CHAPTER IV

RESULTS

4.1 Collection of soil samples from corn fields in Thailand

Soil samples (approximately 500 g) were collected from fifteen (15) corn fields in Northern Thailand including Chaingrai (5 fields), Pare (5 fields), and Payao (5 fields) provinces, fourteen corn fields in (14) Central Thailand including Nakornsawan (3 fields), Pitsanulok (6 fields), Lopburi (2 fields), Saraburi (1 fields), and Bangkok (2 fields) provinces, and four corn fields in (4) Northeastern Thailand including Nakornrajchasima (2 fields), Sakornnakorn (1 fields), and Khonkan (1 fields) provinces. Chaingrai, Payao, Nakornsawan, Pitsanulok, Lopburi, and Nakornrajchasima provinces are a major corn cultivating areas of Thailand with corn fields more than 40,000 acres in 2005 (Figure 4.1). The samples were collected between September and November 2005, at temperature between 27 to 32 ^{O}C (Table 4.1 to 4. 3).

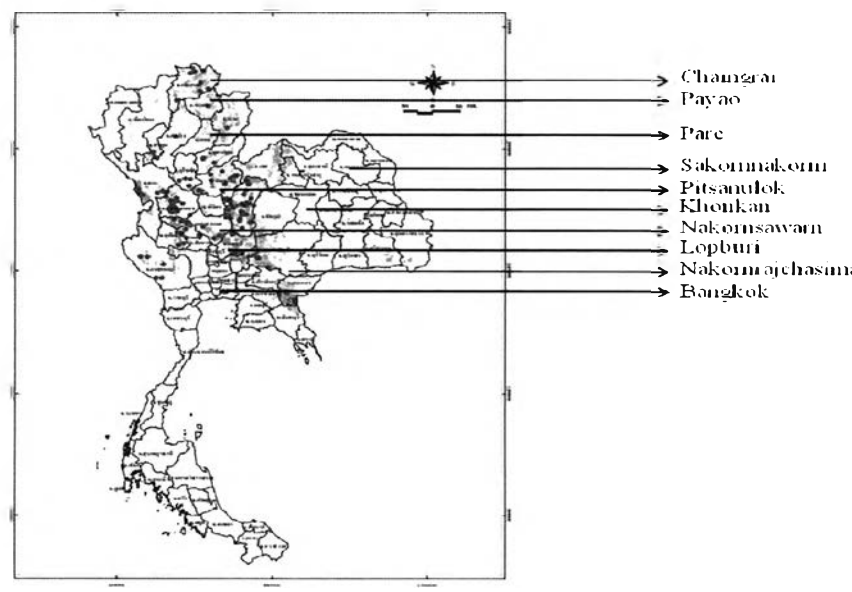


Figure 4.1 Soil collection areas in this study; green spot showed corn producing area, and red spot showed corn trading area.

Table 4.1 Collection sites in Northern Thailand and member of soil collected with local temperature at collecting time and average temperature time between September to November, 2005.

Province	District	Field	Temperature (^{O}C)	Average temperature in province in 2005* (^{O}C)
Chaingrai	Maejun	1	30	28.0
	Maesai	2	29	
	Chaingsan	1	38	
	Chaingkhong	1	31	
Payao	Dokkamtai	4	30	28.4
	Jun	1	31	
Pare	Song	1	30	29.3
	Rongkwang	1	31	
	Soongmen	1	31	
	Den chai	2	32	

* http://www.tmd.go.th (Thai Meteorological Department)

Table 4.2 Collection sites in Northeastern Thailand and member of soil collected with local temperature at collecting time and average temperature time between September to November, 2005

Province	District	Field	Temperature (^{O}C)	Average temperature in province in 2005* (^{O}C)
Nakornrajchasima	Khaoyai	1	27	29.4
	Pakchong	1	27	
Khonkan	-	1	29	28.9

* http://www.tmd.go.th (Thai Meteorological Department)

Table 4.3 Collection sites in Central Thailand and member of soil collected with local temperature at collecting time and average temperature time between September to November, 2005

Province	District	Field	Temperature (^{O}C)	Average temperature in province in 2005* (^{O}C)
Nakornsawan	Payuhakiri	3	32	29.7
Pitsanulok	Phompiram	6	28	29.1
Lopburi	Praputtabat	2	29	29.4
Saraburi	Banmo	1	29	no data
Bangkok	Patumwan	2	29	29.9

* http://www.tmd.go.th (Thai Meteorological Department)

4.2 *Aspergillus* section *Flavi* isolation

Soil samples from corn fields of Thailand were inoculated onto Aspergillus Flavus and Parasiticus Agar (AFPA) to isolate for *A. flavus* and *A. parasiticus*. Both *A. flavus* and *A. parasiticus* were identified in *Aspergillus* section *Flavi* which dominantly produced green conidia. *Aspergillus* members in the section *Flavi* produced conidia with dominantly green to brownish green color on the media including PDA, CMA, and Czapekapek Dox medium. In this study, some species of isolated *Aspergillus* with green to brownish green conidia color *(Aspergillus* section *Flavi*) showed abilities to produce orange color in the reserve phase of the colony when viewed on the reverse. However, some species of *Aspergillus* section *Flavi* produced different degree of yellow orange color in the reverse colony (Table 4.4, and Figure 4.2 to 4.3). They were identified into five *Aspergillus* section *Flavi* groups based on color reaction on AFPA (Klich et al., 1988 and Pitt et al., 1983) including

a. dark brown reverse, and produced brownish green conidia

b. pale brown reverse, and produced brownish green conidia

c. pale yellow reverse, and produced green to brownish green conidia

d. pale yellow reverse, and produced sparsely green to brownish green codinia

e. dark yellow reverse, and produced green conidia

Table 4.4 Isolated *Aspergillus* section *Flavi* from corn fields in Thailand cultivated on AFPA medium at 30 ^{O}C for 7 days

Group	Reverse color	Conidia color	Total member of isolates
A	dark brown	brown-green	4
B	pale brown	brown green	14
C	pale yellow	green to brownish green	12
D	pale yellow	sparsely green to brownish green conidia	12
E	dark yellow	green	205

When five groups of isolated *Aspergillus* were compared with the standard strains of *Aspergillus* section *Flavi*, group A showed dark brown reverse colony color similar *A. tamarii*, and group E showed yellow orange reverse colony color similar *A. flavus, A. parasiticus* and *A. oryzae* (Figure 4.2 to 4.3).

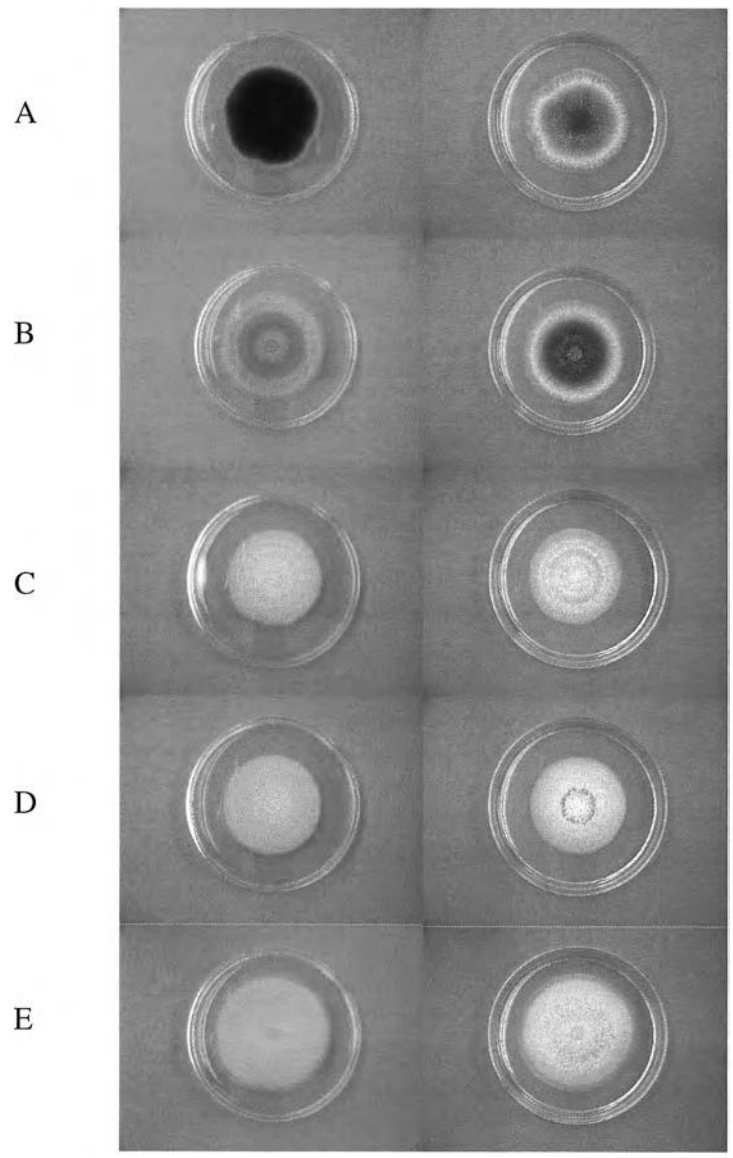


Figure 4.2 Isolates of *Aspergillus* section *Flavi* (A to E) from corn fields in Thailand cultivated on AFPA medium at 30 ^{O}C for 7 days.

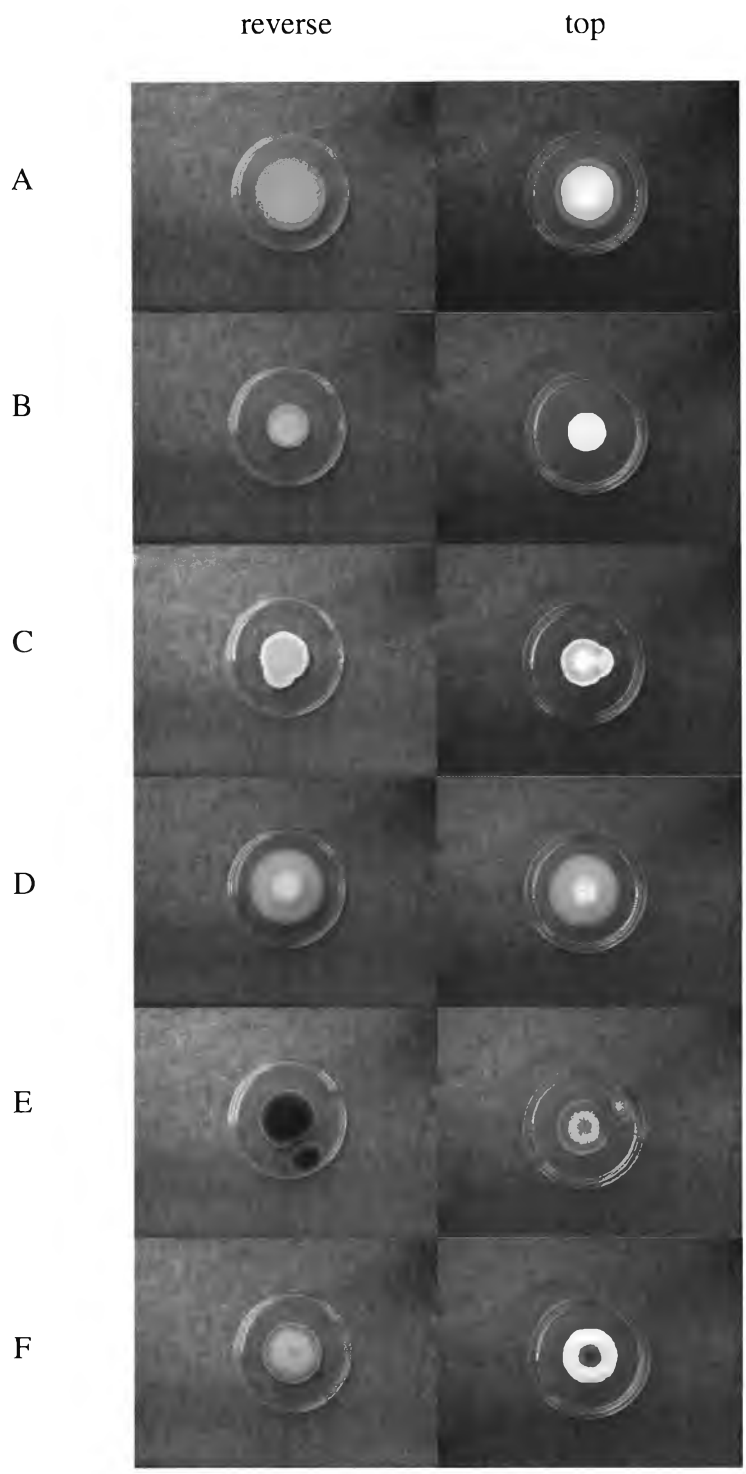


Figure 4.3 standard strains of *Aspergillus* section *Flavi* on AFPA medium at 30 ^{O}C for 7 days (1X); (a) *A. flavus* NRRL 3357, (b) *A. parasiticus* SRRC 75, (c) *A. oryzae* SRRC 2044, (d) *A. nomius* SRRC 2075, (d) *A. pseudotamarii* SRRC 2420, and (e) *A. tamarii* SRRC 99)

Spore suspension of each isolated *Aspergillus* was inoculated onto 5% V8 agar (pH 5.5). The cultures were incubated overnight at 30 ^{O}C in the dark, 7 days for a single colonization. A single colony of *Aspergillus* presumably devised from a single conidium was streaked onto 5%V8 agar (Figure 4.4). The cultures were incubated at 30 ^{O}C in the dark, 7 days for sporulation. At the result, the conidia on this plate was suspended in 1 ml H_2O_2 in 5 ml screw cap tube and keep its at 4 ^{O}C in refrigerator.

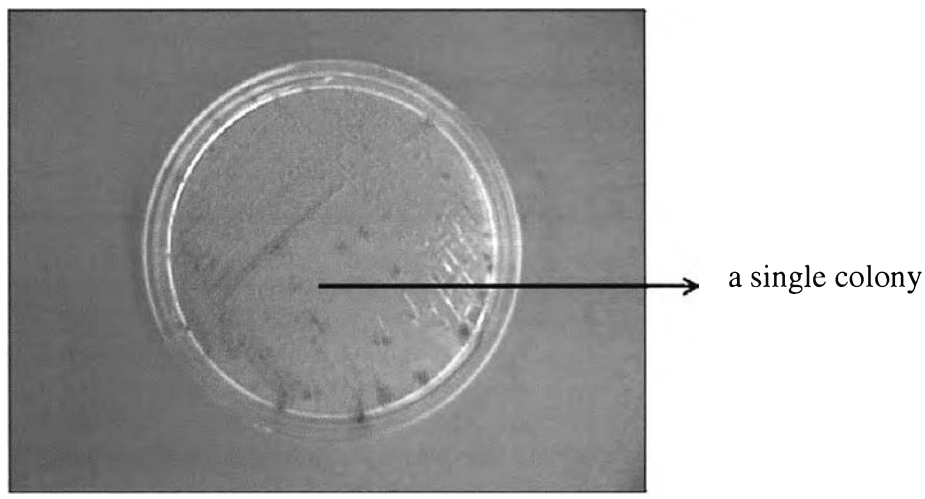


Figure 4.4 Single sporulation of *Aspergillus* on 5% V8 agar pH 5.5 at 30 ^{O}C in the dark

4.3 *Aspergillus* section *Flavi* identification

Five groups (A to E) of isolated *Aspergillus* section *Flavi* were identified based on color reaction on AFPA and colony morphology on media (Klich et al., 1988, Klich, 2002 and Pitt et al., 1983). *Aspergillus* section *Flavi* also produced green, deep green to brown conidia on Czapek Dox medium. Although *A. flavus* showed colony morphology similar to that of *A. parasiticus*, *A. flavus* was separated from *A. parasiticus* by producing finely large to smooth walled conidia (*A. parasiticus* dominantly produced rough walled conidia). *A. tamarii* produced dark green, and had

rough walled conidia (also bigger than *A. parasiticus* and *A. flavus*). *A. nomius* produced large bullet shaped sclerotium on V8 medium, hardly produced more conidia in Czapek. (Figure 4.5 to 4.10).

Group A and B

Fungi produced brownish green or yellowish brown conidia on AFPA, young white mycelium, dark or pale brown reverse colony. On Czapek, and V8 medium, all isolates showed brownish green conidia, colorless reverse colony (Figure 4.5). All isolates showed colony diameters more than 33 mm on Czapek at 30 ^{O}C in the dark for 7 days. All isolates did not produce scleotium on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days. When observed under the microscope, they produced biserate conidial heads, globose conidia, and dominantly rough and thick walled conidia. These *Aspergillus* isolates were identified as *A. tamarii* when compared with the standard (Figure 4.10).

Group C

Fungi produced green conidia on AFPA, young white mycelium, pale yellow reverse colony. On Czapek, and V8 medium, all isolated showed green conidia, colorless reverse colony (Figure 4.6). All isolates showed colony diameters more than 33 mm on Czapek at 30 ^{O}C in the dark for 7 days. All isolates did not produce sclerotium on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days. When observe under microscope, biserate conidia head, globose conidia, dominantly rough walled conidia. These *Aspergillus* isolates were identified as *A. parasiticus/sojae* when compared with the standard (Figure 4.10). However, *A. parasiticus* was separated from *A. sojae* by producing aflatoxins.

Group D

Fungi produced sparsely green conidia on AFPA, young white mycelium, pale yellow reverse colony. On Czapek, and V8 medium, all isolates showed green conidia, colorless reverse colony (Figure 4.7). All isolates showed colony diameters more than 33 mm on Czapek at 30 ^{O}C in the dark for 7 days. All isolated did not produce sclerotium on AFPA, but they produced black bullet shape of sclerotium (400-800 μm) on Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days. When observe under microscope, biserate conidia head, globose conidia, dominantly rough walled conidia. These *Aspergillus* isolates were identified as *A. nomius* when compared with the standard (Figure 4.10).

Group E

Fungi produced green conidia on AFPA, young white mycelium, dark yellow reverse colony. On Czapek, and V8 medium, all isolates showed green conidia, colorless reverse colony (Figure 4.8). All isolates showed colony diameters more than 33 mm on Czapek at 30 ^{O}C in the dark for 7 days. All isolates did not produce sclerotium on AFPA, but some produced black sphere shape of sclerotium on Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days. When observe under microscope, biserate conidia head, globose conidia, dominantly rough walled conidia. These *Aspergillus* isolates were identified as *A. flavus* when compared with the standard (Figure 4.10).

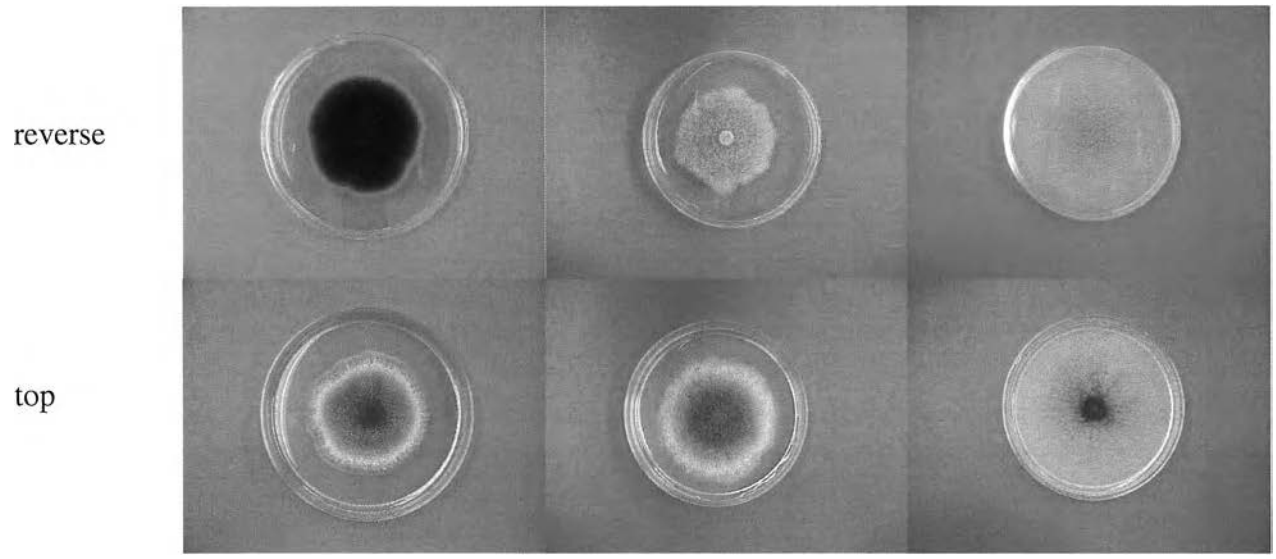


Figure 4.5 *A. tamarii* on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days

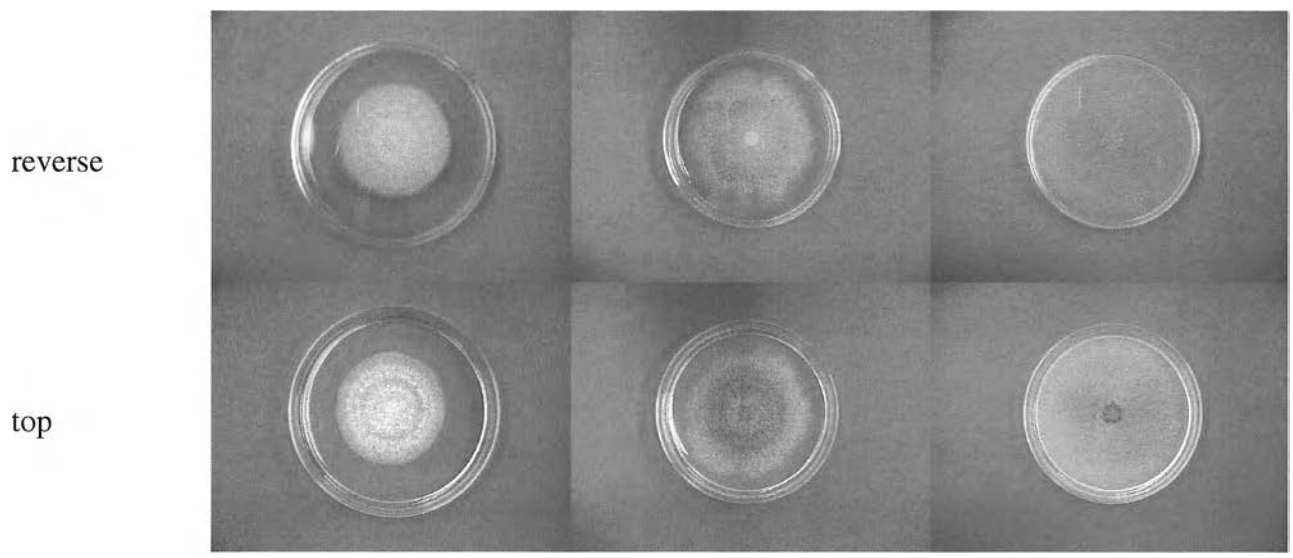


Figure 4.6 *A. parasiticus/sojae* on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days

