Pathogenicity of Local Isolates Entomophatogenic Fungi To *Diaphorina citri* Kuwayama Vector of CVPD Disease

Nadrawati

Faculty of Agriculture, University of Bengkulu, Indonesia

ABSTRACT

Diaphorina citri (Hemiptera: Psyllidae) is a major pest of citrus and CVPD disease vectors. Entomopathogenic fungi are one agent that can be used to control pests. The screening results have been found 3 Bengkulu local isolate potential for controlling *D. citri*. The purpose of this study was to determine the pathogenicity of entomopathogenic fungi isolates was indicated by the LC 50 and LC 80 on *D. citri*. The experimental design used was completely randomized design of 2 factors with 10 combinations, each repeated three times. The first factor is the type of entomopathogenic fungi and the second factor is the concentration of fungi conidia, that control (non-conidia), 10^{10} conidia/ml, 10^{9} conidia/ml, 10^{8} conidia/ml. The results showed that entomopathogenic fungi of *Metarrhizium anisopliae* and *Beauveria bassiana* were effective to *D. citri* nymph with 80-90% mortality on the eighth day after the application, the LC 50 and LC 80 *M. anisopliae* each others 0.1 x 10^{7}; 0.1 x 10^{10} conidia/ml, and *B. bassiana* 0.4 x 10^{7}; 0.8 x 10^{10} conidia/ml. Characterization of colony diameter and germination for all three isolates showed significantly different, the highest isolates of the colony diameter and germination found in *Verticillium lecanii*.

Key words: Pathogenicity, entomopathogenic fungi, *Diaphorina citri*

INTRODUCTION

Diaphorina citri is a major pest in citrus due to its role as a disease vector CVPD (Citrus Vein Phloem Degeneration). CVPD caused by Gram-negative bacteria *Candidatus liberibacter* transmitted asiaticus *D. citri* insect vectors and through the eye patch on citrus nurseries (Wirawan *et al*., 2000). Once the trees are infected, their production rapidly declines to render the infected trees unproductive in a few years. Adult *D. citri* are small (2.7 to 3.3mm long) with mottled brown wings. The adults are active, jumping/flying insects and can readily fly short distances when disturbed. Adults may be found resting or feeding on leaves with their heads at the leaf surface and their bodies held at a 45° angle from the leaf surface (Figure 2).

The psyllid is a sucking insect and thus inserts its mouthparts into plant tissue to feed. Adults feed on young stems and on leaves of all stages of development. Oviposition and development of immature *D. citri* are confined to young, tender flush. Nymphs feed on young leaves and stems, continuously secreting copious amounts of honeydew from the anus and a thread-like waxy substance from circumanal glands and black sooty mold develops on the honeydew deposited on lower leaves. The first instar nymphs were docile and moved only when disturbed or over-crowded (Tsai and Liu, 2000). *Metarrhizium spp, Beauveria bassiana, Nomurae rileyi, Hirsutella citiformis, Lecanicillium lecanii* fungi have been researched and can cause disease in insects pests. *Metarrhizium* spp isolated from S*podoptera exempta* can be lethal to 90% *Spodoptera litura* (Nadrawati, 2008); *B. bassiana* was able to kill whitefly *Bemisia tabaci* with mortality reaching 50% at 108 conidia/ml (Juniawan *et al*., 2013). *L. lecanii* with 10^{7} conidia/ml lethal 100% *B. tabaci* (Prayogo, 2012), and *H. citiformis* with conidia 10^{8} conidia/ml can be lethal to 50% of adult *D. citri* at 11.72 days (Dwiastuti and Kurniawati 2007). Local isolates, in general, will be better able to control local pests, and based on the need to obtain local isolates were able to sporulate in vivo and in vitro as indicated by the fungus on LC80 on *D. citri*. This is done in relation to the specific nature of entomopathogenic fungi on the host and its potential to be reproduced on a large scale and widespread after forming conidia on the target host. The purpose of this study was to determine the pathogenicity of entomopathogenic fungi isolate was indicated by the LC 50 and LC 80 on *D. citri*.

