Identification, Drymass and Spore Count of Entomopathogenic *Metarhizium* Fungi from Infected Insects

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Abstract- In the present study, the isolate fungus from infected insects of mini-farm was studied of cultural, microscopic morphology, antifungal susceptibility test and the growth condition concerned with biomass and spore counting. The isolate fungus was close to *Metarhiziumspecies* that confirmed based on cultural and microscopic morphology. The biomass of fungi was studied that the highest dry weight was 0.0667 at 5 days. And the logarithmic number of spore per milliliter was increased till 9.6 at 5 days. The two growth curves have short time stationary phases and they were gradually increased before around 5 days. Ketoconazole was the highest antifungal effect on the isolate strain of *Metarhizium sp.*
Index Terms- *Keywords:* cultural, morphology, infected insect, growth curve, *Metarhizium species*, biomass.

I. Introduction

The kingdom Fungi contains a diverse range of taxa with an estimated 1.5 million species and about 700 species from 90 genera have been described as insect pathogens and plant pathogens (Roberts and Humber 1981).Fungal species that infect mites and insects are known as entomopathogens. Entomopathogenic fungi are common natural enemies and epizotics of agricultural and forest pests (Roberts and St. Leger 2004).Entomogenous fungi are being used worldwide for the control of many pests of agricultural importance. Entomopathogenic fungi comprise a heterogenous group of cover 100 genera with approximately 750 species, many of which offer greater potential in pest management. They belong to zygomycotina, ascomycotina, basidiomycotina and deuteromycotina. Compatibility of entomopathogenic fungi with pesticides used in commercial crop protection systems is critical, if these fungi are to be utilized for insect control. Biologial control with pathogenic fungi might provide long-lasting insect control without damage to the environment and human. Since many fungicides have broad spectra of activity, the suppression of entomopathogenic fungi by fungicides is of particular concern (WakumaBayissaHundessa, 2016). Entomopathogenic fungi infect host insects by means of asexually produced conidia, which germinate and penetrate the host exoskeleton under favourable environmental conditions. The insect cuticle is made of chitin and other protein components that provide protection and structure to the insect (Richard et al. 2010). Among the most studied insect pathogenic fungi species are Hypomycetes *Beauveria bassiana, Metarhizium anisopliae* and *Verticillium lecanii* that are biocontrol agents. Biocontrol is the use of living organisms to kill target organisms or to create a disease epidemic that spreads and kill target organisms. Entomopathogenic fungi are among the first organisms to be used as biocontrol agents against pests (Roberts and St. Leger 2004). The Metarhizium genus comprises mostly entamopathogenic fungi. *M.anisopliae*, is a generalist and is known to infect insects from more than seven orders, while *M.acridum* is a specialist (Kimberly Moon San Aw and SeowMun Hue, 2017). The classification of *Metarhizium*could be subdivided on morphological charactristics that decriminated between *M. anisopliae* and *M.flavoride* and *M.album* etc. It can be divided the *M. anisopliae* into short-spore isolates (ranging up to 8µ m in length), which she called variety anisopliae, and long-spore isolates (10 µm up to 14 µm). It is referred to as the "Green muscardine fungi" because of the green color of its spores and has been used to control a variety of insect pests such as termites, mosquitoes and other arthropods. *M. anisopliae*is soil borne fungus and infects over 200 hosts indicating a need to evaluate compatibility with non-targets, with pesticides and natural enemies. *M. anisopliae*and *Beauveria bassiana* are both compatible with many commonly used pesticides and are not toxic to human beings (Burges, 1981). Isoiates of *Metarhiziumspp* have widely differing host range and will become important aspect control intensifies. It can be infected rare on Cleopetera or Hemiptera. *M. anisopliae* was the first fungus worldwide to be mass produced and utilized for insect-pest control. Strains of *Metarhizium* differ in their host range, necessitation selection of the most virulent strain against a target insect (Zimmerman 1993). There is variation in germination triggers between different strains, which may be related to host species (St. Leger *etal*. 1994a). Penetration of the cuticle is thought to occur by a combination of enzymatic degradation and physical pressure. Evidence for enzymatic degradation includes the disappearance of the wax layer beneath appressoria of *M. anisopliae*on wireworm cuticle. *M. anisopliae*holds great potential as a biopesticide for managing aphids' species on okra and crucifer plants. Oil formulated *M. anisopliae*was effective in suppressing aphid populations, resulting in