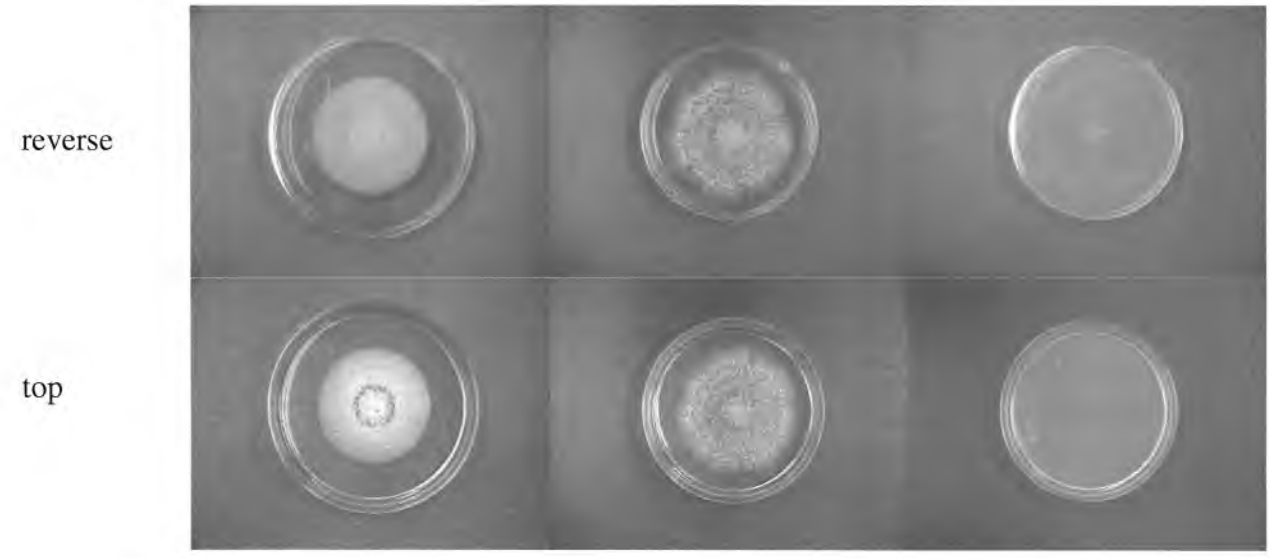


Figure 7 *A. nomius* on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days

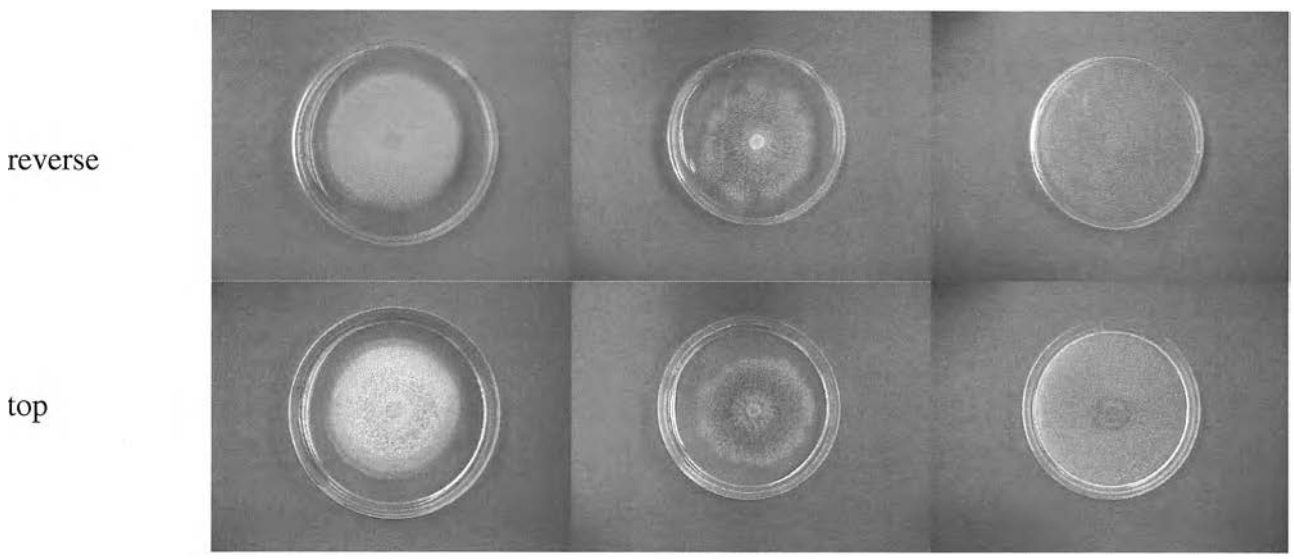


Figure 4.8 *A. flavus* on AFPA, Czapek, and V8 medium at 30 ^{O}C in the dark for 7 days

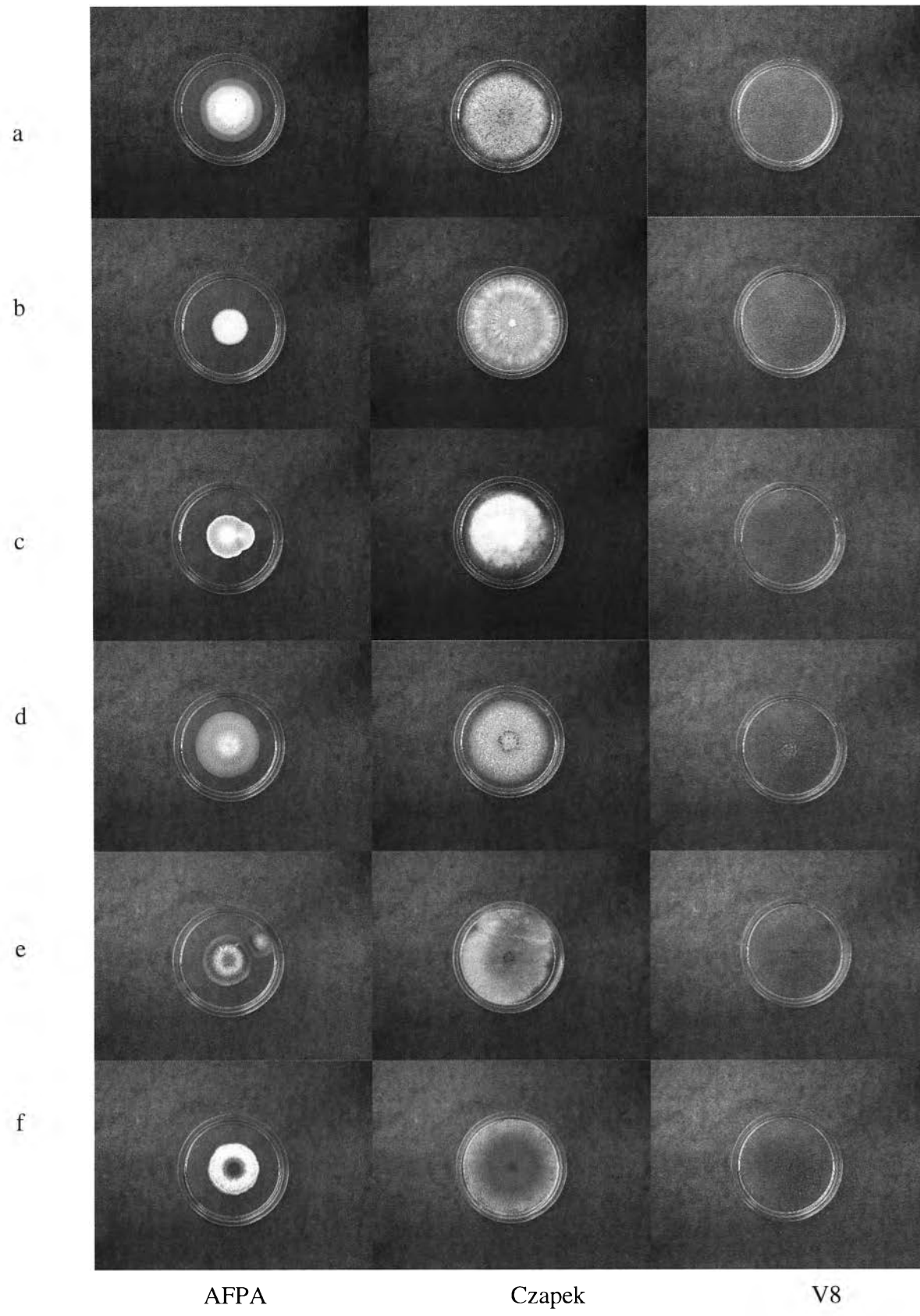


Figure 4.10 standard strains of *Aspergillus* section *Flavi* on AFPA, Czapek, and V8 medium at 30 ^{O}C for 7 days (1X); (a) *A. flavus* NRRL 3357, (b) *A. parasiticus* SRRC 75, (c) *A. oryzae* SRRC 2044, (d) *A. nomius* SRRC 2075, (d) *A. pseudotamarii* SRRC 2420, and (f) *A. tamarii* SRRC 99

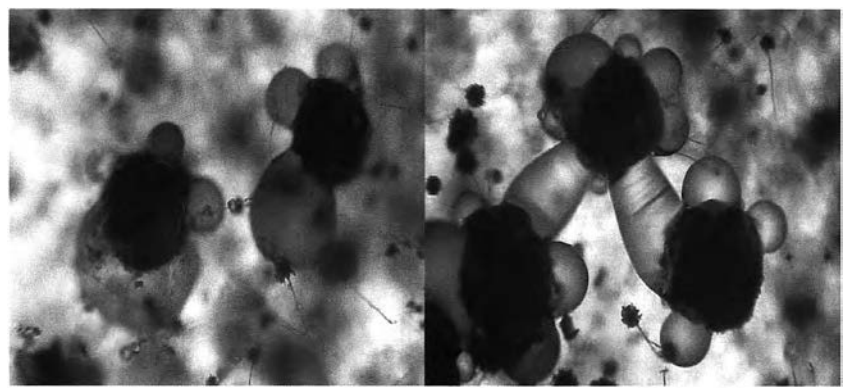
All 33 soil samples in 10 provinces collected in Northern, Northeastern, and Central Thailand, *Aspergillus* section *Flavi* was detected in all ten provinces. *A. flavus* was the most commonly isolated member of section *Flavi* with 206 isolates (83.06%). *A. parasiticus A. nomius* and *A. tamarii* made up only 4.84, 4.84, and 7.26 % of section *Flavi* isolates collected respectively. The highest incidence of section *Flavi* isolates was found in Payao province (29.44%) (Table 4.5). .

Table 4.5 *Aspergillus flavus* and the others in section *Flavi* from soils of different geographic corn fields in Thailand

Province (fields)	*Aspergillus* section *Flavi* (number of the isolates)			
	A. flavus	*A. parasiticus/sojae*	*A.nomius*	*A. tamarii*
Chaingrai (5)	11	2	3	4
Payao (5)	68	2	3	0
Pare (5)	33	6	2	0
Khonkan (1)	11	0	0	1
Nakornrajchasima (2)	7	1	0	2
Lopburi (2)	23	0	0	0
Saraburi (1)	4	1	1	10
Nakornsawan (2)	11	0	3	0
Pitsanulok (6)	15	0	0	0
Bangkok (2)	23	0	0	1
Total	206	12	12	18
%	83.06	4.84	4.84	7.26

4.3.1 Droplet formation in *Aspergillus* section *Flavi*

Fungal spores of each isolated of *Aspergillus* section *Flavi* including *A. flavus*, *A. parasiticus/sojae*, *A. nomius*, and *A. tamarii* were inoculated on Czapek medium. The cultures were incubated in the dark at 30 ^{O}C, 7 days for colonization. Only *A. flavus* and *A. nomius* produced droplets on the surface of the medium (Figure 4.11). Red to red-brown droplets was observed on *A. nomius*, and coloress droplets were observed on *A. flavus* respectively.



a b

Figure 4.11 Droplets color of *A. nomius* b2; (a) and *A. flavus* a3 (b) on Czapek at 30^{O}C

4.3.2 Conidial head morphology of *Aspergillus* section *Flavi* on different media types.

Defined media including AFPA, and Czapek, and organic media including PDA, CMA, and 5% V8 were used in the study. All *Aspergillus* section *Flavi* including *A. flavus, A. parasiticus/sojae*, *A. oryzae*, *A. nomius*, *A. tamarii*, and *A. pseutotamarii* produced conidial head and appeared to same when compared between the same media with or without light. However, medium affected the fungus conidial

head shapes. Immature *A. flavus* conidial head from young mycelia on all media showed radiate globosely shapes, and vary in size depended on media. Mature *A. flavus* mycelia predominantly produced columnar shaped conidial head on PDA, and CMA but did not show columnar conidiophores on Czapek, and AFPA (Figure 4.12). At the results, *A. flavus* conidial head production was activated by media types.

A. flavus was distinguished from the other isolates in section *Flavi* by produced columnar shaped of mature conidial head on PDA, CMA, and V8. All *A. parasiticus/sojae*, *A. oryzae*, *A. nomius*, *A. tamarii*, and *A. pseutotamarii* did not produce columnar conidial head on these media (Figure 4.12 to 4.13). At the result, columnar conidiophores of *A. flavus* on V8 were used as a new facile method for *A. flavus* identification

4.3.3 Sclerotium producing strains of *Aspergillus*

Fungal spores of each isolated of *Aspergillus* section *Flavi* including *A. flavus*, *A. parasiticus/sojae*, *A. nomius*, and *A. tamarii* were inoculated on V8 agar. The cultures were incubated in the dark at 30 ^{O}C for sclerotium formation. *A. flavus* showed sphere shaped sclerotium, and were separated into three groups including large sclerotium (L strains), small sclerotium (S strains), and non sclerotium producing strains. All *A. tamarii* showed large bullet shaped sclerotium bigger than *A. nomius*. However *A. tamarii* hardly produced scleortia within 7 days (*A. tamarii* needed more incubating times; appoximately than 10 days). All isolated strains of *A. parasiticus/sojae* did not show scleortia in the medium (Figure 4.14 and 4.15).

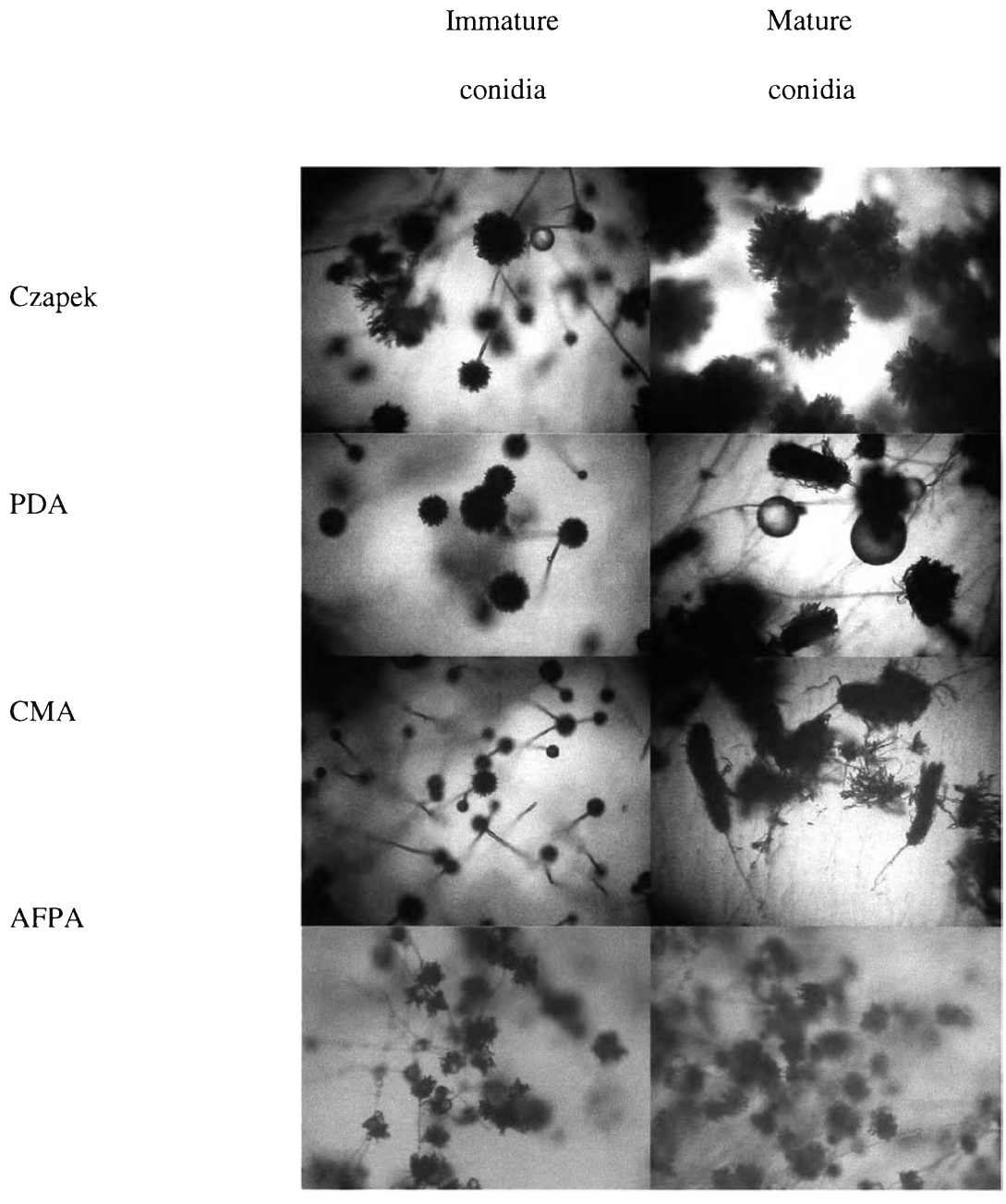


Figure 4.12 *A. flavus* a3 conidiophores and conidial heads on different types of media at 30 ^{O}C for 7 days in the dark

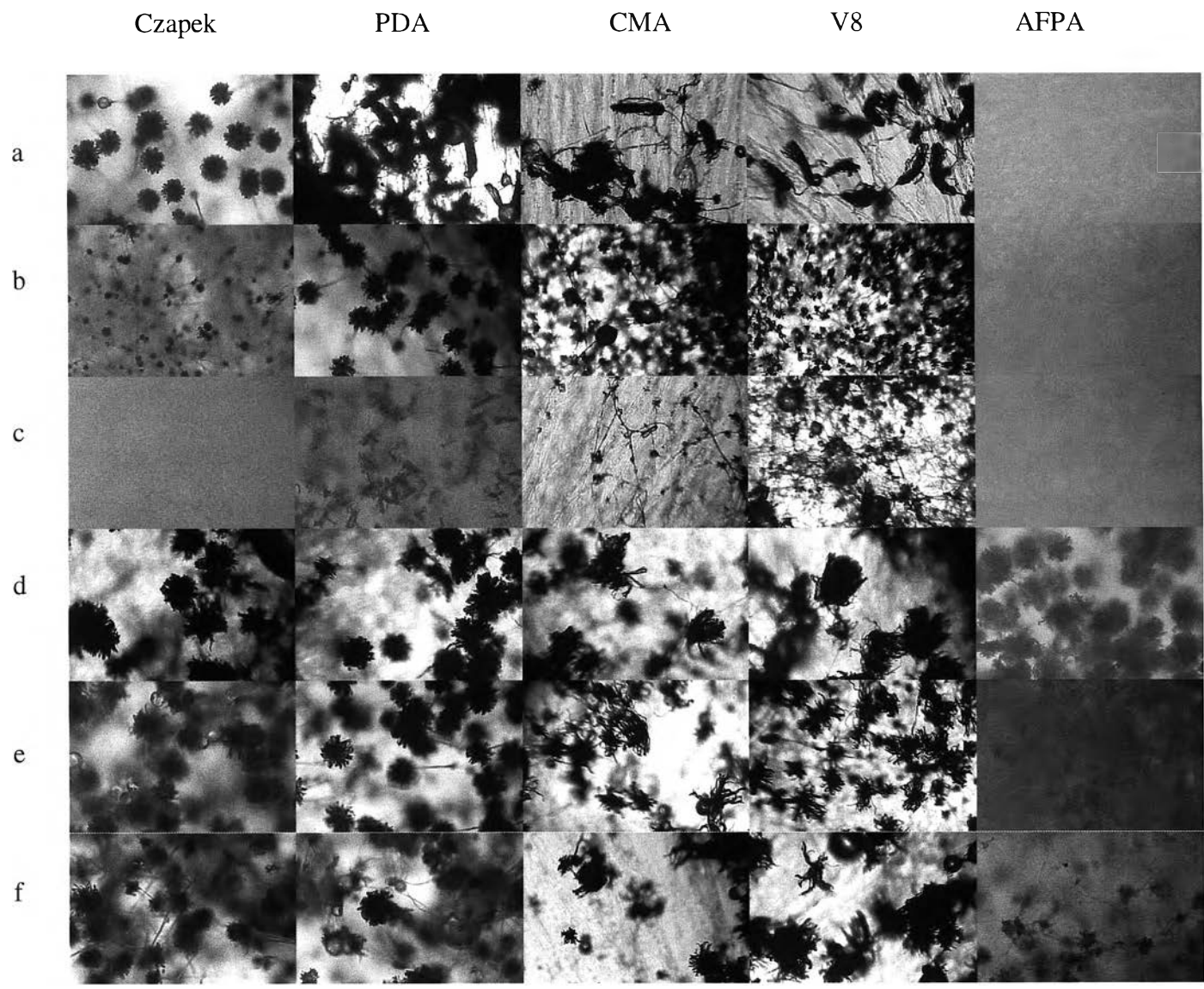


Figure 4.13 Standard strains of *Aspergillus* section *Flavi* on different type media at 30 ^{O}C for 7 days; (a) *A. flavus* NRRL 3357, (b) *A. parasiticus* SRRC 75, (c) *A. oryzae* SRRC 2044, (d) *A. nomius* SRRC 2075, (d) *A. pseudotamarii* SRRC 2420, and (e) *A. tamarii* SRRC 99

