CHAPTER V

DISCUSSION AND CONCLUSIONS

5.1 Collection of the soil samples from different corn fields of Thailand.

Thailand was ranked the 10th major corn producers in the world export in 2005 (Department of foreign trade of Thailand, www.dft.moc.go.th). Northern is geographically mountainous more than Northeastern and Central of Thailand and almost of corn cultivating areas is irrigated regions. (Thai Meteorological Department,www. tmd.go.th). Corns were cultivated in northern Thailand with more than 50% of the total area. Some provinces including Chaing Rai, Pa Yao, Tak, Pitsanulok, Petchaboon, and Nakotnrajchasima provinces are the major corn producing areas with more than 100,000 rai per province (6.25 rai = 1 hectare). Southern Thailand is not corn producing area. In 2005, corn producing area in Thailand (6.607 X 10^6 rai) became lower than the last year (7.040 X 10^6 rai) because corns were substitute cultivated to rubber trees, cassava, and sugar cane by farmer and some corn producing areas did not have water for corn cultivation. Soil samples from these regions were collected in September to November, 2005 because the highest yields of corns producing could be observed in these months (Office of agricultural economics of Thailand, www.oae.go.th). The average temperature in these months was 27 to 31 ^{O}C.

5.2 *Aspergillus* section *Flavi* isolation and identification

Aflatoxin producing *Aspergillus* species were observed in Thailand (Ehrlich et al., 2007). However, all sampling did not show major corn producing area. In this study, samples from different geographic areas of major corn fields in Thailand were

collected to determine for *Aspergillus* section *Flavi* distribution. AFPA medium was used because both *A. flavus*, and *A. parasiticus* produced yellow orange when viewed from the reversed side of the plate. Yellow orange color were found because the medium help improved color production on the reverse of the plate due to the optimal concentration of a more soluble iron salt and the addition of yeast extract (Pitt et al., 1983). *Aspergillus* section *Flavi* including *A. parasiticus*, *A.* oryzae, *A. flavus* and *A. nomius* showed range of color on reverse colony. *A. tamarii* showed pale to dark brown reverse colony. This results similarity as Ito et al (1999) and Samson and Frisvad (1991) which reported *A. tamarii* showed pale to dark brown reverse phase of colony on AFPA medium. Isolated strains of *A. parasiticus* and *A. nomius* form Thailand produced pale yellow reverse colony on AFPA. In this reported, *A. parasiticus* might be separated from *A. flavus* by reverse colony observation on AFPA. However, standard *A. parasiticus* SRRC 75 showed yellow orange reverse colony same as *A. flavus*. The different of reverse colony might be depended on strains of fungi. *A. flavus* also showed dark yellow reverse colony on AFPA (Pitt et al., 1983). In addition, orange color of reverse colony was observed in some strains of *Aspergillus* section *Circumdati* but they were separated from *A. flavus* by producing yellowish brown conidia. Mostly *Aspergillus* section *Flavi* (*A. flavus*, and *A. oryzae*) hardly produced conidia in AFPA at 30 ^{O}C for 7 days except *A. parasiticus*, *A. nomius*, *A. tamarii* and *A. pseudotamarii*. However, conidia were better developed when incubated longer (more than 2 weeks) at 30 ^{O}C in the dark. Damann et al (2004) showed AFPA medium did not have supported sporulation by *A. flavus*.

Conidia observation on Cz, isolated *A. tamarii* had brownish green color, dark brown in maturity and had colony diameter more than 33 mm (Ito et al., 2001), roughened thick wall conidia (Murakami, 1971 and Klich and Pitt, 1988). Horn