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high mortality of adults of *B. brassicae, L. pseudobrassica*eand *A.*gossypii.It should reduce dependence on broad spectrum synthetic insecticides, promotebiodiversity conservation and environmental quality effect on non-target organisms and is compatible with other natural biological approaches necessary for IPM.
In this research work, these *metarhiziumspp* was isolated from infected insects from minifarm at Department of Biotechnology, Mandalay Technological University, Myanmar and grow on Potato-Dextrose Agar media. The growth rates of isolate were determined by measuring the dried mycelia and fungal spore counting. Moreover, it could be studied for drug sensivity test that can be affected on human or not. The aim of this research is to identify and select candidate isolates of entomopathogenic fungus *Metarhiziumspp* as a part of searching for alternatives to environmentally determined chemical insecticides and to assess the interaction between selected fungal isolate and coccinellid aphid predators.

2.MATERIALS AND METHODS

2.1. Collection of Sample

Samples collected from infected insect that got from minifarm at Department of Biotechnology, Mandalay Technological University,Patheingyi Division and Mandalay, Myanmar.

2.2. Growth Conditions

The isolated strain of inoculatewas inoculated in PDA media. The plates were then incubated at 28˚C for 7 days. Slide preparations of mature spore 7-12d cultures were mounted for detailed examination of condial morphology and size.

2.3. Generation of Growth Curves for *Metarhiziumsp*

2.3.1. Cultivation of *Metarhiziumsp*

The fungal colonies were inoculated in PDA media. The broth culture had been cultivated in water bath shaker at 168±2 rpm and 28˚C. Culture broth 10ml of isolate at time intervals were pumped up with sterilized pipette. The inoculum was periodically tested at every 24 hours for various growth parameters as provided below.

2.3.2. Determination of Mycelia Dry Weight

The amount of mycelia dry weight in 10ml of freely suspended cultures at time intervals was determined by filtering mycelia through tare filter paper (Whatman No.1) , the mycelia pellet was repeatedly washed with distilledwater and dried at constant weight at 100˚C for 2 hours. The dried mycelia on each filtered paper was weighed and recorded to determine the growth rate of *Metarhizium sp.*
Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)

2.4. Spore Counting Method for *Metarhiziumsp*

2.4.1. Media Preparation

PDA media for *Metarhiziumsp* were accurately weighed, dissolved in distilled water, heated with magnetic stirrer and sterilized at 130˚C for 15 minutes. After sterilization, these media were placed into Laminar Flow Cabinet to warm and then mixed with ros Bengal.

2.4.2. Serial Dilution for Fungal Spore Count

The fungal spore population at time interval of incubation had been counted by serial dilution method. Dilution was started by mixing one millimeterof sample solution with nine millimeter of sterile normal saline (0.9% NaCL solution) in a sterile test tube to make a 10^{-1} dilution. Serial nine fold dilution was thoroughly made until $10^{-8}$and 10^{-9} dilution of suspension was obtained. After making the serial dilution, 1ml of diluted samples was dropped onto a plate and mixed with 15ml of above rose Bengal media. Drying was accomplished at room temperature. After drying, the plates were incubated at 28˚C and fungal colonies were counted after 5 days.

2.4.3. Spore Counting Technique

In the case of viable cell counts, the number of colonies formed was counted from a suitable partition. The result was expressed as colony forming cells per samples (CFU/ml). The number of viable cell population at time interval was recorded and $1x10^7$ no. of spore forming units per ml.

3.5. Antifungal Susceptibility Test

Antifungal susceptibility testing of the isolates was performed by three antifungal drugs Ketoconazole, Clotrimazole and Grisoefulvin. The tested two doses of every drugs are 50 and 100µg.
Antifungal susceptibility testing was performed by disk diffusion method using Mueller-Hinton Agar + 2% Glucose and 0.5µg/ml Methylene Blue Dye (GMB) Medium as per CLSI guidelines (C.L.S.I. document M44-A2, 2009.). The antifungal susceptibility of the isolates was interpreted with the diameter of clear zone.