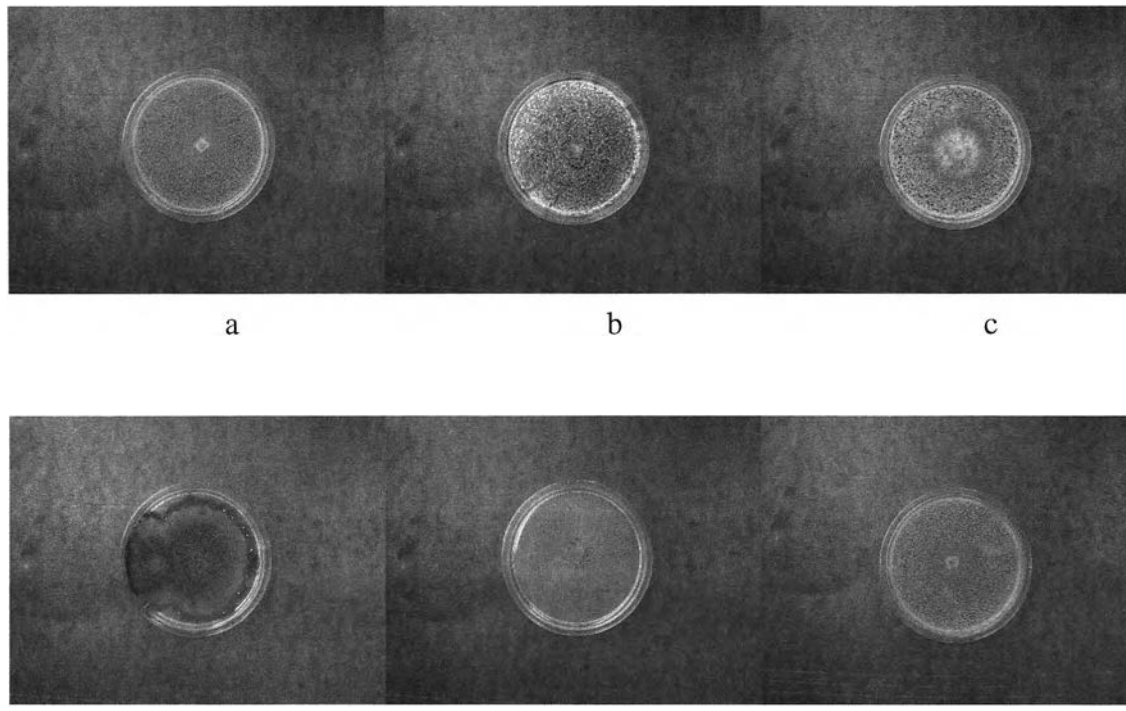


Figure 4.14 *Aspergillus* on 5%V8 agar at 30^{O}C for 14 days; (a) non sclerotium producing strains of *A. flavus* a13, (b) sclerotium producing strains (L strains) of *A. flavus* b11-2, (c) sclerotium producing strains (S strains) of *A. flavus* 168, (d) *A. tamarii* c1, and (e) *A. nomius* b1

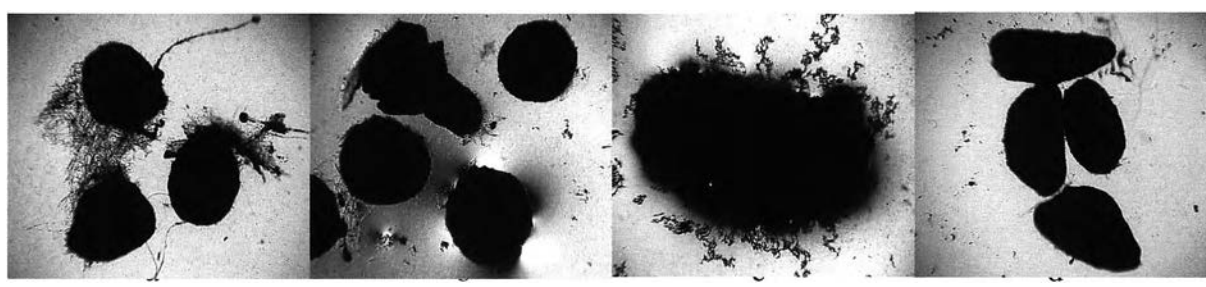


Figure 4.15 *Aspergillus* sclerotium (4X); (a) S strains of *A. flavus* 168, (b) L strains of *A. flavus* b11-2, (c) *A. tamarii* c1, and (d) *A. nomius* b1

Two hundred and six (206) isolated *A. flavus* were separated into three groups including L strain (sclerotium size more than 400 mm), S train (sclerotium size less than 400 mm), and N strain (no sclerotium). All L, S and N strains of *A. flavus* were found in 7 provinces except Lopburi, Pare, and Saraburi provinces. *A. flavus* isolates from Lopburi, and Pare provinces exhibited frequency of N strains when compared with the others places. However, two hundred and six (206) isolated *A. flavus* were found to be N (43.20%), L (28.64%) and S (28.16%) strains respectively (Table 4.16 to 4.26).

Table 4.16 Sclerotium producing strains of 11 *A. flavus* isolates from soils in Chaingrai, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
162			+
163			+
164	+		
165	+		
166			+
167		+	
168	+		
a13			+
b11-1		+	
b11-2		+	
b12		+	
total	3	4	4
%	27.27	36.36	36.36

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.17 Sclerotium producing strains of 68 *A. flavus* isolates from soils in Payao, Thailand

Funagal isolate	Scleortium type		
	L	S	N
5		+	
6		+	
7		+	
8		+	
9		+	
10	+		
11			+
12		+	
69	+		
70		+	
71		+	
72		+	
73		+	
74	+		
75		+	
81	+		
82	+		
83	+		
84	+		
89		+	
90			+
91		+	
92	+		
97			+
98			+
99			+
100	+		
101			+
102			+

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.17 Sclerotium producing strains of 68 *A. flavus* isolates from soils in Payao, Thailand (continue)

Funagal isolate	Scleortium type		
	L	S	N
103		+	
104	+		
105	+		
106	+		
107		+	
108			+
113		+	
114	+		
115	+		
122	+		
131		+	
132			+
133	+		
134		+	
135		+	
136			+
138		+	
139	+		
140			+
149			+
150	+		
171		+	
172		+	
178		+	
a3		+	
a4		+	
c5	+		
c6		+	
d5	+		
e17	+		
e3		+	
e5			+
e7		+	

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.17 Sclerotium producing strains of 68 *A. flavus* isolates from soils in Payao, Thailand (continue)

Funagal isolate	Scleortium type		
	L	S	N
e9		+	
f5		+	
f6		+	
f7			+
g5			+
g6	+		
total	22	31	15
%	32.35	45.59	22.06

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.18 Sclerotium producing strains of 33 *A. flavus* isolates from soils in Pare, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
13			+
14			+
15			+
16			+
17			+
18			+
19			+
20			+
25			+
26			+
27			+
28			+
37			+
38			+
39			+
40			+
123			+

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.18 Sclerotium producing strains of 33 *A. flavus* isolates from soils in Pare, Thailand (continue)

Fungal isolate	Sclerotium type		
	L	S	N
124			+
125			+
126		+	
127			
154			+
155			+
156			+
157			+
b9			+
c17			+
c9			+
d17			+
g7			+
h5			+
h6			+
h7			+
total	0	1	32
%	0	3.03	96.97

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.19 Sclerotium producing strains of 7 *A. flavus* isolates from soils in Nakornrajchasima, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
49	+		
50			+
51		+	
52	+		
158		+	
b5			+
h16		+	
total	2	3	2
%	28.57	42.86	28.57

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.20 Sclerotium producing strains of 11 *A. flavus* isolates from soils in Khonkan, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
116	+		
117	+		
118	+		
119	+		
120	+		
121		+	
128	+		
129	+		
130	+		
d1		+	
d9			+
total	8	2	1
%	72.73	18.18	9.09

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.21 Sclerotium producing strains of 23 *A. flavus* isolates from soils in Lopburi, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
46			+
47			+
48			+
53			+
54			+
55			+
56			+
57			+
58			+
59			+
60			+

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.11 Sclerotium producing strains of 23 *A. flavus* isolates from soils in Lopburi, Thailand (continue)

Fungal isolate	Sclerotium type		
	L	S	N
61			+
62			+
63			+
176			+
177			+
a5			+
a6			+
f4			+
g3			+
g4		+	
h3			+
h4			+
total	0	1	22
%	0	4.35	95.65

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.22 Sclerotium producing strains of 4 *A. flavus* isolates from soils in Saraburi, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
173	+		
174	+		
175	+		
a17	+		
total	4	0	0
%	100	0.00	0.00

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.23 Sclerotium producing strains of 11 *A. flavus* isolates from soils in Nakornsawan, Thailand

Fungal strain	Sclerotium type		
	L	S	N
1		+	
2		+	
3			+
4	+		
160			+
161			+
169		+	
a16		+	
b16			+
f16			+
f17	+		
total	2	4	5
%	18.18	36.36	45.45

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.24 Sclerotium producing strains of 15 *A. flavus* isolates from soils in Pitsanulok, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
88		+	
145		+	
146			+
147			+
148		+	
151			+
152	+		
153		+	
159			+
c11		+	
c12			+
c15			+

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.24 Sclerotium producing strains of 15 *A. flavus* isolates from soils in Pitsanulok, Thailand (contimue)

Fungal isolate	Sclerotium type		
	L	S	N
e14-1	+		
e14-2	+		
f14			+
total	3	5	7
%	20	33.33	46.67

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.25 Sclerotium producing strains of 23 *A. flavus* isolates from soils in Bangkok, Thailand

Fungal isolate	Sclerotium type		
	L	S	N
21	+		
22		+	
23	+		
24	+		
29	+		
30	+		
31		+	
32	+		
33	+		
34	+		
35	+		
36			+
64	+		
65	+		
66	+		
67		+	
68		+	
a11		+	
a12	+		

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.25 Sclerotium producing strains of 23 *A. flavus* isolates from soils in Bangkok, Thailand (continue)

Fungal isolate	Sclerotium type		
	L	S	N
g10	+		
g9	+		
h10		+	
h9		+	
total	15	7	1
%	65.22	30.43	4.35

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

Table 4.26 Summary of sclerotium producing strains of 206 *A. flavus* isolates from soils in ten provinces, Thailand

Province	Sclerotium type			Total
	L*	S**	N**	
Chaningrai	3	4	4	11
Payao	22	31	15	68
Pare	0	1	32	33
Knonkan	8	2	1	11
Nakornrjachasima	2	3	2	7
Lopburi	0	1	22	23
Saraburi	4	0	0	4
Nakornsawan	2	4	5	11
Pitsanulok	3	5	7	15
bangkok	15	7	1	23
Total	59	58	89	206
%	28.64	28.16	43.20	

L; sclerotium size less than 400 mm, S; sclerotium size more than 400 mm, N; no sclerotium

4.3.4 Effect of different surfactant types on fungal colonization

Anionic surfactant (deoxycholic acid, and Niaproof), nonionic surfactants (digitonin, Triton X-100, and Tween 40), and cationic surfactant (CTAB) were used in the study. When using 0.01% (w/v) surfactant to prepare spore suspension, all *Aspergillus* section *Flavi* including *A. flavus*, *A. parasiticus/sojae*, *A. nomius*, and *A. tamarii* produced colonies and mycelia on the Czapek Dox medium. However, all isolates were inhibited when using 0.1% (w/v) CTAB to prepare spore suspension.

4.3.5 Identification of *A. flavus* by synnema formation on Czapek Dox agar containing Avid®

When fungal spores of each strain were inoculated on Czapek medium containing with 40µl/L of Avid®, and incubated at 30 ^{O}C, for 7 days in the dark. All isolated of *A. flavus* and all standard strains of *A. flavus* including *A. flavus* NRRL 3357, NRRL 21882, SRRC 1000E, TX 9-8, F3W4, Af 53, and Af 13 formed synnemata or coremia; clusters of erect fungus filaments that are joined together to form a column and that bear asexual spores (Fig. 4.16). All isolated *A. tamarii* (18 isolates), *A. parasiticus/sojae* (12 isolates), and *A. nomius* (12 isolates) did not form synnemata on Czapek with Avid®. *A. tamarii* showed deep green to brownish green conidial color compared to *A. parasiticus/sojae*. Eight isolates of *A. nomius* and some of sclerotiuml producing *A. flavus* had ability to change color on reverse phase to red (Figure 4.16).

When incubated longer (30 days) at 30 ^{O}C in the dark but some strains did have colony color change from green to brownish green. *A. parasiticus* and *A. tamarii* did have deep brownish green and dark brown conidial color respectively. All *A. nomius* hardly produced conidia, and had sparse mycelia in the medium (Figure 4.16

and 4.17). Observation under microscope, *A. flavus* synnemata showed a cluster of erect fungus filaments that are joined together to form a column and that bear asexual spores with yellow to green yellow radiate conidial heads (Figure 4.16 and 4.19).

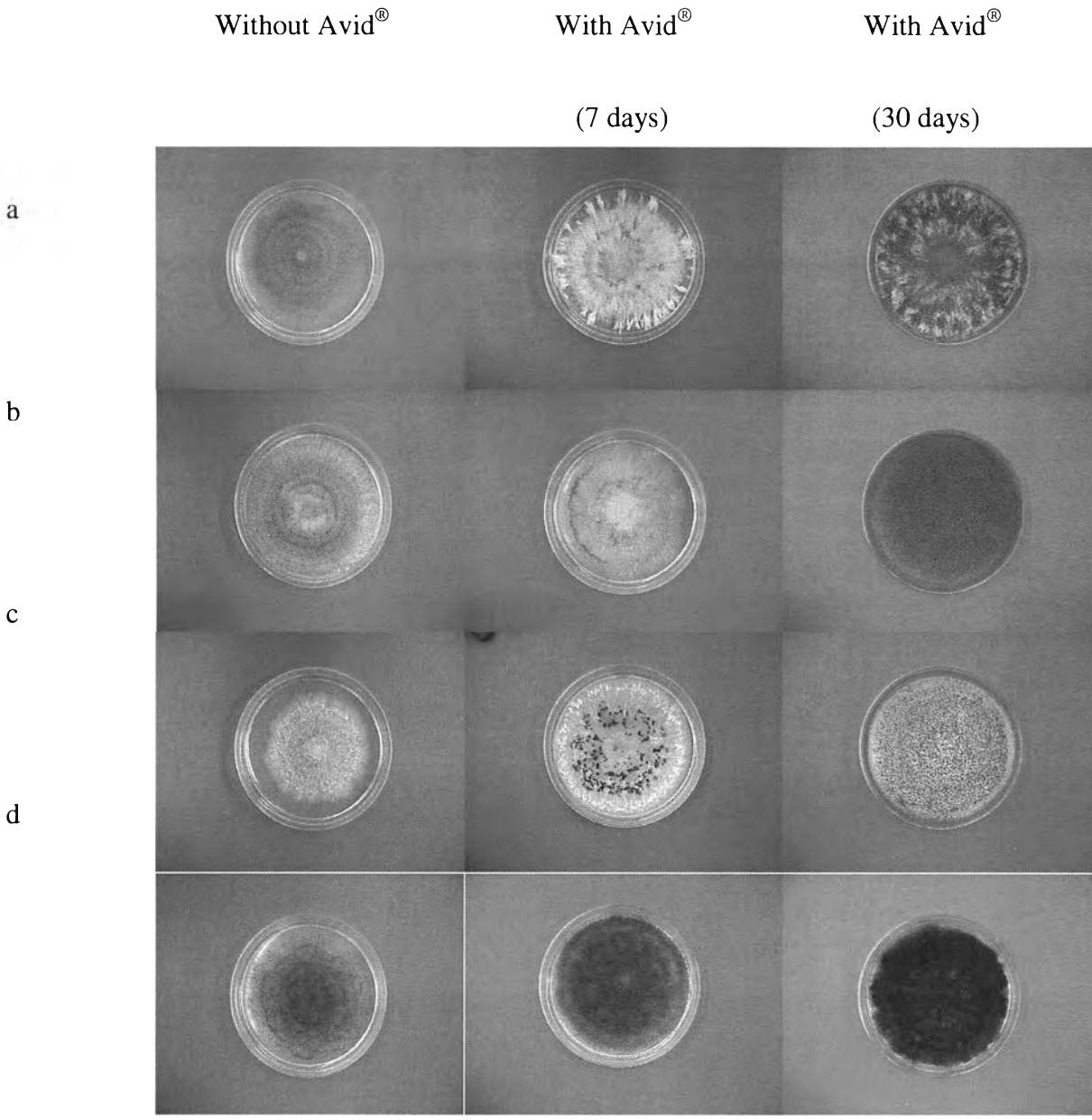
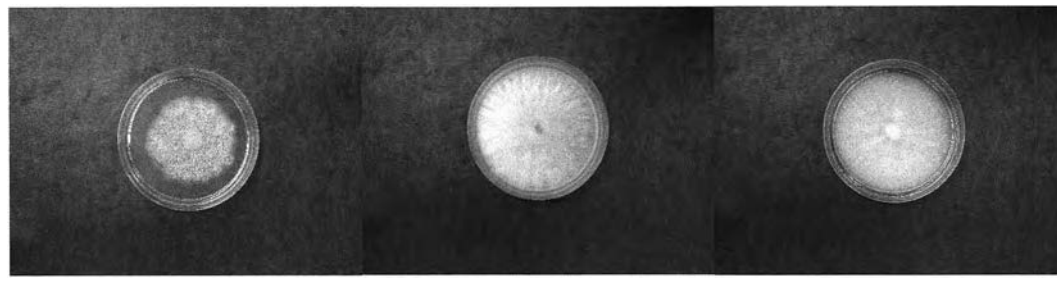
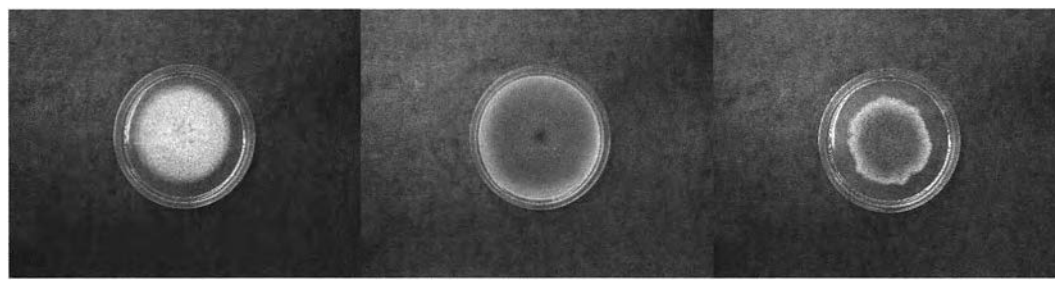


Figure 4.16 Isolated strains of *Aspergillus* section *Flavi* incubated in Czapekapek medium with or without Avid® at 30 ^{O}C in the dark); (a) *A. flavus* a13, (b) *A. parasiticus/sojae* a1, (c) *A. nomius* b3, and (d) *A. tamarii* c1



a b c



d e f

Figure 4.17 Standard *Aspergillus* section *Flavi* (NRRL, and SRRC, USA) incubated in Czapekapek medium without Avid® at 30 ^{O}C in the dark for 7 days; (a) *A. flavus* NRRL 3357, (b) *A. parasiticus* SRRC 75, (c) *A. oryzae* SRRC 302, (d) *A. nomius* SRRC 375, (e) *A. tamarii* SRRC 99, and (f) *A. pseudotamarii* SRRC 2420

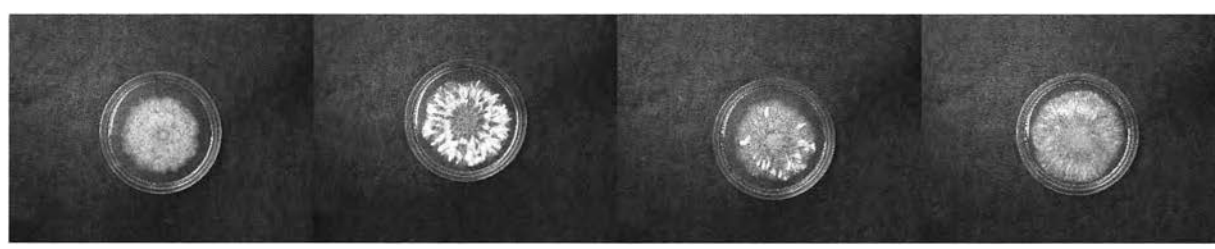
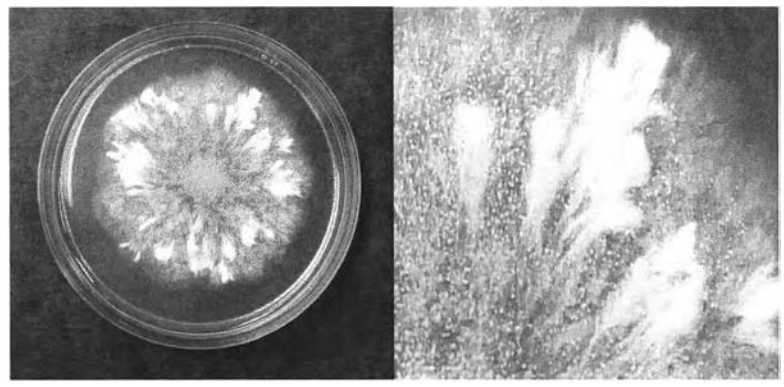
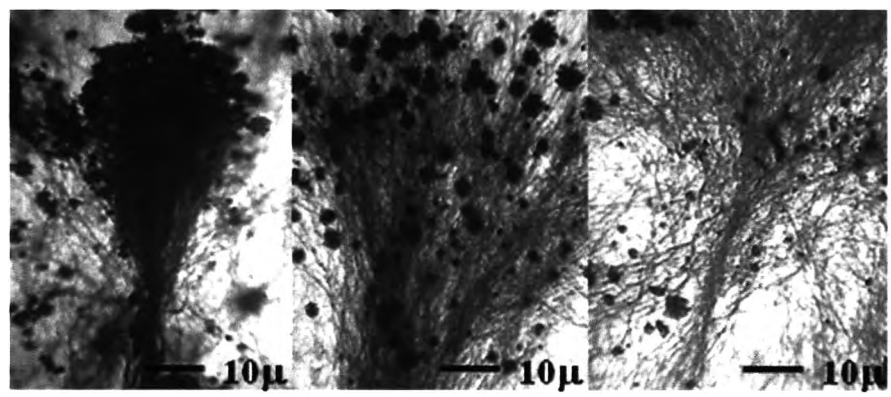


Figure 4.18 different characteristic of synnemata producing strains of *A. flavus*; (a) without synnemata (Czapek medium without 40µl/L of Avid®), and (b, c, and d) different types formation of synnemata (Czapekapek Dox medium containing with 40µl/L of Avid®)



a b



c d e

Figure 4.19 Morphological structures of *A. flavus* on modified Czapek medium containing Avid® at 30 ^{O}C in the dark for 10 days;(a.) *A. flavus* colony, (b.) synnemata, (c.) synnemata, and (d.) synnemata head, and (e) synnemata base

4.3.5.1 Effect of light and dark on by *Aspergillus* synnemata formation

Fungal spores of each isolated of *Aspergillus* section *Flavi* including *A. flavus*, *A. parasiticus/sojae*, *A. nomius*, and *A. tamarii* were inoculated on Czapek medium containing with 40µl/L of Avid®. The cultures were incubated in the dark and light at 30 ^{O}C for 10 days. *A. flavus* produced synnemata in the dark. Under the light, *A. flavus* did not form synnemata. *A. nomius* produced few condia in the dark when

compared with the culture under the light. All isolated strains of *A. parasiticus/sojae*, and *A. tamarii* were incubated in the dark, produced conidia color darker than under the light (Figure 4.20).