MATERIALS AND METHODS

The study was conducted in the laboratory and screen houses of Plant Protection, Faculty of Agriculture, University of Bengkulu on April to October 2013. All entomopathogenic fungi used in the laboratory assays were obtained from the entomopathogenic fungi the previous collection (Nadrawati *et al*., 2014). *B. bassiana* was initially isolated from a *Leptocorisa acuta* cadaver, found in Curup. Both *M. anisopliae* and *V. lecanii* were isolated from soil samples with the *Galleria melonella* bait method (Zimmerman, 1986).

Multiplication of *D. citri*

Both adult and nymph of *D. citri* were collected from calamasi citrus. The multiplication of *D. citri* was carried out on the plant Murraya paniculata in polyester cages (40 x 40 x 60) cm with an opening covered by mesh for ventilation. These were kept at controlled greenhouse condition until they were used in the bioassays.

Preparation of conidia suspension *M. anisopliae* and *B. bassiana*

Corn rice steamed 15 minutes, put in a glass bottle and then sterilized for 30 minutes. The fungus was inoculated into the corn rice medium and incubated for 30 days. Culture is stirred by adding 250 ml of sterile water and filtered using sterile nylon membrane to remove mycelium. Subsequently, the concentration of the conidia suspensions was calculated by using haemocytometer. The concentration of conidia was adjusted to $1x10^{10}$ conidia/ml, $1x10^{9}$ conidia/ml, $1x10^{8}$ conidia/ml, and control. This suspension was used as inoculums for further experiments.

Pathogenicity test of entomopathogenic fungi on *D. citri*

The tests conducted on 3-4 instar nymphs using a completely randomized design with two factors. The first factor is the type of isolates / species, and the second factor is the concentration of entomopathogenic fungi conidia. The concentration of treatment was 10^{10}, 10^{9}, and 10^{8} conidia / ml, with three replications. At each experimental unit as many as 30 nymphs already invested on Murraya plant in polybags and sprayed with the concentration of the treatment. Mortality is calculated every day for 8 days after spraying. Mortality data was processed by analysis of variance and if there is a significant difference between the treatment continued with DMRT 5%.

The percentage mortality of nymph is calculated using the formula: M = A / B x 100%

Description :

M = Percentage mortality

A = Number of dead insects infected with fungus

B = Number of insects tested

To determine the pathogenicity of entomopathogenic fungi with concentration and time lethal to 80% (LC80 and LT80) of each isolate, the data is processed by using probit analysis.

When a nymph died, the cadaver was transferred individually to a petri dish lined with a moistened sterile filter paper to ascertain the involvement of the entomopathogens to the death of nymph. The Petri dishes were kept at room temperature, in darkness, to monitor for external signs of fungi infection.

The ability tests to germination conidia

Observations of germination conidia are calculated by taking a drop of the suspension of each treatment concentration and placed on the sterile object glass and closed with a cover glass, then put into a petri dish that already contain moist filter paper and incubated at 240 C for 12-24 hours. Each treatment was repeated four times. Observations were carried out under a magnification microscope of 400 times, the percentage of germinated conidia were counted from 100 conidia. Conidia have germinated when the germ tubes have appeared longer than the diameter of conidia

The test of diameter colony

Each isolate of *M. anisopliae*, *B. bassiana* and *V. lecanii* was grown on medium PDA (Potato Dextrose Agar) in Petri dish. Medium incubated and colony diameter was measured at 15 days after inoculated fungi. Treatment was three replications. Diameter of colony and germination data were processed by analysis of variance and if there were a significant difference between the treatment continued with BNT 5%.

RESULTS AND DISCUSSION

The pathogenicity test of entomopathogenic fungi on *D. citri* nymp. The examined entomopathogenic fungi caused different levels of mortality to *D. citri*. The mean mortality of nymph differed significantly at different treatment. No mortality was recorded for nymph when treated with the aqueous solution (control treatment).