3. RESULTS AND DISCUSSIONS

3.1. Cultural and MorphologicalCharacteristics of Metarhiziumsp

In cultural morphology, the isolate was incubated on PDA media. At first, the surface on mycelia is white, turning green, yellow-green, brown (often with green tones) to yellow when sporulation has taken place. The reverse was white or pale dried yellow. The colonies of isolate presented a white edge of variable thickness and sporulated, at least in the center.The texture showed circular colonies and thicker, cottony or milky mycelium. The mycelium texture was thin and stuck to the surface of the growth. The size of conidia of isolate was about 5-5.3µm length and 1.7-1.9µm width through dissecting microscope.It was difficult to visualize the arrangement and the structure of conidia for detailed microscopic view. The cultural, microscopic morphologies of spore and mycelium are shown in Fig 1,2 and 3.

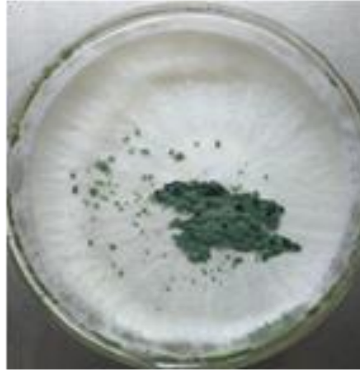

Fig 1: The colonies of Isolate of *Metarhiziumsp*on PDA

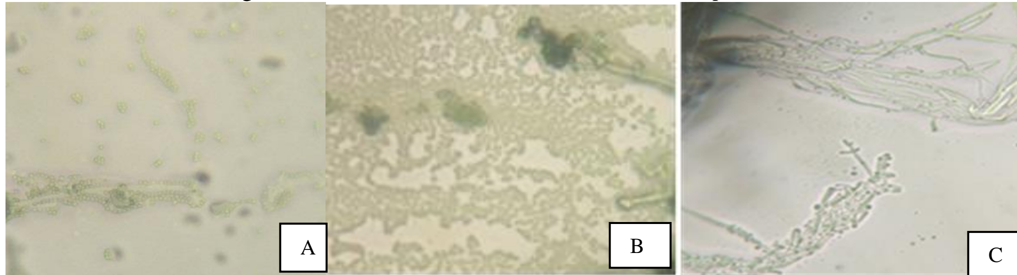

Fig 2: Microscopic Morphology of*Metarhiziumsp*(A and B - Spore of Isolate and (C- Mycelium of Isolate

3.2. Generation of Growth Curve for *Metarhiziumsp*

The isolate strain was cultivated in nutrient broth and filtered and dried. The dried mycelia at everyday were listed in Table 1 and the growth curve of the isolate was shown in Figure 4. In these result, the heaviest amount of dry weight of isolate was 66.7mg at 5 days. The growth curve of isolate was constructed with dry weight of isolate. It was normally increased but it had not almost included the stationary phase. It was observed that the dry weight was continuously decreased after 5 days and then finally reached the dead phase.

Fig 3:Growth curve of of Isolate *Metarhiziumsp*

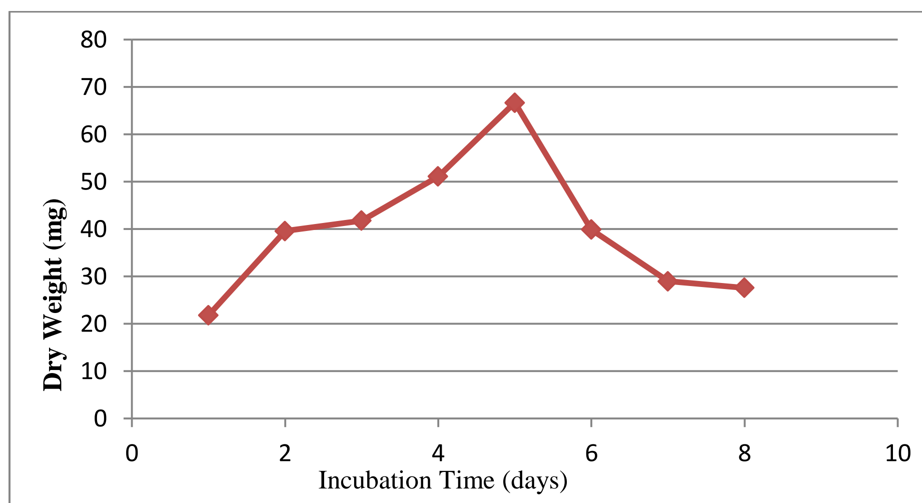

Table1: Dry Weight of Isolate *Metarhiziumsp* Strain in Nutrient Broth

Incubation Time(days)	Dry Weight(g)
1	21.8
2	39.6
3	41.8
4	51.1
5	66.7
6	39.9
7	29.0
8	27.6

