CHAPTER 6

Biomass Culture Preparation of Competent Nematophagous Fungi for Controlling Root-knot Nematodes

6.1 Introduction

The population dynamics of bio-agents is positively correlated with numbers of pests in soil. Soil organisms and their inter-specific interactions, soil abiotic factors including pH, soil moisture and porosity, physical factors (limiting factors), pollutants and formulation of the bio-agents have been shown to affect bio-control potential (Tedford *et al*., 1992; Jaffee, 1999; Jaffee & Zasoski, 2001; Limei *et al*., 2008). Therefore, effective field applications of soil biological control agents require sufficient numbers of micro-agent spores that are virulent, stable and in storable formulations that can establish and control the targeted pests. In addition, enhancement of environmental and nutritional conditions similar to the natural habitat of bio-agents should be concurrently carried out. Generally, fungi develop in three key stages: spore germination, vegetative growth (mycelial growth), and reproduction (spore production). Therefore, spores are a primary component of most fungal bio-pesticides and bio-fertilizers (Bartlett & Jaworski, 1980; Feng *et al.*, 1994; Jenkins *et al*., 1998; Sun *et al*., 2009).

The mycelial growth and sporulation of fungi are influenced by components of the medium and culture conditions. Culture media can be divided into synthetic, semi-synthetic and natural media which have specific advantageous and disadvantageous properties. In addition, media can be grouped into solid or liquid types. Mo *et al*.

(2007); Gao (2011) reported carbon and nitrogen sources, carbon-to-nitrogen ratio (C: N ratio) and initial pH value effected growth and sporulation in liquid culture. Occasionally, researchers reported spore production using a diphasic system; mycelium is produced in liquid culture by incubating in shake flasks or fermenters and is then transferred to a solid substrate containing rice, wheat, or rice bran for conidia production (Jenkins *et al.*, 1998). The majority of industrial fungal production systems involve two-stages in which fungal inocula of mycelium or hyphal bodies is produced in liquid culture and then transferred to a solid substrate for production of aerial conidia (Vimala, 1994). Most studies have been conducted using continuous cultivation on agar plates in the manner of the vegetative growth and sporulation occurring on the same agar plate (Rosenzweig, 1984; Coleman & Hodges, 1990; Jackson *et al.*, 1991; Li & Hololdom, 1995; Elson *et al.*, 1998; Liu & Chen, 2002, 2003; Sun *et al*., 2009). However, the nutritional requirements for fungal growth vary among different micro-agents including water, a source of carbon and energy, a nitrogen source, trace elements and growth factors.

The objectives of this chapter were as follows:

1. To select the optimal culture media for enhancing biomass and spore production of nematophagous fungi competent against root-knot nematodes.

2. To determine a practical method for stimulating sporulation of nematophagous fungi against root-knot nematodes.

6.2 Materials and methods

6.2.1 *In vitro* study of solid state fermentation to enhance biomass and spore production of nematophagous fungi

Solid state fermentation in polypropylene bags (Jerzy *et al*., 2009) sealed with a bottleneck and cotton was used for sporulation of fungi. Each isolate of eight nematophagous fungi, *Arthrobotrys oligospora* isolate DLO1-001, *Arthrobotrys oligospora* isolate MTI2-001, *Arthrobotrys conoides* isolate API3-001, *Arthrobotrys thaumasium* isolate JDI1-001, *Arthrobotrys thaumasium* isolate MPI1-003, *Arthrobotrys musiformis* isolate MSO1-001, *Pochonia* sp. isolate KJO1-003 and *Paecilomyces* sp. isolate WJI1-003 was transferred into three replicated bags containing boiled 125 g solid substrates which were sterilized by autoclaving at 15 pound/inch^2 at 121^{o}C for 25 minutes. Conidial production in seven solid substrate media consisting of corn, soybean, glutinous rice, rice, rice mixed V8 juice (15 ml/bag), corn mixed V8 juice (15 ml/bag) and millet were compared for selecting the optimal culture media of fungi.

