigenic *A. flavus* both in cotton bolls and in liquid medium, and concluded that competitive exclusion contributed to the toxin inhibition effect. The work of P. K. Chang, USDA-ARS-SRRC, New Orleans, LA (personal communication) showed that some atoxigenic isolates could grow well in a culture with toxigenic isolates without reducing

2

aflatoxin accumulation. This suggests that vegetative growth alone does not play a significant role and that there could be other mechanisms involved.

In 2003, Wicklow *et al.* used the suspended disc method to look at the effect of atoxigenic isolates on toxin production by toxigenic isolates. They showed that the final toxin concentration is independent of inoculum level (**Table 1.1)** and the increase of toxin inhibition is greater than the increase in concentration of atoxigenic conidia in the total fungal mixture **(Figure 1.1**). The predicted line for toxin production is based on the assumption of utilization of nutrients in proportion to the ratio of the toxigenic and atoxigenic conidia. The difference between the predicted and calculated suggests there is an unknown biological phenomenon involved in this process. The later finding is supported by previous research (Cotty & Bayman 1993) which showed that the same inhibition level occurred when the ratio of toxigenic isolate and atoxigenic isolate was 1:1 or 1:0.5. These phenomena again can not be explained by competitive exclusion.

Table 1.1 Toxin production of isolate NRRL 32355 at different conidial concentrations in the suspended disc assay.

Concentration	Toxin B1 (μg/ml)	
($\times 10^5$/ml)	Range	Mean
1	1.84–5.98	3.28 a*
0.8	0.58–7.68	2.51 a
0.6	0.75–7.17	3.52 a
0.4	0.86–7.73	3.81 a
0.2	1.55–5.74	2.93 a

*Numbers with the same letter suggest no significant difference (P=0.05) based on Duncan's Multiple Range Test. Data from Wicklow *et al.* (2003).
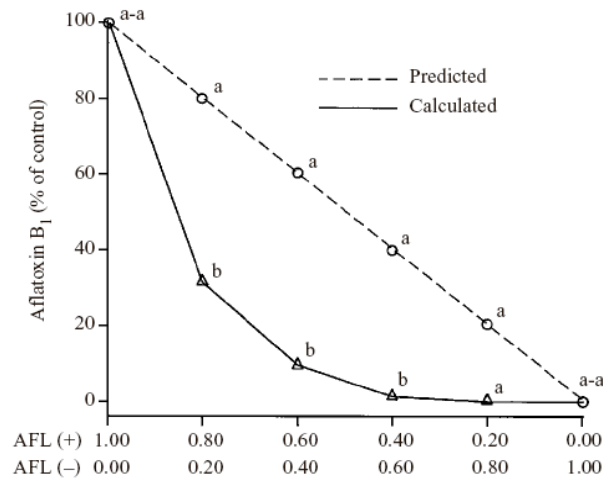
Figure 1.1 Effect of atoxigenic isolates of *Aspergillus flavus* on aflatoxin B1 production in the suspended disc assay (Wicklow *et al.* 2003). Note the discrepancy between predicted and calculated result.

- **Vegetative Compatibility**

Horn *et al.* (2000) used atoxigenic white-conidial mutants of *A. flavus* with toxigenic yellow-conidial mutants belonging to the same vegetative compatibility group (VCG) or different VCGs to test whether vegetative compatibility played an important role in toxin inhibition. They showed that there were no consistent differences in aflatxoin B1 inhibition by atoxigenic isolates in pairings from the same or different vegetative compatibility groups. This may exclude the possible role of VCGs in toxin inhibition. This was also confirmed by Jha *et al.* (2003) using the suspended disc method. She showed that toxin production of a toxigenic isolate can be completely inhibited by different atoxigenic isolates whether they come from the same or different vegetative compatibility groups.

However, Wicklow & Horn (2007) used the suspended disc method to demonstrate a relationship between strength of the vegetative compatibility reaction and aflatoxin production in

A. flavus. They showed that pairing aflatoxin-producing isolates belonging to different VCGs or the same VCG but showing weak compatibility yielded very little aflatoxin. However, combining isolates displaying a strong compatibility reaction produced high levels of aflatoxins. They believed that vegetative compatibility played a very important role in intraspecific toxin inhibition. Therefore this hypothesis is still controversial.

- **Antibiotics**

Acremonium zeae, an endophyte of corn, produces antibiotics which are inhibitory to *Aspergillus flavus* (Wicklow *et al*. 2005). Therefore another possible hypothesis is that atoxigenic *A. flavus* produces antibiotics that inhibit toxin production. However, Cotty & Bayman (1993) reported that aflatoxin production by isolate AF13 was stimulated by culture filtrates and mycelial extracts of *A. flavus* isolate AF36 (an atoxigenic biocontrol isolate), and that there was no evidence of colony inhibition on agar media. Moreover, Wicklow *et al.* (2003) found more fungal growth when toxigenic and atoxigenic isolates were grown together (Wicklow *et al*. 2003). So the antibiotic hypothesis can probably be excluded.

- **Resource Competition**

Inhibition of aflatoxin production may be due to competition for resources, especially nutrients. Wicklow *et al.* (2003) showed that aflatoxin inhibition occurred in conidial mixtures in which both isolates produced aflatoxin B1 or just one isolate did. This means that aflatoxin production will be inhibited when competition exists, no matter whether it is between non-toxigenic strains and toxigenic strains, or between toxigenic strains. No direct evidence to support or exclude this hypothesis has been found.

The mechanism of the intraspecific toxin inhibition is not well understood. Therefore I investigated the mechanism under lab conditions with different techniques in the hope of understanding the intraspecific toxin inhibition mechanism.

1.3 Previous Work

Fifty *A. flavus* cultures were isolated with AFPA selective medium (Pitt *et al*., 1983) from kernels from Louisiana corn fields and divided into two groups: 9 were toxigenic and 41 were atoxigenic. All the atoxigenic isolates were individually evaluated for their ability to inhibit aflatoxin production by a single toxigenic isolate 53 in a suspended disc assay (Jha *et al*. 2005). Eight isolates (42, 43, 45, 46, 48, 50, 51, and 52) completely inhibited aflatoxin production, whereas four (18, 41, 47, 49) were highly inhibitory (**Table 1.2**). Additional analysis found that some of those isolates were in different VCGs and some were in the same VCG as isolate 53 (**Table 1.3**). This result suggests that VCG does not play an important role in intraspecific toxin inhibition.

The intraspecific mechanism studies were done using plate culture, liquid culture or suspended disc assay. These culture methods do not physically separate the toxin producer and toxin inhibitor. Janisewicz *et al*. (2000) used a simple approach with an *in vitro* system closely resembling *in vivo* conditions to successfully prove that competition for nutrients is the mechanism of biological control of fruit decays. The apparatus for this assay is shown in **Figure 1.2**. It consists of 24-well tissue culture plate and 24 inserts with 0.4 µm pore size. Inserts separated *Penicillium expansum* (in insert) and its yeast antagonist (under insert) to exclude the effect of space, and allowed the diffusion of nutrients. This apparatus would be useful for testing whether intraspecific toxin inhibition involves nutrient competition or antibiotic interaction.

Table 1.2 Effect of atoxigenic isolates on toxin production by isolate 53 (Jha *et al.* 2005). Isolate 53 was paired with 41 atoxigenic isolate on suspended disc. Isolates 42, 43, 45, 46, 48, 50, 51, and 52 (in blue) completely inhibited aflatoxin production and isolates 18, 41, 47, 49 were highly inhibitory.

Isolate #	*50:50 (ppb AFB1)	*80:20 (ppb AFB1)	Isolate #	50:50 (ppb AFB1)	80:20 (ppb AFB1)
1	1396	112	31	21	298
3	17	8	32	20	0
4	7	50	33	34	0
13	8	32	34	105	21
14	0	73	35	136	24
15	125	242	36	387	60
16	15	218	37	29	34
17	5	244	38	108	10
18	0	1	39	395	1
19	1	152	40	78	5
20	615	502	41	0	1
21	11	22	42	0	0
22	0	304	43	0	0
23	1	115	45	0	0
25	18	397	46	0	0
26	43	484	47	8	0
27	2	136	48	0	0
28	20	80	49	0.3	0
29	42	199	50	0	0
30	104	321	51	0	0
			52	0	0

* Ratio of conidia from toxigenic and atoxigenic isolates

Table 1.3 Distribution of completely and highly inhibitory isolates across vegetative compatibility groups A, B, C or other (Jha *et al.* 2005). Isolates 42, 43, 45, 46, 48, 50, 51, and 52 (in blue) completely inhibited aflatoxin production by toxigenic isolate 53 and isolates 18, 41, 47, 49 were highly inhibitory.

A	B(53)	C	Other
45	50	42	48
18	51	43	52
		46	
		41	
		47	
		49	

a
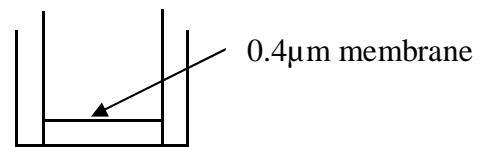
b

Figure 1.2 Filter insert-well plate system from Janisiewicz *et al.* 2000.

a: Cylinder with membrane attached at bottom is inserted into a well of a tissue culture plate containing apple juice and the yeast antagonist. Spacers on the cylinder bottom allow free movement of apple juice underneath to the membrane and diffusion of apple juice through the membrane into the cylinder. A suspension of the *Penicillium expansum* conidia is put inside the cylinder and kept separate from the yeast antagonist in the well (Janisiewicz *et al.* 2000).
b: Schematic diagram to show the structure of insert-well system.

