CHAPTER 5

Selection of the Effective Fungal Pathogens to Two-spotted Spider Mites *Tetranychus urticae* Koch (Acari: Tetranychidae)

5.1 Introduction

Two-spotted spider mite, *T. urticae* (Koch) is considered to be the most polyphagous species of the Tetranychidae. These mites attack a wide variety of plants, including several crops (soybeans, dry beans, alfalfa, and corn, vegetables, ornamentals, and trees). This species is adapted to various environmental conditions and is distributed worldwide, causing loss of quality and yield or the death of the plants by sucking out the contents from the leaf cells (Granham, 1985; Vrie, 1985). Both immature and adults feed by extracting fluids from plant cells. All stages are typically found on leaf undersides. TSSM has a high rate of fecundity and a short developmental time. Mites overwinter as eggs and move to crops from permanent vegetation. Prolonged drought always raises the threat of TSSM (*T. urticae* (Koch)) outbreaks in soybeans and corn. In Manisota, *T. urticae* is often an economic pest of soybean.

Predators and parasites have been used to control this mite. Among them, *Phytoseiulus persimilis* (Athias-Henriot), *Stethorus punctillum* (Weise) and *Scolothrips sexmaculatus* (Perg.) are very much predacious on two-spotted spider mite. In Florida, *P. persimilis* (Athias-Henriot) is a commonly used predatory mite to control TSSM in strawberry fields. However, it is highly sensitive to acaricides and fungicides, and is unable to survive in temperate climates (Easterbrook, 1992;

Escudero and Ferragut, 2005). Natural pathogens of arthropods often play an important role in the regulation of insect and mite populations in agroecosystems (Ignoffo, 1985; Steinkraus, 2007). The acari-specific pathogens *Hirsutella thompsonii* and *Neozygites floridana* are gaining importance as natural regulators of eriophyid and tetranychid mite population. *B. bassiana* also has potential as a mycopesticide on the TSSM (Chandler *et al*., 2000).

Therefore, this experiment was related with the biological control of two-spotted spider mite, *T. urticae* Koch, in soybean by using microbial control agents from natural habitats and culture collection.

5.2 Objectives

5.2.1 To screen the virulence strains of insect pathogenic fungi in controlling *T. urticae* (Koch).

5.2.2 To examine the efficacy of selected strains infection to two-spotted spider mites by spraying in pot experiments.

5.3 Methodology

5.3.1 Culturing two-spotted spider mites

Field collection of *T. urticae* (Koch) were obtained from the bean leaf, and transferred to wild mulberry leaves placed on moistened cotton pads on top of sponges in plastic boxes in order to multiply. Water was added frequently to keep the cotton wet if it is necessary. Leaves were taken out of the rearing unit when started deteriorate, and placed on the top of new ones (Oliveira *et al*., 2009).

5.3.2 Fungal culture and preparing the conidial suspension

Six collected fungal isolates from natural habitat and two isolates obtained from BIOTEC, Bangkok were used to evaluate their pathogenicity assay two spotted spider mite, *Tetranchus urticae* Koch. Conidial suspensions were carried out as mentioned in Experiment (2). The viability of conidia was examined by spread-plating 100 µl of suspension on PDA plates.

5.3.3 Screening of virulence fungal isolates to *T. urticae*

The laboratory bioassays were done with young adult female of *T. urticae*. Multiple doses of conidial solution for six isolates (10^5 - 10^8 conidia ml^{-1}) and the fungal body suspension for two species of *Cordycpes* were used as *in vitro* screening assay. Ten milliliters of conidia solution was sprayed on both sides of soybean leaf (5.12×3.84 cm in diameter) by viral sprayer. Leaves were air dried in a laminar flow for 10-20 min., and placed on wet cotton wool in Petri dishes (Fig. 5.1). Ten young adult females were then placed onto the treated leaves and incubated in incubator at 25˚C and 70%RH (Bugeme *et al.*, 2009). Control treatment was done by using sterilized distilled water with 0.1% Tween-80. Mortality was recorded daily for 7 days. Dead mites showing external mycelium were re-isolated in PDA to confirm the infested organisms after washing with sterilized distilled water. Each experiment was repeated four times.