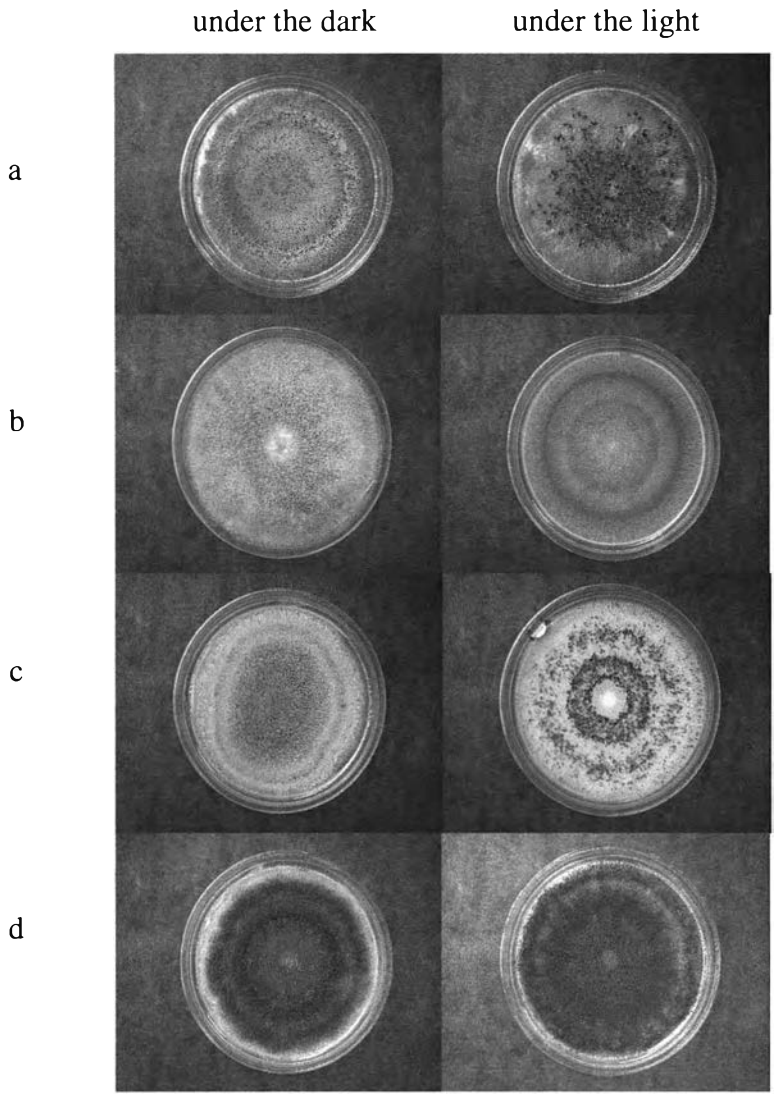


Figure 4.20 Isolates of *Aspergillus* section *Flavi* growing on Czapekapek medium with or without Avid® at 30 ^{O}C in the dark and under the light (1.8X); (a) *A. flavus* a3, (b) *A. parasiticus/sojae* a1, (c) *A. nomius* b1, and (d) *A. tamarii* c1

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

A. flavus colonization and synnema formation were observed. All strains of *A. flavus* did not form synnema on all media including Czapekapek, AFPA, CMA, and PDA without Avid®. Only *A. flavus* produced synnema on Czapek containing 40 µl/L of Avid® (Figure 4.21). In addition, *A. flavus* conidial head shape was not affected by Avid®.

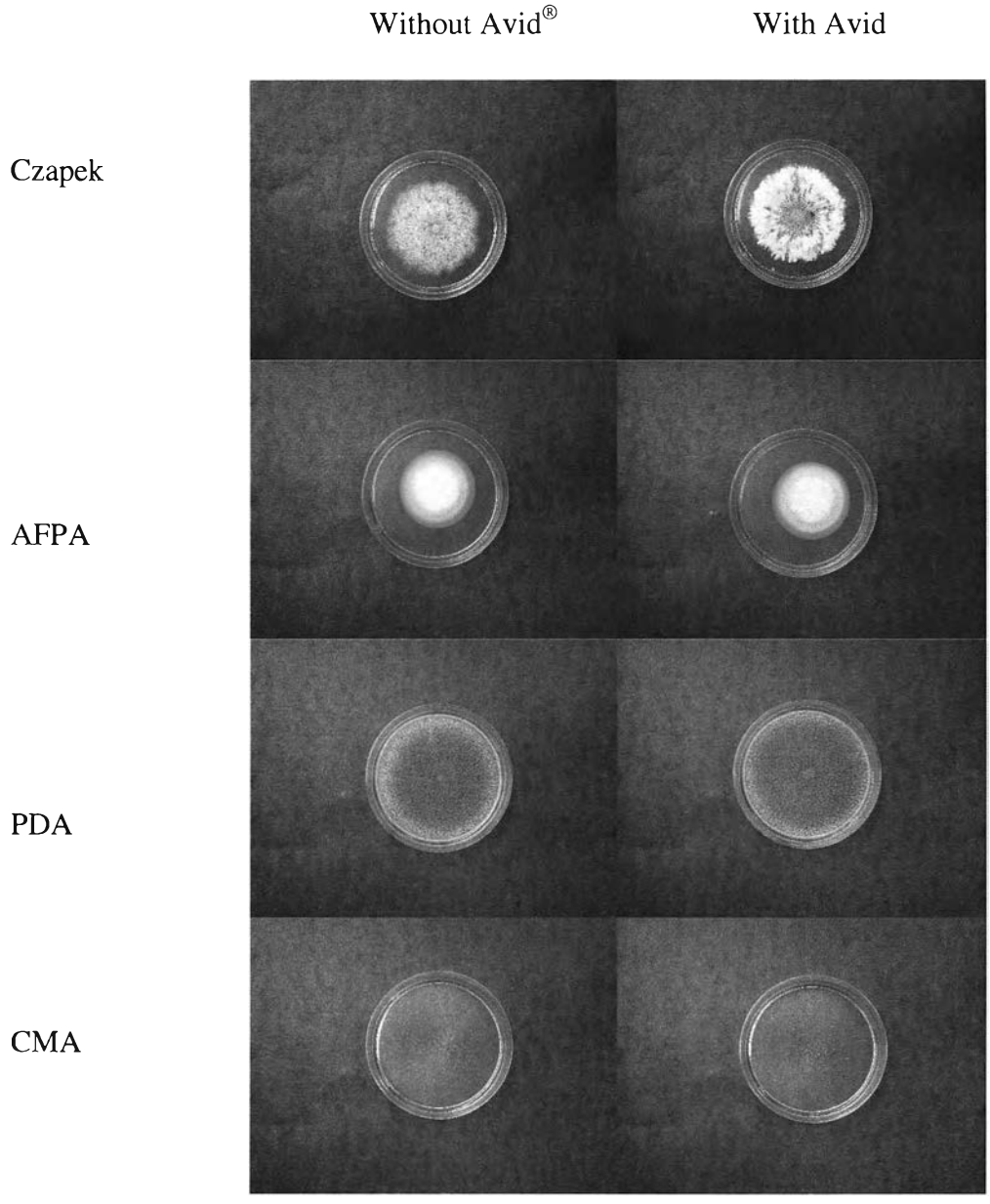


Figure 4.21 isolated strains of *A. flavus* NRRL 3357 on different media types with or without 40 µl/L of Avid® at 30 ^{O}C for 7 days in the dark (1X)

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

Czapekapek medium containing Avid® was modified by replacing sodium nitrate with either 0.3% ammonium sulphate, 0.3% ammonium tartrate, or 0.3% peptone. Spore suspensions of each isolated of *A. flavus* was inoculated on the media, and incubated in the dark at 30 ^{O}C for 7 days. *A. flavus* colonization and synnema formation were observed. All *A. flavus* formed mycelia and conidia on the Czapek medium containing ammonium tartrate and peptone than sodium nitrate, and ammonium sulphate (Figure 4.21 and 4.22). *A. flavus* did not formed synnema on all media without Avid®. However, *A. flavus* produced synnema on Czapek containing 40 µl/L of Avid® with sodium nitrate as the sole nitrogen source. The failure to form synnema when other source of nitrogen were substituted for sodium nitrate suggest, that nitrogen metabolism in concert with Avid® effects synnema induction (Figure 4.22 and Table 4.27).

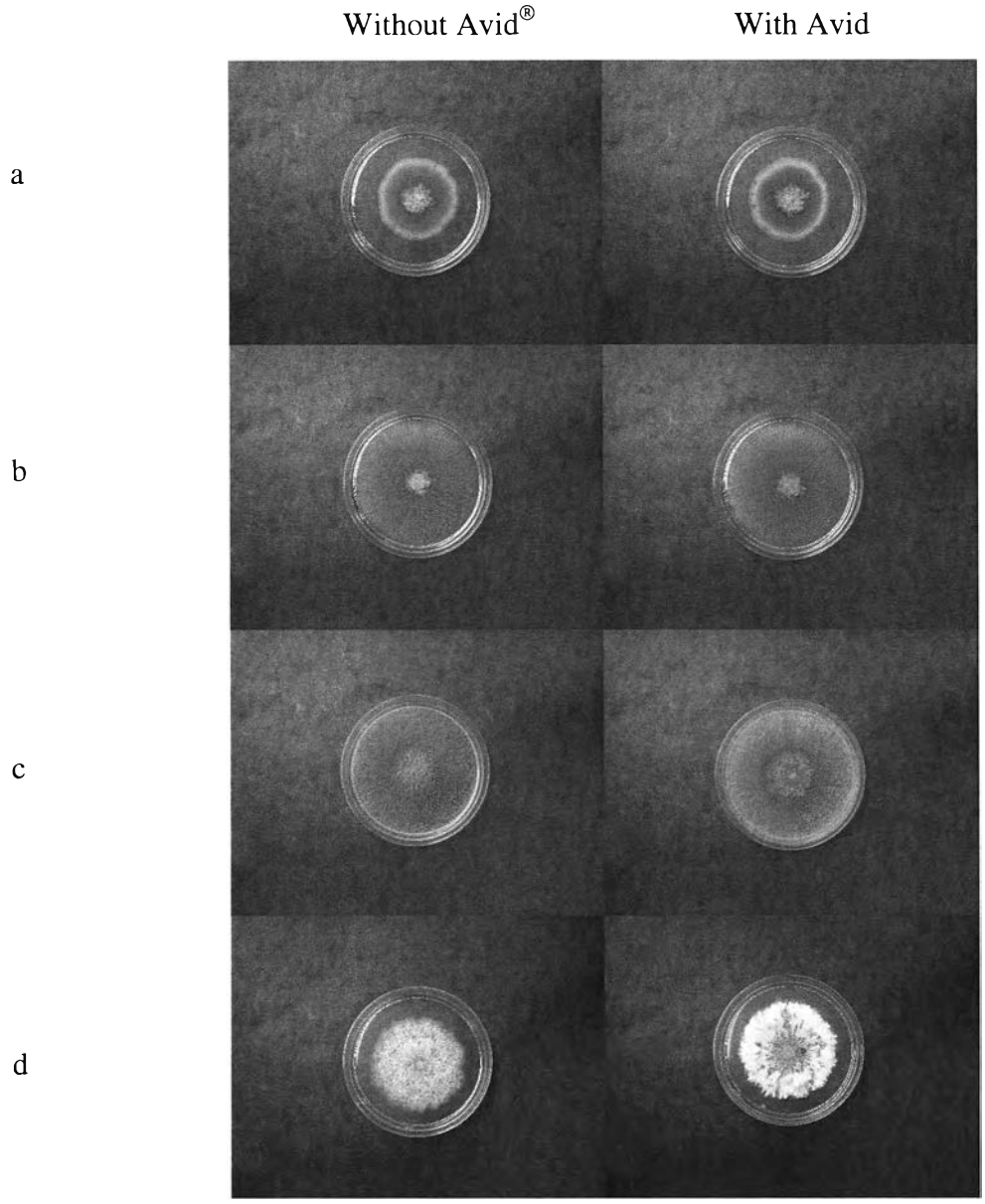


Figure 4.22 *A. flavus* incubated in Czapekapek medium with or without Avid®, and replacing sodium nitrate with either ammonium sulphate, ammonium tartrate, or peptone at 30 ^{O}C in the dark for 7 days; (a) ammonium sulphate, (b) ammonium tartarte, (c) peptone, and (d) sodium nitrate

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

Spore suspensions of each isolated of *Aspergillus* was inoculated in the Czapek medium containing different (0, 20, 40, 80, 200, 500, and 1,000 µl/L) Avid®

concentrations. The cultures were incubated in the dark at 30 ^{O}C for 7 days. High level of Avid® concentration did not induce synnema formation by *A. tamarii*, *A. nomius*, and *A. parasiticus* (Figure 4.23 to 4.25). Only *A. flavus* specifically produced synnema in Czapek with Avid®. However, synnema formation numbers of *A. flavus* were decreased at the high Avid® concentration (1,000 µl/L) (Figure 4.26).

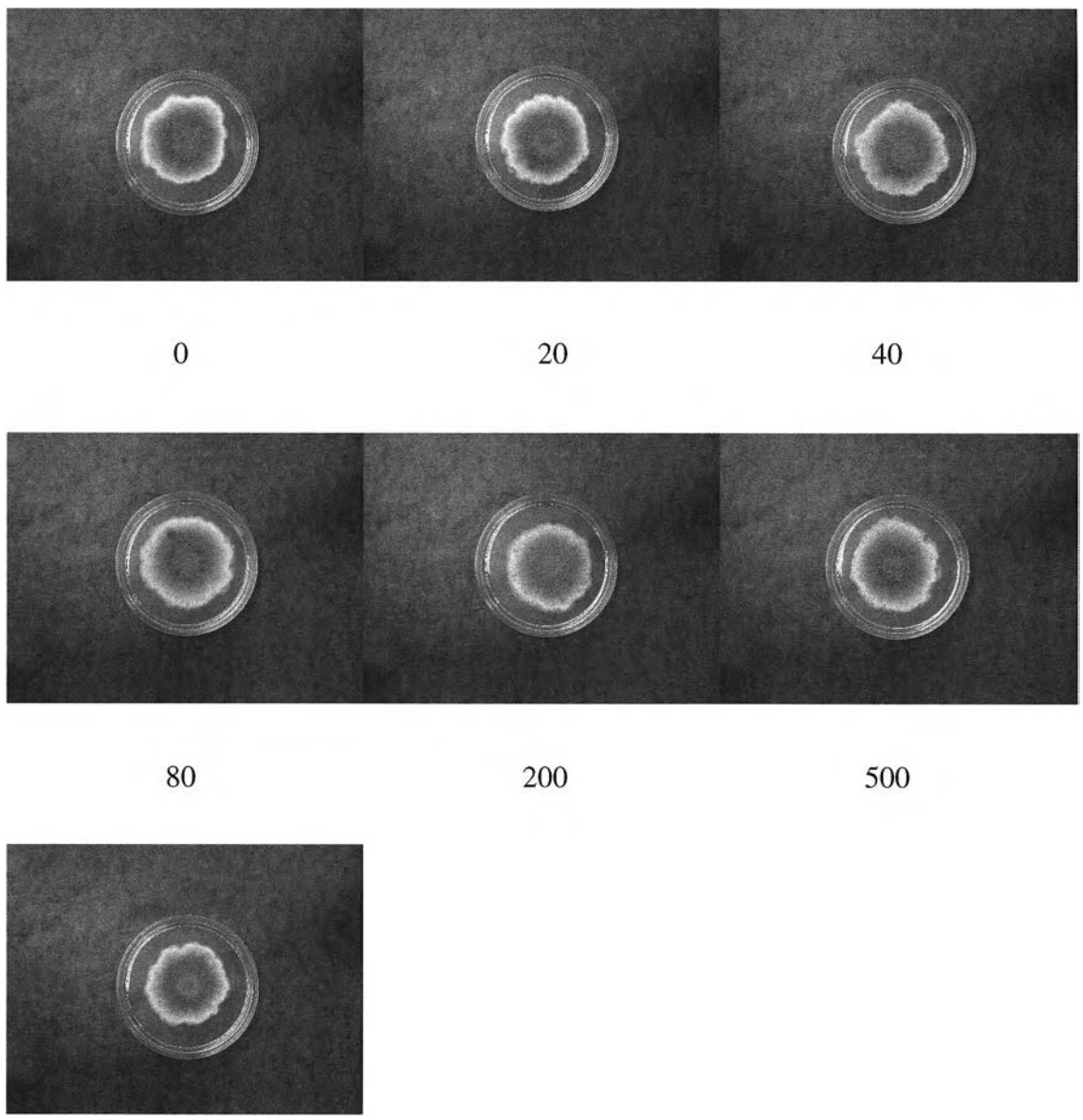
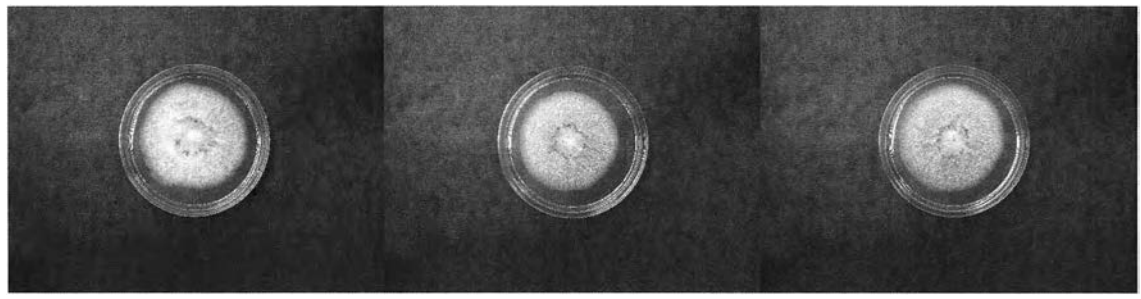
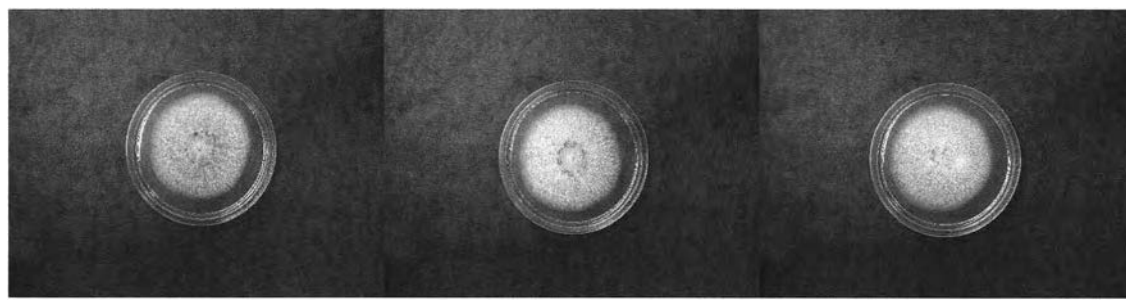


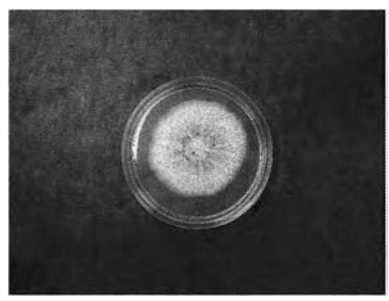
Figure 4.23 *A. tamarii* c1 on Czapek medium containing different (0, 20, 40, 80, 200, 500, and 1,000 µl/L) Avid® concentration in the dark at 30 ^{O}C for 7 days.



0 20 40

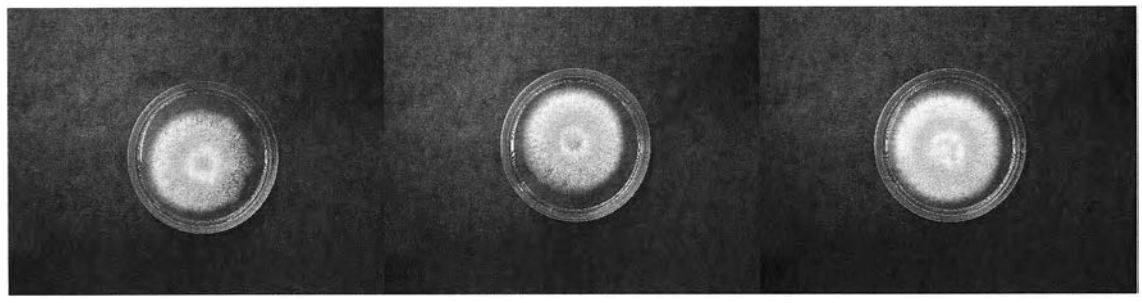


80 200 500

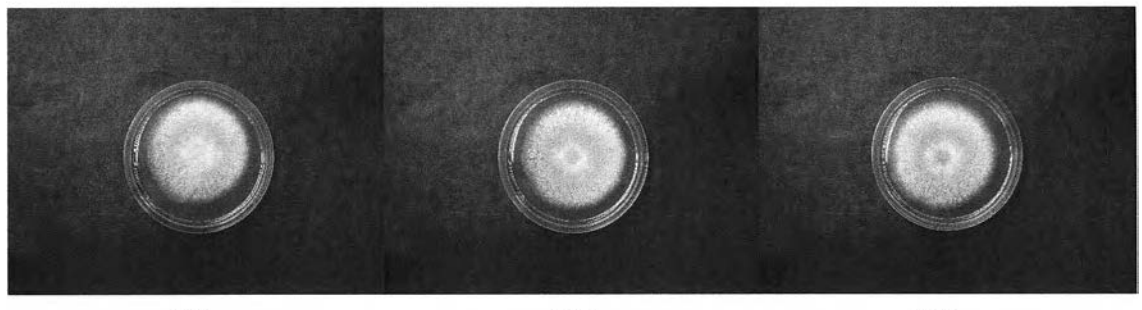


1,000

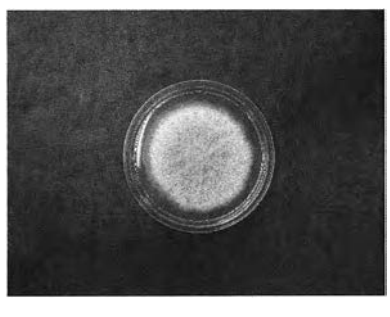
Figure 4.24 *A. nomius* b1 on Czapek medium containing different (0, 20, 40, 80, 200, 500, and 1,000 µl/L) Avid® concentration in the dark at 30 ^{O}C for 7 days.