Table 1. The mean mortality of *D. citri* nymph after treated with various types and concentrations of entomopathogenic fungi

The entomophatogenic fungi	The mortality of D. citri nymph (%)					
M. anisopliae (10^{10} conidia/ml)	90.00	a				
M. anisopliae (10^{9} conidia/ml)	73.33		b			
M. anisopliae (10^{8} conidia/ml)	73.33		b			
B. bassiana (10^{10} conidia/ml)	80.00	a	b			
B. bassiana (10^{9} conidia/ml)	73.33		b			
B. bassiana (10^{8} conidia/ml)	63.33		b	c		
V. lecanii (10^{10} conidia/ml)	50.00			c		
V. lecanii (10^{9} conidia/ml)	46.67			c	d	
V. lecanii (10^{8} conidia/ml)	30.00					e
Control	0.00					f

Description: Digit number followed by the same small letter are not significantly different according DMRT 5%

Based on the test of entomopathogenic fungi in this study indicate the level of conidia showed a significant reaction against *D. citri* nymphs mortality. In general, there is a correlation between the level of conidia with mortality. The more the conidia treated was showed *D. citri* nymphs mortality are high, the fungi*M. anisopliae* and *B. bassiana* with a concentration of 10^{8} -10^{10} conidia / ml lethal > 70% nymph of *D . citri* (Table 2). In this case the more conidia were applied to the nymphs, allowing contact conidia with the body of the nymph in greater numbers. This situation gives a better chance for the conidia to germinate and penetrate the body of *D. citri* nymphs unless treated with *V. lecanii*. The toxin produced by M. anisoplie like destruxin A, B and E killed nymphs to stimulate or spur slump insect tissues thus losing the integrity of the membrane structure and eventually dehydrates the cell. And may also occur closing of spiracles which may cause death before the attack on hemocoel. While the *B. bassiana* containing toxins beauvericin, beauverolit, isorolit and oxalic acid. According to Soetopo and Indrayani (2007), that *B. bassiana* beauvericin produces toxins that can cause damage infected tissue as a whole so that it can lead to death in insects.

Besides mortality, ability to sporulate on the host to be very important for the purpose of transmission to other insect life, because hyphae or conidia that arise from the dead insects will spread with the help of wind or water. Three days after inoculation of conidia, nymph of *D. citri* were a progressive symptom of sluggishness (slow movement) when compared with control. These were no visible external fungal development on the nymph before death occurred. Mortality of nymph was present of black melanization spot on the cuticle. Melanization spots were observed around the thoracic and abdominal segment. *D. citri* that had been treated with conidia of *M. anisopliae*, white mycelial emergence was observed 3-5 days after the death of nymph. Sporulation occurred 3-6 days after mycelial emergence on *D. citri* nymph (Figure 1.)

M. anisopliae *M. anisopliae* *B. bassiana* *V. lecanii*

Figure 1. The examined entomopathogenic fungi sporulated on cadavers of *D. citri* nymph after several days of conidia inoculation

Characterization of Physiology Fungus On Media PDA: The ability to germinate and Diameter Colonies

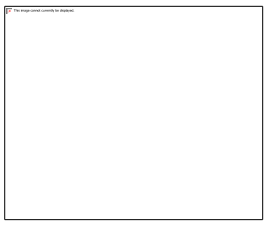
The ability of the fungus to grow and thrive on artificial media or hosts will be important if the fungus is propagated on a large scale for commercial purposes. But in general, entomopathogenic fungi with high pathogenicity can be recommended in future studies although there are differences in germination and colony diameter.