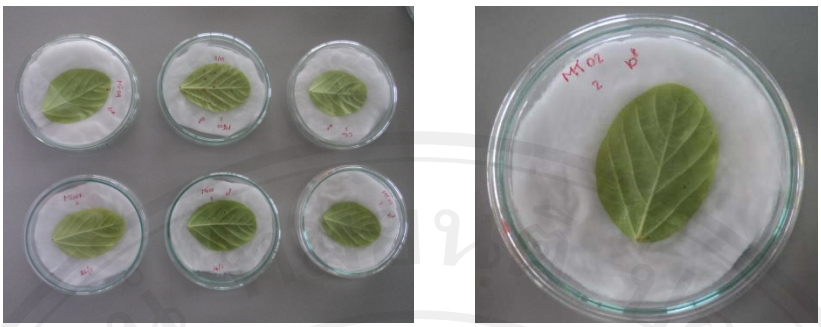

Figure 5.1 *In vitro* screening of fungal isolates infecting *T. urticae* with different conidial concentrations spraying on the surface of soybean leaf

5.3.4 Plant growing

Soybean *Glycine max* (L.) Merrill, variety CM60, was used in pot experiment. The seeds were surface-sterilized by soaking in 95% ethyl alcohol for 30 seconds and 5% sodium hypochloride solution for one minute and after that rinsed with the sterilized distilled water three times. The surface sterilized seeds were soaked in sterilized distilled water for 4-6 hours and then germinated in sterile Petri-dish (Fig. 5.2) containing moist tissue paper (Soe *et al*., 2010).

Three germinated seeds were grown in the pot (18×13×14 cm) containing autoclaved soil. The soil was autoclaved at 121º C for 1 hour before using in each pot trial. All pots were kept under the screen net. When first true leaf was observed, soybean plants in each pot were thinned out leaving one plant per pot.

5.3.5 Pot experiments

Pot experiments were carried out during April-May, 2011 with slight modification of Chandler *et al*., 2005. Selected virulence fungal isolates were subjected to test their infectivity against *T. urticae* in soybean. Each isolate was

applied to each pot with a randomized complete block design intended to one pot per treatment. Adult female *T. urticae* were released onto marked leaflet with 20 per leaflet using a fine piece of bamboo (toothpick). Seven days later, there was a further release of 20 mites onto the same marked leaflet. Each plant was enclosed with a cylinder made of translucent plastic film, and it was fasten with the soil to prevent the escape of mites and entering the foreign insects. The open top of the cylinder was closed with nylon cloth. A holes was bored about one third of the cylinder for additional ventilation, and covered with nylon cloth (Fig. 5.2). Suspensions of fungal conidia were sprayed to run-off onto the leaflet on which the mites were released including marked one by using viral sprayer. In order to investigate whether time of spraying will affect the control of mite population, two ways of sprays were performed: one time spraying 7 days after the second time released of mites; and conidia suspensions were applied twice, with a 7-day interval. Soybean plants were irrigated to maintain the humidity and stimulate a whole crop spray throughout the experimental period. Seven days after sprays, leaves were picked; five leaves per plant, to determine the numbers of motile *T. urticae* (nymphs/adults) and eggs per leaf, and dead mites.

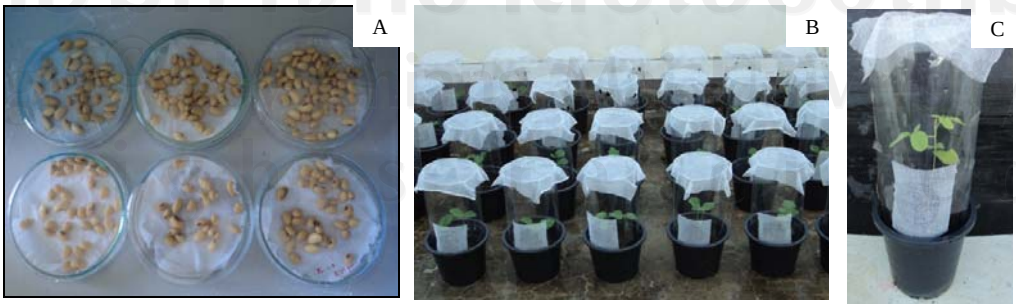

Figure 5.2 Pot experiment. A. soybean germination; B+C growing of soybean in pot

5.3.6 Statistical analysis

Log_{10} transformation was used for the number of motile *T. urticae* before analysis. Corrected percent mortality data (Abbott, 1925) for each bioassay were subjected to one-way ANOVA at the $P = 0.05$. Mean values were separated Tukey's post-hoc test $P<0.05$ (SPSS 16.0).