The periphery of a 7-day-old pre-cultures on the potato carrot agar (PCA) medium of each fungus was cut with a sterilized cork borer and transferred in the center of substrate contained in polypropylene bag with a sterilized inoculation needle. One disc was transferred in each bag. After 21 days of incubation at 30±1^{o}C and 12 hour dark following 12 hour light regime for inducing growth and sporulation respectively, the colonized grain was mixed thoroughly in each bag and 1 g was added to 10 ml water containing 0.05% Tween 80 and centrifuged to release the spores. Concentration of conidia in each was counted using a haemocytometer; three replications of each treatment. The experiment was done by two factors factorial in

Completely Randomized Design (CRD). Factor A represented isolates of nematophagus fungi, A1 = *A. oligospora* isolate DLO1-001, A2 = *A. oligospora* isolate MTI2-001, A3 = *A. conoides* isolate API3-001, A4 = *A. thaumasium* isolate JDI1-001, A5 = *A. thaumasium* isolate MPI1-003, A6 = *A. musiformis* isolate MSO1-001, A7 = *Paecilomyces* sp. isolate WJI1-003, A8 = *Pochonia* sp. isolate KJO1-003 and Factor B represented solid substrate media where B1= corn, B2= soybean, B3= glutinous rice, B4= rice, B5= rice mixed V8 juice, B6= corn mixed V8 juice and B7= and millet.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) "Two factors factorial in Completely Randomized Design (CRD) by 8×7 factorial arrangement and Duncan's New Multiple Range Test (DMRT) for comparison of the means of each treatment.

6.2.2 *In vitro* study of liquid state fermentation to enhance biomass and spore production of nematophagous fungi

Eight liquid media formulations:

1) ½ adamek-lösung (3% maltzin, 0.5% yeast extract, 0.3% $CaCo_3$, 2% agar, 20% V8)
2) enteromophthoraceae media (4.8% malt extract agar, 0.3% $CaCo_3$, 20% V8)
3) gemüsesaft-malzextrakt media (2% yeast extract, 2% glucose, 1.5% corn steep liquor, 1.9% Tween 80 10%)

4) beef corn media (2% beef extract, 1.5% sucrose, 2% corn powder, 0.2%$CaCo_3$)
5) coconut media (3% coconut milk, 2% protein hydrolysis)
6) egg corn media (2% egg yolk, 1.5% sucrose, 1% corn powder)
7) V8 rice media (2% sucrose, 5% V8, 2% rice powder, 0.5% corn steep liquor)
8) V8 media (20% V8, 0.2% $CaCo_3$)

All media were tested to select the optimal liquid culture media on tree isolates of effective nematophagous fungi (*A. oligospora* isolate MTI2-001, *A. conoides* isolate API3-001 and *Paecilomyces* sp. isolate WJI1-003) which selected as a result of their sporulation ability in solid substrate media.

Conical flasks containing 100 ml liquid media sterilized by autoclaving at 15 pound/inch^2 at 121^{o}C for 15 minutes were transferred. The periphery of 7-day-old pre-cultures on the potato carrot agar (PCA) medium of each fungus was cut with a sterilized cork borer and transferred in flasks with a sterilized inoculation needle. One fungal agar plug was transferred in each flask with seven replications and incubated in a growth chamber at 30±1^{o}C together with vertical shaking at 180 rpm for 14 days. This experiment was designed by two factors factorial in Completely Randomized Design (CRD). Factor A represented isolates of nematophagus fungi, A1 = *A. oligospora* isolate MTI2-001, A2 = *A. conoides* isolate API3-001 and A3 = *Paecilomyces* sp. isolate WJI1-003. Factor B represented liquid culture media where B1 = ½ adamek-lösung, B2 = enteromophthoraceae media, B3 = gemüsesaft-

malzextrakt media, B4 = beef corn media, B5 = coconut media, B6 = egg corn media, B7 = V8 rice media and B8 = V8 media.

Data collection: The three replication measurements of fresh weight and dry weight of biomass and ten replications of sporulation of each fungus were compared. To quantify the spore and biomass production, cultures were agitated vigorously and filtered through Whatman NO.2 filter paper to obtain the fresh weight of fungal biomass. The fungal mat was oven-dried at 40-45ºC until a consistant weight was achieved (approximately 2 days) to obtain the dry mycelia weights. The filtrate was diluted in a known quality of water and the number of spore ml^{-1} was assessed with a haemacytometer (Ali *et al*., 2008).

Statistical analysis: The data were analyzed by analysis of variance (ANOVA) “Two factors factorial in Completely Randomized Design (CRD) by 3×8 factorial arrangement and Duncan's New Multiple Range Test (DMRT) for comparison of the means of each treatment.