0 20 40



80 200 500



1,000

Figure 4.25 *A. parasiticus/sojae* a1 on Czapek medium containing different (0, 20, 40, 80, 200, 500, and 1,000 µl/L) Avid® concentration in the dark at 30 ^{O}C for 7 days.

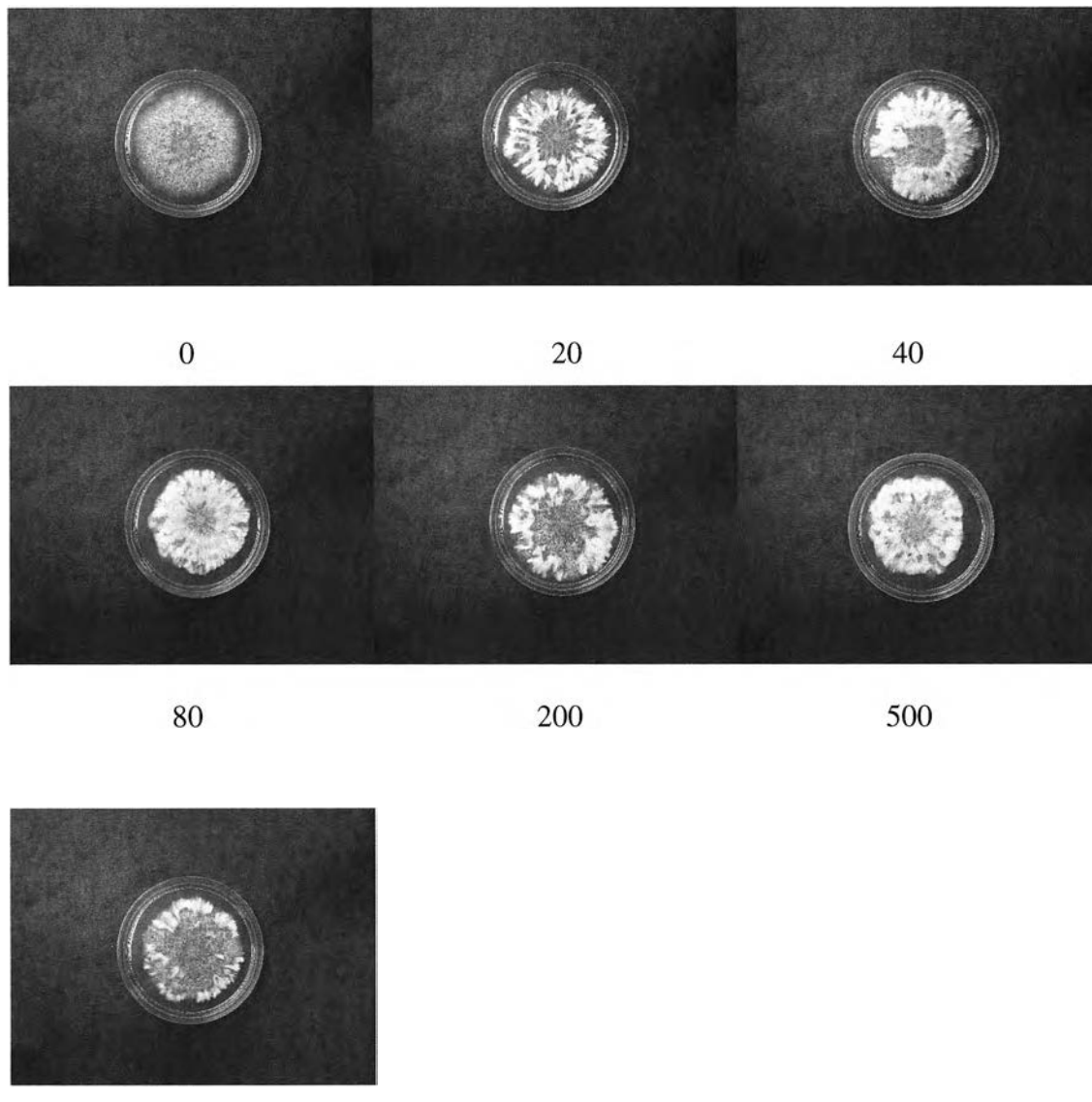


Figure 4.26 *A. flavus* on Czapek a3 medium containing different (0, 20, 40, 80, 200, 500, and 1,000 µl/L) Avid® concentration in the dark at 30 ^{O}C for 7 days.

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. flavus NRRL 3357	+	-	-	-
A. flavus NRRL 21882	+	-	-	-
A. flavus TX 9-8	+	-	-	-
A. flavus F3W4	+	-	-	-
A. flavus Af 53	+	-	-	-
A. flavus Af 13	+	-	-	-
A. flavus a11	+	-	-	-
A. flavus a12	+	-	-	-
A. flavus a13	+	-	-	-
A. flavus a16	+	-	-	-
A. flavus a3	+	-	-	-
A. flavus a4	+	-	-	-
A. flavus a5	+	-	-	-
A. flavus a6	+	-	-	-
A. flavus b11-1	+	-	-	-

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 °C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 °C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. flavus b11-2	+	-	-	-
A. flavus b12	+	-	-	-
A. flavus b16	+	-	-	-
A. flavus b5	+	-	-	-
A. flavus b9	+	-	-	-
A. flavus c11	+	-	-	-
A. flavus c12	+	-	-	-
A. flavus c15	+	-	-	-
A. flavus c17	+	-	-	-
A. flavus c5	+	-	-	-
A. flavus c6	+	-	-	-
A. flavus c9	+	-	-	-
A. flavus d1	+	-	-	-
A. flavus d17	+	-	-	-
A. flavus d5	+	-	-	-

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SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. flavus d9	+	-	-	-
A. flavus e14-1	+	-	-	-
A. flavus e14-2	+	-	-	-
A. flavus e17	+	-	-	-
A. flavus e3	+	-	-	-
A. flavus e5	+	-	-	-
A. flavus e7	+	-	-	-
A. flavus e9	+	-	-	-
A. flavus f14	+	-	-	-
A. flavus f16	+	-	-	-
A. flavus f17	+	-	-	-
A. flavus f4	+	-	-	-
A. flavus f5	+	-	-	-
A. flavus f6	+	-	-	-
A. flavus f7	+	-	-	-

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SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. flavus g10	+	-	-	-
A. flavus g3	+	-	-	-
A. flavus g4	+	-	-	-
A. flavus g5	+	-	-	-
A. flavus g6	+	-	-	-
A. flavus g7	+	-	-	-
A. flavus g9	+	-	-	-
A. flavus h10	+	-	-	-
A. flavus h16	+	-	-	-
A. flavus h3	+	-	-	-
A. flavus h4	+	-	-	-
A. flavus h5	+	-	-	-
A. flavus h6	+	-	-	-
A. flavus h7	+	-	-	-
A. flavus h9	+	-	-	-

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. parasiticus SRRC 75	-	ND	ND	ND
A. parasiticus SRRC 143-A	-	ND	ND	ND
A. parasiticus a1	-	ND	ND	ND
A. parasiticus a2	-	ND	ND	ND
A. parasiticus c13	-	ND	ND	ND
A. parasiticus c3	-	ND	ND	ND
A. parasiticus c4	-	ND	ND	ND
A. parasiticus d13	-	ND	ND	ND
A. parasiticus f1	-	ND	ND	ND
A. parasiticus f2	-	ND	ND	ND
A. parasiticus g1	-	ND	ND	ND
A. parasiticus g2	-	ND	ND	ND
A. parasiticus h12	-	ND	ND	ND
A. parasiticus h17	-	ND	ND	ND

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. nomius SRRC 375	-	ND	ND	ND
A. nomius SRRC 362	-	ND	ND	ND
A. nomius b1	-	ND	ND	ND
A. nomius b13	-	ND	ND	ND
A. nomius b2	-	ND	ND	ND
A. nomius b3	-	ND	ND	ND
A. nomius e1	-	ND	ND	ND
A. nomius e2	-	ND	ND	ND
A. nomius g14	-	ND	ND	ND
A. nomius g15-1	-	ND	ND	ND
A. nomius g15-2	-	ND	ND	ND
A. nomius h1	-	ND	ND	ND
A. nomius h14	-	ND	ND	ND
A. nomius h2	-	ND	ND	ND

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. tamarii SRRC 99	-	ND	ND	ND
A. tamarii SRRC 1088	-	ND	ND	ND
A. tamarii c1	-	ND	ND	ND
A. tamarii c2	-	ND	ND	ND
A. tamarii c14	-	ND	ND	ND
A. tamarii c17-2	-	ND	ND	ND
A. tamarii a14	-	ND	ND	ND
A. tamarii b14	-	ND	ND	ND
A. tamarii b15	-	ND	ND	ND
A. tamarii d11	-	ND	ND	ND
A. tamarii d15	-	ND	ND	ND
A. tamarii e11	-	ND	ND	ND
A. tamarii e12	-	ND	ND	ND
A. tamarii f9	-	ND	ND	ND

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

Table 4.27 Synnema formation of *Aspergillus* section *Flavi* isolates on modified Czapek Dox medium containing (40 µl/L) Avid® supplemented with (3%w/v) nitrogen sources at 30 ^{O}C in the dark (continue)

Fungal species	Czapek medium containing Avid® at 30 ^{O}C in the dark			
	Sodium nitrate	Ammonium sulphate	Ammonium tartrate	Peptone
A. tamarii f11	-	ND	ND	ND
A. tamarii f12	-	ND	ND	ND
A. tamarii f13	-	ND	ND	ND
A. tamarii g11	-	ND	ND	ND
A. tamarii g12	-	ND	ND	ND
A. tamarii h13	-	ND	ND	ND
A. pseudotamarii SRRC 2420	-	ND	ND	ND
A. pseudotamarii SRRC 2428	-	ND	ND	ND
A. oryze SRRC 302	-	ND	ND	ND
A. oryze SRRC 2085	-	ND	ND	ND
A. oryze SRRC 480	-	ND	ND	ND
A. oryze SRRC 2079	-	ND	ND	ND
A. oryze SRRC 2044	-	ND	ND	ND

NRRL: Northern Center for Agricultural Utilization Research (NCAUR), Peoria, IL, USA,

SRRC: United States Department of Agiculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA,

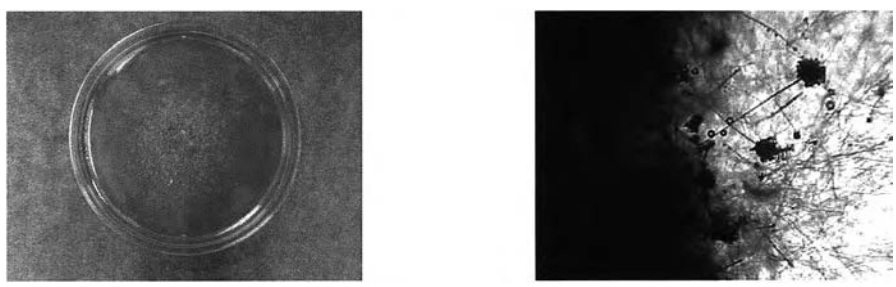
Positive test for synnema development (+), negative test for synnema development (-), and not determined (ND)

4.4 Determining Vegetative compatibility groups (VCGs) of *A. flavus*

nit mutant selection

Mycelia of each isolated of *A. flavus* from 5% V8 agar were inoculated in Rose Bengal-chlorate medium, and incubated at 30 ^{O}C in the dark for approximately 2 weeks for *nit*-nonutilizing mutaion. Morphological structures of wild type strains with nitrate reductase activity exhibited very restricted dense and dark purple color mycelium and with radian less than 1 cm from the point of inoculation (Figure 4.27). *nit* mutant (cell unable to reduce nitrate; without nitrate reductase) were not affected by chlorite, and had outgrown (white sparsely hyphae) the wild type mcelia.

Two hundred and two (206) wild types isolated of *A. flavus* were determined. One hundred and eighty (87.38%) of *nit* mutant strains of *A. flavus* were collected. However, some isolated of *A. flavus* did not have outgrown mycelium. So, twenty six strains (12.62%) did not outgrow wild type (Table 4.28).



a b

Figure 4.27 *A. flavus* on Rose Bengal-chlorate medium at 30 ^{O}C for 10 days in the dark ; (a)1.8X, and (b). 25X

A. flavus colonies on Rose Bengal-chlorate medium were separated into 3 groups including (1) sparsely mycelia, with small colony (dark pink color nearby colonies), (2) restricted dense mycelia, with green conidia (media was changed to

slightly red), and (3) restricted dense mycelia, without conidium (medium was changed to slightly red). (Figure 4.28)

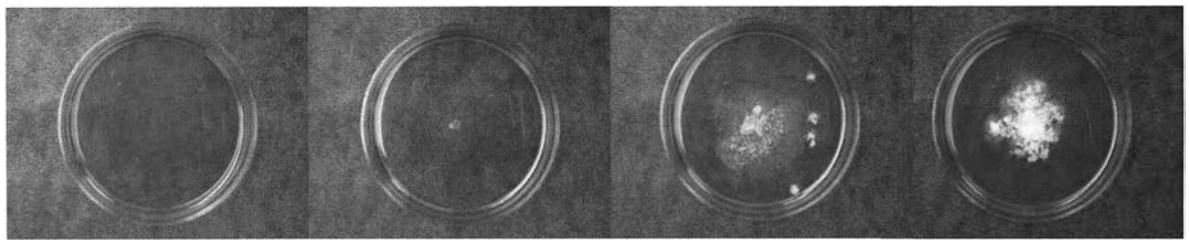


Figure 4.28 *Nit* mutants of *A. flavus* on Rose Bengal-chlorate medium at 30 ^{O}C for 10 days in the dark for 10 days; (a) no fungi, (b) sparsely mycelia, with small colony (dark pink color nearby colonies), (c) restricted dense mycelia, with green conidia (media was changed to slightly red), and (d) restricted dense mycelia, without conidium (medium was changed to slightly red).

Table 4.28 *nit* mutant strains of *A. flavus* from soils of different corn fields in Thailand

Province	nit mutant	wild type	Total
Chaningrai	9	2	11
Payao	62	6	68
Pare	27	6	33
Knonkan	7	4	11
Nakornrjachasima	7	0	7
Lopburi	18	5	23
Saraburi	4	0	4
Nakornsawan	10	1	11
Pitsanulok	14	1	15
bangkok	22	1	23
Total	180	26	206
%	87.38	12.62	

Nitrate-nonutilizing (*nit*) mutant selection

Nitrate-metabolism mutant selection media were used to identify three different types of *nit* mutant (*niaD*, *nirA*, and *cnx*). *nit* mutant strains of *A. flavus* were inoculated on three types of Nitrate-metabolism mutant selection medium, and incubated at 30 ^{O}C in the dark for 7 days. The mutant strains of *A. flavus* that formed profusely or cloudy mycelia on all types of Nitrate-metabolism mutant selection media were identified as the *nia*D mutant. The mutant strains of *A. flavus* that formed sparsely mycelia only on Nitrate-metabolism mutant selection medium containing sodium nitrite, were identified as the *nirA* mutant (Figure 4.29).

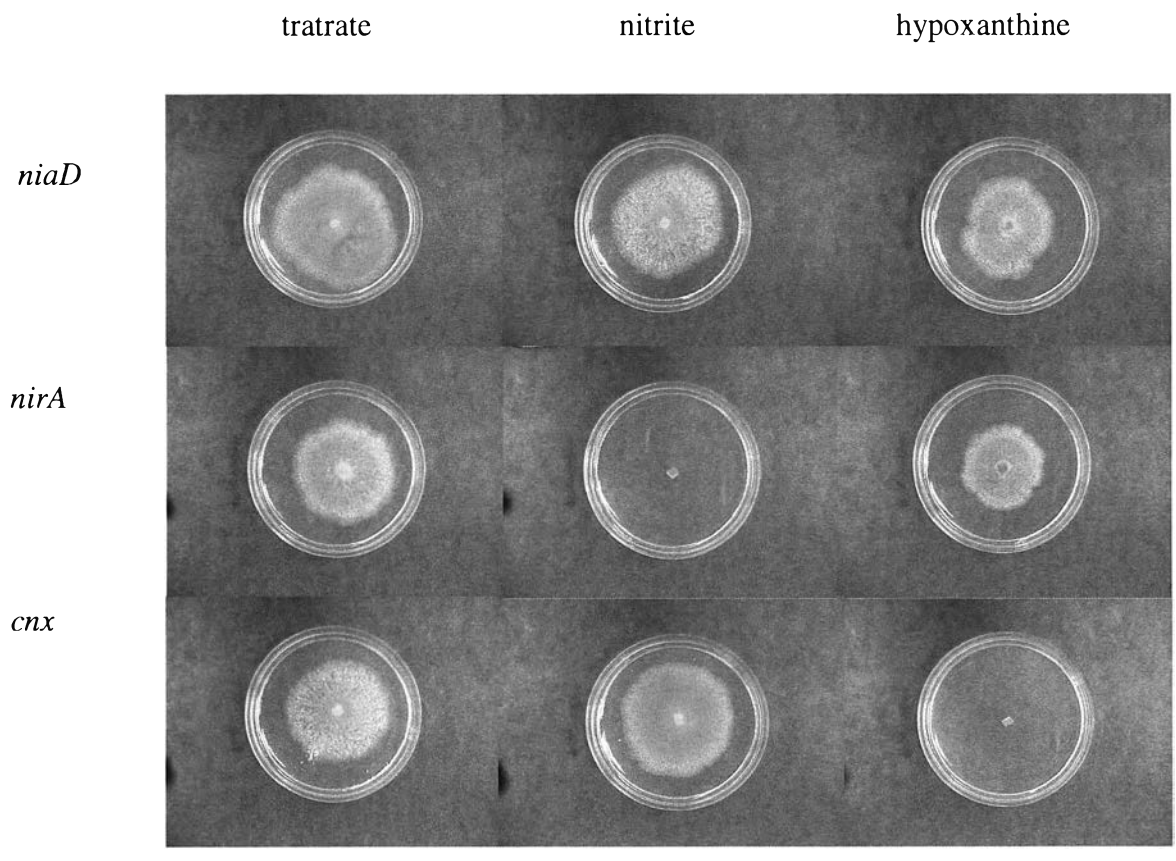


Figure 4.29 *niaD*, *nirA*, and *cnx* mutant of *A. flavus* on Cz containing each ammonium tratrate, nitrite, and hypoxanthine as sole nitrogen sources.

The mutant strains of *A. flavus* that formed sparsely mycelia on Nitrate-metabolism mutant selection medium containing hypoxanthine, were identified as the *cnx* mutant (Figure 4.29). Isolates *A. flavus* in all ten provinces of Thailand exhibited high member of *niaD* (50-80% approximately) more than *nirA*, and *cnx* mutant (Table 4.29 to 4.38). However, one hundred seventy six (176) of *nit* mutants were identified to be *niaD* mutants, 26 *nirA* mutants and 15 *cnx* mutants respectively (Table 4.38).