5.4 Results

5.4.1 Susceptibility of *T. urticae* to fungal isolates

Two-spotted spider mite, *T. urticae* was susceptible to all tested fungal isolates at all concentrations in screening bioassay. Though all tested isolates caused infection to *T. urticae*, the highest mortality was observed especially at highest conidial concentration. For all isolates, mite mortality started two days after inoculation at the concentration of 10^7 and 10^8 conidia ml^{-1}. Among them *M. flavoviride, P. lilacinus* and 2 species of *Beauveria* were highly pathogenic to *T. urticae* (Table 5.1). The adult mite mortality was 39.76% (F= 39.74; d.f= 6, 20; $P< 0.0001$), 55.94% (F= 23.54; d.f= 6, 20; $P< 0.0001$), 75.99% (F= 23.84; d.f= 6, 20; $P< 0.0001$) and 43.94% (F= 10.24; d.f= 6, 20; $P< 0.0001$) for *M. flavoviride, P.lilacinus* and two species of *Beauveria* isolates CMUCDMF03 and CMUCDMG03, respectively at the dose of 1×10^8 conidia ml^{-1}.

Mortality in control treatment was about 10-15%. However, the mortality from the control treatment was not observed until 5 days of post treatment. Significant lower mortality was recorded when *I. tenuipes, M. anisopliae, C. militaris* and *C. pseudomilitaris* were investigated for the pathogenic activity against two-spotted

spider mite. The number of mites was significantly reduced at 10^7 and 10^8 conidia ml^{-1} than other two concentrations (10^5 and 10^6 conidia ml^{-1}).

Table 5.1 Percent mortality of adult *Tetranychus urticae* Koch after 7 days exposure of six fungal isolates with various conidial concentrations

Species	Percent mortality *T. urticae*			
	1×10^5 (conidia ml^{-1})	1×10^6 (conidia ml^{-1})	1×10^7 (conidia ml^{-1})	1×10^8 (conidia ml^{-1})
M. flavoviride (CT01)	6.67b	7.14ab	30.77ab	40.00a
P. lilacinus (MT02)	6.67b	10.71b	50.00a	56.00a
I. tenuipes (MF02)	3.33b	3.57ab	7.69ab	20.00a
B. bassiana (MF03)	10.00c	14.29bc	53.85ab	76.00a
M. anisopliae (MF04)	3.33a	0.00a	11.54a	12.00a
B. bassiana (MG03)	10.00c	7.14bc	34.62ab	44.00a

Note: The results are mean of three replicates. Data with different letters within row indicates a significant difference at $P<0.001$ according to Tukey's HSD Post-hoc test within the same treatment.

The percent mortality and mean number of alive *T. urticae* infected by two species of *Cordyceps* are shown in Table 5.2. This less virulence of *C. militaris* and *C. pseudomilitaris* may be due to the application pattern of fungal solution in controlling insect. There was a significant difference in mortality of developmental stages of *T. urticae* in all tested fungal isolates (Fig. 5.3).

Table 5.2 Percent mortality and mean number of *Tetranychus urticae* Koch after infection with the mycelial suspension of two species of *Cordyceps* 7 days post inoculation

Species	% Mortality	Mean number of alive *T. urticae*		
		Adult	Nymph	Egg
C. pseudomilitaris BCC70	7.92	7.67	3.00	1.33
C. militaris BCC91	3.96	8.00	2.33	3.00

Beauveria bassiana MF03 showed minimum lethal concentration to kill 50% of *T. urticae* at 7 days after inoculation followed by *P. lilacinus*, *B. bassiana* MG03 and *M. flavoviride*. Moreover, time taken to kill 50% of mites was similar to those of lethal concentration (Table 5.3).