6.3 Result

6.3.1 *In vitro* study of solid state fermentation to enhance biomass and spore production of nematophagous fungi

Analysis of variance by factorial treatment effects and interaction of eight fungi sporulating on seven solid substrate media at 21 days after inoculation indicated fungal isolate, solid media and their interaction were significantly different at P=0.01. The statistical analysis data is shown in Table 36 of appendix.

Almost all grains used in this study for solid substrate are available, low-priced or have similar nutritional components. Field corn grain substrate was the least expensive followed by millet and striped rice which are 14, 18 and 25 baht per kilogram of raw material, respectively (Table 82 of appendix). General record of nutrient content of major staple foods from Wikipedia (2012h) showed rice had the highest carbohydrate amount and their estimation of carbon (C): nitrogen (N) ratio was 10:1, 8:1 for field corn and millet grain (data was not show). Generally, fat, carbohydrate, fiber and sugar were grouped in carbon source while nitrogen source was included as protein.

In general, the solid substrates were completely covered with fungal hyphae and consistent sporulation after inoculation. Representative characteristics of solid state fermentation in polypropylene bag containing grains coated with fungal filament are displayed in Figures 6.1 and 6.2. *Paecilomyces* sp. isolate WJI1-003 produced the highest quantity of conidia in all solid substrates followed by *Arthrobotrys oligospora* isolate MTI2-001, but sporulation by the other isolates was greatly inhibited.

Corn grain showed significantly promoted the sporulation of nematophagous fungi followed by rice mixed V8 juice, corn mixed V8 juice and millet and were not

significantly different at P=0.01. Other grains including soybean, glutinous rice and rice did not support detectable levels of conidia (Table 6.1).

The result indicated that the highest conidial production of *Paecilomyces* sp. isolate WJI1-003 (3.42×10^7spore/ml), *A. oligospora* isolate MTI2-001(1.93×10^4 spore/ml) and *Arthrobotrys conoides* isolate API3-001 (0.40×10^4 spore/ml) was observed on corn grain medium. Rice mixed with V8 and corn mixed with V8 media were second and were not significantly different (P=0.01). Millet was third, but only supported sporulation of *Paecilomyces* sp. isolate WJI1-003 and *A. oligospora* isolate MTI2-001. Adding V8 to corn and rice grains caused lower sporulation of *A. oligospora* isolate MTI2-001 when contrasted with no amendments to those media. A similar result was seen with *Paecilomyces* sp. isolate WJI1-003, reproduction in corn was higher than in corn mixed with V8.

Table 6.1 Effect of seven solid substrate media on sporulation of nematophagous fungi at 21 days after incubation

Solid substrate media	Sporulation on media (×10^4 spore/ml)[1/]						
	Corn	**Soybean**	**Glutinous Rice**	**Rice**	**Rice mixed V8 juice**	**Corn mixed V8 juice**	**Millet**
DLO1-001 *A. oligospora*	0.00 h[2/]	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
MTI2-001 *A. oligospora*	1.93 g	0.00 h	0.00 h	1.53 g	0.40 h	0.20 h	0.33 h
API3-001 *A. conoides*	0.40 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
JDI1-001 *A. thaumasium*	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
MPI1-003 *A. thaumasium*	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
MSO1-001 *A. musiformis*	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
WJI1-003 *Paecilomyces* sp.	3420.00 a	231.00 e	209.33 f	476.00 d	2149.70 b	1971.70 bc	846.60 c
KJO1-003 *Pochonia* sp.	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h	0.00 h
CV %[3/]	1.54						

[1/] Mean of conidia of each fungus calculated from three replications.
[2/] Means followed by the same letter are not significantly different by DMRT at P= 0.01.
[3/] CV% = coefficient of variation 99%.

Figure 6.1 Seven solid substrates in polypropylene bags at 21 days after incubation.