Table 2. Characterization physiology entomopathogenic fungi species: The diameter of the colony and germination

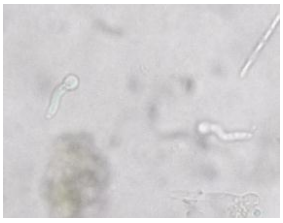
Isolates/Species	Source of inoculum	Diameter of colony (cm)	Germination (%)
M. anisopliae	Soil	5,1 a	24,00 a
B. bassiana	*Leptocorisa acuta*	4,5 b	42,25 b
V. lecanii	Soil	9,0 c	82,50 c

The numbers followed by the same small letter in the same column are not significant according to BNT 5%

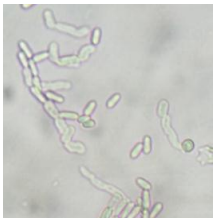
Diameter colonies of each species after 15 days ranging from 4.5 cm - 9.0 cm, and based on statistical analysis of the three species were significantly different. Germination of each species showed *V. lecanii* has a high ability to germinate compared with *M. anisoplie* and *B. bassiana* (Table 2). The ability of conidia to germinate is an important factor for successful penetration on the host, but in this study turned out to *V. lecanii* that have a high germination ability is not effective for controlling *D. citri*, while for *M. anisopliae* and *B. bassiana* showed lower of colony diameter with a thick growth, and this may be the cause of this fungus species are capable of *D. citri* lethal. Entomopathogenic germination tested each contained in Figure 2.



V. lecanii



B. bassiana



M. anisopliae

Figure 2. Some entomopathogenic fungi conidia which is germinating (10 x 40)

Lethal Concentration (LC).

This research showed the value of LC 50 and 80 *M. anisopliae* and *B. bassiana* were the lowest compared to *V. lecanii* isolate (Tabel 3). This indicates that *M. anisopliae* and *B. bassiana* have the highest degree of pathogenicity against *D. citri* nymphs.

Tabel 3. Lethal concentration (LC) of entomopathogenic fungus to *D. citri* nymph

Entomoathogenic fungi	LC	
	50%	80%
M. anisopliae	0.1×10^{7}	0.1×10^{10}
B. bassiana	$4{,}1 \times 10^{7}$	0.8×10^{10}
V. lecanii	4.0×10^{7}	-

Pathogenicity tests of entomopathogenic fungi local isolates indicated that *M. anisopliae* and *B. bassiana* was more pathogenic than *V. lecanii*. LC50 of the isolates were 0.1×10^{7} and LC 80 0.4-0.8 $\times 10^{10}$ conidia/ml respectively and LC80 *V. lecanii* was not detected. Several studies have pointed out entomopathogen fungus has the specific host. This result is in agreement with reports by Moorhouse *et al. (*1993) who tested vine weevil larval, *Otiorhynchus sulcatus* with two strains of *M. anisopliae* and found that var. majus were pathogenic but less virulent than the var. anisopliae. Kramm and West (1982) working on the termite, *Reticulitermes* sp. found that 100% mortality occurred within one day

after exposure to the whole culture of *M. anisopliae* and within five days in the case of *B. bassiana*. Many researchers suspected that the rapid kill by M. anisopliae on its host could be caused not only through a direct physical invasion of the hyphae but also possible due to some enzymatic mechanisms or toxic metabolites produced by the fungus (Jiang et al, 2002, 2003). This view is supported by Moorhouse (1993) who reported activity of the immune system of the host and the fungal response is likely to be another important factor determining pathogenicity. Robert (1982) suggested the secondary metabolites, such as destruxin, might depress the cellular immune reaction.

CONCLUSION

The bioassay results showed local isolates of *M. anisopliae* and *B. bassiana* have promised as microbial control agents against nymph of *D. citri* compared with *V. lecanii*. Both of these isolates can be deadly *D. citri*nymph 70 to 90%. LC50 and LC80 M. anisopliae respectively 0.1 x 10^7 and 0.1 x 10^{10} conidia/ml, and *B. bassiana*0.4 x 10^7 and 0.8 x 10^{10} conidia/ml. This study shows that *M. anisopliae* and *B. basssiana* are suitable to infect the nymph of *D. citri* in greenhouse condition and has the potential to be developed as a microbial agent for controlling the *D. citri*

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