UDC 632

# STUDY OF ENTOMOPHATOGENIC FUNGUS TO CONTROL VECTOR INSECT OF CITRUS TRISTEZA VIRUS ON CITRUS

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## ABSTRACT
Citrus Tristeza Virus (CTV) disease is a silent killer, which threatens to decrease productivity, quality and even death of citrus plants and the erosion of genetic resources. Spreading in the field very quickly by the intermediate insect vector pest, aphid *(Toxoptera citricida, T. Aurantii and A. Gosypii).* The microbes studied for potential biopesticide candidates are: *Beauveria bassiana and Hirsutella citriformis, and Metarhizium anisopliae* (Metch) Sorokin previously reported to control *Diaphorina citri* pests resulting effectiveness of > 25% and was able to suppress yield loss up to 10%. The objectives of the study examined the effectiveness of entomopathogen in controlling the pest of CTV vector, *Toxoptera citricida,* in the laboratory and screen house, to findout the physiological, biochemical and molecular physiology of entomopathogen. The results showed that the best entomopathogen suspension concentration was *B.bassiana* $10^6$ followed by *H. citriformis* $10^6$ and *M. anisopliae* $10^{6}$. Entomopatogen *B. bassiana and H. citriformis* effectively controled the CTV vector pest in the laboratory. In the semi-field experiments at the screen house, the most effective result was *H.citriformis* $10^6$ and the combination of *H.citriformis* $10^6$ + *B.bassiana* $10^6$, killing up to 50% and 100% on day 7th *H.citriformis* had the most physiological character, was able to develop optimally at a temperature of 20-40$^0$C and humidity between 60-80%. The biochemical character of the entomopathogenic fungus *B.bassiana* contained cellulase enzyme and phosphate solvent and IAA hormone, at most compared to the others. *H.citriformis* had not been found to contain enzymes and hormones. The molecular biochemical characterization of entomopathogenic fungi using FS1 and NS2 primers more clearly distinguished isolates and entomopathogenic species.

## KEY WORDS
Citrus, *B.bassiana, M. Anisopliae, H.citriformis*.

The ASEAN global market that begins in 2016, demands high-competitiveness agricultural products, high quantity and international standards of eco-labeling attributes, nutritional attributes and food safety attributes. Therefore, citrus products demanded that can compete in terms of price and quality associated with health that must meet the maximum residual limit (MRL) of pesticides and sanitary and phytosanitari measures (Gardjito 2014). Related to the citrus program which is proclaimed in one of Nawacita program of agriculture field, citrus area is now strengthened to 58 citrus centre. The government's efforts should be guarded with high vigilance against the threat of infectious diseases that could reduce the continuity of production and quality through environmentally friendly controls.

Citrus Tristeza Virus (CTV) is one of the infectious diseases of citrus known as silent killer, resulting in decreased productivity, quality and even death of citrus plants and the erosion of citrus genetic resources. CTV is idespread in citrus centers. CTV reported attacks more than 80% of commercial citrus crops with Japanese Citroen rootstock, and marcotting plant (Roesmiyanto et al., 1986). The incidence of CTV is known to have multiple strains, often in the field along with complex HLB disease (Dwiastuti 2011, Dwiastuti 1993a, Dwiastuti & Triwiratno 1994). Transmission of diseases through infected plant material and insect aphid vector *Toxoptera citricida* (Kirkaldy), T. *Aurantii* (Boyer de Fonscolombe), *A. Gosypii* (Glover) and *Myzus persicae* (Sulzer), CTV vector (Dwiastuti 1993b, Dwiastuti et al.).

Currently farmers' control is still based on chemical pesticides with an average of 28 times / year or 2 times per month.

The utilization of entomopathogenic fungi as bioinsecticidal natural ingredients have developed among others H. citriformis (Dwiastuti et al., 1994), *B. bassiana* and *M.anisopliae* (Nurhadi & Whittle, 1988; Dwiastuti, 2003). Its potential as a biological control for *Diaphorina citri* was naturally 30-82.9% in the imago stadia (Dwiastuti *et al*., 1994; Subandiyah *et al.,* 2000). *M. anisopliae* controls the nymph stadia (Raharjo *et al.,* 2000) and *B. Bassiana* in the imago and nymph stadia. In limited field in vivo studies with concentrations of $10^8$ conidia / ml were effective in controlling *D.citri* (Dwiastuti & Kurniawati, 2007). *H citriformis* products in the form of suspension and wetable powder can be kept at 4ºC (Widyaningsih & Dwiastuti 2007; Dwiastuti & Widyaningsih 2011). Another potential that has been investigated is that H.citriformis infection can be performed with two other entomopathogenic types and result in higher *D.citri* mortality than single infections alone (Dwiastuti *et al*., 2007). Applications in the field should be considered in order not to use together with bupimirit chemical fungicide but can still be used with profenofos insecticide (Dwiastuti & Iqbal, 2014).