Table 4.29 *nit* mutant strains of *A. flavus* corn fields in Chaingrai, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*nirA*	*cnx*	
162				+
163	+			
164	+			
165	+			
166				+
167	+			
168	+			
a13	+			
b11-1	+			
b11-2	+			
b12	+			
total	9	0	0	2
%	81.82	0.00	0.00	18.18

Table 4.30 *nit* mutant strains of *A. flavus* corn fields in Payao, Thailand

Funagal isolate	*nit* mutant			Wild type
	NiaD	*nirA*	*cnx*	
5	+			
6	+			
7	+			
8	+			
9	+			
10	+			
11				+
12	+			
69	+			

Table 4.30 *nit* mutant strains of *A. flavus* corn fields in Payao, Thailand (continue)

Fungal isolate	*nit* mutant			Wild type
	NiaD	*nirA*	*cnx*	
70	+			
71	+			
72			+	
73	+			
74	+			
75		+		
81		+		
82	+			
83	+			
84	+			
89	+			
90			+	
91		+		
92	+			
97	+			
98	+			
99			+	
100	+			
101	+			
102	+			
103		+		
104	+			
105	+			
106				+
107	+			
108	+			
113	+			
114	+			
115	+			
122	+			
131		+		
132		+		
133	+			
134	+			
135	+			
136				+

Table 4.30 *nit* mutant strains of *A. flavus* corn fields in Payao, Thailand (continue)

Fungal isolate	*nit* mutant			Wild type
	NiaD	*nirA*	*cnx*	
138	+			
139	+			
140			+	
149				+
150				+
171	+			
172	+			
178	+			
a3			+	
a4			+	
c5	+			
c6		+		
d5	+			
e17	+			
e3				+
e5		+		
e7	+			
e9	+			
f5	+			
f6	+			
f7	+			
g5	+			
g6	+			
total	48	8	6	6
%	70.59	11.76	8.82	8.82

Table 4.31 *nit* mutant strains of *A. flavus* corn fields in Pare, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
13		+		
14	+			
15	+			
16				+
17				+
18	+			
19				+
20	+			
25		+		
26	+			
27		+		
28		+		
37	+			
38	+			
39	+			
40	+			
123	+			
124	+			
125				+
126	+			
127	+			
154	+			
155	+			
156	+			
157				+
b9				+
c17	+			
c9	+			
d17	+			
g7	+			
h5	+			
h6		+		
h7	+			
total	22	5	0	6
%	66.67	15.15	0.00	18.18

Table 4.32 *nit* mutant strains of *A. flavus* corn fields in Nakornrajchasima, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
49	+			
50	+			
51		+		
52	+			
158		+		
b5	+			
h16			+	
total	4	2	1	0
%	57.14	28.57	14.29	0.00

Table 4.33 *nit* mutant strains of *A. flavus* corn fields in Khonkan, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
116			+	
117				+
118	+			
119			+	
120				+
121	+			
128			+	
129				+
130				+
d1	+			
d9	+			
total	4	0	3	4
%	36.36	0.00	27.27	36.36

Table 4.34 *nit* mutant strains of *A. flavus* corn fields in Saraburi, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
173	+			
174	+			
175			+	
a17	+			
total	3	0	1	0
%	75.00	0.00	25.00	0.00

Table 4.35 *nit* mutant strains of *A. flavus* corn fields in Lopburi, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
46				+
47	+			
48	+			
53		+		
54	+			
55	+			
56	+			
57	+			
58				+
59	+			
60				+
61	+			
62	+			
63	+			
176	+			
177				+
a5	+			
a6	+			
f4	+			
g3			+	
g4			+	
h3	+			
h4				+
total	15	1	2	5
%	65.22	4.35	8.70	21.74

Table 4.36 *nit* mutant strains of *A. flavus* corn fields in Nakornsawan, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
1	+			
2			+	
3	+			
4	+			
160	+			
161	+			
169	+			
a16	+			
b16		+		
f16				+
f17		+		
total	7	2	1	1
%	63.64	18.18	9.09	9.09

Table 4.37 *nit* mutant strains of *A. flavus* corn fields in Pitsanulok, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
88	+			
145		+		
146	+			
147	+			
148	+			
151	+			
152		+		
153		+		
159	+			
c11	+			
c12	+			
c15				+
e14-1	+			
e14-2	+			
f14	+			
total	11	3	0	1
%	73.33	20.00	0.00	6.67

Table 4.38 *nit* mutant strains of *A. flavus* corn fields in in Bangkok, Thailand

Fungal isolate	*nit* mutant			Wild type
	NiaD	*NirA*	*cnx*	
21	+			
22		+		
23	+			
24	+			
29	+			
30		+		
31		+		
32	+			
33	+			
34	+			
35	+			
36				+
64	+			
65		+		
66	+			
67	+			
68			+	
a11	+			
a12	+			
g10		+		
g9	+			
h10	+			
h9	+			
total	16	5	1	1
%	69.57	21.74	4.35	4.35

Table 4.39 Summary of *nit* mutant strains of *A. flavus* from soils of different corn fields in Thailand

Province	*NiaD*	*nirA*	*cnx*	wild type	Total
Chaningrai	9	0	0	2	11
Payao	48	8	6	6	68
Pare	22	5	0	6	33
Knonkan	4	0	3	4	11
Nakornrjachasima	4	2	1	0	7
Lopburi	15	1	2	5	23
Saraburi	3	0	1	0	4
Nakornsawan	7	2	1	1	11
Pitsanulok	11	3	0	1	15
Bangkok	16	5	1	1	23
Total	139	26	15	26	206
%	67.48	12.62	7.28	12.62	100.00

4.4.1 Complementation between different classes (*niaD*, *nirA*, and *cnx*) of *nit* mutant

Starch medium was prepared, and used in the studied. Spore suspension of each *niaD* mutant strains of *A. flavus* were inoculated in the middle of the plate and surrounded with four different strains of *cnx* and *nirA* mutant strains of *A. flavus*. The plates were incubated in the dark at 30°C for three weeks. The heterokaryons of the fungi were determined by formation of dense mycelia growth between different mutants. The isolates that could be formed heterokaryons were considered to be the same VCG (Figure 4.30).

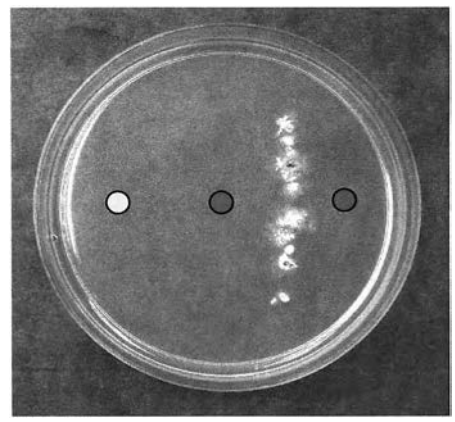


Figure 4.30 Complementation between different types of fungal strains; same VCG (green spot), and different VCG (yellow spot); *A. flavus* a13 (yellow spot), *A. flavus* 97 (green spot; center), and *A. flavus* a4 (green spot; right)

Compatible reactions, resulting in heterokaryon formation, varied in strength based on growth characteristics of aerial mycelium formed at points of contact between two complementary nit mutants. Some pairs produced dense and profuse mycelial growth, followed by production of numerous conidia (Figure 4.30), while a few pairs produced a thin line of aerial mycelia that took up to 3 weeks to sporulate. This variation in the morphology of heterokaryons was also observed for some strains in the self-compatibility tests. Among the 52 *nit* mutants *of A. flavus* tested, 17 VCGs were observed.

Seventeen VCGs were revealed from complementation test, with a total of 52 isolates from 180 *nit* mutants. As previously mentioned, compatibility was identified by a line of wild-type growth at the zone of interaction (Figure 4.30). The remaining isolates (n = 180) could not be assigned to any VCG, because complementary pairs of mutants were not obtained or the isolates pairings failed to generate the heterokaryon (vegetative incompatibility) (Table 4.40).

Table 4.40 *nit* mutant strains of *A. flavus* from soils of different corn fields in Thailand

Province	pairing	unpairing	Total
Chaningrai	0	9	9
Payao	17	45	62
Pare	10	17	27
Knonkan	3	4	7
Nakornrjachasima	0	7	7
Lopburi	3	15	18
Saraburi	0	4	4
Nakornsawan	5	5	10
Pitsanulok	4	10	14
bangkok	11	11	22
Total	52	128	180
%	29.44	70.56	

Complementations between different *nit* mutants (*niaD, nirA*, and *cnx*) were determined. Complementary between *niaD* and *nirA* with *cnx* were found less than *niaD* with *nirA* mutant. Seven teen (17) of different VCGs were observed. However, the remaining 128 isolates did not pair (70.56%). This result indicated that fifty two isolates of *A. flavus* in Thailand were indicated into seventeen groups (the same group showed same strain of *A. flavus*) (Table 4.39 to 4.40 and Figure 4.31). All 128 un-pairing *A. flavus* did not mean different VCGs but difficult to pair with the others. However, *A. flavus* isolates were obtained from the same or nearby areas showed the same VCGs including *A. flavus* 24, 30, 65, a11, and h10 from Bangkok was also observed for the VCGs A (Table 4.41).

Some isolates *A. flavus* that obtained from several areas including were grouped in the same VCGs (VCGs I) including *A. flavus* 128 from Khonkan, 88 from Pitsanulok, 47 from Lopburi, and 98 Payao provinces. In addition *A. flavus* member in the same VCGs showed same morphological structures including S trains of *A. flavus*

a11and h10 in VCGs A, L trains of *A. flavus* 90 and f7 in VCGs H and N trains of *A. flavus* 123 and 124 in VCGs N.

However, when seventeen VCGs of Thai isolates *A. flavus* were tested with sixty three Horn's VCGs (National Peanut Research Laboratory, Dawson, GA). All Thai VCGs did not showed zone of dense of the heterokaryons formation (same VCGS) with Horn's fungi. At the results, all Thai isolated *A. flavus* should be exhibited different strains from Horn's isolates (USA).

Table 4.41Vegetative compatibility groups of isolated *A. flavus* from different geographic areas of corn fields in Thailand

VCGs	Fungal isolate	Province	*nit* mutant			sclerotium type		
			niaD	*nirA*	*cnx*	L	S	N
A	24	Bangkok	+			+		
	30	Bangkok		+		+		
	65	Bangkok		+		+		
	a11	Bangkok	+				+	
	h10	Bangkok	+				+	
B	31	Bangkok		+			+	
	21	Bangkok	+			+		
	91	Payao		+			+	
C	20	Pare	+					+
	28	Pare		+				+
D	26	Pare	+					+
	27	Pare		+				+
E	132	Payao		+				+
	135	Payao	+				+	
	c6	Payao		+			+	
F	153	Pitsanulok		+			+	
	e14-2	Pitsanulok	+			+		
G	126	Pare	+				+	
	138	Payao	+				+	
	140	Payao		+				+

Table 4.41Vegetative compatibility groups of isolated *A. flavus* from different geographic areas of corn fields in Thailand (continue)

VCGs	Fungal isolate	Province	*nit* mutant			sclerotium type		
			niaD	*nirA*	*cnx*	L	S	N
H	152	Pitsanulok		+		+		
	40	Pare	+					+
	115	Payao	+			+		
	90	Payao			+	+		
	f7	Payao	+			+		
I	128	Khonkan			+	+		
	88	Pitsanulok	+				+	
	47	Lopburi	+			+		
	98	Payao	+					+
	99	Payao			+			+
	a3	Payao			+		+	
	a4	Payao			+		+	
J	118	Khonkan	+			+		
	a5	Lopburi	+					+
K	68	Bangkok			+		+	
	g9	Bangkok	+			+		
	g10	Bangkok		+		+		
L	119	Khokkan			+	+		
	e17	Payao	+			+		
M	1	Nakornsawan	+				+	
	2	Nakornsawan			+		+	
	3	Nakornsawan		+				+
	h9	Bangkok	+				+	
	g4	Lopburi			+		+	
N	123	Pare	+					+
	124	Pare	+					+
O	h5	Pare	+					+
	h6	Pare		+				+
P	f5	Payao	+				+	
	f6	Payao	+				+	
Q	a16	Nakornsawan	+					+
	b16	Nakornsawan	+					+
Total	53		29	14	9	16	19	17

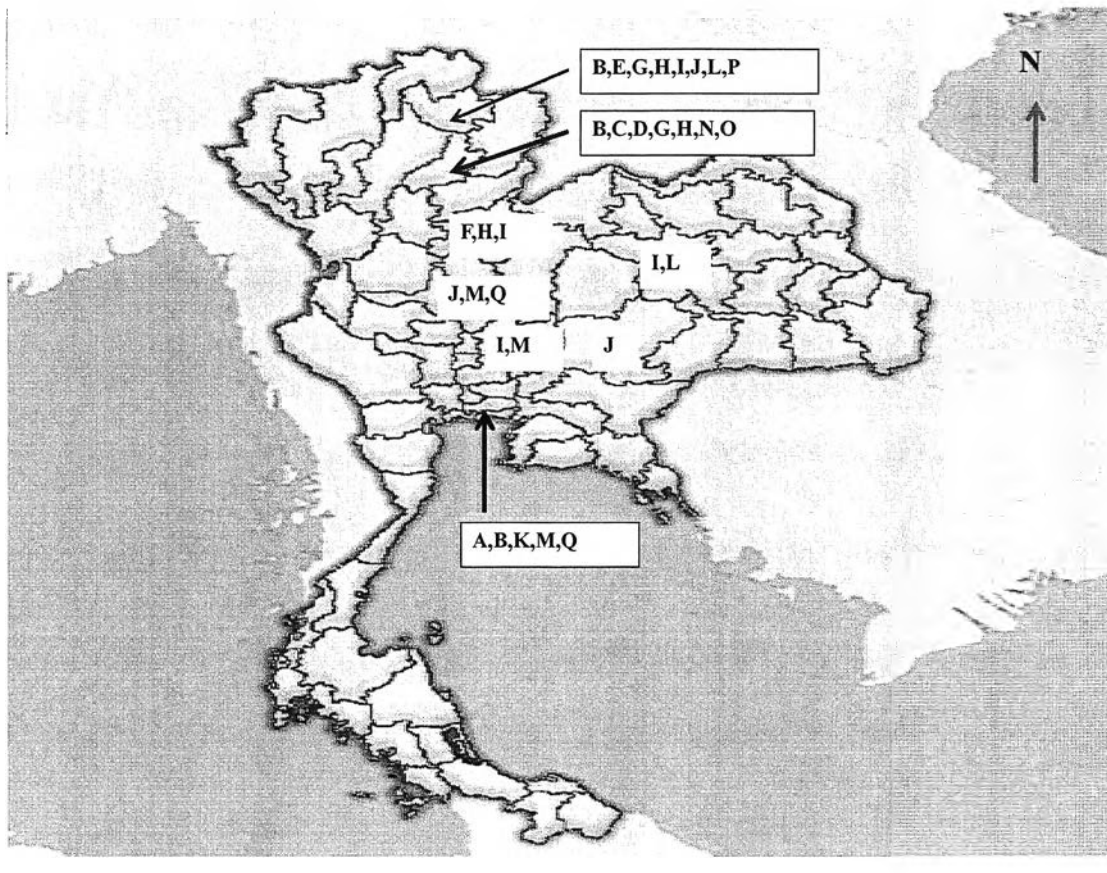


Figure 4.31 Distribution of seventeen VCGs of *A. flavus* in Thailand

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

4.5.1 Qualitative determination of aflatoxin producing strains of *Aspergillus*

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

Isolates *Aspergillus* had ability to utilize citrate, changed the medium to blue (Figure 4.33). Atoxigenic *Aspergillus* strains that did not produce aflatoxin including *A. oryzae* SRRC 2085, 302 and 480, *A. tamarii* SRRC 99 and all isolates *A. tamarii* (9), atoxigenic *A. falvus* including NRRL 21882 except *A. nomius* SRRC 362 and 8 isolates *A. nomius* from Thailand changed medium from green to blue color faster than toxigenic *Aspergillus* strains including *A. flavus* NRRL 3357 and 7 isolates atoxigenic *A. flavus* from Thailand, *A. parasiticus* SRRC 75 and 7 isolates A. *parasiticus* from Thailand, and *A. pseudotamarii* SRRC 2428 and 2020 (Table 4.42 and Figure 4.32).

Table 4.42 Number of *Aspergillus* had ability to use Citrate utilizing medium and change into blue color at 30 ^{O}C and 2 days in dark.

Aspergillus species	green	blue
atoxigenic *A. flavus*	3	7
A. oryzae	0	3
A. tamarii	0	10
toxigenic *A. flavus*	8	2
A. parasiticus/sojae	8	2
A. nomius	1	9
A. pseudotamarii	2	0

a b c d e f g

Figure 4.32 *Aspergillus* in Citrate utilizing medium containing bromthylmol blue at 30 ^{O}C and 2 days in dark; (a) *A. flavus* NRRL 3357, (b) *A. parasiticus* SRRC 75, (c) *A. psedotamarii* (2428), (d) *A. flavus* NRRL 21882, (e) *A. nomius* SRRC 362, (f) *A. tamarii* SRRC 99, and (g) *A. oryzae* SRRC 2085

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

Aflatoxins production mechanisms by fungi needs more enzymes and intermediate substances including xanthone and in anthraquinone (Payne and Brown, 1998). These substances showed yellow to brown color. On Czapek Dox agar, atoxigenic of *Aspergillus* section *Flavi* including *A. tamarii* SRRC 99, 1088 and 10 isolates *A. tamarii* from Thailand, *A. oryzae* SRRC 2085, 302 and 480, *A. nomius* SRRC 362 and 4 isolates *A. nomius* from Thailand and atoxigenic strains of *A. flavus* NRRL 3357, SRRC 1000-E and 13 isolates from Thailands howed coloress when viewing from the side cultural plates. Toxigenic strains of *Aspergillus* section *Flavi*

(except *A. nomius*) including *A. pseuotamarii*, *A. parasiticus,* and toxigenic strains of *A. flavus* changed medium to brown when viewing from the side cultural plates (Figure 4.33 and Table 4.43). At the results, toxigenic strains of *Aspergillus* had ability to convert the medium color from colorless to brown over than atoxigenic strains of *Aspergillus*. This method should be used as a new facile method for atoxigenic/toxigenic *A. flavus* identification

a b

Figure 4.33 *Aspergillus* in Cz medium and incubated at 30 ^{O}C for 7 days in the dark; (a) toxigenic starins of *Asergillus* including *A. flavus* NRRL 3357, SRRC 1000-E, *A. parasiticus* SRRC 143-A, *A. pseudotamarii* SRRC 2420, 2428 and isolates toxigenic *A. flavus*, *A. parasiticus* from Thailand (b) atoxigenic strains of *Aspergillus* including *A. flavus* K49, *A. tamarii* SRRC 1088, 99, *A. oryzae* SRRC 2085 and isolates *A. tamarii* and atoxigenic *A. flavus* from Thailand.

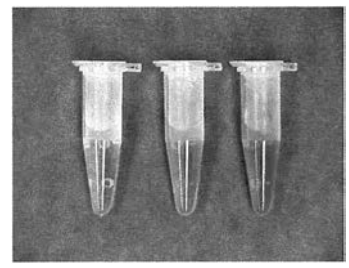
Table 4.43 number of *Aspergillus* had/ had not ability to change Czapek Dox agar to brown when incubated at 30 ^{O}C for 7 days in the dark

Aspergillus species	Brown	Colorless
atoxigenic *A. flavus*	1	7
A. oryzae	0	3
A. tamarii	0	12
toxigenic *A. flavus*	15	0
A. parasiticus	9	0
A. pseudotamarii	3	0
A. nomius	0	5

4.5.2 Quantitative determination of aflatoxin producing strains of *Aspergillus*

Aflatoxins extraction

Almost isolates *Aspergillus* section *Flavi* including *A. flavus, A. parasiticus/sojae*, *A. tamarii*, and, *A. nomius* produced colorless extracts except twenty eight strains of *A. flavus* showed yellowish orange extracted solution (Figure 4.34).



a b c

Figure 4.34 extracts from *Aspergillus* section *Flavi.* Almost extracted solutions did not have color; (a), yellow to yellowish orange extracts of some isolated *A. flavus* (b and c).

Aflatoxin analysis by TLC

Aflatoxins producing *Aspergillus* were tested by TLC method under longwave UV light compare with standards aflatoxin B1, B2, G1, and G2. Aflatoxins were observed with blue spot under longwave UV light (Figure 4.35). Howver, some toxigenic *A. flavus* including A. flavus B12, c11, and 159 that produced low aflatoxins could not determine by this method. So, they were later determined by HPLC.

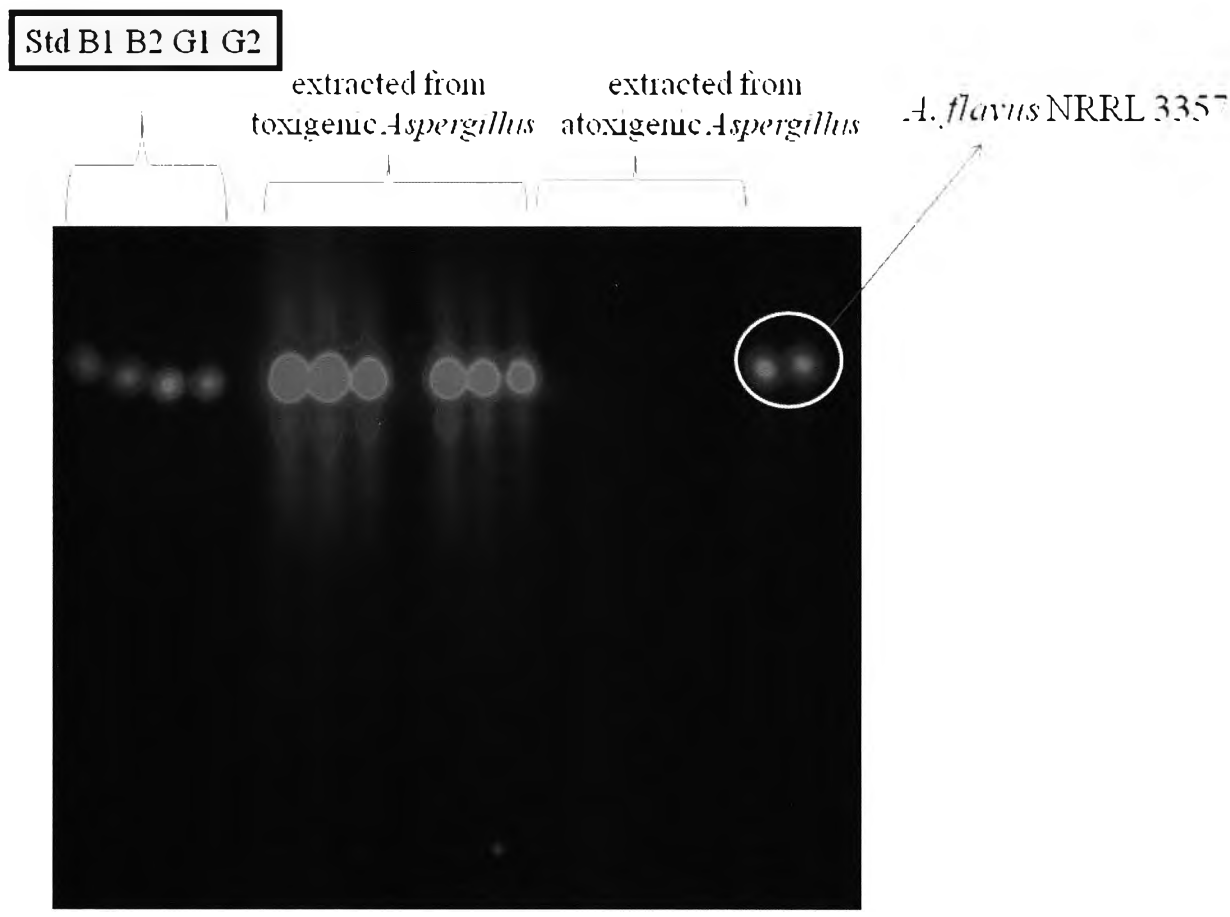


Figure 4.35 Aflatoxins (B1, B2, G1, G2 as aflatoxin standard) were detected on TLC plate under longwave UV light.

2 Aflatoxin Analysis Using HPLC

All *A. tamarii* (18 isolates) did not have ability to produce aflatoxin B1 and B2 in mYES (Table 4.44). *A. nomius* produced two groups of aflatoxin with average total aflatoxins more than 1,000 ng/ml but 7 from 12 strains of the isolates had G1 more than B1 in mYES (Table 4.45). Isolates *A. parasiticus/sojae* were identified to be *A. parasiticus* because all produced aflatoxins (*A. sojae* did not produce aflatoxin). *A. parasiticus* had high ability to produce both B and G groups of aflatoxins similarity as *A. parasiticus* (all dominantly produced aflatoxin type B1 more than G1 in mYES) (Table 4.46).

Table 4.44 Aflatoxins from 18 isolates *A. tamarii* in mYES at 30^{O}C for 7 days.

Isolates	mYES			
	aflatoxins content (ng/ml)			
	B1	B2	G1	G2
	0	0	0	0
	0	0	0	3.703
	0	0	0	0
	0	0	2.3315	2.882
	0	2.4345	1.095	0.959
	0	0	2.144	2.524
	0	0	0	2.233
	0	0	0	0.1009
	0	0	1.3665	0.827
	0	0	0	0.8335
	0	0	0	0
	0	0	4.669	7.668
	0	0	0	5.523
	0	0	2.0425	1.5805
	0	0	0	0
	0	0	0	3.703
	0	0	0	0
	0	0	2.3315	2.882

Table 4.45 Aflatoxins from 12 isolates *A. nomius* in mYES at 30^{O}C for 7 days.