1.4 Laboratory Techniques

1.4.1 Aflatoxin Analysis

Major aflatoxins are aflatoxin B1, B2, G1 and G2 (**Figure 1.3**). *A. flavus* typically produces B1 and B2 (Diener *et al.* 1987). B1 is normally predominant in amount and is usually quantified because it is the most carcinogenic and the only member which is regulated by the FDA. There are different ways to analyze aflatoxin quantatively or qualitatively.
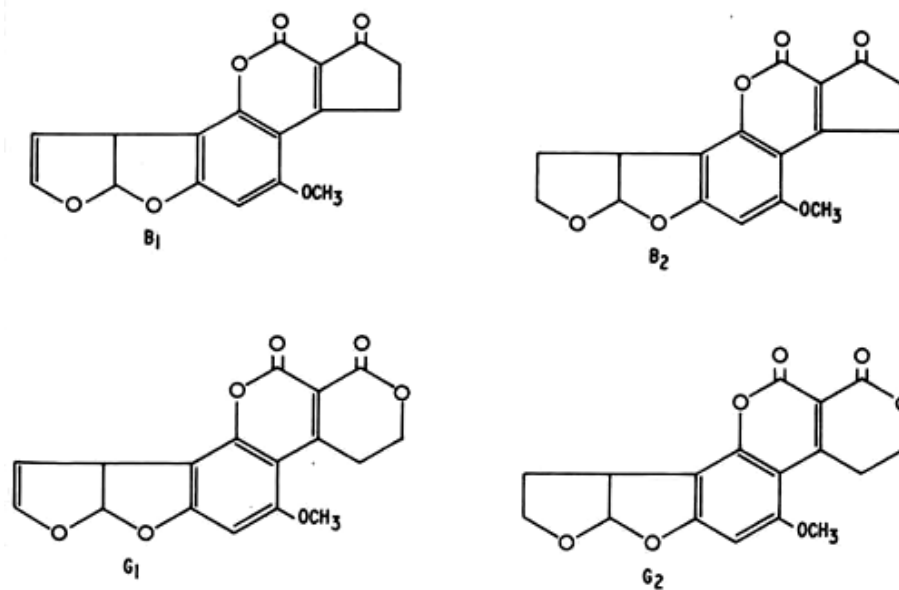
Figure 1.3 The structure of aflatoxins: B_1, B_2, G_1, G_2 (from http://www.aflatoxin.info/aflatoxin.asp).

- **Cultural Techniques**

There are different cultural techniques to detect toxin production by *A. flavus*. Abbas *et al.* (2004) evaluated three culture methods: fluorescence (FL) on β-cyclodextrin-containing media, yellow pigment (YP) formation on potato dextrose agar, and color change after ammonium hydroxide vapor exposure (AV) on potato dextrose agar. FL, YP, and AV responses showed good agreement but were not as sensitive as chemical methods, e.g. HPLC. The advantages of culture techniques are that they do not need chemical extraction and are faster and cheaper than

chemical methods. Disadvantages are that they do not quantify toxin production and are not 100% reliable.

- **Thin Layer Chromatography**

Thin layer chromatography (TLC), is one of the most widely used techniques in aflatoxin analysis. It is the AOAC (Association of Official Agricultural Chemists) official method and is used to identify and quantify aflatoxins at levels as low as 1 ng/g. It can be used in one-dimensional and two-dimensional formats (Hans *et al*. 1986). This method is the basic technology which is used to verify newer techniques.

- **Mini-column Chromatography**

Mini-column chromatography (MC) was first developed by Holaday (1968) and was tested and found to be both rapid and simple to detect and quantify aflatoxin in peanuts. Sensitivity of this method is 5 ppb and one assay can be finished within 15 to 25 min. Compared to TLC, this procedure is less time-consuming, cheap, and simple. It was further improved for determination of aflatoxin in different crops (Velasco 1972; Sashidhar *et al*. 1988; Holaday 1981).

- **Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance**

Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) was developed by Mirghania *et al*. (2001) and was demonstrated as a fast, easy, convenient and accurate way to determine aflatoxin in groundnut and groundnut cake. FTIR can detect small differences in toxin concentration which made the repeatability of the FTIR much better than that of TLC. It was a possible alternative to the standard chemical methods for determination of aflatoxin levels in food and feed. But using this method, Shamsaie *et al*. (2003) could not detect aflatoxin B1 at concentrations as high as 1500 ppb. Therefore, this method is still controversial.

- **Immunochemical Methods**

Highly specific immunochemical methods are available to identify and quantify aflatoxins in food within 10 min. The basis of this method is that antibodies can bind aflatoxins. There are different types of immunochemical methods available, including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA) (Chu *et al*. 1987; Scott & Trucksess 1997).

- **Liquid Chromatography**

Liquid chromatography (LC) was first developed by Paulsch (1988) as an aflatoxin detection method. The limit of detection for aflatoxin B1 is less than 1 ng/g. LC and TLC can complement each other when testing aflatoxin. Usually, a researcher can use TLC for preliminary work to optimize LC separation conditions.

One kind of LC is called High performance liquid chromatography (HPLC). It was found as a simple and sensitive procedure for the analysis of aflatoxins B1, B2, G1 and G2 in cereal and animal feedstuff samples (Diebold & Zare 1977; Pons 1976; Pons 1979; Hetmanski & Scudamore 1989). The procedure was improved by Sobolev & Dorner (2002) and considered as a fast and reliable way to determine aflatoxin in samples. The detection limit for aflatoxin B_1 is 1 ng/g.

1.4.2 Fungal Culture Conditions

1.4.2.1 Media

There are many different kinds of culture media for growing *A. flavus*: **synthetic medium:** glucose salts (GS) medium (Reddy *et al*. 1971, Wicklow *et al.* 2003); **semisynthetic media:** potato dextrose agar (PDA), aflatoxin producing ability medium (APA, Hara *et al.*, 1974), glucose yeast extract agar (GY-Agar, Filtenberg & Frisvad 1980) and coconut agar

medium (CAM, Lin *et al*. 1976); and **natural media**: rice, wheat, and corn. Different media may result in different results for toxin production.

According to Cutuli *et al.* (1991), natural media (rice, wheat) are best for toxin production, CAM is better than GY-Agar and APA for toxin production. Wicklow *et al.* (1981) showed that one of their *A. flavus* isolates was aflatoxin negative on APA medium but produced aflatoxin when grown on autoclaved corn, which also suggests that natural medium is better than semisynthetic medium (e.g., APA medium) for toxin production by *A. flavus*.

Reddy *et al.* (1971) introduced several chemically defined media which allowed high aflatoxin production. They showed that glucose-ammonium nitrate medium (GAN) was not a good synthetic medium for aflatoxin production but allowed high yield of aflatoxin with addition of asparagine. Both synthetic low-salts medium (SL medium) and synthetic high-salts medium support high aflatoxin production: about 30 mg of aflatoxin per 100 ml of medium. Later, Wicklow *et al.* (2003) modified the medium by replacing sucrose with glucose in the suspended disc system.

1.4.2.2 Environmental Factors

Environmental factors that can affect toxin production include temperature, relative humidity or moisture, and CO_2.

- **Temperature**

Sorenson *et al.* (1967) tested the effect of temperature on production of aflatoxin on rice by *A. flavus*. Temperatures of 8°C, 11°C, 15°C, 28 °C and 32°C were tested, and 28°C was the optimum temperature for toxin production. Ogundero (1987) tested the effect of temperature on toxin production and found that the best temperature for aflatoxin production by *A. flavus* was 30 °C but there was no toxin production at 10°C. Northolt *et al.* (1976) showed that at high water activity, the optimum temperature for aflatoxin was around 24°C. Wicklow *et al.* (2003) used 25

12

°C as culture temperature. Maren (2007) concluded that that the optimum temperature for aflatoxin biosynthesis is between 24° and 30°C, with some variation due to strain and substrate.

- **Relative Humidity**

Sanders *et al*. (1968) reported that aflatoxin levels on peanut decreased as relative humidity decreased (from 99% to 86%). Moreno Romo (1986) tested the effect of minimal moisture content for aflatoxin production on mixed feeds medium and found that very low amounts of aflatoxin are accumulated when minimal moisture content is 17% or lower but significant amount of aflatoxin was detected when minimal moisture content is above that. Maren (2007) reviewed other studies about the water activity effect on aflatoxin production and concluded that aflatoxin production was generally higher at relatively high water activities.

- **Air Component**

Sanders *et al.* (1968) reported that aflatoxin levels on peanut decreased as CO_2 concentration increased when other conditions were constant. Landers *et al.* (1967) showed lower O_2 resulted in less toxin production. Epstein *et al.* (1970) tested the effects of controlled atmosphere (10% CO_2, 1.8% O_2 and 88.2% N_2) on toxin production by *A. flavus* versus air (0.0314% CO_2, 20.94% O_2 and 78.084% N_2) in liquid medium and cracked corn. They found that less toxin was produced in the controlled atmosphere (higher CO_2 and lower O_2 content).

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory Facilities and Equipment

This research was conducted in the Department of Plant Pathology and Crop Physiology, Life Sciences Building (**LSB**), Louisiana State University and LSU Agricultural Center, Baton Rouge, Louisiana. Work was done in LSB room A 401, A 403 and A424.

Equipment used is listed by manufacturer in alphabetical order: **American Precision Plastics (Northglenn, CO),** Plastic Plates; **Baker Company Inc. (Sanford, ME),** Edge GARD® Hood (Laminar flow hood); **Costar® Corning Incorporated (Corning, NY)**, 24 Well Cell Culture Cluster and Netwell TM Mesh (74μm and 200 μm) and Plates System; **Dionex Corporation (Houston, TX)**, Summit HPLC system with a Photochemical Reactor for Enhanced Detection (PHRED, **Aura Industries, NY**); **Eppendorf (Germany),** Eppendorf tubes (1.5 ml); **Lab-line Instruments Inc. (Melrose Park, IL)**, Low Incubator; **Leica Corporation (Bannockburn, IL),** Confocal Laser Scanning Microscope; **Millipore (Bedford, MA)**, Culture Plate Inserts (Pore Size: 0.4 μm; 3 μm; 12 μm); **Olympus Corporation (Center Valley, PA),** IMT2 Inverted Fluorescence Microscope; **Tuttnauer Corporation (Hauppauge, NY)**, Horizontal Autoclave; **VWR Company (VWR Scientific Model 2015)**, Low Temperature Incubator.

2.1.2 Chemicals

Chemicals used for the research are listed in alphabetical order of suppliers: **Aaper Alchohol and Chemical Co. (Shelbyville, KY)**, ethanol (EtOH); **Becton, Dickinson and Co. (Sparks, MD),** Potato Dextrose Agar (PDA); **Curtin Matheson Scientific Inc. (Houston, TX)**, Potassium Phosphate monobasic (KH_2PO_4); **EMD Chemicals Inc. (Gibbstown, NJ)**, Dextrose, Anhydrous (Glucose), Acetonitrile, Methanol; **Fisher Scientific Co. (Fair Lawn, NJ),**

Manganous Chloride 4-Hydrate ($MnCl_2 \cdot 4H_2O$), Ammonium Sulfate (($NH_4)_2SO_4$); **J. T. Baker Chemical Co. (Phillipsbirg, NJ)**, Magnesium Sulfate 7-Hydrate ($MgSO_4 \cdot 7H_2O$), Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$); **Mallinckrodt® (St Louis, MO)**, Ammonium Molybdate (($NH_4)_6Mo_7O_{24} \cdot 4H_2O$), Ferrous Sulfate 7-Hydrate ($FeSO_4 \cdot 7H_2O$)**; Sigma Chemical Co. (St. Louis, MO)**, Triton X-100; **The Coleman & Bell Co. (Norwood, OH)**, Zinc Sulfate 7-Hydrate ($ZnSO_4 \cdot 7H_2O$), Sodium Tetraborate Decahydrate ($Na_2B_4O_7 \cdot 10H_2O$).