(1997) reported *A. tamarii* did not produce sclerotia on medium. However, all isolated strains of *A. tamarii* from Thailand showed large bullet shaped sclerotia formation in Cz medium when incubated in the dark more than 10 days. *A. parasiticus* produced green conidia, and had conidia size and shape same as *A. flavus* but did have dominantly rough walled conidia (Klich, 2002, and Pitt and Hocking, 1997). Raper and Fennell (1965) reported, sometimes smooth walled conidia of *A. parasiticus* were observed. *A. parasiticus* showed some morphological structures similarity as *A. sojae*. However *A. parasiticus* was separated from *A. sojae* by showed pink color reverse colony on Cz medium containing 0.05% anisaldehyde Murakami et al., 1982), and *A. sojae* did not produce aflatoxin B and G (Samson et al., 2006). *A. nomius* was the first discovered in soil samplings form Thaialnd, did not as soils from corn producing area, by Ehrlich et al (2006). *A. nomius* did have morphologically structures similarity as *A. parasiticus* but they dominantly showed large bullet shape sclerotia on 5%V8 medium. However, sometimes bullet shape scleorotia were produced by uncommon strains of *A. parasiticus* (Frisvad et al., 2006, Chang et al., 2001, Kurtman et al., 1987, and Barros et al., 2007). *A. flavus* produced green conidia, same as *A. parasiticus* but did have dominantly entire rough to smooth walled conidia (Klich, 2002). Sclerotia occasionally produced, black spherical shape on medium (Klich, 2002, Gao et al., 2007 and Pildain et al., 2004). Some strains of *A. flavus* hardly separated from the others in section *Flavi*. So, several methods are used together to distinguish *A. flavus* from the others including conidiophores, conidial morphology, and aflatoxin production (Klich 2002). It requires more time, and further observation for identification. However, *A. flavus* was found to be distinguished from each other strains in section *Flavi* by produced quantity columnar shapes of conidiophores but *A. parasiticus*, *A. nomius*, *A. oryze*, *A. tamarii*, and *A. pseudotamarii* produced radiated

shapes of conidiophores on V8 medium. This result might be used to the facile method for *A. flavus* identification.

5.3 Mode of action of miticide (Avid®) on morphological structures of *Aspergillus* section *Flavi*

Several fungi in *Aspergillus* section *Flavi* produced green to brownish green conidia on the media including PDA, Cz, and MEA. The important species in this group are *A. flavus, A. parasiticus, A. nomius*, and *A. pseudotamarii* since produce afaltoxins. Observation under the microscope *A. flavus* was separated from *A. parasiticus* because *A. flavus* produced smooth walled conidia but *A. parasiticus* usually produced rough walled conidia. Avid® is the miticide, a pesticide used to control mites commonly use in soil to the biological control of these arthropods on several economically important crops (Carruthers & Hural 1990, Van Der Geest *et al.* 2000). The primary structures of Avid® are Avermectin B1a and B1b. However, this miticide showed the side effect on the vegetative growth and conidia germination in the fungus *Beauveria bassiana*. In medium containing Avid®, *Beauveria bassiana* was reduced 23.8% germination, 22.7% vegetative growth and 40.8% conidia number respectively but did not have significant anti-fungal activity (Olieviera & Neves 2004). However, there is little information regarding the effects of Avid® on fungi. Spores of each strain of *Aspergillus* section *Flavi* including *A. flavus*, *A. parasiticus*, *A. tamarii*, and *A. nomius* were inoculated on Cz containing Avid®. The medium was incubated at 30 ^{O}C in the dark. All *Aspergillus* showed ability to grow similarity as in the medium without Avid®. Only all *A. flavus* produced synnemata in the Cz medium containing (20-1,000 µl/L) Avid® while the others species in section *Flavi* did not. However, synnemata formation was reduced in the Cz medium containing high level

Avid® concentration. Types of media and nitrogen sources had important factors for synnemata formation by *A. flavus*. Although the others media including AFPA, CMA, and PDA containing Avid® were used to determine synnemata formation. All *A. flavus* did not produce synnemata in all media. It might be affected by media types. Damann et al (2004) showed AFPA medium did not have supported sporulation by *A. flavus*. However, Cz medium containing Avid® and replacing sodium nitrate with either ammonium sulphate, ammonium tartrate, or peptone did not induce synnemata formation. Failure to form synnemata when others source of nitrogen were substituted for sodium nitrate suggest that nitrogen metabolism, in concert with Avid®, effects synnemata induction. It is difficult to opine what the specific effect is on *A. flavus* that results in synnematal development. In mYES medium, this miticide did not affect on aflatoxin production by *A. flavus, A. parasitiucs*, and *A. nomius*. However, the effect of Avid® might be used to distinguish *A. flavus* from the other species in section *Flavi* by synnmeata formation. Another strain of *Aspergillus* section *Flavi* including *A. caelatus* produced synnemata in Cz medium without Avid® (McAlpin 2001). However, it was distinguished from *A. flavus* because *A. caelatus* produced rough walled conidia, lacked yellow reverse colony on AFPA, and failed to grow at 42°C (Ito et al. 1999). McAlpin (2001) showed *A. flavus* NRRL 29254 (mutant from NRRL 3357) had ability to produce synnemata on Murashige-Skoog agar.In conclusion, this novel yet readily prepared culture medium permits differentiation of *A. flavus* from related species in section *Flavi*.