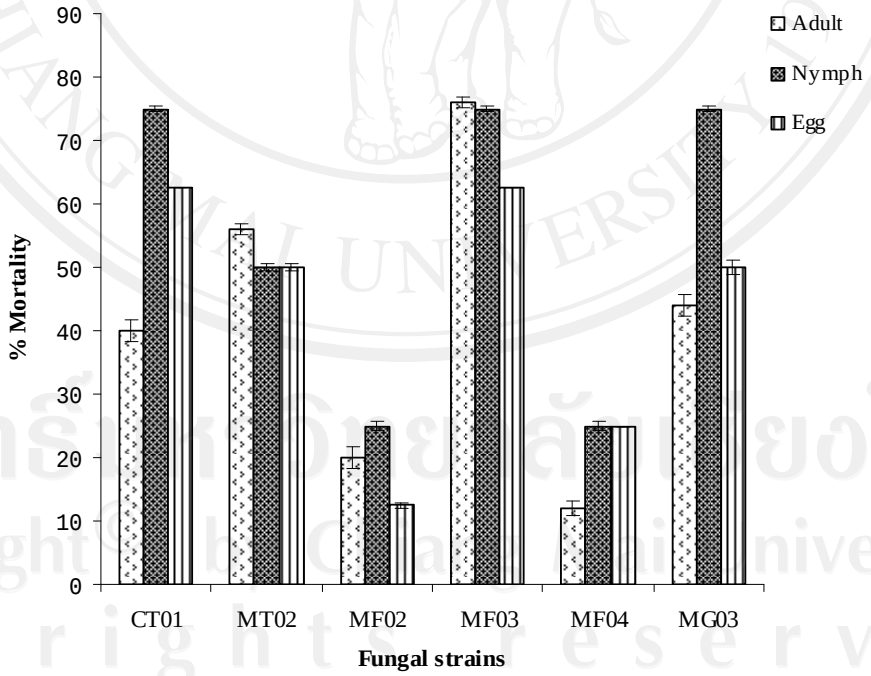

Figure 5.3 Percent mortality of the growth stages of *Tetranychus urticae* Koch infected by six insect pathogens 7 days after inoculation with 10^8 conidia ml^{-1}. Bars represent the standard deviation of three replicated means

Table 5.3 Lethal concentrations and lethal times of each tested isolates infected two-spotted spider mite, *Tetranychus urticae* (Koch) 7 days post inoculation

Isolates	LC_{50} log(conidia ml^{-1})	LC_{90} log(conidia ml^{-1})	LT_{50} (days)	LT_{90} (days)
M. flavoviride (CT01)	6.73×10^{10}	5.61×10^{19}	6.74	10.38
P. lilacinus (MT02)	2.38×10^{8}	6.82×10^{14}	5.96	8.99
I. tenuipes (MF02)	8.51×10^{24}	2.64×10^{47}	8.13	11.99
B. bassiana (MF03)	2.21×10^{7}	4.67×10^{12}	5.25	7.71
M. anisopliae (MF04)	5.34×10^{23}	2.67×10^{45}	9.08	13.56
B. bassiana (MG03)	9.58×10^{9}	1.42×10^{18}	6.68	9.95

The infection of fungal isolates to *T. urticae* was confirmed by the presence of fungal mycosis on the dead body, and immotile body when touched (Fig. 5.4).

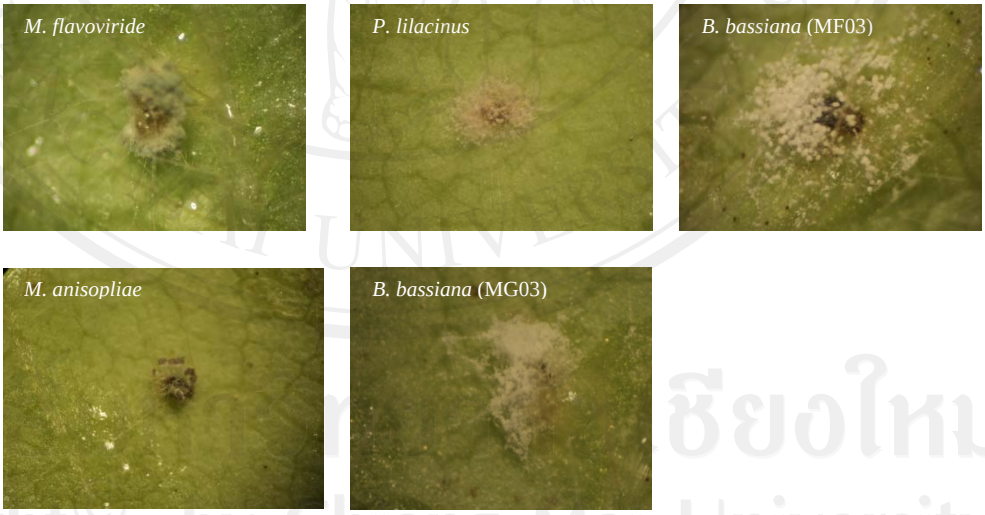

Figure 5.4 Fungal mycosis showed on the dead body of two-spotted spider mite (*Tetranychus urticae*, Koch)

The number of motile spider mites *T. urticae* examined 7 days after treated with conidial suspensions are showed in Table 5.4.