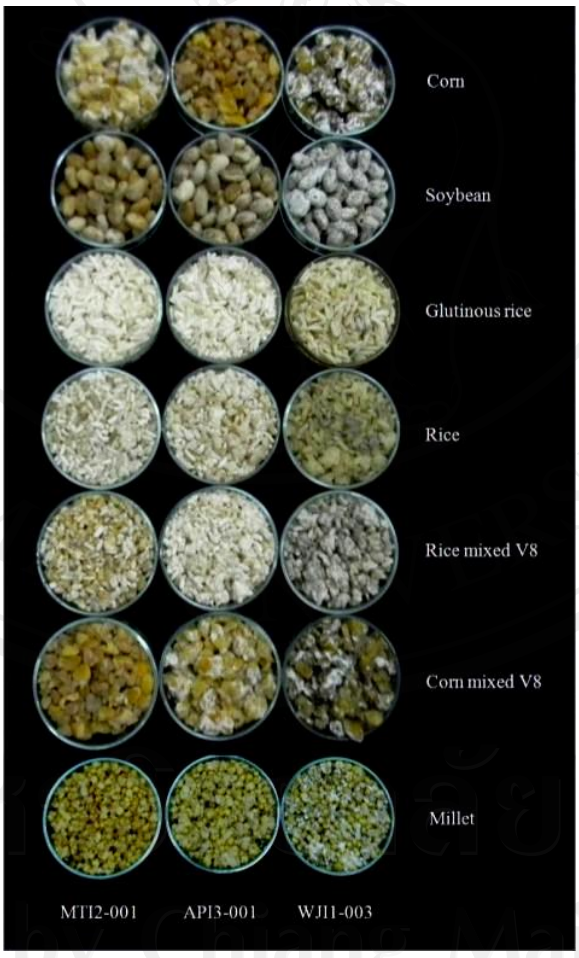

Figure 6.2 Fungal characterizations on seven solid substrates at 21 days after incubation.

6.3.2 *In vitro* study of liquid state fermentation to enhance biomass and spore production of nematophagous fungi

Because *Arthrobotrys oligospora* isolate MTI2-001, *Arthrobotrys conoides* isolate API3-001 and *Paecilomyces* sp. isolate WJI1-003 had growth and sporulation in solid state fermentation similar to the previous time and showed high percentage of damaged nematodes in previous results, they were selected for further observations in liquid state fermentation.

Analysis of variance table by factorial treatment effects and interaction of fungal fresh weight biomass, dry weight and conidial production on eight liquid media at 14 days after incubation indicated type of liquid media, isolate and their interaction influenced the fresh weight biomass, dry weight and conidial production of fungi. All of factors had highly significant differences at 99%. The statistical data are displayed in Tables 37-39 of appendix.

Enteromophthoraceae, ½ adamek-lösung and V8 had complete source of carbon, nitrogen, phosphorus, trace elements and growth factors. The cost of enteromophthoraceae medium, the best medium for sporulation after incubating the biomass of fungi, was 64.26 baht per liter while ½ adamek-lösung was 107.70 baht and V8 was 32.79 baht. The data showed beef corn was an expensive medium (406.80 baht per liter) followed by gemüsesaft-malzextrakt medium (119.86 baht per liter) whereas egg corn medium was low in price (2.70 baht per liter). The nutritional sources of eight liquid media and their cost were displayed in Table 83 of appendix.

In general, enteromophthoraceae, V8 rice and gemüsesaft-malzextrakt medium induced fresh weight biomass of nematophagous fungi and were significantly different at the highest probability. However, each fungus varied with respect to its

optimal medium. *Arthrobotrys oligospora* isolate MTI2-001 had highest fresh weight biomass by incubating in gemüsesaft-malzextrakt medium (3.63 g), enteromophthoraceae (3.43 g) and V8 media (3.48 g) which are not significantly different (P=0.01). *Arthrobotrys conoides* isolate API3-001 grew and produced the highest amount of hyphae in ½ adamek-lösung followed by beef corn and egg corn media; the fresh weights-biomass in test media was 4.35 g, 4.12 g and 3.88 g, respectively. *Paecilomyces* sp. isolate WJI1-003 had the significantly highest fresh weight biomass when incubated in enteromophthoraceae media and V8 rice. This trial showed that coconut media supported only poor hyphal production of *A. oligospora* isolate MTI2-001 and *A. conoides* isolate (Table 6.2).

The data showed V8 rice, enteromophthoraceae and ½ adamek-lösung medium were general medium to induce dry weight biomass. Different results were observed with media effect on dry weight biomass which was lower than fresh weight biomass. Beef corn medium was the best mediumry biomass production of *A. oligospora* isolate MTI2-001 followed by egg corn, enteromophthoraceae and gemüsesaft-malzextrakt medium, each of which produced a significantly lower dry biomass. *A. conoides* isolate API3-001 produced the highest level of dry biomass on egg corn (1.16 g), ½ adamek-lösung (1.06 g) and beef corn media (0.92 g), but they were significantly different. However, the effect of media on the fresh weight and dry weight biomass of WJI1-003 were very similar because it had a large dry weight biomass in V8 rice medium followed by enteromophthoraceae and egg corn media (Table 6.3).