2.1.3 Isolates

Five atoxigenic isolates (4, 20, 42, 45 and 51) and one toxigenic isolate (53) were isolated from kernels collected from corn fields in Louisiana. AF70s-GFP was a transgenic toxigenic isolate which can fluoresce green (509 nm) under the excitation of 488 nm light was provided by Dr. Jeff Cary, USDA-ARS-SRRC, New Orleans, LA. Isolate NRRL 21882 was from Dr. Joe Dorner, USDA-ARS-NPL, Dawson, GA. Isolate NRRL 3357 was obtained from USDA-ARS-SRRC, New Orleans, LA and has been sequenced (http://www.aspergillusflavus.org/). Isolate K49 was obtained from Dr. Hamed Abbas, USDA-ARS, Stoneville, MS. Isolate Af Papa 827 were acquired from Shannon Betz, USDA-ARS-SRRC, New Orleans, LA, and two Australian isolates, Af 4-2 (Group II, small sclerotia) and Af 5-1 (Group I, large sclerotia), from Dr. David Geiser, Penn State Univ.(Geiser *et al*. 2000). Results from Jha *et al.* (2005) using the suspended disc assay procedure of Wicklow *et al.* (2003) showed that isolate 42, 45 and 51 completely inhibited aflatoxin production by isolate 53 whereas isolate 20 was only partially inhibitory and isolate 4 was non-inhibitory to isolate 53.

2.2 Methods

2.2.1 Media

Two media were used for culturing *A. flavus*. Potato dextrose agar (PDA) was used as solid medium for conidia production. The isolates were grown in liquid glucose salts (GS)

medium to measure toxin production and intraspecific inhibition of toxin production. GS medium was prepared as follows: 2.5-times (2.5X) salts solution (3.5g $(NH_4)_2SO_4$, 750mg KH_2PO_4, 350mg $MgSO_4 \cdot 7H_2O$, 75mg $CaCl_2 \cdot 2H_2O$, 10mg $ZnSO_4 \cdot 7H_2O$, 5mg $MnCl_2 \cdot 4H_2O$, 2mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2mg $Na_2B_4O_7 \cdot 10H_2O$, 2mg $ZnSO_4 \cdot 7H_2O$ per L) and 2.5-times (2.5 X) glucose solution (125g glucose per L) were prepared and autoclaved (121°C for 20 min) separately. The two solutions were mixed together in a 1:1 ratio.

2.2.2 Conidia Preparation

All the isolates were cultured on PDA plates for 7 days, at 30°C, in dark. Sporulating plates were flooded with 5 ml sterile 0.01% Triton X-100 and conidial suspensions were collected. Finally, all the conidial suspensions were diluted to 5×10^5 conidia/ml with 0.01% Triton X-100. Conidial suspensions were mixed with GS medium at 1:4 ratios which resulted in conidia-medium mixture with a concentration of 1×10^5 conidia / ml. Controls for the conidial-medium mixture were prepared by mixing 0.01% Triton X-100 with GS medium at 1:4 ratios (TX-medium mixture).

2.2.3 Filter Insert-plate Well System

The filter insert-plate well system was modified from Janisevicz *et al* (2000) to study the mechanism of intraspecific toxin inhibition. It was composed of tissue culture plate with 24 wells (Costar, Corning Inc., Corning, NY) and Millicell inserts (Millipore Corp., Bedford, MA). An insert is a polystyrene cylinder with a membrane attached to the bottom of the cylinder. Inserts with different pore sizes (0.4, 3, 12, 74 and 200 µm) and composition (hydrophilic polytetrafluoroethylene, polycarbonate and polyester mesh) were used in this study. The diffusion of the solution though the hydrophilic (PTFE) membrane was determined by Janisevicz *et al.* (2000) using crystal violet solution. They stated that movement of the dye from one side of insert to the other was observed within 3 min. Therefore, this kind insert was used in the first

experiment to test whether competition for nutrients was involved in intraspecific toxin inhibition or not.

Every experiment in this system was done by putting 400 µl isolate conidia-medium mixture (1×10^5 conidia/ml) in the well and 400 µl in the insert with 4 replicates. Plates were wrapped with parafilm and incubated at 25°C in the dark. In all cases, the toxigenic isolate was allowed to grow for a total of 5 days. Controls were done by replacing the atoxigenic isolate conidia-medium mixture with TX-medium mixture.

Aflatoxin samples were prepared by withdrawing 240 µl liquid by inserting the pipette tip into the space between the insert and well wall. This was combined with 240 µl acetonitrile in an Eppendorf tube and vortexed. The entire sample was passed through an alumina column (Sobolev & Dorner 2002) into an HPLC autosampler vial (2 ml, 8-425). The vial was closed with a cap containing a PTFE/silicone slit septa and aflatoxin B1 was determined by HPLC.

Some experiments were done in the plate without an insert (plate well system) by combining 200 µl atoxigenic isolate conidia-medium mixture (1×10^5 conidia/ml) and 200 µl toxigenic isolate conidia-medium mixture (TX-medium mixture as the control) in the well.

2.2.4 Tube System

Eppendorf tubes (1.5 ml) were used to study the timing of intraspecific toxin inhibition. For these experiments, toxigenic isolate 53 conidia-medium mixture (100 µl of 1×10^5 conidial/ml) was paired with the same volume and concentration of atoxigenic 51 conidia-medium mixture in Eppendorf tubes (5 replicates) and incubated at 25 °C in the dark. The control was done by mixing 100 µl of isolate 53 conidia-medium mixture and 100 µl TX-medium mixture in Eppendorf tubes. In each case, isolate 53 was allowed to grow for a total of 5 days.

Aflatoxin samples were prepared by adding 400 µl acetonitrile to each experimental tube and vortexed. The entire sample was passed through an alumina column (Sobolev & Dorner 2002) into an HPLC autosampler vial (2 ml, 8-425). The vial was closed with a cap containing a PTFE/silicone slit septa and aflatoxin B1 was determined by HPLC.

2.2.5 Aflatoxin Analysis Using HPLC

The final concentrations of aflatoxin were determined by a Dionex Summit HPLC system. This 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 365nm and detected emission of 440nm. Each sample was run for 20 min with the aflatoxin B1 peak emerging at approximately at 16.9 min. The amount of aflatoxin B1 (ppb) was calculated by comparison with previously run standards using Chromeleon software.

2.2.6 Microscopic Observation

Rajasekaran *et al*. (1999) engineered *A. flavus* isolate Af70s to express the green fluorescent protein (Af70s-GFP) in order to visualize the inhibitory effect of a purified antifungal peptide on conidial germination and subsequent fungal growth. This isolate is toxigenic and can be easily distinguished, therefore it was used to microscopically study the interaction. Atoxigenic isolate K49 was used to inhibit toxin production by Af70s-GFP. They were paired by combining 200µl Af70s-GFP conidia-medium mixture (1×10^5 conidial/ml) with the same amount of K49 conidia-medium mixture in wells of Corning Costar 24 well plates with 4 replicates. The plate was incubated at 25^oC in the dark for 24 hours. The growth of the two isolates was observed with

an Olympus IMT2 inverted fluorescence microscope. The excitation wavelength for GFP (green fluorescent protein) is 488nm and the emission wavelength is 509nm. Digital images of the sub-cellular structure of the Af70s-GFP were acquired by using Leica Confocal Laser Scanning Microscope (63X objective).

2.2.7 Statistical Analysis

Statistical analyses were performed using one way analysis of variance (ANOVA) with Tukey's Studentized Range (HSD) Test at significance level $p < 0.05$ except experiment **3.4.3** in which Duncan's Multiple Range Test was used.

CHAPTER 3. RESULTS

3.1 Effect of Culture Volume on Aflatoxin B1 Production in the Filter Insert-plate Well System

Different conidia-medium mixture volumes both in the well and in the insert were compared, from 100 µl, 200 µl, 300 µl, 400 µl to 500 µl to find the optimal volume for maximum toxin production in the filter insert-plate well system (**Table 3.1**). The conidial concentration was 1×10^5 conidia/ml. All the treatment had four replicates and the experiment was not repeated. In this experiment, 100 µl of the liquid was withdrawn from the well and mixed with 300 µl acetonitrile, vortexed and passed through the alumina column as previously described. The results showed that 100 µl both in the well and in the insert did not allow toxin production, and the best volume for producing the largest amount of toxin with a relatively low standard deviation was 400µl. Toxin production with 500µl decreased. Therefore, the volume of 400 µl + 400 µl was used in the experiments with inserts and 400 µl was used in those without inserts.

Table 3.1 The volume effect on toxin production by isolate 53 in the filter insert-plate well system.

Volume composition	Mean amount of B1(ppb)	Standard deviation
100µl+100µl [1]	0[b2]	0
200µl+200µl	9.74[b]	±17.56
300µl+300µl	183.42[b]	±119.87
400µl+400µl	487.88[a]	±101.34
500µl+500µl	246.79[a]	±168.45

[1]100µl+100µl means that 100 µl 53 conidia-medium mixture both in and under the filter.
[2]Means followed by the same letter are not significantly different at the $\alpha = 0.05$ level.