Isolates	mYES			
	aflatoxins content (ng/ml)			
	B1	B2	G1	G2
	1377.95	121.585	441.576	114.914
	338.398	26.3195	1778.19	5.263
	2300.18	163.471	1246.23	82.337
	1125.19	91.745	1839.31	153.195
	689.319	100.772	340.832	16.22
	155.817	12.8405	245.371	1.420
	461.651	75.201	941.361	56.061
	657.041	56.027	1888.83	0
	158.493	12.997	86.385	10.038
	736.704	48.188	133.126	0
	460.13	43.343	948.27	123.268
	943.534	79.226	1108.89	0

Table 4.46 Aflatoxins from *A. parasiticus* in mYES at 30^{O}C for 7 days.

Isolates	mYES			
	aflatoxins content (ng/ml)			
	B1	B2	G1	G2
	2173.95	180.249	797.092	0
	2018.2	153.221	746.051	0
	920.046	96.153	1004.22	164.137
	893.972	71.978	468.998	45.875
	1158.06	95.34	348.193	48.432
	1720.84	103.027	420.732	42.793
	8252.8	675.977	7698.49	0
	7620.91	473.613	2806.29	0
	3399.64	278.122	0	858.022
	2013.59	182.432	633.622	76.544
	7056.65	524.71	1280.99	0
	7059.35	428.219	4247.93	0

Aflatoxins producing by *A. flavus* showed wide range of aflatoxin types and contents in the medium. Toxigenic strains of *A. flavus* with afaltoxin B were found more than approximately 60% isolates. Twenty three atoxigenic isolates A. flavus were found and characterized. However, they were summarized in the table depend on collecting site (10 provinces) (Table 4.47 to 4.56).

Chaingrai province

Ten toxigenic (more than 54% isolates produced both aflatoxins B and G) and one (1) atoxigenic isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxin producer with capacity to produce total aflatoxin in range 1-500 ng/ml (63.64%), 501-1,000 ng/ml (0%), and over than 1,001 ng/ml (27.27%) respectively (Table 4.47).

Table 4.47Aflatoxins from isolates *A. flavus* (11 isolates) obtained from different corn soils fields in Chaingrai province in mYES at 30^{O}C for 7 days.

Isolates	aflatoxin (ng/ml)			
	B1	B2	G1	G2
162	33.521	0	0	0
163	57.556	1.043	0	0
164	59.055	0	0	0
165	33.548	0	0	0
166	1400.972	28.431	0.4921	0
167	1619.585	43.611	0	10.572
168	1468.935	37.777	0.761	0
a13	0	0	0	0
b11-1	30.672	2.2095	14.496	3.688
b11-2	10.367	0	0	1.3085
b12	9.741	0	8.1845	6.495
total	10	5	4	4
%	90.91	45.45	36.36	36.36

Payao province

Fourty seven toxigenic (more than 76.47% isolates produced both aflatoxins B and G) and eleven atoxigenic (11) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxin producer with capacity to produce total aflatoxin in range 1-500 ng/ml (55.88%), 501-1,000 ng/ml (2.94%), and over than 1,000 ng/ml (25%) respectively (Table 4.48).

Table 4.48 Aflatoxins from isolated *A. flavus* (68 isolates) obtained from different corn soils fields in Payao province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
5	859.162	40.313	6.436	0
6	1329.11	86.044	35.012	0
7	19.34	2.407	0.746	0
8	948.993	50.29	4.204	0
9	830.652	43.162	2.542	0
10	1547.896	97.569	39.754	0
11	1426.553	77.975	13.06	0
12	1287.566	72.489	28.177	0
69	1390.971	41.013	8.565	0
70	1582.144	116.907	38.465	0
71	2659.726	203.468	52.385	0
72	196.757	122.144	31.79	0
73	2184.875	178.427	40.945	0
74	2259.99	201.812	51.223	0
75	2267.74	201.654	38.721	0
81	1098.262	21.245	6.42	0.269
82	52.51	0.206	0	0
83	1476.92	59.394	17.297	0
84	30.18	0.911	0.283	0
89	0	0	0	0
90	0	0	0	0
91	0	0	0	0
92	1.453	0.932	0	0
97	1.319	0	0	0.675
98	1.769	0	0	0.857

Table 4.48 Aflatoxins from isolated *A. flavus* (68 isolates) obtained from different corn soils fields in Payao province in mYES at 30^{O}C for 7 days. (continue)

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
99	0	0	0	0
100	10.23	0	0	0.718
101	4.981	0	0	0.523
102	189.836	5.433	0.786	0
103	524.076	6.392	23.653	0
104	169.197	4.586	0.962	0
105	0	0	0	0
106	0	0	0	0
107	20.96	0	0	1.279
108	1.326	0	0.54	0
113	124.3	16.353	0.412	0
114	6.853	0	0.852	0
115	9.652	0	0.46	0
122	0	1.282	0	2.409
131	129.772	1.291	2.322	0
132	372.828	11.044	2.822	0
133	0	0	0	0
134	300.159	2.389	4.908	0
135	368.041	9.657	1.45	0
136	31.787	0.313	0	0
138	101.798	0.515	0	0
139	85.177	2.036	0	0
140	244.556	2.85	0	0
149	87.779	0.544	0	0
150	36.135	0	0	1.244
171	0	0	0	6.239
172	0	0	0	1.139
178	0	0	0	0
a3	0	0	0	0
a4	0	0	0	0.309
c5	89.0075	6.542	18.2535	10.153
c6	84.143	3.5445	13.0995	3.802
d5	0	0	0	6.29
e17	0	0	0	0
e3	22.666	0.699	0	0
e5	0	0	0	4.942
e7	81.523	7.378	17.3435	2.3045

Table 4.48 Aflatoxins from isolated *A. flavus* (68 isolates) obtained from different corn soils fields in Payao province in mYES at 30^{O}C for 7 days. (continue)

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
e9	12.7285	0	0	0
f5	2678.82	207.925	454.477	156.739
f6	1636.64	134.303	162.759	78.2015
f7	29.028	1.827	2.481	4.2995
g5	2543.71	177.837	429.81	120.304
g6	2889.73	350.786	884.939	179.074
total	44	45	37	21
%	64.71	66.18	54.41	30.88

Pare province

Thirty toxigenic (75.76% isolates produced both aflatoxins B and G) and three atoxigenic (3) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (63.64%), 501-1,000 ng/ml (18.18%), and over than 1,001 ng/ml (9.09%) respectively (Table 4.49).

Table 4.49 Aflatoxins from isolated *A. flavus* (33 isolates) obtained from different corn soils fields in Pare province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
13	2.395	0	0	0.185
14	31.004	0	0	0
15	3.5924	0.001	0	0
16	1.449	0.543	0	5.159
17	1436.889	28.504	49.696	0
18	1.828	0.055	0	0
19	1574.409	34.911	13.35	0
20	1469.864	49.634	5.492	0
25	469.378	11.71	1.775	0.182
26	314.104	14.564	0	0.369

Table 4.49 Aflatoxins from isolated *A. flavus* (33 isolates) obtained from different corn soils fields in Pare province in mYES at 30^{O}C for 7 days. (continue)

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
27	333.921	11.2	0.535	0.153
28	648.469	13.062	1.362	0.223
37	1.384	0.167	0	0
38	0	0	0	0
39	1.47	0	0.421	0
40	0	0	0	0
123	531.075	10.345	4.872	0.699
124	412.428	14.372	2.317	1.301
125	736.898	12.834	8.166	5.132
126	665.199	9.817	3.862	3.485
127	666.717	9.441	2.065	1.095
154	2.853	0	0.714	0
155	3.144	0	0	0.567
156	0.985	0	0	0.425
157	0.973	0	0	0.256
b9	0	0.188	0	12.1085
c17	89.384	7.2695	14.0495	0
c9	0	0	0	0
d17	56.917	2.4435	6.878	14.1605
g7	0	0	0	0
h5	219.316	21.0665	52.7015	11.1945
h6	556.579	64.698	182.325	30.516
h7	0	4.235	0	5.2965
total	27	22	17	19
%	81.82	66.67	51.52	57.58

Nakornrajchasima province

Seven toxigenic (28.57 % isolates produced both aflatoxins B and G) and one atoxigenic (1) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (85.71%), 501-1,000 ng/ml (0%), and over than 1,000 ng/ml (0%) respectively (Table 4.50).

Table 4.50 Aflatoxins from isolated *A. flavus* (7 isolates) obtained from different corn soils fields in Nakornrajchasima province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
49	60.874	0.37	0	0
50	44.596	0.881	0	0
51	12.918	0.131	0	0
52	0	0	0	0
158	362.935	6.953	3.473	2.27
b5	347.39	28.6185	79.228	24.2665
h16	0	0	0	4.552
total	5	5	2	3
%	71.43	71.43	28.57	42.86

Khonkan province

Nine toxigenic (72.73 % isolates produced both aflatoxins B and G) and two atoxigenic (2) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (45.45%), 501-1,000 ng/ml (0%), and over than 1,000 ng/ml (36.36%) respectively (Table 4.51).

Table 4.51 Aflatoxins production from isolated *A. flavus* (11 isolates) obtained from different corn soils fields in Khonkan province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
116	1521.326	57.612	20.397	0
117	1497.95	51.716	16.799	0
118	1055.526	23.157	11.759	0.632
119	1020.214	17.722	9.151	0.259

. Table 4.51 Aflatoxins from isolated *A. flavus* (11 isolates) obtained from different corn soils fields in Khonkan province in mYES at 30°C for 7 days. (continue)

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
120	108.524	0.699	1.345	0
121	0	0.736	0	1.897
128	0	0	0	0.789
129	0	0	0	0
130	0	0	0	0
d1	0	1.0185	463.614	5.7805
d9	293.709	27.5215	81.8135	26.3485
total	6	8	7	6
%	54.55	72.73	63.64	54.55

Pitsanulok province

Fourteen toxigenic (80 % isolates produced both aflatoxins B and G) and one atoxigenic (1) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (46.67%), 501-1,000 ng/ml (0%), and over than 1,000 ng/ml (46.67%) respectively (Table 4.52).

Table 4.52 Aflatoxins from isolated *A. flavus* (15 isolates) obtained from different corn soils fields in Pitsanulok province in mYES at 30°C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
88	1887.07	115.388	200.655	0
145	102.87	1.874	14.485	0
146	0	0	0	0
147	1960.467	127.613	18.784	0
148	1874.752	147.992	14.638	0
151	69.854	0.062	0.381	0
152	19.345	0.137	4.043	0
153	15.461	0.093	0	0
159	1.034	0	0	0.485

Table 4.52 Aflatoxins from isolated *A. flavus* (15 isolates) obtained from different corn soils fields in Pitsanulok province in mYES at 30^{O}C for 7 days. (continue)

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
c11	9.404	0	0	0
c12	10.8305	0	0	0
c15	1452.13	112.198	273.228	24.92
e14-1	1865.67	202.782	482.333	406.677
e14-2	823.211	67.483	164.981	44.8855
f14	4495.94	312.703	1431.63	427.224
total	14	11	10	5
%	93.33	73.33	66.67	33.33

Saraburi province

Three toxigenic (75 % isolates produced both aflatoxins B and G) and one atoxigenic (1) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (0%), 501-1,000 ng/ml (0%), and over than 1,000 ng/ml (75%) respectively (Table 4.53).

Table 4.53 Aflatoxins from isolated *A. flavus* (4 isolates) obtained from different corn soils fields in Saraburi province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
173	1885.96	69.167	13.516	0
174	1568.913	41.829	6.225	0
175	0	0	0	0
a17	1578.52	90.67	245.65	33.337
total	3	3	3	1
%	75.00	75.00	75.00	25.00

Nakornsawan province

Ten toxigenic (63.64 % isolates produced both aflatoxins B and G) and one atoxigenic (1) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (45.45%), 501-1,000 ng/ml (36.36%), and over than 1,000 ng/ml (9.09%) respectively (Table 4.54).

Table 4.54 Aflatoxins from isolated *A. flavus* (11 isolates) obtained from different corn soils fields in Nakornsawan province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
1	824.15	28.381	4.785	0
2	8.532	46.753	5.047	0.685
3	896.54	13.469	7.549	0
4	859.698	20.158	3.832	0.1901
160	0	0	0	0.439
161	0	0	0	0
169	401.572	3.214	0	0
a16	130.605	10.857	24.5605	0
b16	0	0	0	5.456
f16	642.523	37.851	84.1975	70.425
f17	1657.37	154.277	395.153	50.7515
total	8	8	7	6
%	72.73	72.73	63.64	54.55

Lopburi province

Twenty one toxigenic (65.22 % isolates produced both aflatoxins B and G) and two atoxigenic (2) isolated *A. flavus* were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (78.26%), 501-1,000 ng/ml (0%), and over than 1,000 ng/ml (13.04%) respectively (Table 4.55).

Table 4.55 Aflatoxins from isolated *A. flavus* (23 isolates) obtained from different corn soils fields in Lopburi province in mYES at 30^{O}C for 7 days.

Isolates	afaltoxin (ng/ml)			
	B1	B2	G1	G2
46	2007.034	174.147	35.792	0
47	43.007	0.598	0	0.301
48	1558.857	74.05	23.122	0
53	17.738	0.178	2.852	0
54	84.68	0.795	2.717	0
55	18.285	0.211	0.324	0
56	104.039	0.615	2.687	0
57	22.957	0.254	0.693	0
58	192.113	10.743	1.162	0
59	14.936	0.033	0	0
60	107.095	0.647	1.872	0
61	1522.389	71.758	8.406	0
62	0	0.827	0	0.332
63	0	19.929	3.107	0
176	50.919	0.117	0	0
177	92.005	1.495	0	0
a5	0	0	0	0
a6	0	0	0	0
f4	0	0	0	1.49
g3	270.208	69.454	200.669	19.2005
g4	0	0.3635	0	1.246
h3	9.4575	0	0	0
h4	0	0.347	0	0
total	17	19	12	5
%	73.91	82.61	52.17	21.74

Bangkok province

Twenty three toxigenic (100 % isolates produced both aflatoxins B and G) were determined. Toxigenic isolated *A. flavus* members were found to be aflatoxins producer with capacity to produce total aflatoxin in range 1-500 ng/ml (13.04%), 501-1,000 ng/ml (26.09%), and over than 1,000 ng/ml (60.87%) respectively (Table 4.56).

Table 4.56 Aflatoxins production from isolated *A. flavus* (23 isolates) obtained from different corn soils fields in Bangkok province in mYES at 30^{O}C for 7 days.

Strains No.	afaltoxin (ng/ml)			
	B1	B2	G1	G2
21	1352.268	27.133	11.316	0
22	1728.568	49.951	18.809	0
23	845.125	26.01	19.855	0.275
24	1238.254	30.42	4.672	0
29	1254.089	34.832	3.125	0
30	1344.889	25.277	6.869	0
31	1103.447	30.17	2.481	0.082
32	321.546	16.555	0.573	0.393
33	953.543	9.762	2.643	0
34	630.755	20.152	0.855	0.213
35	425.377	2.916	0.584	0.234
36	755.486	10.994	5.241	0
64	96.136	0.74	1.518	0
65	1164.192	28.006	5.148	0
66	1412.613	39.11	10.766	0
67	1387.694	36.491	8.024	0
68	1434.393	39.174	10.512	0
a11	521.518	44.5885	125.882	74.438
a12	424.686	25.1455	66.533	30.138
g10	4281.75	369.149	998.393	279.427
g9	1237.1	83.3025	134.15	39.647
h10	3196.09	220.859	606.523	106.931
h9	1237.94	91.283	267.295	50.5305
total	23	23	23	11
%	100.00	100.00	100.00	47.83

4.6 Relationship between fungal morphology, VCG, and aflatoxin production.

Seven teen groups of VCG (A to Q) with Fifty two of nit mutants were characterized for relationship between fungal morphology, VCG, and aflatoxin production. In the same VCG, *A. flavus* showed wide range of *nit* mutant types including *A. flavus* 24 (niaD), and *A. flavus* 30 in VCG A. Most of the isolates grouped in the same VCG produced the same combination of aflatoxins and sclerotium production, with the exception of some VCGs including B, I, M, L, and Q, which differed in the combination of mycotoxins and sclerotium types (Table 4.57).

Table 4.57 VCGs of *A. flavus* with fungal morphology (sclerotium types), and aflatoxins.

VCG	Fungal isolate	Province	*nit* mutant			sclerotium type			Aflatoxin (ng/ml)			
			niaD	*nirA*	*cnx*	L	S	N	B1	B2	G1	G2
A	24	Bangkok	+			+			1238.25	30.42	4.67	0
	30	Bangkok		+		+			1344.88	25.27	6.86	0
	65	Bangkok		+		+			1164.19	28.00	5.14	0
	a11	Bangkok	+				+		521.51	44.58	125.88	74.43
	h10	Bangkok	+				+		3196.09	220.85	606.52	106.93
B	31	Bangkok		+			+		1130.44	30.17	2.48	0.08
	21	Bangkok	+			+			1352.26	27.13	11.31	0
	91	Payao		+			+		0	0	0	0
C	20	Pare	+					+	1469.86	49.63	5.49	0
	28	Pare		+				+	648.46	13.06	1.36	0.22
D	26	Pare	+					+	314.10	14.56	0	0.36
	27	Pare		+				+	333.92	11.20	0.53	0.15
E	132	Payao		+				+	372.82	11.04	2.82	0
	135	Payao	+				+		368.04	9.65	1.45	0
	c6	Payao		+			+		84.14	3.54	13.09	3.80
F	153	Pitsanulok		+			+		15.46	0.09	0	0
	e14-2	Pitsanulok	+			+			823.21	67.48	164.98	44.88
G	126	Pare	+				+		665.19	9.81	3.86	3.48
	138	Payao	+				+		101.79	0.51	0	0
	140	Payao		+				+	244.55	2.85	0	0

Table 4.57 VCGs of *A. flavus* with fungal morphology (sclerotium types), and aflatoxins. (continue)

VCG	Fungal isolate	Province	*nit* mutant			sclerotium type			Aflatoxin (ng/ml)			
			niaD	*nirA*	*cnx*	L	S	N	B1	B2	G1	G2
H	152	Pitsanulok		+		+			19.34	0.13	4.03	0
	40	Pare	+					+	0	0	0	0
	115	Payao	+			+			9.65	0	0.46	0
	90	Payao			+	+			0	0	0	0
	f7	Payao	+			+						
I	128	Khonkan			+	+			0	0	0	0.789
	88	Pitsanulok	+				+		1887.07	115.38	200.65	0
	47	Lopburi	+			+			43.00	0.59	0	0.30
	98	Payao	+					+	1.769	0	0	0.67
	99	Payao			+			+	0	0	0	0.85
	a3	Payao			+		+		0	0	0	0
	a4	Payao			+		+		0	0	0	0.309
J	118	Khonkan	+			+			1055.52	23.15	11.75	0.63
	a5	Lopburi	+					+	0	0	0	0
K	68	Bangkok			+		+		1434.39	39.17	10.51	0
	g9	Bangkok	+			+			1237.10	83.30	134.15	39.64
	g10	Bangkok		+		+			4281.75	369.14	998.39	279.42
L	119	Khokkan			+	+			1020.21	17.72	9.15	0.25
	e17	Payao	+			+			0	0	0	0
M	1	Nakornsawan	+				+		824.15	28.38	4.78	0
	2	Nakornsawan			+		+		8.53	46.75	5.04	0.68
	3	Nakornsawan		+				+	896.54	13.46	7.54	0
	h9	Bangkok	+				+		1237.94	91.28	267.29	50.53
	g4	Lopburi			+		+		0	0.36	0	1.24
N	123	Pare	+					+	531.07	10.34	4.87	0.69
	124	Pare	+					+	412.42	14.37	2.31	1.30
O	h5	Pare	+					+	219.31	21.06	52.70	11.19
	h6	Pare		+				+	556.57	64.69	182.32	30.51
P	f5	Payao	+				+		2678.82	207.92	454.47	156.73
	f6	Payao	+				+		1636.64	134.30	162.75	78.20
Q	a16	Nakornsawan	+					+	130.60	10.85	24.56	0
	b16	Nakornsawan	+					+	0	0	0	5.45

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

4.7.1 Inhibition of aflatoxins production from toxigenic strains of *A. flavus* by the same strain of atoxigenic

A. flavus NRRL 3357 was used as the model of toxigenic strains of *A. flavus* in this study. Spore suspension (15 µl of 10^8 spore/ml) of toxigenic *A. flavus* NRRL 3357, and atoxigenic *A. flvus* were co-inoculated into the mYES medium (30 µl final concentration). The cultures were incubated at 30^{O}C, in the dark for 7 days. After incubation, aflatoxins were extracted and determined. *A. flavus* NRRL 3357 showed high ability to produce Aflatoxin B1 (1601.34 ng/ml) in the medium. However, *A. flavus* NRRL 3357 lost activity to produce aflatoxins when co-inoculated with atoxigenic *A. flavus*. The highest antagonistic activity was detected with an atoxigenic *A. flavus* A13 (Figure 4.39 bar 4). They were observed between 0 to 7 days of incubation for aflatoxin B1 production Conidia were germinated and Aflatoxin B1was produced in the secondary day of incubation. In this study, *A. flavus* NRRL 3357 produced aflatoxin B1 in the second days and had the highest amount of aflatoxin B1 after six days of incubation. However, atoxigenic *A. flavus* A13 showed ability to inhibit aflatoxin B1 production when co-inoculation with toxigenic *A. flavus* NRRL 3357 in the first days of incubation (Figure 4.40 to 4.41). It is hard working to visually measure the relative degree of colonization following incubation because atoxigenic strains of *A. flavus* are indistinguishable from toxigenic strains in the medium. However, *A. flavus* NRRL 3357 showed conidial characteristics with conidial chains on conidiophores shorter than *A. flavus* A13 (Figure 4.39).