Table 5.4 Population of two-spotted spider mite *Tetranychus urticae* (Koch) 7 days after treated with different conidial concentrations

Isolates	Concentration (conidia ml^{-1})	Number of developmental stages *T. urticae*		
		Adult	Nymph	Egg
M. flavoviride (CT01)	1×10^5	9.33	1.67	1.67
P. lilacinus (MT02)		9.33	1.33	2.00
I. tenuipes (MF02)		9.67	2.00	2.33
B. bassiana (MF03)		9.00	1.33	2.00
M. anisopliae (MF04)		9.67	1.67	2.33
B. bassiana (MG03)		9.67	1.33	2.33
M. flavoviride (CT01)	1×10^6	8.67	1.67	2.00
P. lilacinus (MT02)		8.33	1.33	2.00
I. tenuipes (MF02)		9.00	2.00	2.33
B. bassiana (MF03)		8.00	1.33	1.33
M. anisopliae (MF04)		9.67	2.00	2.33
B. bassiana (MG03)		9.00	1.67	1.67
M. flavoviride (CT01)	1×10^7	6.00	0.67	1.67
P. lilacinus (MT02)		4.33	1.00	1.33
I. tenuipes (MF02)		9.00	1.67	3.33
B. bassiana (MF03)		4.00	1.00	1.00
M. anisopliae (MF04)		7.67	1.33	2.00
B. bassiana (MG03)		5.67	1.00	2.00
M. flavoviride (CT01)	1×10^8	5.00	0.33	1.00
P. lilacinus (MT02)		3.67	0.67	1.33
I. tenuipes (MF02)		6.67	1.00	2.33
B. bassiana (MF03)		2.00	0.33	1.00
M. anisopliae (MF04)		7.33	1.00	2.00
B. bassiana (MG03)		4.67	0.33	1.33

From the *in vitro* screening, *M. flavoviride*, *P. lilacinus* and 2 isolates of *B. bassiana* were found to be highly virulence to all developmental stages of *T. urticae* and selected for further experiments.

5.4.2 Control of *Tetranychus urticae* (Koch) in pot experiments

The selected fungal strains in pot experiments showed the greatest efficacy only in two-times sprayed trial. The population of mites in all treated pots by one-time spraying with fungal conidia suspensions was not differed significantly from those in control treatment (Fig. 5.5).