3.2 Kinetics of Aflatoxin B1 Production in the Plate Well System

Toxigenic isolate 53 (400µl, 1×10^5 conidia/ml) was cultured in 24-well plates (4 replicates) for 1, 2, 3, 4, 5 days and the amount of toxin produced was quantified (**Figure 3.1**). This experiment was not repeated. It was shown that toxin production appeared on the 3rd day and reached a peak on the 4th day. Therefore toxin production was quantified on the fifth day which was not significantly different from the fourth day.
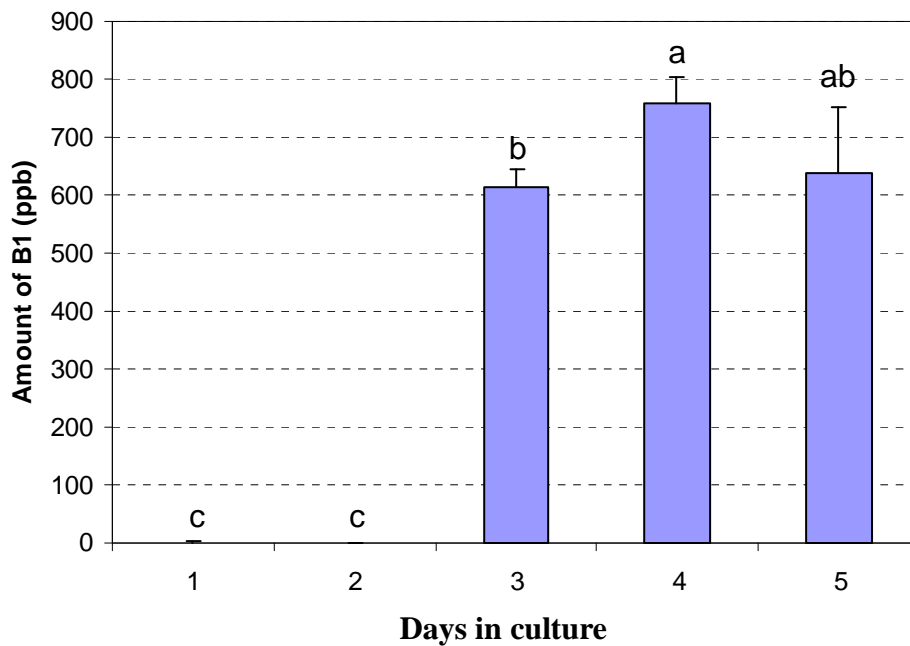
Figure 3.1 Kinetics of aflatoxin B1 production by isolate 53. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the $\alpha = 0.05$ level.

3.3 Touch or Intimate Growth is Needed for Intraspecific Toxin Inhibition

3.3.1 Effect on Toxin Inhibition of Growing Together or Separated by a 0.4 µm Membrane

Isolate 53 was paired with isolate 51 in the filter insert/plate well system two different ways: together or separated by a 0.4 µm filter membrane. The result (**Figure 3.2**) showed that when isolate 53 and isolate 51 were separated by a 0.4 µm filter membrane, 575.82±39.11 ppb aflatoxin was produced, which was even more than the control (430.10±66.38 ppb). However,

very little toxin (15.21±9.52 ppb) was produced when the two isolates were cultured together. This experiment was repeated with similar results.
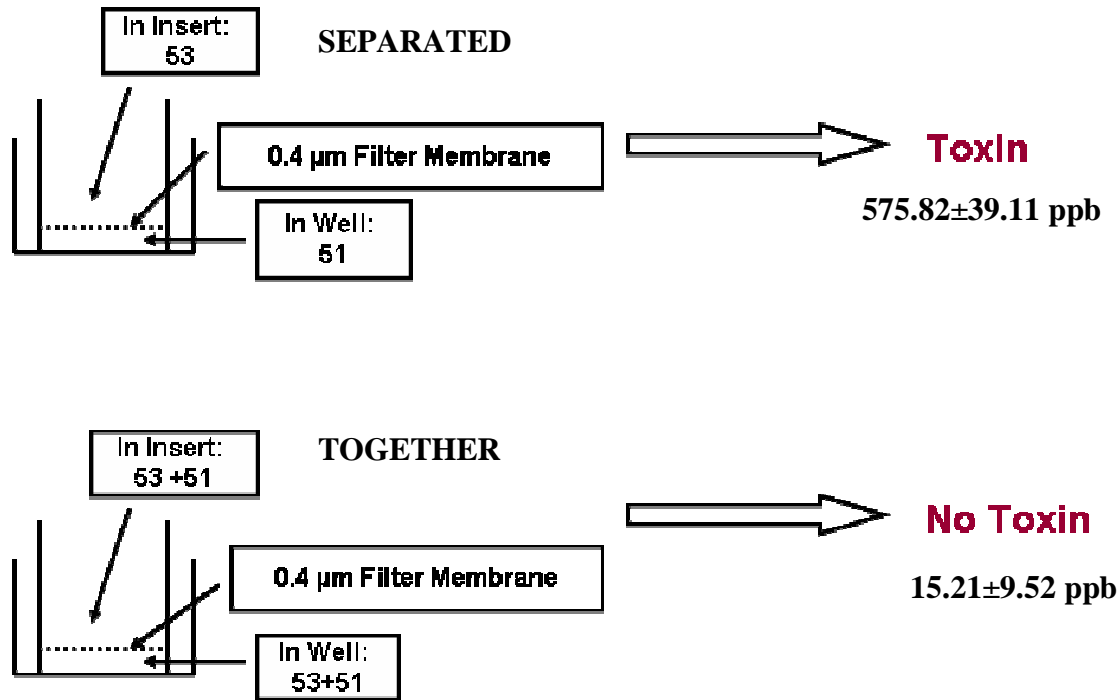
Figure 3.2 Toxin produced by isolate 53 paired with isolate 51.

3.3.2 Generalize the Result to Other Atoxigenic Isolates

Isolate 53 was individually paired in two ways (together or separated) with four atoxigenic isolates: 42, 45, NRRL 21882 and 20 in the GS medium with 0.4 µm pore size filter. The results (**Table 3.2**) showed significant differences between the two treatments: mixed together or separated by the membrane. Intraspecific inhibition occurred when toxigenic and atoxigenic isolates were together. Whether the toxigenic isolate is in the insert or in the well did not make any significant difference. The conclusion was that touching or close physical interaction was needed for the intraspecific inhibition of toxin production. Nutrient or space

competition are not involved in toxin inhibition by atoxigenic isolates as they were identical when together and separated. This experiment was not repeated.

Table 3.2 Aflatoxin B1 production by isolate 53 grown together or separated from atoxigenic isolates.

	Together	Separated	
	Mix[1] (ppb)	T/A[2] (ppb)	A/T[3] (ppb)
51+53	15.21±9.52 [d*]	575.82±39.11 [a]	
42+53	110.77±85.90 [dc]	638.47±67.91 [a]	
45+53	81.80±84.54 [dc]	635.96±89.48 [a]	
21882+53	36.54±34.96 [d]	587.32±10.90 [a]	546.52±52.09 [a]
20+53	193.18±206.81 [c]	665.80±25.90 [a]	
Check[4]		430.10±66.38 [b]	

[1] Mix: 53 and atoxigenic isolates mixed together in and under filter
[2] T/A: 53 in filter and atoxigenic isolate under filter
[3] A/T: atoxigenic isolates in filter and 53 under filter
[4] Check: 53 in filter and only medium under filter
* Means followed by the same letter are not significantly different at the $\alpha = 0.05$ level

3.3.3 Effect of Pore Sizes on Inhibition

In order to test the conclusion that contact was essential for inhibition, inserts with different pore sizes (0.4, 3, 12, 74 and 200 µm) were used. The hypothesis was that inhibition occurs only when conidia, germ tubes or hyphae are allowed to pass through the membrane and grow together. Isolate 51 which has the highest inhibitory ability among the isolates tested was chosen for this experiment. Treatments with filter pore sizes 0.4, 3 and 12 µm were done together and those with pore size 74 and 200 µm were done 10 days later. There were four replicates and the experiment was not repeated. The result (**Table 3.3**) showed that composition of the membrane has little effect on the inhibitory function and as predicted pore size was important. Twelve µm was the critical pore size which should allow some passage of *A. flavus* conidia and hyphae with diameters between 3.5-7.0 µm. An approximately 50% inhibition

occurred with 12 µm pore size and higher inhibition with larger pore sizes, which supported the

conclusion that touching or physical interaction was necessary in intraspecific toxin inhibition.

Table 3.3 The effect of the pore size on toxin inhibition in separated culture system. The critical point is 12 µm pore size, which is shown in red.

Treatment	Pore Size	Membrane Material	Mean Amount of B1 (ppb)± SD
Check(53/0.01%TX)	0.4 µm	Hydrophilic PTFE	605.93±45.78 a*
	0.4 µm	Polycarbonate	425.22±82.60 bc
	200 µm	Polyester Mesh	528.02±80.38 ab
Separated (53/51)	0.4 µm	Hydrophilic PTFE	507.54±87.49 ab
	0.4 µm	Polycarbonate	586.10±44.52 ab
	3 µm	Polycarbonate	511.34±115.68 ab
	12 µm	Polycarbonate	317.59±102.09 c
	74 µm	Polyester Mesh	1.82±1.65 d
	200 µm	Polyester Mesh	0.02±0.02 d
Together(53+51/53+51)	0.4 µm	Hydrophilic PTFE	10.65±2.18 d
	0.4 µm	Polycarbonate	0.21±0.14 d
	200 µm	Polyester Mesh	0.01±0.01 d

*Means followed by the same letter are not significantly different at the $\alpha = 0.05$ level.

3.4 Timing of Intraspecific Toxin Inhibition

3.4.1 Does the Addition Time of Atoxigenic Spores Affect the Inhibition?

In order to see when the recognition event occurred, isolate 53 was grown in Eppendorf

tubes for 0, 1, 2, 3 or 4 days before adding isolate 51 conidia-medium mixture. Toxin was

quantified on isolate 53's fifth day of growth. The result (**Figure 3.3**) showed that no toxin was

produced when isolate 51 was added at time 0 and they grew concurrently. Toxin was not

significantly different from the control when isolate 51 was added 1 day later. There were five

replicates and this experiment was repeated with similar results. Short intervals were tested by

adding atoxigenic isolate 51 at 0, 4, 8, 12, 16, 20 or 24 hours after isolate 53's growth was

initiated. The result (**Figure 3.4**) showed that the inhibition only occurred when the addition of

isolate 51 was within the first 16 hours' growth of isolate 53.