3.3. Determination of Fungal Spore Count

The growth curve of the isolate of *Metarhiziumsp* was constructed with logarithmic number of viable spores versus incubation periods on Rose Bengal media and presented in Table 2 and Figure 5.The effect of Rose Bengal can prevent bacteria and restrict size of colonies. In this growth curve, the greatest logarithmic number of spore per millimeter was 9.6 at 5 days but it was not obviously different with 4 and 6 days. The spore count of growth curve was gradually increased and decreased. But the stationary phase was short time in also spore count growth curve.

Fig 4: Growth Pattern of Spore of Isolate *Metarhiziumsp*

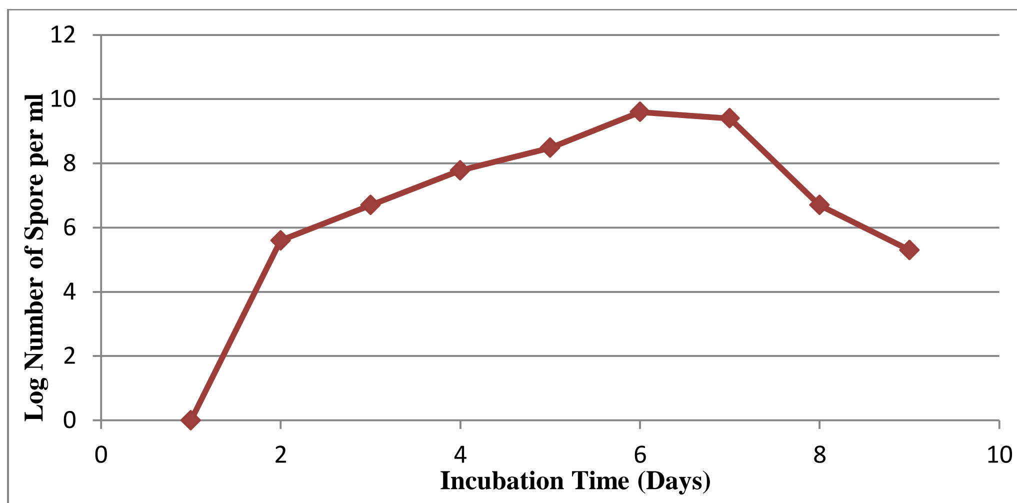

Table 2: The Viable Spore Count of The Isolate *Metarhizium.sp*

Incubation Time (Days)	Number of Spore Forming Units per ml	Log No. of Spore per ml
1	$4x10^5$	5.6
2	$5x10^6$	6.7
3	$6x10^7$	7.78
4	$3x10^8$	8.48
5	$4x10^8$	9.6
6	$3x10^9$	9.4
7	$5x10^6$	6.7
8	$2x10^5$	5.3

3.4. Studying of Antifungal Susceptibility Test

Metarhiziumsp was attacked by threes antifungal drugs; Ketoconazole, clotrimazole and griseofluvin. Aamong them, Ketocnazole was the most affected (2.9 and 3.5 mm) to isolate. The table showed that the 100µg had the high diameter of clear zone. It was observed that Ketokconazole was a little different with clotrimazole but Griseofluvin was the least strength of antifungal effect to isolate of *Metarhizium sp*.

Table 3: Clear Zone Diameter (mm) of Drug Susceptibility Test

Types of Antifungal	Clear zone of Diameter (mm)	
	1	2
Ketoconazole	2.9	3.5
Clotrimazole	2.5	2.8
Griseofulvin	1.9	2.3

*1 = 50 µg and 2=100µg.

4. CONCLUSION

Agriculture is the main industry in Myanmar and biopesticides are necessary in agriculture. In this research, isolates of the entomopathogenic fungi *Metarhiziumsp* was based on cultural, microscopic morphologies and studied of their biomass and number of spore. Native isolate strain was appropriate for our country. *Metarhiziumsp* has minimal effect on non-target organisms and is therefore compatible with other natural biological approaches necessary for IPM. Hence, promote conservation of arthropod biodiversity. The future work will be studied to assess the interaction between selected fungal isolate and the sucking insect, Aphids.

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