A great number of sporulation of tested nematophagous fungi was observed on ½ adamek-lösung, beef corn and coconut media. The results on eight liquid media on

each fungus indicated that ½ adamek-lösung was a suitable medium for *A. conoides* isolate API3-001 and *P. lilacinus* isolate WJI1-003; moreover, beef corn medium also induced a high level of conidial production of *P. lilacinus* isolate WJI1-003. However, enteromophthoraceae and V8 rice media supported a low level of conidial production by *P. lilacinus* isolate WJI1-003 in contrast with fresh and dry weight biomass results. There was no sporulation by *A. oligospora* isolate MTI2-001 on any of the liquid media and *A. conoides* isolate API3-001 only produced spores on the ½ adamek-lösung medium (Table 6.4).

This study showed fungal sporulation in liquid media was poor so that incubation of each fungal biomass was continued at 30±1ºC to observe the density of sporulation following Sun *et al*, 2009 who stated the general stages of fungal development. Enteromophthoraceae medium could induce the highest density of all conidia. In addition, *A. oligospora* isolate MTI2-001 and *A. conoides* isolate API3-001 showed a high sporulation density on ½adamek-lösungmedium. For *Paecilomyces* sp. isolate WJI1-003, gemüsesaft-malzextrakt medium was the best for sporulation. All data of the density of fungal sporulation were presented in Table 6.5 and Figures 6.3-6.5.

Table 6.2 Effect of eight liquid media on fresh weight biomass of three nematophagous fungi at 14 days after incubation

Fungal isolates	Fresh weight biomass on media (g)[1/]							
	½ adamek-lösung	Beef corn	Coconut	Egg corn	Enteromoph-thoraceae	Gemüsesaft-malzextrakt	V8 rice	V8
MTI2-001 *A. oligospora*	2.66 i[2/]	3.08 fg	1.21 k	2.67 i	3.43 e	3.63 e	2.78 hi	3.48 e
API3-001 *A. conoides*	4.35 c	4.12 cd	1.22 k	3.99 d	3.55 e	3.55 e	2.96 f-h	3.16 f
WJI1-003 *Paecilomyces* sp.	2.86 g-i	1.69 j	3.45 e	1.85 j	5.81 a	2.91 f-i	5.44 b	3.13 f
CV %[3/]	3.77							

[1/] Mean of fresh weight biomass of each fungus calculated from three replications.
[2/] Means followed by the same letter are not significantly different by DMRT at P= 0.01.
[3/] CV% = coefficient of variation 99%.

Table 6.3 Effect of eight liquid media on dry weight biomass of three nematophagous fungi at 14 days after incubation

Fungal isolates	Dry weight biomass on various media (g)[1/]							
	½ adamek-lösung	Beef corn	Coconut	Egg corn	Enteromoph-thoraceae	Gemüsesaft-malzextrakt	V8 rice	V8
MTI2-001 *A. oligospora*	0.72 gh[2/]	0.83 f	0.49 l	0.79 fg	0.76 fg	0.76 fg	0.52 kl	0.62 ij
API3-001 *A. conoides*	1.06 d	0.92 e	0.53 kl	1.16 c	0.54 j-l	0.58 i-k	0.48 l	0.54 j-l
WJI1-003 *Paecilomyces* sp.	0.79 fg	0.56 j-l	0.93 e	0.52 kl	1.76 b	0.72 gh	2.54 a	0.65 hi
CV %[3/]	4.75							

[1/] Mean of dry weight biomass of each fungus calculated from three replications.
[2/] Means followed by the same letter are not significantly different by DMRT at P= 0.01.
[3/] CV% = coefficient of variation 99%.

Table 6.4 Effect of eight liquid media on sporulation of three nematophagous fungi at 14 days after incubation

Fungal isolates	Sporulation on various media (×10^4 spore/ml)[1/]							
	½ adamek-lösung	Beef corn	Coconut	Egg corn	Enteromoph-thoraceae	Gemüsesaft-malzextrakt	V8 rice	V8
MTI2-001 *A. oligospora*	0.00 f[2/]	0.00 f	0.00 f	0.00 f	0.00 f	0.00 f	0.02 f	0.00 f
API3-001 *A. conoides*	6.03 m	0.00 f	0.00 f	0.00 f	0.00 f	0.00 f	0.00 f	0.00 f
WJI1-003 *Paecilomyces* sp.	119.90 a	117.10 a	96.70 b	69.20 c	50.00 d	62.40 c	49.50 d	8.90 e
CV %[3/]	3.56							

[1/] Mean of conidia of each fungus calculated from ten replications.
[2/] Means followed by the same letter are not significantly different by DMRT at P= 0.01.
[3/] CV% = coefficient of variation 99%.