Figure 3.3 Timing of the recognition event for intraspecific toxin inhibition. Isolate 53 was grown for days indicated prior to adding isolate 51. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the α = 0.05 level.
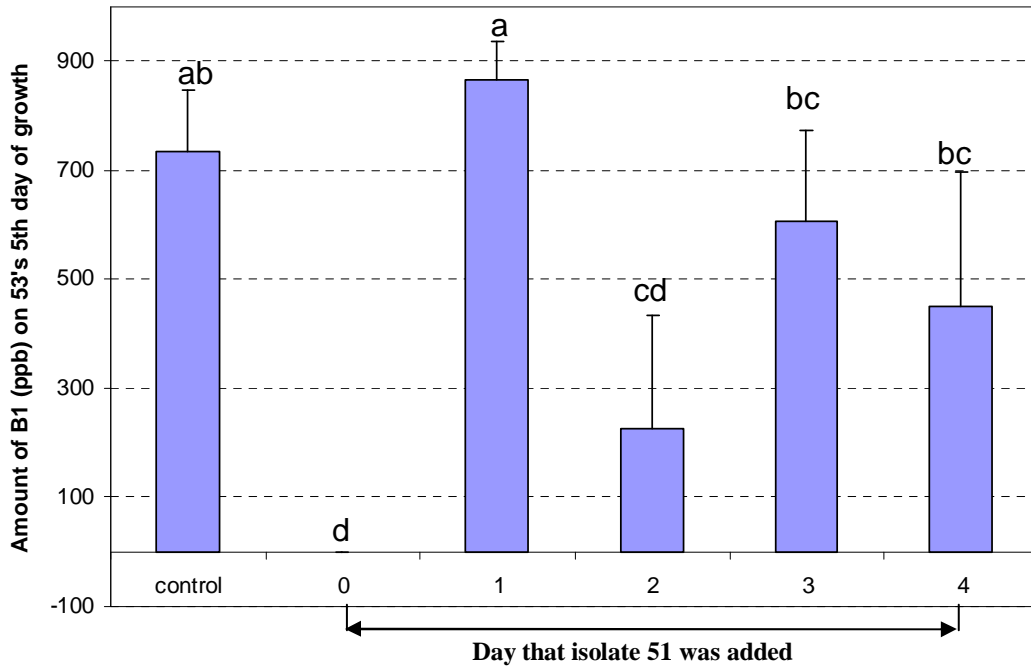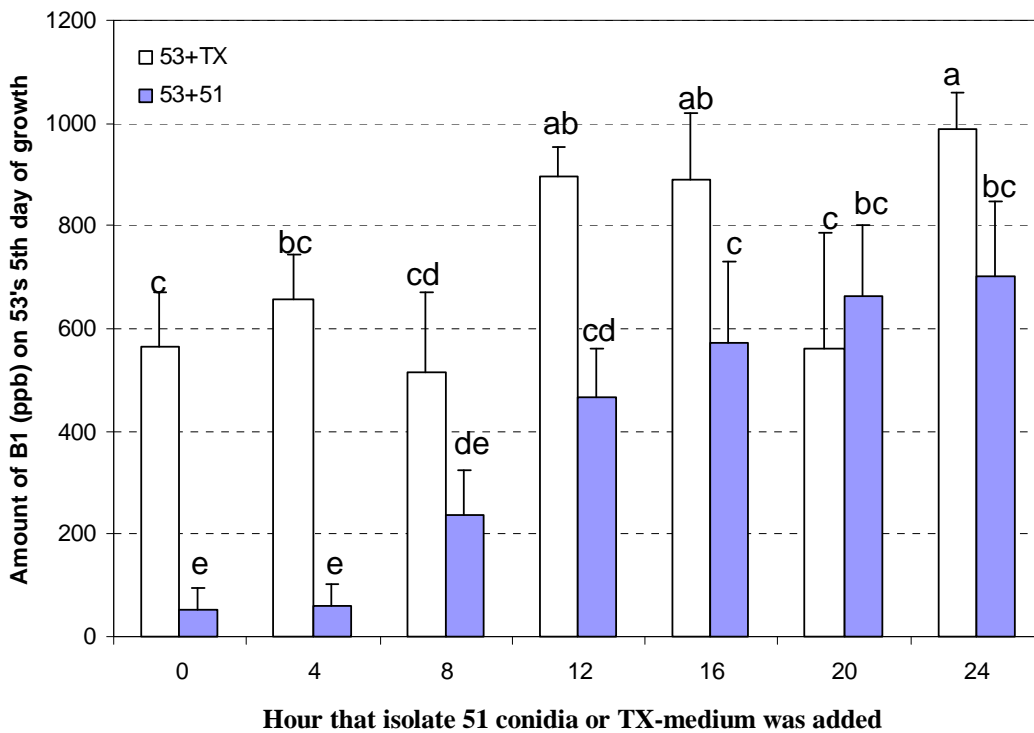

Figure 3.4 Precise timing of the recognition event for intraspecific toxin inhibition. Isolate 53 was grown for the hours indicated prior to adding of isolate 51 or TX-mixture. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the α = 0.05 level.

25

3.4.2 Does the Time after Germination of Atoxigenic Spores Affect the Inhibition?

In order to see whether the time after germination of atoxigenic spores affected the inhibition, isolate 51 was grown for 0, 1, 2, 3 or 4 days before the addition of isolate 53 to Eppendorf tubes. Toxin was quantified on isolate 53's fifth day of growth. There were 5 replicates and the experiment was not repeated. The result (**Figure 3.5**) showed that there is no difference between toxin productions by isolate 53 when it was challenged by different growth stages (0, 1, 2, 3 or 4 days) of isolate 51. Toxin production of isolate 53 was almost totally suppressed by all the different growth stages of isolate 51. It appears that isolate 51 is always competent to inhibit isolate 53's toxin production as long as isolate 51 is present in the first 16 hours of isolate 53's growth.
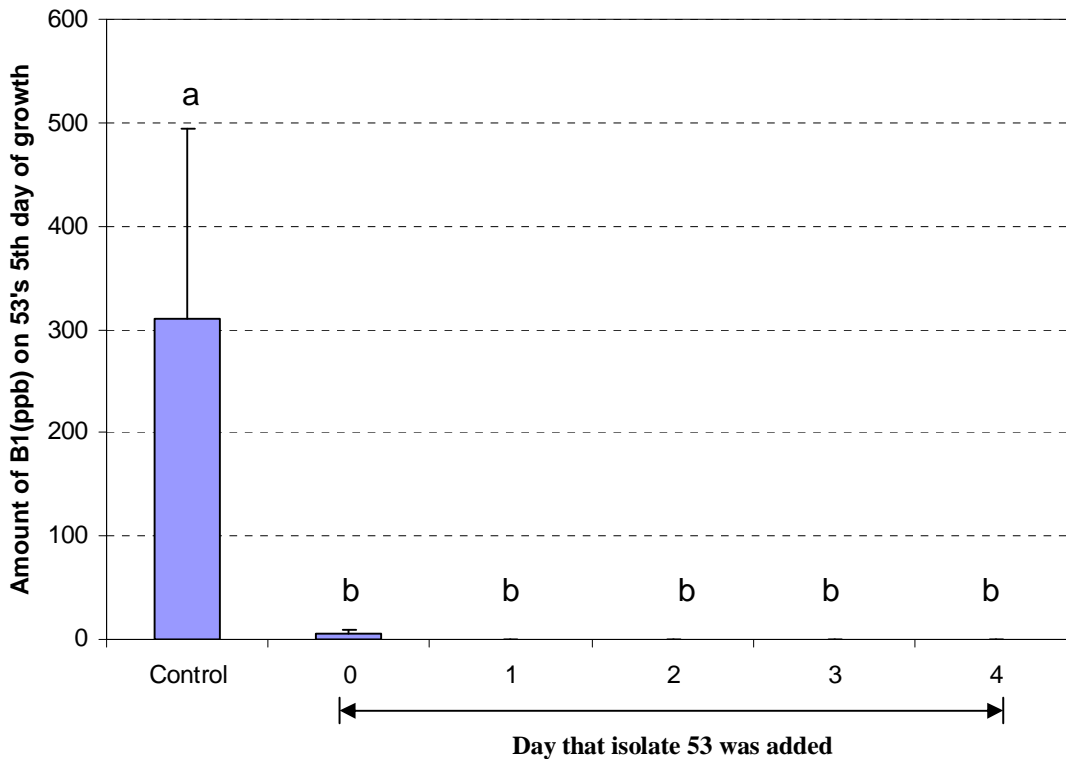
Figure 3.5 Toxin production by isolate 53 which was challenged by different growth stages (0, 1, 2, 3, 4 days) of isolate 51. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the $\alpha = 0.05$ level.

3.4.3 Does Inhibition only Occur within the First 16-hour's Growth of Toxigenic Isolate?

In order to determine whether inhibition only occurs within the first 16-hours growth of the toxigenic isolate, isolate 53 and 51 were grown separately for 1, 2, 3 and 4 days and then combined. The result showed that toxin inhibition can occur even on the 2nd day (**Figure 3.6**). Moreover, isolate 51 was grown for 24 hours and then was combined with different ages (0, 24, 36, and 48 hours) of isolate 53. The result showed that 24-hour old isolate 51 can inhibit isolate 53 with different ages (0-48 hours) of (**Figure 3.**7).
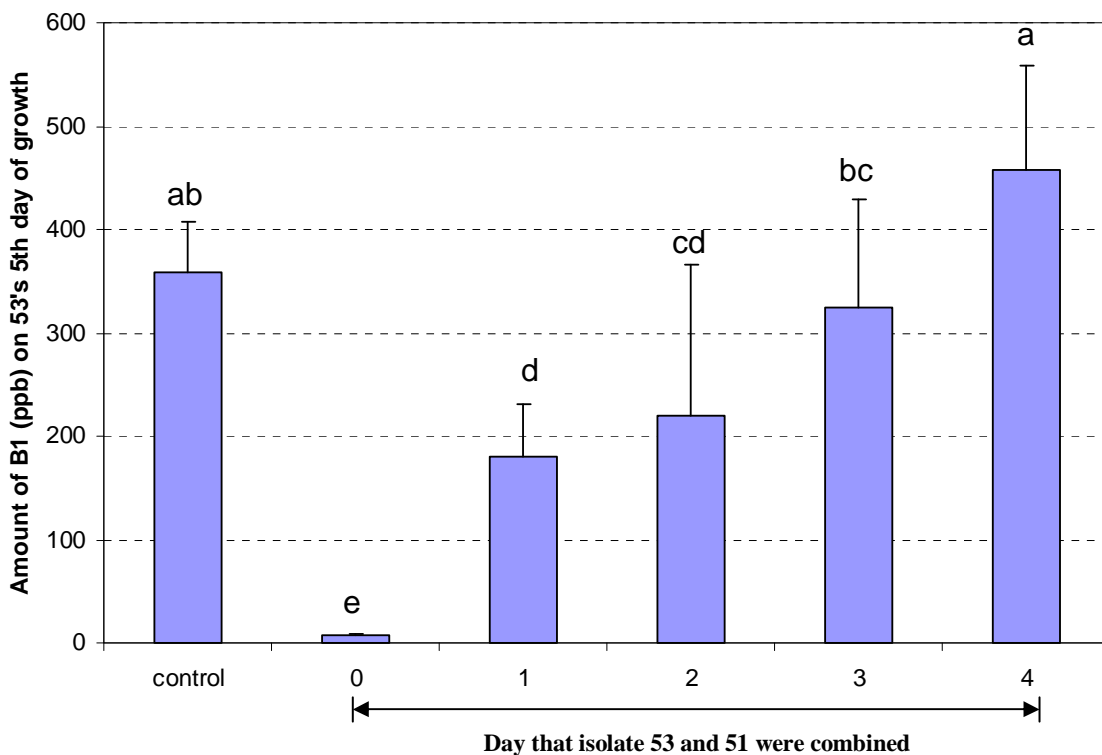