5.4 Vegetative Compatibility Groups test (VCGs) of *A. flavus*

Mutation in any one of several genes can produce the nit phenotypes. *Nit* mutant also showed sparsely colony on Cz agar, which contain nitrate as sole the

nitrogen source. *Nit* mutant can complement each the other when paired on the medium; the zone of complementation resembles the luxuriant growth of wild type mycelium. As a rule, complementation occurs between non-allelic nit mutants of a single isolate, and between non-allelic *nit* mutants of different isolates isogenic at a series of loci governing vegetative compatibility (Bayman and Cotty, 1991). *A. flavus* also did not have sexual stage. They cloud be grouped by Heterokaryon compatibity test or Vegetative Compatibility Groups test (VCGs) (Papa, 1986). However, heterokaryons of fungi only cloud be formed among complementary auxotroph (Couteaudier and Viaud, 1997 and Leslie, 1993). Rose Bengal-chlorate medium, which containing $NANO_3$ and potassium chlorate (Elias and Cotty, 1994), was selected and used to produce auxotroph or nitrate non utilizing strains (*nit*) mutant of fungi. Cells able to reduce nitrate to nitrite by nitrate reductase also reduce chlorate to chlorite (a fungal toxin) (Cove, 1976b, Bayman, and Cotty, 1991, Bayman, and Bennett, 1998, and Chuan-qing and Ming-guo, 2004). At the rule, wild type with nitrate reductase, also showed very restricted dense mycelium less than 1 cm from the point of inoculation. However, *Nit* mutant (cell unable to reduce nitrate) will be not killed by chlorite, and will outgrow (sparsely hyphae) wild type (Elias and Cotty, 1994). Starch medium was used to test VCG pairing because complementation was inhibited by reduced agar concentration medium. So the starch medium helped to resolve ambiguous reactions, simplify vegetative compatibility grouping, and expand the number of *A. flavus* VCGs that could be tracked with vegetative compatibility analyses (Cotty and Tylor, 2003). In this work, Two hundred and two Nitrate-nonutilizing mutants (*Nit*) were determined from 208 isolated *A. flavus* from different geographic areas of corn fields in Thailand. Twenty six strains of *A. flavus* did not show *Nit* mutant. One hundred forty two (142) were identified to be *niaD*, 26 were

identified to be *nirA* mutants, and 14 were identified to be *cnx* mutants respectively. This results similarity as Horn et al (1995 and 1996) that showed *nit* mutant of *A. flavus* was found to be *niaD*, *nirA*, and *cnx* mutants respectively. Complementation tests between different *Nit* mutant types (*niaD*, *nirA*, and *cnx*) were determined. Seven teen (17) of different VCGs were observed. However, the remaining 125 isolates did not pair. Horn and Greene (1995) showed *nit* mutant did not react easily, they need to be crossed as many as ten times to form a heterokaryon. This result indicated that fifty seven strains of *A. flavus* in Thailand were separated into seventeen groups (the same group showed same strain of *A. flavus*).

5.5 Metabolites products from *Aspergillus*

Citrate utilizing *Aspergillus* was observed by inoculated *Aspergillus* in citrate medium complementary with bromothymol blue as indicator. *Aspergillus* that ability to utilize citrate as sole carbon source turned the medium to blue color because it had affected of alkali reaction (Atlas and Snyder, 2006). *Aspergillus* did not have ability to produce aflatoxin including *A. oryzae, A. tamarii*, atoxigenic *A. falvus* including NRRL 21882 except *A. nomius* used the medium and changed medium to blue faster than toxigenic *Aspergillus* including *A. flavus* NRRL 3357, *A. parasiticus*, and *A. pseudotamarii*. However, aflatoxins were not induced by only citrate as the sole carbon source because aflatoxin formations had one or more of the enzymes which is regulated by the availability of specific readily-metabolizable carbon sources (Abdollahi and Buchanan, 1981).