Table 6.5 Effect of eight liquid media on sporulation of three nematophagous fungi at 7 days after pouring to sterilized Petri dishes and incubation at 27±1°C

Liquid media	Sporulation density on media							
	½ adamek-lösung	Beef corn	Coconut	Egg corn	Enteromoph-thoraceae	Gemüsesaft-malzextrakt	V8 rice	V8
MTI2-001 *A. oligospora*	++++	+++	-	+	++++	++	++	+
API3-001 *A. conoides*	++++	+++	-	+	+++++	++	++	+
WJI1-003 *Paecilomyces* sp.	+	+	+++	++++	+++++	+++++	++++	++

+++++ = highest density.
++++ = high density.
+++ = moderate density.
++ = a few density.
+ = lowest density.
- = absent.

Figure 6.3 Characterizations of *Arthrobotrys oligospora* isolate MTI2-001 in eight liquid media at 14 days after incubation (Top). Incubating suspension of *A. oligospora* isolate MTI2-001 in eight liquid media after 7 days (Bottom-left) and conidial forming 20× (Bottom-right).

Figure 6.4 Characterizations of *Arthrobotrys conoides* isolate API3-001 in eight liquid media at 14 days after incubation (Top). Incubating suspension of *A. conoides* isolate API3-001 in eight liquid media after 7 days (Bottom-left) and conidial forming 20× (Bottom-right).