Figure 3.6 Timing of the recognition event for toxin inhibition when isolate 53 and 51 were combined at the days indicated. Isolate 53 and 51 were grown separately for the days indicated prior to being combined. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the $\alpha = 0.05$ level.
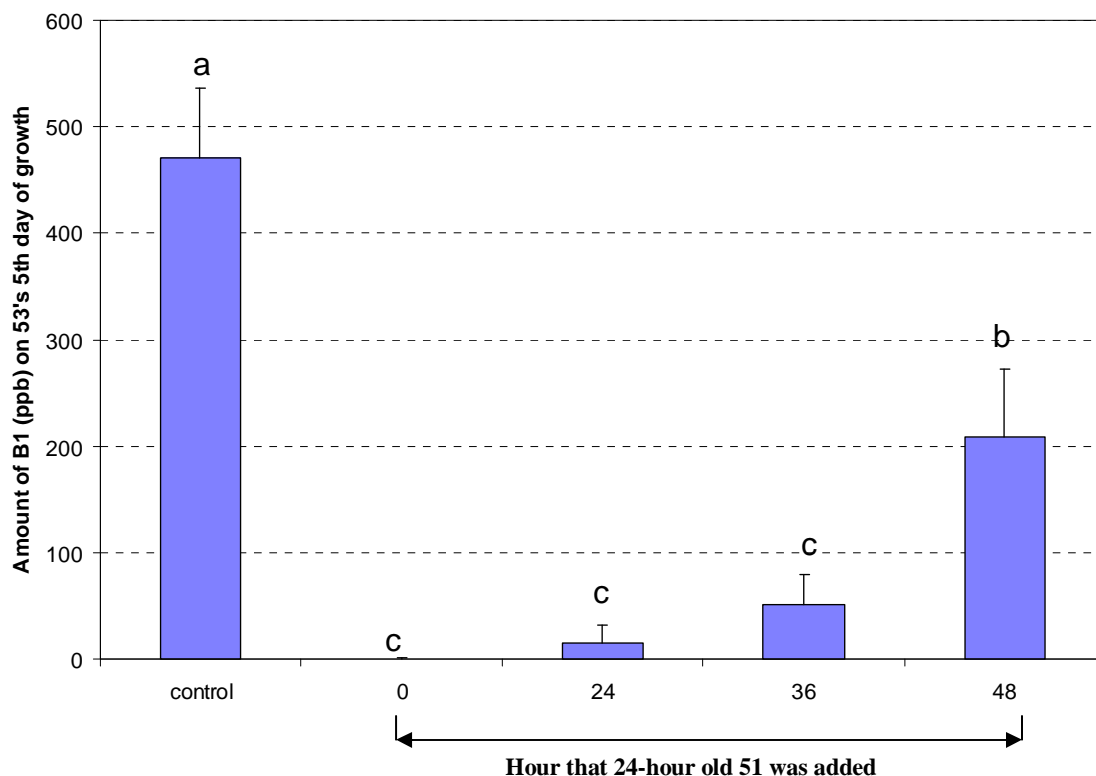
Figure 3.7 Timing of the recognition event for inhibition of toxin production of isolate 53 by 24-hour old isolate 51. Isolate 53 was grown for the hours indicated prior to adding of 24-hour old isolate 51. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the $\alpha = 0.05$ level.

3.5 Specificity of Intraspecific Toxin Inhibition

3.5.1 Inhibitory Ability of Different Atoxigenic Isolates to Toxin Production by Af70s-GFP

Af70s-GFP was acquired to microscopically observe the toxin inhibition interaction. Six different atoxigenic isolates (42, 45, 51, NRRL 21882, 4, Af Papa 827), four of which (42, 45, 51 and NRRL 21882) were previously shown to inhibit isolate 53, were tested to see whether they could inhibit toxin production by Af70s-GFP in the plate well system. The result (**Figure 3.8**) showed that none of the six isolates could inhibit toxin production by Af70s-GFP. It seems that there is specificity in toxin inhibition.

Figure 3.8 Toxin production by Af70s-GFP when it was alone or paired with atoxigenic isolates (42, 45, 51, NRLL 21882, 4 and Af Papa827). Error bars represent the standard deviation.
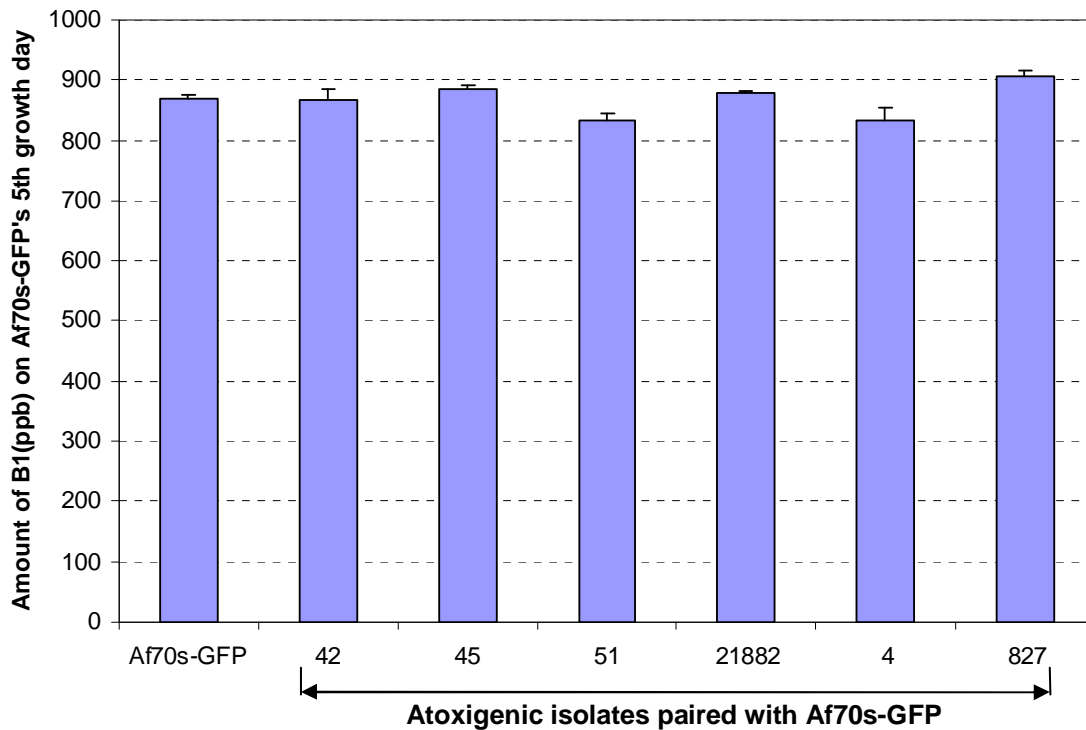
3.5.2 Intraspecific Toxin Inhibition Patterns among Isolates 53, Af70s-GFP and NRRL3357

Toxigenic isolates (53, Af70s-GFP, and NRLL 3357) and atoxigenic isolates (42, 45, 51, NRRL 21882, 4, Af Papa 827, K49, Af 4-2 and Af 5-1) were selected to test whether atoxigenic isolates produce the same inhibitory pattern on toxigenic isolates using the plate well system. The data was from experiments done on different dates. The experiment for the inhibition of 42, 45, 51 and NRRL 21882 to toxin production by toxigenic isolate 53 began on March 6th, 2006. The experiment for the inhibition of isolates 42, 45, 51 and NRRL 21882 to toxin production of Af70s-GFP began on May 15th, 2006. The experiment for the inhibition of isolates Af Papa 827 and 4 to toxin inhibition of isolates 53 and Af70s-GFP was done at the end of May, 2006. The experiment for the inhibition of isolate 42, 45, 51, NRRL 21882, 4, Af Papa 827 and K49 to toxin production by NRRL 3357 began on July 30th, 2006. The experiment for the inhibition of isolate Af 4-2 and Af 5-1 to toxin production by AF70s-GFP and NRRL 3357 began on August

29

15th, 2006. The experiment for the inhibition of isolate Af 4-2 and Af 5-1 for the inhibition of isolate 53 began on October 5th, 2006. All the experiments were done in the plate system without inserts and have not been repeated. The results showed that the inhibitory profiles of the three toxigenic isolates 53, Af70s-GFP and NRRL 3357 were different. Isolates 42, 45, 51, NRRL 21882, K49 and Af 5-1 can inhibit 53 more than 80% while 4, Af Papa 827 and Af 4-2 can hardly inhibit 53 (**Figure 3.9 a**). Af70s-GFP can only be inhibited by K49, Af 5-1 and Af 4-2 but not the other atoxigenic isolates (**Figure 3.9 b**). The profile pattern for NRRL 3357 appears similar to Af70s-GFP (**Figure 3.9 c**).