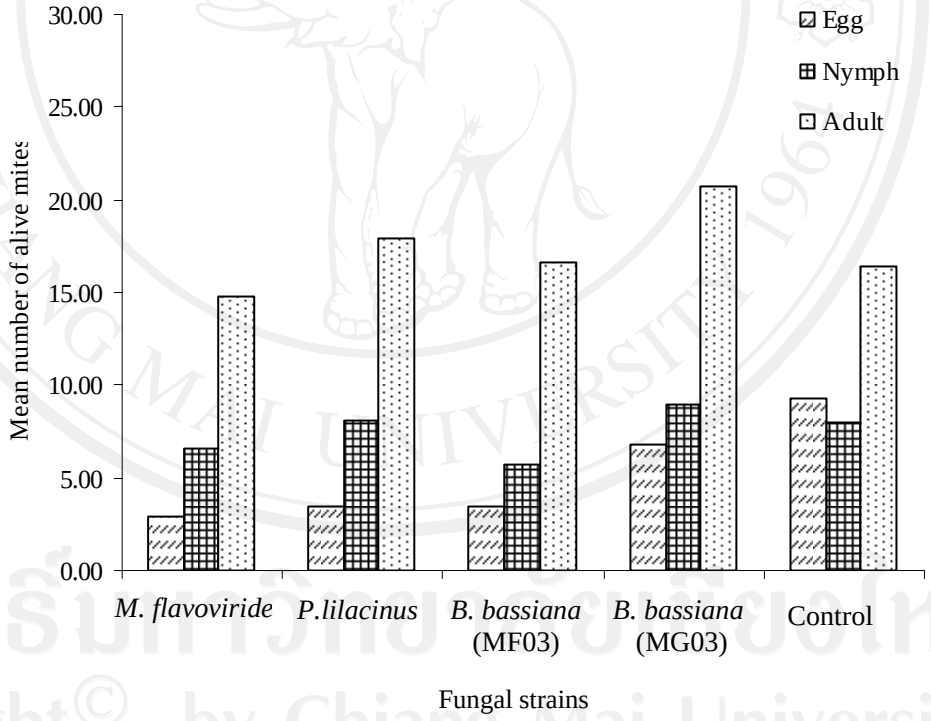

Figure 5.5 Comparison of the mean number of alive two-spotted spider mite between treated fungal conidial suspensions and untreated control after 7 days exposure of in one-time spray trial

All tested fungal isolates killed completely all developmental stages of two-spotted spider mites when fungal conidia were sprayed two times interval (Fig**.** 5.6). There was no significant difference between the infectivity of fungal isolates in killing *T. urticae*.

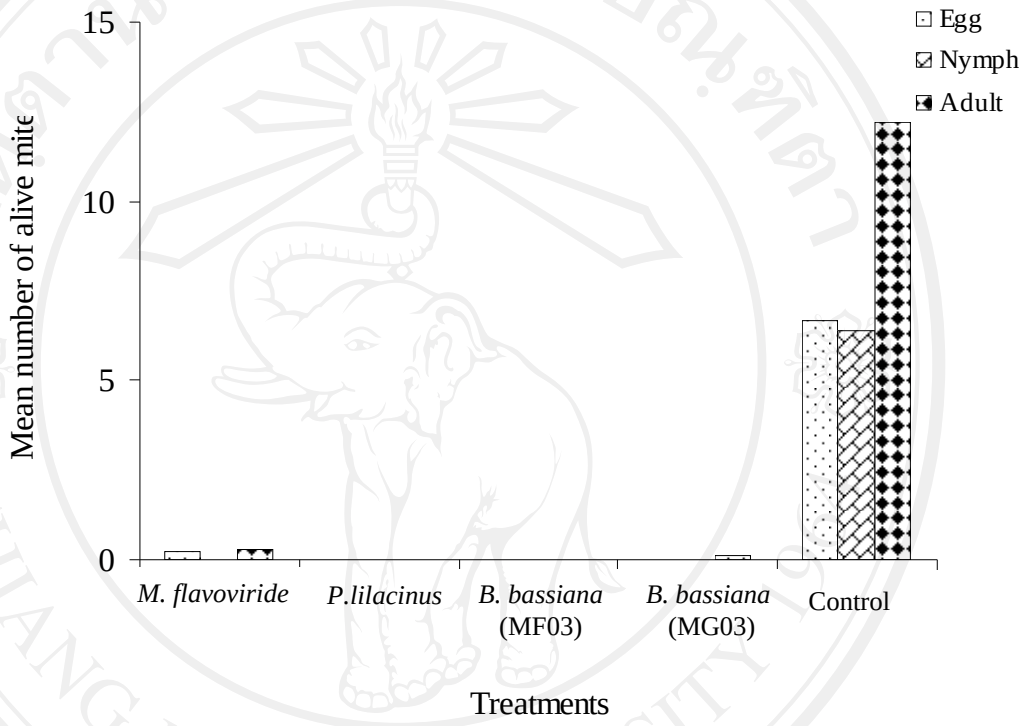

Figure 5.6 Mean number of alive *Tetranychus urticae* (Koch) in two-time spray trial 7 days after post inoculation

5.5 Discussion

The most promising microbial control agents of *T. urticae* (Koch) are entomopathogenic fungi, which invade their hosts by growing through the cuticle (Chandler *et al.,* 2005). *In vitro* and pot experiments were carried out in order to investigate the microbial control of two-spotted spider mite *T. urticae* by using native insect pathogenic fungal isolates in soybean. Furthermore, there was a comparison of

pathogenic activity two species of *Cordyceps* in controlling two-spotted spider mite in this experiment. Many studies have been proved that the efficacy of microbial control agents in controlling spider mites by fungal pathogens (Chandler *et al*., 2000, 2005; Van der Geest *et al*., 2000; Maniania *et al*., 2008). Some selected isolates of the insect pathogens have proven potential for spider mite control. Sprays of *B. bassiana* and *M. anisopliae* have resulted in significant control of spider mites on eggplants (Batta, 2003), citrus (Shi and Feng, 2006) and cotton (Shi *et al*., 2008a).