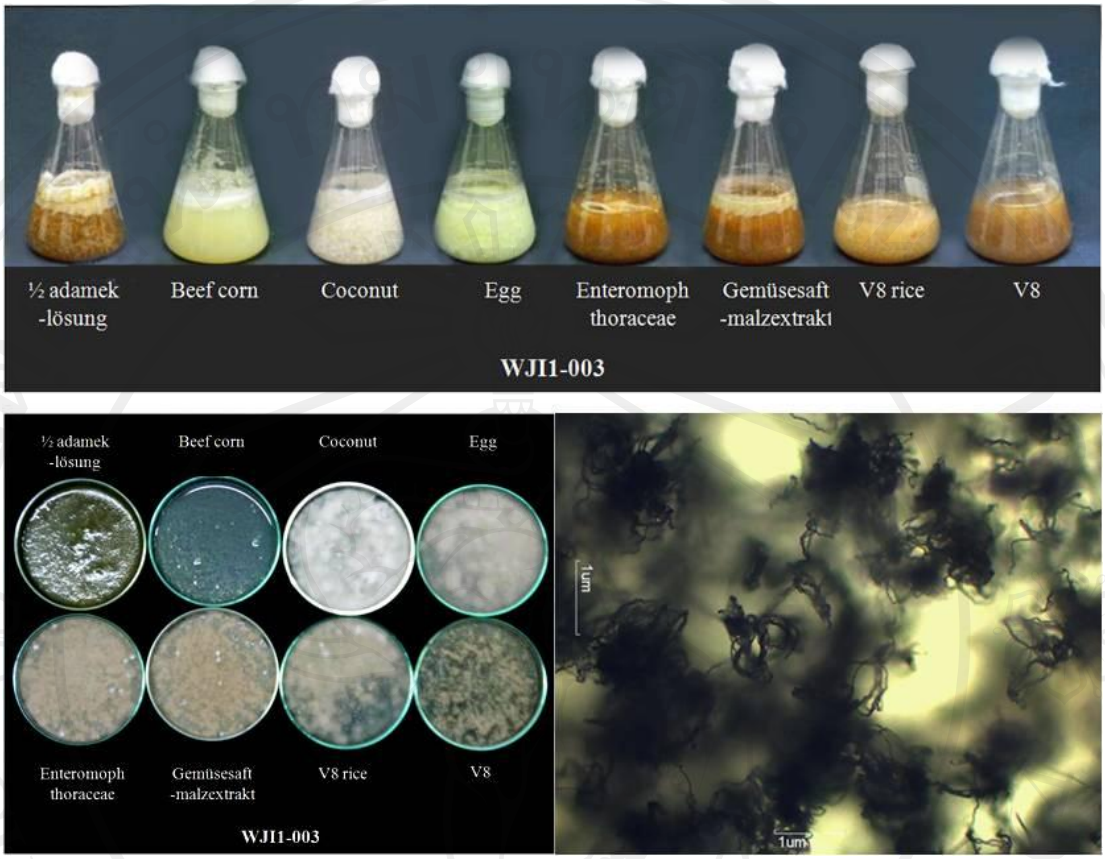

Figure 6.5 Characterizations of *Paecilomyces* sp. isolate WJI1-003 in eight liquid media at 14 days after incubation (Top). Incubating suspension of *Paecilomyces* sp. isolate WJI1-003 in eight liquid media after 7 days (Bottom-left) and conidial forming 20× (Bottom-right).

6.4 Discussion

University of Sydney (2004) reported that a typical pattern of fungal growth and sporulation (sexual or asexual spores) followed a response to the nutrients in the environment, modified by other environmental factors. The environmental factors affecting sporulation included low concentrations of nutrients, light, oxygen, optimal pH level and temperature. Response of fungi to nutrients was highly variable; some fungi were unable to sporulate under conditions of high nutrients. The current research confirmed previous reports especially with reference to the individual requirements for hyphal and conidial production of selected nematophagous fungi that directly involved optimal culture media. For example, *A. oligospora* isolate MTI2-001 grew well and produced the highest fresh weight biomass in liquid media gemüsesaft-malzextrakt, enteromophthoraceae and V8, but isolate API3-001 produced the highest quantities in ½ adamek-lösung, beef corn and egg medium, respectively. In addition, the statistical analysis of this research also showed a high factorial interaction on fungal growth and sporulation effect including fungal isolate, media (liquid medium and solid substrate).

For optimum sporulation, a medium is required where extensive mycelial growth is followed by spore production. A nutrient rich medium would not stimulate sporulation while a nutrient poor medium would not support extensive mycelial growth (Diogo *et al*., 2009). The source of isolated fungi and their original habitation should correlate with their performance on artificial media; the competent nematophagous fungi in this study which were isolated from highland areas with low organic debris and a low quantity of nutrients; accordingly should grow and sporulate well in a nutritionally impoverished medium such as the crushed corn substrate. On

the other hand, those fungi that settled in soil with higher levels of organic matter which have transformational nutrient cycles cause alternative states for their existence such as saprophytic or parasitic action and resting stage should produce maximal mycelial growth and sporulation in a rich medium such as rice mixed with V8.

In the cases of *A. oligospora* isolate DLO1-001 and isolate MTI2-001, the response of sporulation on various solid substrates showed different results. It may be caused from hereditary characteristic (sub-species) and topography of original isolate are as organic matter obtaining, soil pH and type of soil which correlated with specific requirements. These reasons were also confirmed by previous results showing in Chapter 5 on the effect of media and pH level to growth and sporulation of eight nematophagous fungi including colony characterizations. Many data indicated sporulation occurred upon nitrogen depletion in the presence of carbohydrate as Gao *et al*. (2007), Gao (2011) and Engelkes *et al*. (1997) reported. In this case, the result of estimating C:N ratio from component of amount nutrition (Wikipedia, 2012h) of tested raw grains showed 10:1 occurred with rice, 8:1 with corn and millet grain which correlated with University of Sydney (2004) that stated sporulation of some fungus was favored by a low C:N ratio.

Liquid media stimulated a larger number of fungal hyphae than conidia whereas solid substrate induced a huge number of conidia and a low biomass. These results may be caused by the concentration of the medium. Almost all liquid media in this research consisted of a nutrient-rich component. They consisted of carbon, nitrogen, phosphorus, trace elements and growth factor sources except coconut medium which mostly had saturated fat but a low amount of sugar or glucose (a good carbon source) and protein (nitrogen source). Moreover, the submersion of inoculated

fungal discs in a liquid medium and incubation together with shaking to oxygenate the culture was a good method for mycelia growth but not for sporulation. Therefore, the ability of liquid media including ½ adamek-lösung, beef corn, egg, gemüsesaft-malzextrakt, enteromophthoraceae, V8 rice and V8 excluding the coconut medium to stimulate production of fungal biomass was higher than solid substrates.