All isolated strains of *A. tamarii*, *A. parasiticus*, *A. flavus*, and *A. nomius* produced kojic acid in Rice medium. Kojic acid was observed by appearance of red color because kojic acid had metabolite reaction with ferric chloride and had red color

in medium (Murakami, 1971). These results were commonly reported for *Aspergillus* section *Flavi* because mostly *Aspergillus* section *Flavi* did have ablility to produce kojic acid (Robert et al., 2006). *A. melleus* and *A. ochraceus* did not have ability to produce kojic acid on rice medium.

Aflaxins producing fungi were detected. Aflatoxins in each fungal culture were extracted by Chloroform because it did has common solution widely used to extracted mycotoxins from fungi and had high capacity to resolve mycotoxins including 85% for aflatoxin B1, 98% for o-methylsterigmatocystin (OMST), and 99% for cyclopiazonic acid (CPA) in medium (Leslie, 1993 and Horn et al., 1996). Extracts from *Aspergillus* section *Flavi* also did have colorless except all isolated *A. melleus/ochraceus* and little number of *A. flavus* showed yellowish orange extracted solution. Hessentine et al (1966) reported yellow pigments were produced during fungal growth and were especially noticeable in rice.

Preliminary study of aflatoxins productions by each isolates *Aspergillus* was detected. TLC analysis was used to determine aflatoxins contents in solution. Low aflatoxins content in extracted solutions hardly developed blue spot under UV light (Horn et al., 1996). It must be quantified by another method including HLPC.

All *A. tamarii A. ochracues*, and *A. melleus* did not produced aflatoxin B1 and B2 (Ito et al., 1999, 2001). *A. parasiticus* produced 2 groups of aflatoxins (B and G) (Koehler et al., 1975, Horn et al., 1996, and Samson, 2006), and had dominantly produced aflatoxin type B1 more than G1 in medium (Lin et al., 1980). *A. nomius* produced four groups of aflatoxin similarity as *A. parasiticus* but 7 of 12 strains of these isolates showed G1 contents more than B1 in mYES (Horn et al., 1998). Many reports showed *A. flavus* produced only aflatoxin B (Koehler et al., 1975, Cotty and Bhatnagar, 1999, Goto et al., 1996, Yu et al., 2002, Abyaneh et al, 2006). However,

isolated strains of *A. flavus* from Thailand showed widely range groups (B and G) and contents (atoxigenic to highly toxigenic). High level of corn producing areas did not as the specifically areas for aflatoxin producing strains of *A. flavus* because some aflatoxin producing strains of *A. flavus* were obtained from some areas without corn cultivation including Bangkok, and Khonkan provinces. The information of this report will be helpful for further study on biocontrol of toxigenic stains of *A. flavus* by atoxigenic strains of the same strain in corn fields in Thailand.

5.6 Inhibition of aflatoxins production from toxigenic strains *A. flavus* NRRL 3357 by the same strain of atoxigenic

A. flavus loosed activity to produce aflatoxins when co-inoculated with atoxigenic *A. flavus*. The highest synergic activity was detected with strains of atoxigenic *A. flavus* A13. 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. It is hard working to visually measure the relative degree of aflatoxin inhibition following. However, here are many reports showed relationship between toxigenic and atoxigenic fungi for aflatoxin inhibition including

1. Degradation

Degradation of aflatoxin by several fungi had been demonstrated, and even some strains of *A. flavus*, and *A. parasiticus* partially degrade aflatoxin after the biosynthesis had stop (Bhatnagar et al., 1991 and Bol, and Smith, 1989)

2. Competitive exclusion

The competitive exclusion principle suggests that when two species directly compete for the same limiting resource, one species can eliminate the other. In reality, the competitive exclusion principle suggests that no two organisms can exploit the

same resources at the same time and place. The competitive exclusion of a toxigenic strain of *A. flavus* by an atoxigenic strain was developed by Peter J Cotty at SRRC, ARS, USDA