a. Toxigenic isolate 53
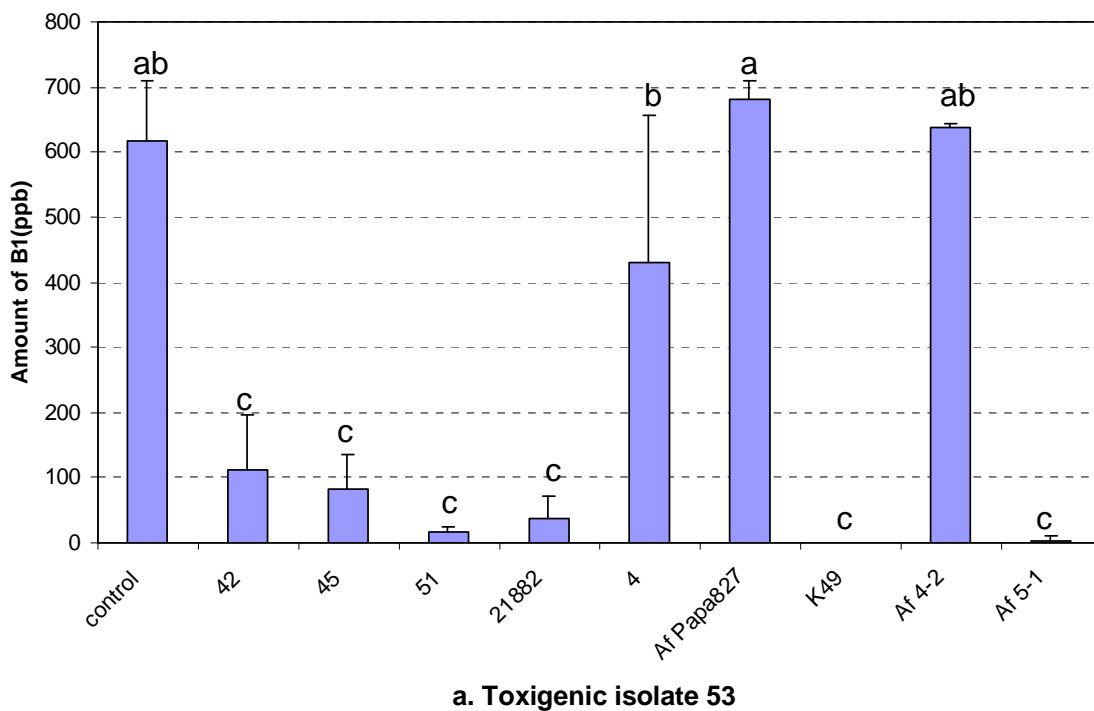
Figure 3.9 Aflatoxin production by three toxigenic isolates of *Aspergillus flavus* after 5 days incubation with nine different atoxigenic isolates of *A. flavus*: a) toxigenic isolate 53, b) toxigenic isolate Af70s-GFP, and c) toxigenic isolate NRRL 3357. Error bars represent the standard deviation. Bars with the same letter are not significantly different at the α = 0.05 level.
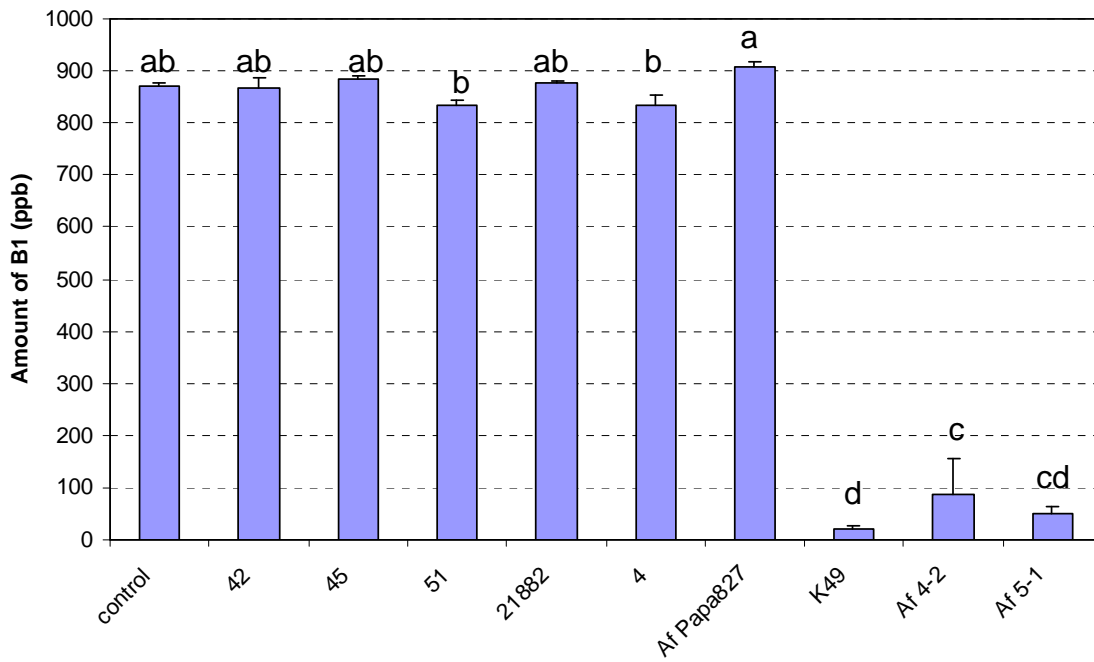
b. Toxigenic isolate Af70s-GFP
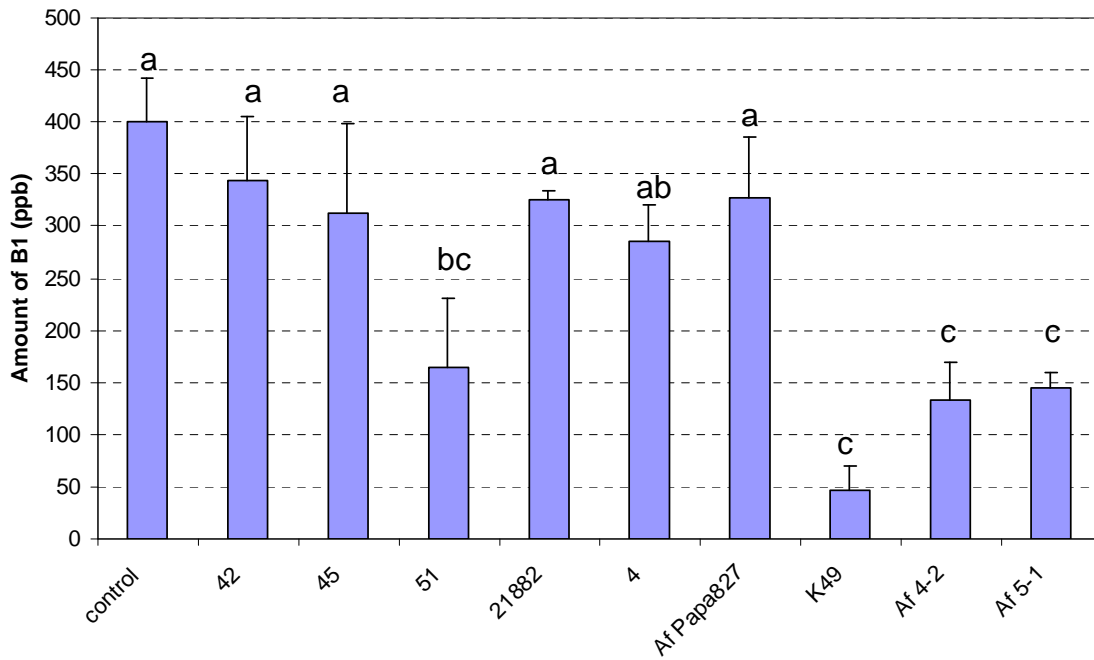
c. Toxigenic isolate NRRL 3357

Figure 3.9 (continued)

A preliminary experiment was performed to test whether inhibition profiles are stable with age. Isolate 53 and isolate 51 conidia were regenerated on PDA. Isolate 53 was paired with isolate 51 and old conidia of previously inhibitory isolates (NRRL 21882, 45 and 42) in plate well system. The result (**Figure 3.10**) showed that old conidia of the three isolates did not inhibit toxin production by newly prepared conidia of isolate 53. However, newly prepared conidia of isolate 51 maintained inhibitory ability.
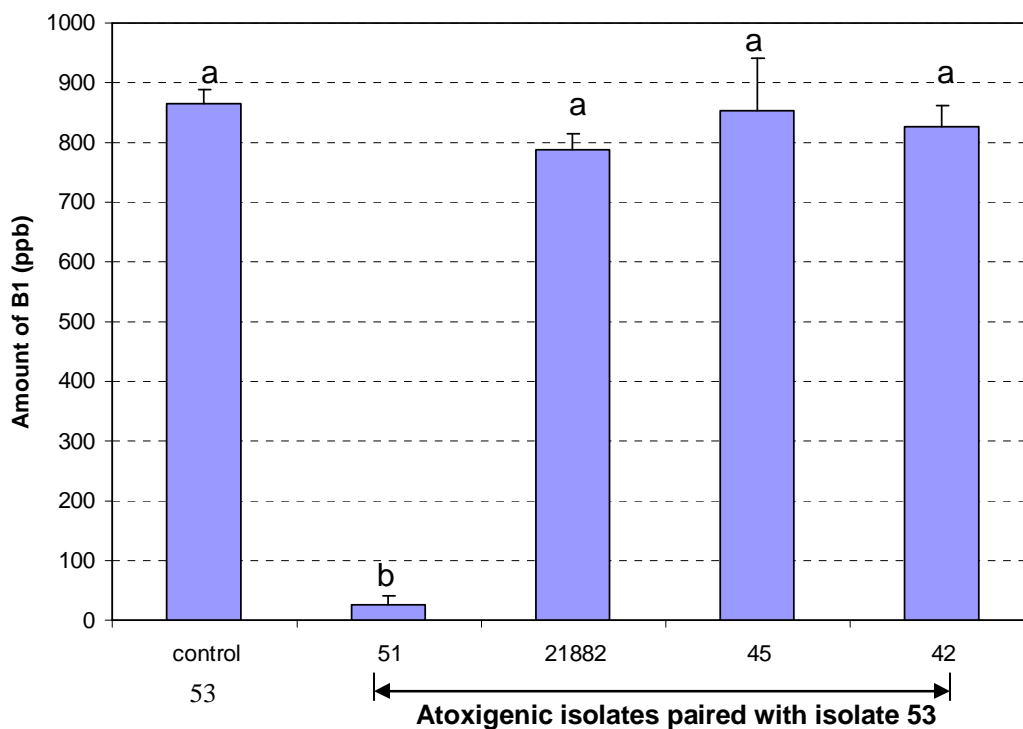
Figure 3.10 Effect of conidial age on toxin inhibition. Newly harvested isolate 53 conidia were paired with newly harvested isolate 51 conidia and old conidia of three atoxigenic isolates (NRRL 21882, 45, and 42). Error bars represent the standard deviation. Bars with the same letter are not significantly different at the $\alpha = 0.05$ level.

3.6 Microscopic Observation of Intraspecific Competition

Toxigenic isolate Af70s-GFP (Rajasekaran *et al*. 1999), a transgenic isolate which produces green fluorescent protein, was paired with its effective toxin inhibitor isolate K49, to microscopically observe the inhibitory effect. The result was observed with the Olympus IMT2

inverted fluorescence microscope. It appeared that Af70s-GFP grew much less when it was paired with K49 than when it was grown alone: fewer spores germinated, and the germ tubes were much shorter in the Af70s-GFP with K49 than in Af70s-GFP alone. No fusion phenomena were observed.

Sub-cellular structure of Af70-GFP was observed using a Leica Confocal Laser Scanning microscope. The objective magnification was 63X and zoom in magnification was adjusted according to the size of germlings. Vacuoles in Af70s-GFP were very clear when it was grown alone but were not observed when paired with K49 (**Figure 3.11**) in GS liquid medium.