Though all the tested fungal strains were pathogenic to *T. urticae*, there were significant differences between isolates and concentrations in this work. Among all examined fungal strains, *M. flavoviride*, *P. lilacinus* and two isolates of *B. bassiana* (MF03 and MG03) caused higher mortality to mites than others. It is conquered with the finding of Fiedler and Sosnowska (2007) that *P. lilacinus* was able to reduce 60% of red spider mite population at temperature below 10˚C. In Poland, a domestic strain of this fungus species was investigated as a potential biological agent against root-knot nematodes in greenhouses (Sosnowska, 2003). Moreover, Atkins *et al.*, 2005 pointed out that it is only commercially available fungal formulation to control nematode pests in Europe, and commercial strain 251 is registered for sale in several countries.

However, rest of the fungal strains; *I. tenuipes*, *M. anisopliae*, *C. pseudomilitaris* BCC70 and *C. militaris* BCC91 were found to be less-pathogenic to mites.

There was a linear relationship between mite mortality and conidial concentrations. In the concentration of 10^5 and 10^6 conidia ml^{-1}, mites bred and the population was not significantly differing from control treatment. Based on LD_{50} and

LT_{50}, the highest pathogenicity was found in *B. bassiana* (MF03). In our finding, it was clearly found that infection of fungal isolates to mites were dose-dependent manner with highest mortality recorded with the highest concentration. However, there was no significant difference in the mortality of *T. urticae* between the killing concentration 10^7 and 10^8 conidia ml^{-1}.

A direct application bioassay enables a precise dose of conidia to be presented, which is thought to be important for reducing variability and enabling results to be repeated. However, the use of a direct application bioassay could have led to mistakes in isolate selection and the indirect bioassay would appear to be preferable (Goettel and Inglis, 1997; Butt and Goettel, 2000). Though there was accordance with the significant effect of direct application of collected fungal conidia against *T. urticae*, the insignificant effect was observed when two species of *Cordyceps* were investigated. It may be due to the application pattern of fungal suspension to hosts. Sato and Shimazu (2002) demonstrated that cuticle infection by fungal ascospore require dipping into the fungal solution. Moreover, these two species grown very slow in agar medium with luck of aerial spore formation, and it might be another reason of poor infection activity.

In our finding, it was recorded that adult mites were more susceptible than egg and nymph. Susceptibility of a host to a fungal pathogen is influenced by many factors including the host and pathogen properties as well as environmental factors (Inglis *et al*., 2001; Benz, 1987). Among the host factors, developmental stage has been reported to affect host susceptibility to entomopathogenic fungi (Ferron, 1985). There was an agreement that the immature stages of the spider mites *T. urticae* Koch and *Mononychellus tanajoa* (Bondar) (Acari, Tetranychidae) were reported to be less

susceptible to infection with *B. bassiana* and *Neozygites floridana* (Fisher) (Entomophthorales, Neozygitaceae), respectively, than the adults (Oduor 1995; Irigaray *et al*. 2003).

Wekesa *et al*. (2006) stated that the reduced fecundity of spider mites, *T. evansi* was significantly affected by the sublethal concentration of *B. bassiana* and *M. anisopliae*. Recently, Shi and Feng (2009) demonstrated that the infection of *B. bassiana, P. fumosoroseus* and *M. anisopliae* to *T. urticae* female and the fecundity. Here even the fecundity of spider mite was not studied, less population in the treated mites assumed that fungal infection affected on the fecundity during the experiment.

In pot experiments, the twice of the conidial suspension are necessary in order to complete control of spider mite. Our finding was in accordance with the results of Chandler *et al*. (2005). Chandler *et al*. (2000) stated that the insect pathogenic fungi (eg. *Beauveria* spp. and *Metarhizium* spp.) penetrated the body of the mite and then secrete toxic compounds. In our case, mortality of *T. urticae* was recorded by the presence of fungal mycosis on the dead body after washing with sterilized distilled water.

In the next experiments, we evaluated the suitable medium for inocula production of native fungal isolates by using cereal grains with cost-effective ways and determined the presence of extracellular toxins and enzymes that are important for the degradation of host cuticle during infection.