Cotty and Bayman (1993) reported some atoxigenic *A. flavus* did not degrade aflatoxin. Diffusion factors from atoxigenic strain including *A. flavus* AF36 might be significantly involved to inhibit aflatoxin production by toxigenic strain. Diffusion factors did not show aflatoxin inhibitors because it helps stimulated aflatoxin production. For the aflatoxin biosynthesis pathway, he found some atoxigenic *A. flavus* did not produce aflatoxin because failure to synthesis the enzymes in aflatoxin partway. However some of them including *A. flavus* AF36, which produced many of enzymatic activity in aflatoxin synthesis, did not produce aflatoxin (Cotty, and Bhatnagar, 1994). Horn et al (2000), showed on evidenced that inhibition of aflatoxin production by competing strains of A. parasiticus was due to factors other than competition for nutrients. At the result, aflatoxin production from toxigenic strain of *A. flavus* might be reduced by competition for nutrients of the same strain of atoxigenic (Cotty, and Bayman, 1993 and Chang, and Hua, 2006).

3. Intraspecific competition

The intraspecific competition was developed by Wicklow et al (2003). Intraspecific competition can be stronger than competition between different species because genetically distinct strains of the same species are competing for the same resources.

3.1 inhibition of aflatoxin yield was greater than predicted by the model, indirect evidence that some biological phenomenon was associated with intraspecific competition between genotypes and not with initial levels of *A. flavus* strains inoculums.

3.2 conidial mixtures of both toxigenic and atoxigenic strains showed a large quantitative inhibition of aflatoxin yield. The results could suggest that atoxigenic strains are more effective in capturing nutrients than the aflatoxin producing strains.

3.3 conidial mixtures of different strains of toxigenic strains showed the result similarity as conidial mixtures of both toxigenic and atoxigenic strains (3.2).

3.4 Interference with aflatoxin production may also result, in part, from the failure of aflatoxin producing *A. flavus* genotypes to form a cooperative mycelia network when infecting the same resource.

4. Relationship between fungal development and metabolite

During the exponential, or log phase, many precursors of primary metabolism accumulate. The secondary metabolites may start appearing due to the presence of some nondividing cells. In *A. parasiticus*, this phase lasts up to 50 hours of growth after inoculation. It is probable that the accumulation of pyruvate during the exponential phase of the toxigenic strain coincides with the onset of aflatoxin production. During stationary phase atoxigenic strain showed higher tricarboxylic acid cycle activity more than toxigenic strain (Maggon et al., 1977).

Maggon et al (1977) reported atoxigenic strain showed higher tricarboxylic acid (TCA) cycle activity more than toxigenic strain. So atoxigenic strain more used of primary metabolism intermidate for TCA cycle, and did have no intermediate for aflatoxin production by toxigenic strain. The results could be suggested and cleared for many reported including Cotty and Bayman (1993) about "diffusion factors" from atoxigenic strain (it might be significantly involved to inhibit aflatoxin production by toxigenic strain) because diffusion factors may had more TCA cycle activity. In additional, agitation culture showed more activity to inhibit aflatoxin more than stationary culture. However, these investigators also determined that aflatoxin

production by toxigenic strain was stimulated by culture filtrates and mycelia extracts of atoxigenic strain because diffusion factors might loss the TCA activity after sterilization, and have more fungal metabolite intermediate. Wicklow and Horn (2007) and Martins et al (2008) supplicate reported, interaction between synergic or vegetative compatible toxigenic fungi showed more aflatoxin production than antagonist or vegetative incompatible toxigenic fungi.

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. Chang and Hua (2006) reported that 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.

Yu et al (2002) reported fungi are deficient in sporulation were unable to produce aflatoxins. In additional, agitation culture of *A. flavus* NRRL 3357 (low number of conidia) had aflatoxin yield nearby stationary condition of co-inoculation *A. flavus* NRRL 3357 with *A. flavus* A13.

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. These results did not support for Wicklow et al (2003) that showed inhibition of aflatoxin yield for

inoculum mixtures results from the failure of spore germlings to establish a cooperative network, Cotty & Bayman (1993) that reported about competitive exclusion of toxigenic by atoxigenic. However, these results supported for Cotty & Bayman (1993) reported that inhibition of aflatoxin production by atoxigenic involves diffusible factors, and Maggon et al (1977) reported that during stationary phase, atoxigenic showed higher TCA enzymatic activities than toxigenic, so less of intermidiates for the aflatoxin synthesis by toxigenic. Summarize, inhibition of aflatoxin production from toxigenic *A. flavus* by atoxigenic strain involves solution factors more than living cell of fungi (mycelial network, or touching).