(a) AF70s-GFP alone
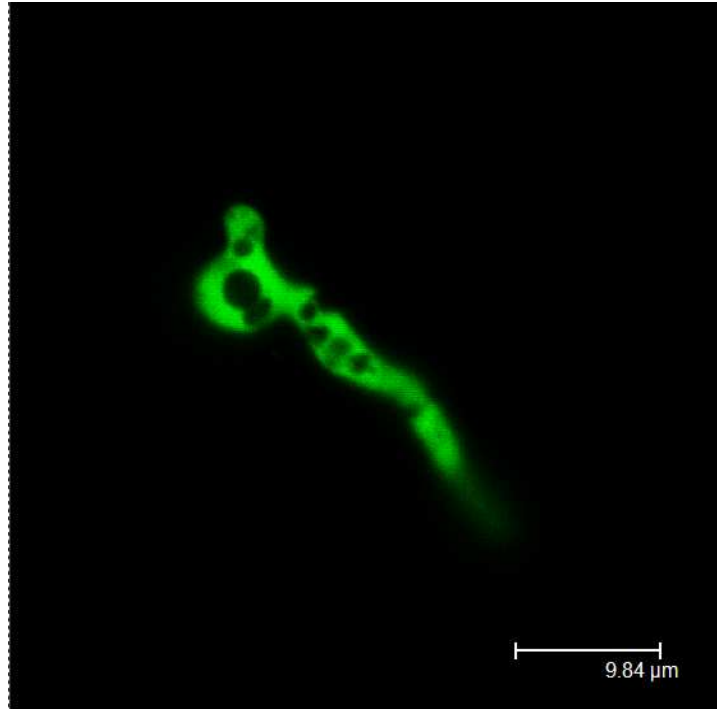
(b) Af70s-GFP paired with K49
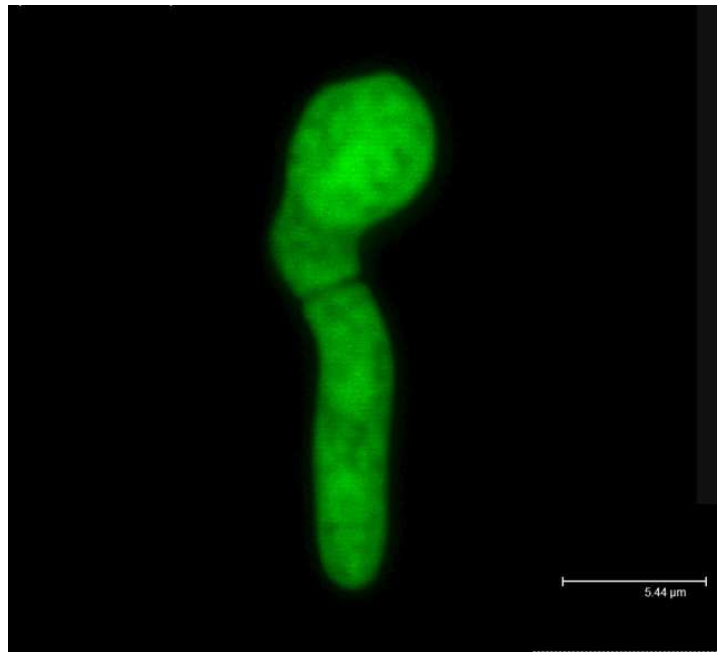
Figure 3.11 The appearance of Af70s-GFP alone or with K49 under Leica Confocal Microscope (63X objective with unknown zoom magnification). There were a lot of clear vacuoles in Af70s-GFP mycelia when it was grown alone (a), while no vacuoles were observed when Af70s-GFP was paired with K49 (b).

CHAPTER 4. DISCUSSION

Results showed the amount of aflatoxin produced when isolate 53 and 51 were separated by 0.4 µm membrane was not statistically different from isolate 53 alone. While almost no toxin was produced when those two isolates were grown together. This result was generalized by pairing isolate 53 with four other atoxigenic isolates (42, 45, NRRL 21882 and 20) and the same trend was observed. Because the 0.4 µm filter in filter insert-well plate system separates fungus but not nutrients, toxin inhibition should occur if the mechanism of toxin inhibition is due to nutrients competition. This suggests that nutrient competition does not explain the intraspecific toxin inhibition and touching or close physical interaction is needed. Wicklow *et al.* (2003) used a suspended disc assay to look at the effect of atoxigenic isolates on toxin production by toxigenic isolates and suggested that nutrient competition should be at least one of the mechanisms of intraspecific toxin inhibition. This is the first direct evidence against nutrient competition as the basis of intraspecific toxin inhibition.

The touching or close physical interaction requirement was further supported by the test with different filter insert pore sizes. No toxin inhibition occurred when isolate 53 and 51 were separated by 0.4 µm membrane, approximately 50% inhibition occurred when they were separated by a 12 µm membrane, and complete inhibition occurred when a 74 µm membrane was used. Because the critical pore size is 12 µm and the diameters of *A. flavus* conidia and hyphae are between 3.5-7.0 µm, this suggests that inhibition only occurs when the toxigenic isolate and atoxigenic isolate can contact each other or grow within one compartment. Zummo (1991) inoculated a white conidial isolate and a green conidial isolate of *A. flavus* in a corn field and found that an individual kernel could be infected by both isolates. Therefore, in nature, toxigenic strains and atoxigenic strains can grow together in one corn kernel and toxin inhibition will occur, which is the true basis of biological control.

Chang & Hua (2006) reported that their atoxigenic TX 9-8 did not affect aflatoxin accumulation by toxigenic isolates when it was inoculated 24 h later than the toxigenic isolate and it seems that there is a 24-hour window for intraspecific toxin inhibition. However, Cotty & Bayman (1993) reported that 48-hour old mycelial balls of an atoxigenic isolate could inhibit toxin production by 48-hour old mycelial balls of a toxigenic isolate. Therefore this is a controversial issue in intraspecific toxin inhibition. Our results from experiment **3.3** and **3.4** supported P. K. Chang's study and showed that inhibition only occurred when adding atoxigenic isolate 51, no matter what the initial growth stage is, within first 16-hour growth of toxigenic isolate 53. However, the result from experiment **3.5** showed that isolate 51 can inhibit toxin production by two-day-old isolate 53 if they are at the same growth stage (**Figure 3.6**), the same as the result of Cotty & Bayman (1993) and 24-hour old mycelia of isolate 51 can inhibit toxin production by different growth stage (from 0 to 48 hours) of isolate 53 (**Figure 3.7**). This suggests that there is a 16-hour "window" for the conidia inhibition ability but for mycelia, the "window" is expanded to 48 hours.

The different inhibition patterns of 53, Af70s-GFP and NRLL 3357 from this study showed that there is specificity in the toxin inhibition. This conclusion was also supported by the study of Bandyopadhyay & Cardwell 2004. They reported that American atoxigenic isolate AF36 was effective against the American toxigenic isolate AF13, but not the toxigenic African S-strain, BN40. African atoxigenic L-strain BN30 was the only isolate that reduced toxin production by the toxigenic African S-strain, BN40. Due to the specificity in toxin inhibition and diversity of toxigenic *A. flavus* strains in the field, it is unlikely that application of a single atoxigenic biocontrol isolate will be able to eliminate aflatoxin contamination of crops, and probably a mixture of atoxigenic isolates will be required for effective biocontrol.

A preliminary experiment (**Figure 3.10**) to test whether inhibition profiles are stable with age showed that fresh conidia of toxigenic isolate 53 can be inhibited by new isolate 51, as before, but not by old conidia of isolates 42, 45 and NRRL 21882 which were inhibitory in previous profile. This suggested that inhibition was affected by age of conidia. However the complete interaction of conidial age and toxin inhibition was not thoroughly tested. An applicable experimental arrangement is shown in **Table 4.1**.

Table 4.1 Experimental arrangement for testing the effect of conidial age on intraspecific toxin inhibition.

Toxigenic isolate	Inhibitory atoxigenic isolate		Non-inhibitory atoxigenic isolate	
	New	Old	New	Old
New	New+ New	Old + Old	New+ New	Old + Old
Old	Old+ New	New + Old	Old+ New	New + Old

Though inhibitory profiles of isolate 51 shown in **Figure 3.9** and **Figure 3.10** are different, both of them are valid. This difference opened the door to the secret of conidial age as it relates to the toxin inhibition interaction/mechanism. This triggers two other questions. What changes within the conidia with age? Do these age related changes occur in the field situation? The answer to these questions should contribute to a better understanding of biocontrol.

Conidial germination and germ-tube growth of Af70s-GFP appeared to be inhibited when it was paired with its competitor isolate K49, which suggests that the presence of atoxigenic isolates inhibits growth of toxigenic isolates as well as toxin production. Measurement of these parameters is needed. This is an important point for the understanding of intraspecific toxin inhibition. Wicklow *et al.* (2003) showed that total mycelial dry weight of the fungal mixture, toxigenic and atoxigenic, was actually greater than when grown separately. A phenomenon they referred to as compensatory growth. However in this study, less vegetative growth of the

toxigenic isolate (Af70s-GFP) was observed when paired with K49 but the growth of atoxigenic isolate was not determined.

Microscopic observation of sub-cellular structure of *A. flavus* by confocal microscopy showed that vacuoles in the cell of Af70s-GFP were not seen when Af70s-GFP was paired with K49 but were very distinct when Af70s-GFP was grown alone. This suggests that vacuole production in Af70s-GFP was inhibited, or the GFP protein could diffuse into vacuole in the presence of atoxigenic isolate K49, or the vacuole membrane dissociated. The relationship between vacuole disappearance and toxin production has not been reported before. Recent literature suggests that vacuoles may be responsible for fungal growth (Weber, 2002). Therefore, a possible explanation is that the presence of K49 disrupted vacuoles in Af70s-GFP which resulted in the inhibition of growth and toxin inhibition.

Kinetics of aflatoxin B1 production by 400 µl isolate 53 (1×10^5 conidia/ml) in plastic plates showed that this fungus starts to produce toxin after the second day and reaches a peak on the fourth day. This result is slightly different from a previous study (Mellon *et al*, 2002) in which aflatoxin B1 production increased after 36 h, with a peak on the fourth day. This probably is due to the difference in medium (simulating corn kernel) and culture conditions (31^oC, shaking).

Future work will involve clarification of the effect of toxigenic and atoxigenic conidial age on intraspecific inhibitory ability as it relates to specificity profiles. Growth measurements (germination rate; germ tube length; biomass) of Af70s-GFP and K49 when grown together or separated in the filter insert-well plate system are needed. A series of specific chemical signal inhibitors should be used in an attempt to further understand the signaling involved in the touch inhibition phenomenon.

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