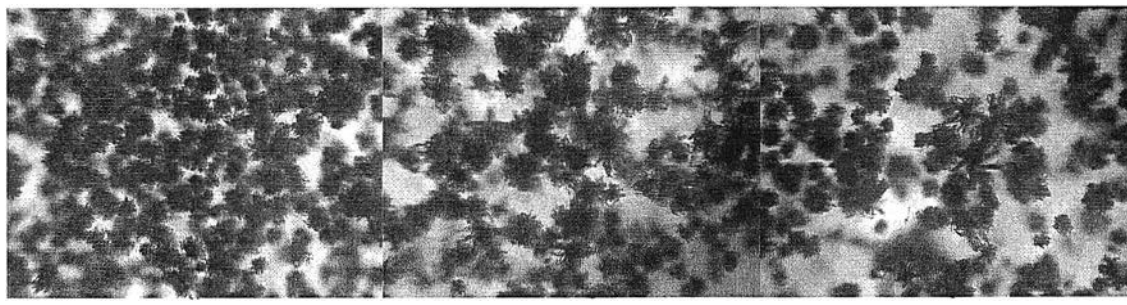


Figure 4.39 *Aspergilus* colonization in mYES at 30^{O}C, in the dark for 7 days; (a) only *A. flavus* NRRL 3357, (b) only *A. flavus* A13, and (c) co-inoculation between *A. flavus* NRRL 3357 with *A. flavus* A13.

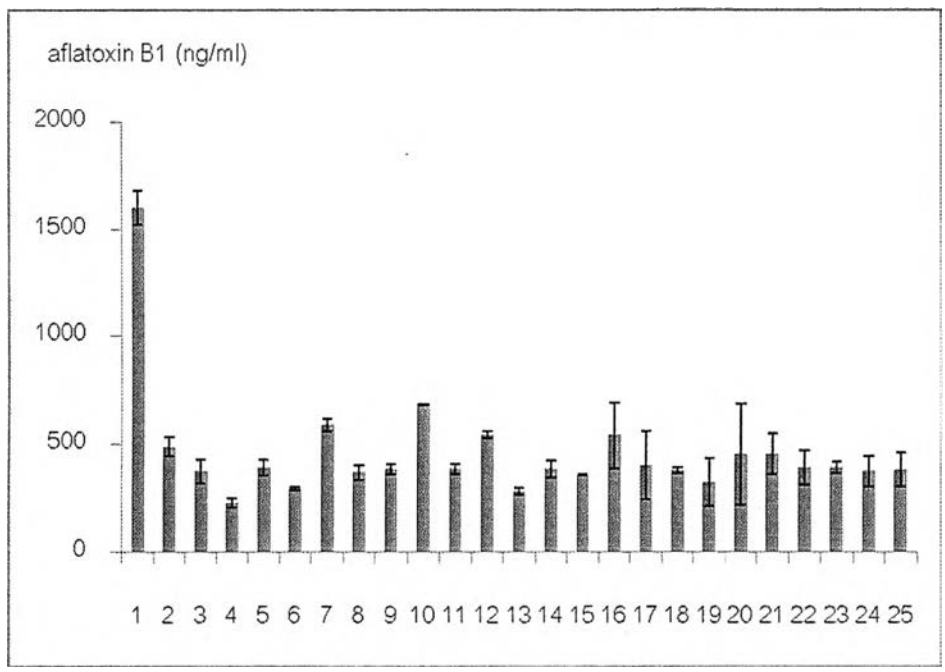


Figure 4.40 Co-inoculation of *A. flavus* NRRL 3357 with each isolated atoxigenic *A. flavus* from Thailand in mYES at 30^{O}C, in the dark for 7 days; Only *A. flavus* NRRL 3357 (bar no.1), co-inoculation with atoxigenic *A. flavus* NRRL 21882 (bar no.2) and co-inoculation with atoxigenic *A. flavus* from Thailand (bar no. 3 to 25).

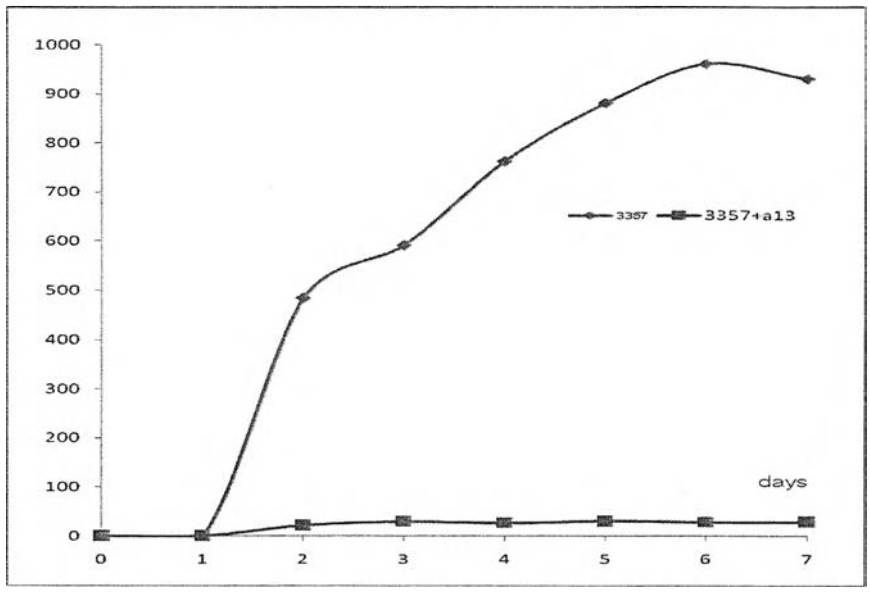


Figure 4.41 aflatoxin B1 production from *A. flavus* NRRL 3357 and co-inoculation of *A. flavus* NRRL 3357 with atoxigenic *A. flavus* A13 in mYES at 30^{O}C, in the dark for 7 days

4.7.2 Effect of the addition time of atoxigenic culture of *A. flavus* A13 on aflatoxins production by *A. flavus* NRRL 3357

The result (Figure 4.43) showed that low level aflatoxin B1 was produced when *A. flavus* A13 culture was added at the beginning of incubation (0 day). Atoxigenic *A. flavus* A13 loosed activity to inhibit aflatoxin B1 from *A. flavus* NRRL 3357 when adding atoxigenic *A. flavus* culture after the 1 day of incubation. The results showed that the inhibition only occurred when the addition of *A. flavus* A13 culture was within the first day of incubation (Figure 4.44).

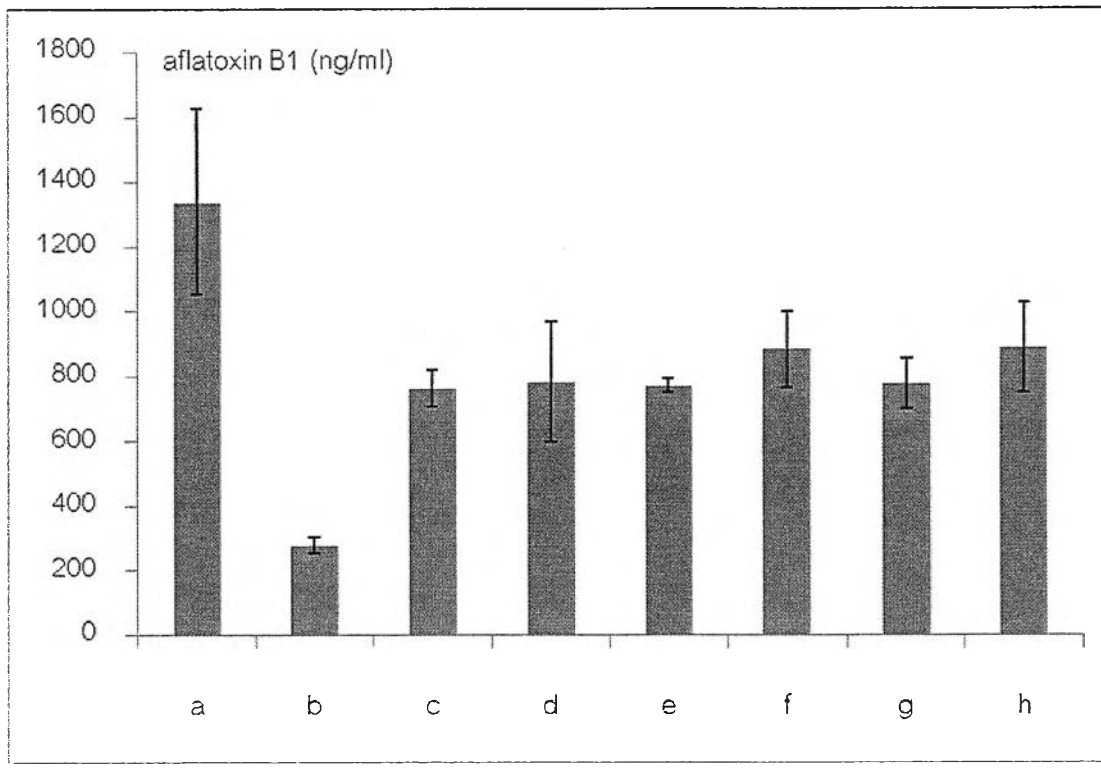


Figure 4.44 aflatoxin B1 production from *A. flavus* NRRL 3357 and addition timing of atoxigenic *A. flavus* A13 culture in mYES at 30^{O}C, in the dark for 7 days; (a) only *A. flavus* NRRL 3357, (b) 0 day, (c) 1 day, (d) 2 days, (e) 3 days, (f) 4 days (g) 5 days ,and (h) 6 days.

4.7.3 Effect of shaking or stationary condition on aflatoxins production and inhibition by co-inoculated toxigenic *A. flavus* NRRL 3357 with the same strains of atoxigenic

Toxigenic strains of *A. flavus* NRRL 3357 and atoxigenic strains of *A. flavus* A13 were used to the model in this study. They were co-inoculated into 10 ml mYES medium (300 □l final concentration). The cultures were separately incubated at stationary or shaking (150 rpm), at 30^{O}C, in the dark for 7 days. After incubation, aflatoxins were extracted and determined. *A. flavus* NRRL 3357 showed the highest aflatoxin B1 content when using stationary incubation. However, agitation culture of *A. flavus* NRRL 3357 with low number of conidia had aflatoxin yield nearby

stationary condition of co-inoculation *A. flavus* NRRL 3357 with a13 (Figure 4.45). In additional, fungi are deficient in sporulation were unable to produce aflatoxins.

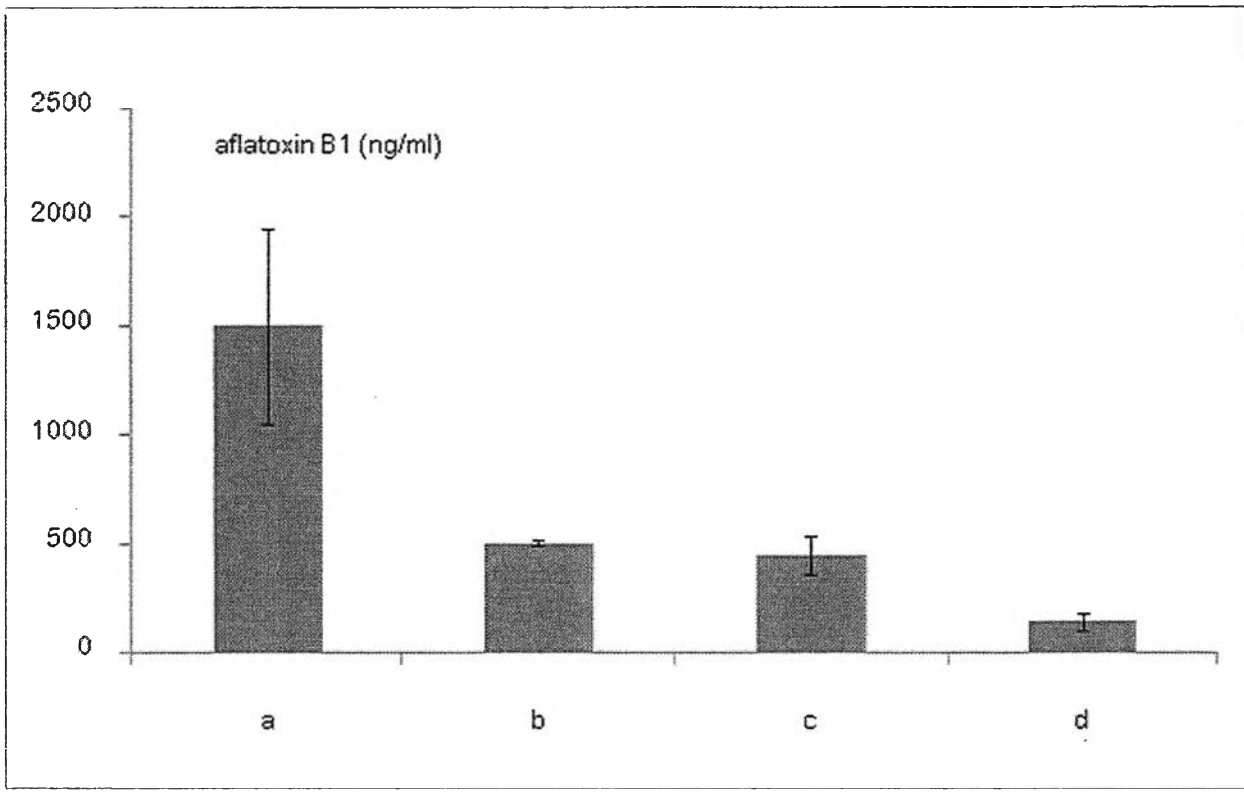


Figure 4.45 Aflatoixin yield by co-inoculation (50:50 of 150 □l of 10^8 spores) of *A. flavus* NRRL 3357 with *A. flavus* A13 in 10 ml YES medium in the dark at 30 ^{O}C; (a) stationary culture of *A. flavus* NRRL 3357, (b) agitation culture of *A. flavus* NRRL 3357condition, (c) stationary culture of co-inoculation, and (d) agitation culture of co-inoculation.

4.7.4 Inhibition of aflatoxin production from toxigenic *A. flavus* by atoxigenic strain involves living cell of fungi (mycelial network, or touching) or solution factors.

Toxigenic strains of *A. flavus* NRRL 3357 and atoxigenic strains of *A. flavus* A13 were used to the model in this study. *A. flavus* NRRL 3357 was paired with *A.*

flavus A13 in the filter insert/plate well system two different conditions. The results showed that when *A. flavus* NRRL 3357 were separated from *A. flavus* A13 in the same well plate, aflatoxin B1 was produced but even less than the control (only *A. flavus*) However, very little toxin was produced when the two isolates were cultured together (Figure 4.46). At the results, Inhibition of aflatoxin production from toxigenic *A. flavus* by atoxigenic strain involves solution factors more than living cell of fungi (mycelial network, or touching)

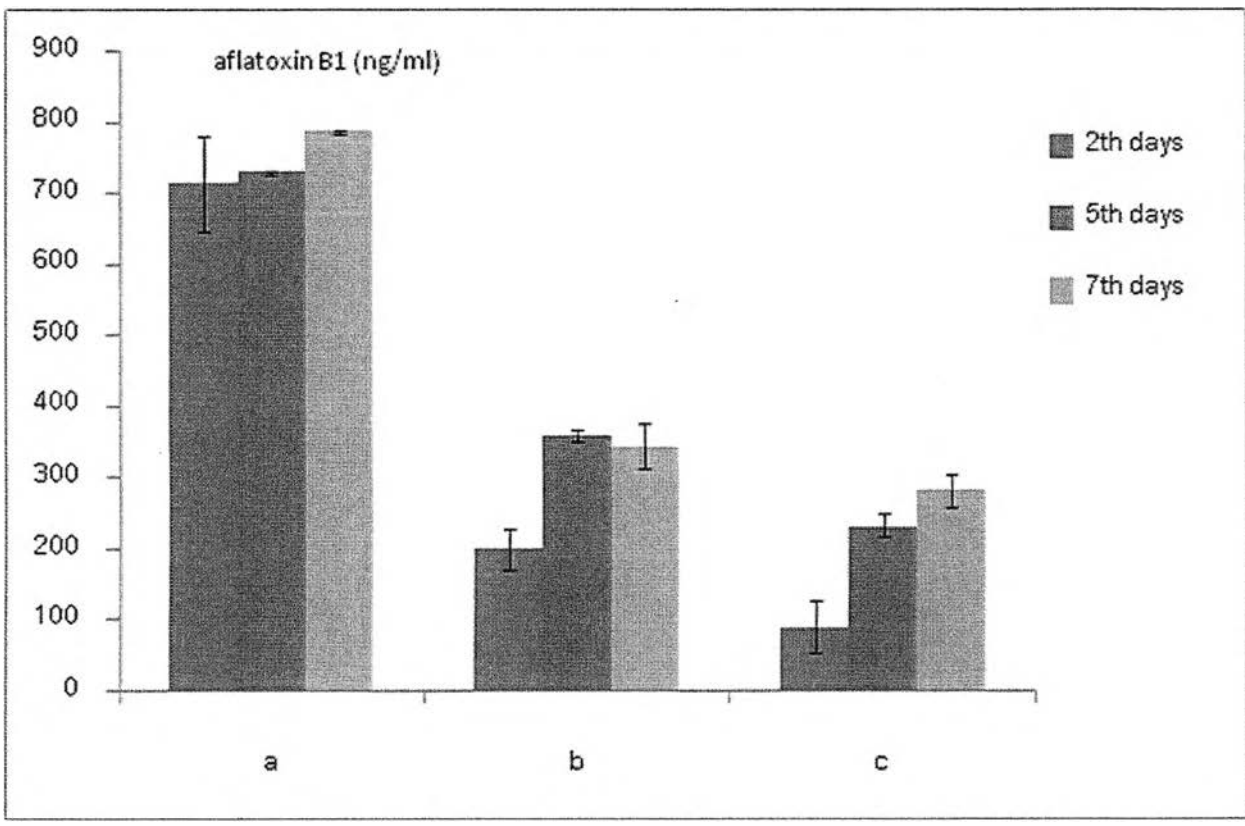
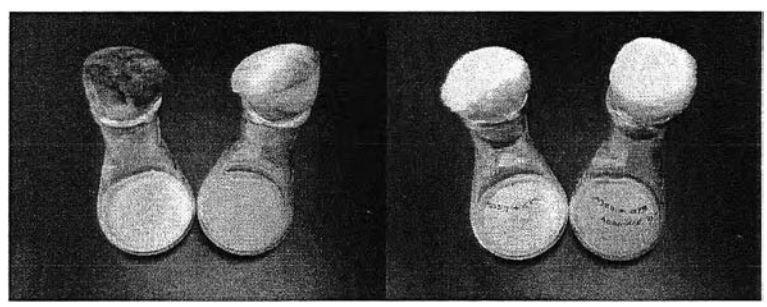


Figure 4.46 Aflatoxin B1 from growing together or separated between *A. flavus* NRRL 3357 with *A. flavus* A13 in mYES medium at 30 ^{O}C; (a) only 3357, (b) separated culture, and (c) growing together culture.

4.7.5 Effect of gallic acid on aflatoxins production and inhibition by co-inoculated toxigenic *A. flavus* NRRL 3357 with the same strains of atoxigenic

Atoxigenic and toxigenic strains of *A. flavus* produced conidia during the second days of incubation. *Aspergillus* in the medium containing gallic acid showed conidia color stronger than medium without gallic acid (Figure 4.47). Gallic acid (100 and 1,000 □l of 10 mM gallic acid/10 ml medium) did not help to inhibit aflatoxin production by toxigenic strains of *A. flavus* NRRL 3357 in mYES at 30^{O}C, in the dark. It might be affected of the low gallic acid concentration. However atoxigenic strains of *A. flavus* showed more aflatoxin inhibition in the medium containing gallic acid (Figure 4.48).



a b

Figure 4.47 *Aspergillus* colonization in mYES at 30^{O}C in the dark for 3 days; (a) *A. flavus* 3357 (left: without gallic acid, and right: with gallic acid), (b) co-inoculation (left: without gallic acid, and right: with gallic acid)

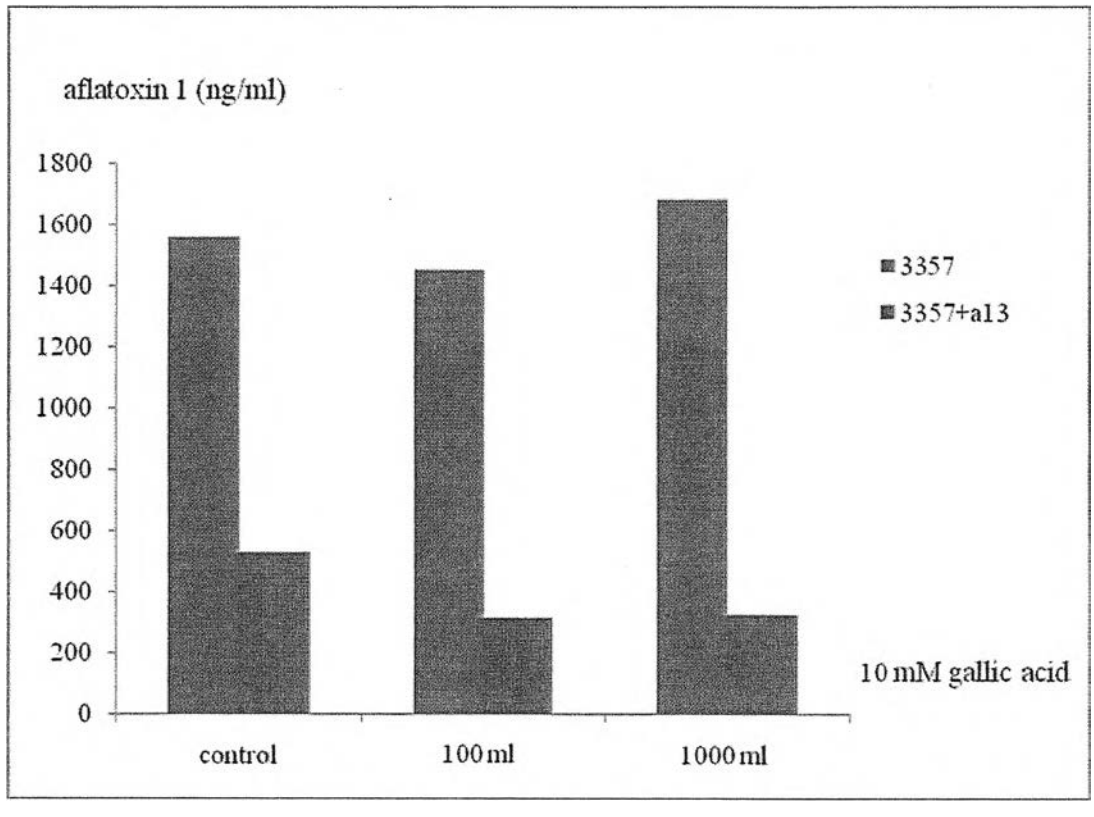


Figure 4.48 Aflatoxin B1 from *Aspergillus* culture in mYES medium with or without gallic acid.