However, the cost of many liquid media was high so that researchers adapted the formulation especially to replace an expensive component. Leena *et al.* (2003) evaluated mass production of *Paecilomyces farinosus* and *Paecilomyces lilacinus* on sugarcane molasses, spent wash and other agro-industrial wastes. Sugarcane press mud at a lower cost supported significantly greater growth as well as spore production of both species compared to other agro-industrial by-products and wastes tested.

Manipulating fungal sporulation by nutritional and environmental conditions during the solid stage could shorten the period of solid cultivation and thus reduce the cost (Rosenzweig, 1984; Coleman & Hodges, 1990; Jackson *et al*., 1991; Li & Hololdom, 1995; Elson *et al.*, 1998; Liu & Chen, 2002, 2003; Sun *et al*., 2009). Therefore, commercial production of conidia is usually done on solid substrates that can consist of cereal grains, rice or other starch-based substrates (Diogo *et al*., 2009; Goettel & Roberts, 1992). For example, rice, corn bran, and wheat bran (low-cost substrates) were used as solid substrates to grow *Trichoderma harzianum*, *Trichoderma viride*, *Trichoderma koningii* and *Trichoderma polysporum*, soil fungi (Rosane *et al*., 2008). These researchers found that similarly to this research crushed corn grain substrate supported high conidial production followed by rice substrate. Much research has shown that spore production of each fungus was mostly affected

by specific different solid substrate and culture conditions. However, this research found crushed corn grain substrate could culture all of the fungi tested.

The continual incubation of fungal biomass after the mature vegetative stage under dry conditions, alternative light and low relative humidity caused increased conidial production; this process may be a good technique for inducing sporulation by liquid media or solid substrates.

6.5 Conclusion

Both experiments presented a correspondence of statistical analysis which indicated fungal isolate, media type and their interaction had an effect to growth and conidial production.

The results of *in vitro* solid state fermentation indicated the crushed corn grain substrate, which had low cost of raw material (14 baht per kilogram), was effective for inducing conidial production of all competent fungi against root-knot nematodes. In addition, *Arthrobotrys oligospora* isolate MTI2-001 may also be cultured in rice (25 baht per kilogram) and rice mixed with V8 (41 baht per kilogram), whereas *Paecilomyces* sp. isolate WJI1-003 produced a large amount of spore in rice mixed with V8 and crushed corn grain mixed with V8 media (30 baht per kilogram).

The culturing of fungi in liquid media was effective for hyphal biomass production of three competent nematophagous fungi against root-knot nematodes (*A. oligospora* isolate MTI2-001, *A. conoides* isolate API3-001 and *Paecilomyces* sp. isolate WJI1-003), but was generally a very poor substrate for sporulation when compared with culturing on a solid substrate. The data showed that each fungus, varied with respect to its optimal medium. Using gemüsesaft-malzextrakt,

enteromophthoraceae and V8 media were suitable resources for inducing fresh weight biomass of *A. oligospora* isolate MTI2-001. *Arthrobotrys conoides* isolate API3-001 produced an increasing quantity of hyphae in ½ adamek-lösung, beef corn and egg media, respectively while WJI1-003 responded best to enteromophthoraceae and V8 rice media. However, the media producing the greatest dry weight biomass were not all the same as those producing the greatest fresh weight biomass. In this case, egg, enteromophthoraceae and gemüsesaft-malzextrakt media were optimal for *A. oligospora* isolate MTI2-001. Egg, ½ adamek-lösung and beef corn media were optimal for *A. conoides* isolate API3-001 while *Paecilomyces* sp. isolate WJI1-003 produced a high amount of dry weight biomass in V8 rice medium, enteromophthoraceae and egg media. Because fungal sporulation in liquid media was poor incubation of each fungal biomass was continued at 30±1ºC to observe the density of sporulation. Enteromophthoraceae medium (64.26 baht per liter) could induce the highest density of all conidia. In addition, *A. oligospora* isolate MTI2-001 and *A. conoides* isolate API3-001 showed a high sporulation density on ½adamek-lösungmedium (107.70 baht per liter). For *Paecilomyces* sp. isolate WJI1-003, gemüsesaft-malzextrakt medium was the best for sporulation.

This research identified effective methods for inducing a large amount of conidia by competent nematophagous fungi including: (1) culture on certain solid substrates (corn or rice grain) in polypropylene bags and (2) biomass culture in a liquid medium (enteromophthoraceae medium) followed by biomass incubation at 30±1ºC for 7 days.