Mechanisms of Fungus Attacks Common Entomopathogen is a pathogenic fungus enters the body of an insect directly into the body through the skin or an integument, then, the fungus multiplies itself through the formation of hyphae in the epidural tissue, epidermis, hemocoel, and other tissues. In the end all the tissues are filled by Mycelia fungus. The process of development of the fungus in the host body until the dead host goes about 7 days. After the host is killed, the fungus forms a primary and secondary conidia in appropriate weather conditions spreading the spores through wind, rain, water, etc. (Untung, 2006). The results of research Deciyanto (2009) entomopathogenic fungus Beauveria bassiana produces Beauvericin which leads to disruption of hemolimfa function and host nucleus of insect cells. As commonly B. Bassiana fungus infects host insects by physical contact, that is by attaching conidia to the integument. Conidial germination occurs within 1-2 days and grows its miscelin in the host's body. Infected insects will usually stop eating causing their immunity to decline, 3-5 days later die by marking the growth of konidia on the integument. While Novianty (2005) states that entomopathogenic fungus Metarhizium anisopliae is parasitic to insects and is saprophytic in soil or organic matter.This fungus penetrates into the body of the insect by contact with the skin between the segments of the body. The penetration mechanism begins by attaching conidia On the cuticle or insect's mouth. This conidia further germinate by forming the body of the sprouts. The precursor is first formed by penetrating the epicycule, further penetrating deeper tissues.

The technique of formulating a microbial product must have started from the microbial production process to the application on the target pest. Often the formulation of a microbial product is less optimally supported by a good production process, so the microbial active ingredient produced is less ideal for formulation because it is less stable during storage. According to Weinzier et al. (2005), the formulation has the following benefits: (1) stabilizing the organism of the agensia during the process of production, distribution, and storage, thereby extending its life (Batista Filho et al., 2001); (2) facilitating the handling of the product during the field application (3) protect the pathogens of active ingredients from environmental hazards while in the field, thus increasing their persistence, and (4) enhancing the activity of pathogens against target pests by increasing their activity, reproduction, contact and interaction with target organisms. According to Robert & Yendol (1971), the formulation is also one of two important parameters to achieve effective applications, as well as the proper use of application equipment.

The development of microbial product utilization of entomopathogenic fungus is highly dependent on product availability and formulation. Improving the stability and pathogenicity of a microbial product can not be done through the addition of chemicals at the time of application, but must be done before the microbes are formulated, for example by providing an appropriate environment for growth during the production process, appropriate use of production media, post-production handling, Such as optimal drying, appropriate storage time and temperature before formulation, and the addition of certain additives to maintain stability. Similarly, the effectiveness of entomopathogen in controlling pests in the field is

often decreased, because it is highly influenced by the environment, solar radiation, temperature and humidity, based on the above matters need to be done more research on application of entomopathogen in aphids singly or together with *D.citri*, With the final target of obtaining an active biopesticide formulation of rough secondary metabolites of entomopathogen so that it is more effective in controlling CVPD and CTV vector pests without fear of losing its effectiveness. The product formulation can be produced in a very simple way to obtain a mixed material that can be utilized in one or more vector pest targets.

The objectives of the study were to obtain the best entomopathogenic type and concentration in controlling the pests of Toxoptera citricida in the laboratory. Obtaining single or joint entomopathogen species that effectively control the semi-field, physiological, biochemical and molecular characters of entomopathogenic fungus H. citriformis, B. bassiana and M.anisopliae.

## MATERIAL AND METHODS OF RESEARCH

*Effectiveness Test 3 entomopathogenic fungi in several concentrations to control T. citricida in the laboratory.* The study was conducted in entomology laboratory. The entomopathogen isolates H. citriformis, B. bassiana and M.anisopliae used in this test were collections already stored in the laboratory and reococted in Aphis gossypii (insect passage) Fungus The suspension of entomopathogenic fungi, each isolate for treatment was obtained by adding Aquades as much as 10 ml poured into a petri dish containing H.citriformis, B bassiana and M. anisopliae cultures that were one to ten days old. Ose needle is gently slid on the surface of PDA media. After homogenous spores with aquades, the mushroom suspension is transferred into a sterile reaction tube. To obtain the desired concentration of the fungus the suspension is taken sufficiently with the dropper, then dripped on the hemocytometer and covered with the glass cover. Spore observation on the haemocytometer box (400x magnification).The calculation of the number of spores performed under a light microscope for ten days, using the equation of Gabriel & Riyanto, 1989 in Herlinda, 2006):

$$S = \frac{t.d}{n.0.25} x\ 10$$

Where: S = number of spore, t = Total number of spore in observed sample box, d = level dilution, n = number of sample boxes observed, 0,25 correction factor for the use of small-scale sample boxes in *haemocytometer.*

Equation of spore density:

$$C = \frac{t}{N} \text{ x } 10^6$$

Where: C = spore density per ml larutan, t = total number of spores in sample box observed; n = number of sample boxes observed; 0,25 correction factor for the use of small-scale sample boxes in *haemocytometer.*

*T.citricide* insects are prepared from collections from the field which are then reared to obtain a population of uniform age and quantity. Rearing is done in the home screen using citrus plant *Japanse citroen* (JC) that is sprouting. Experiments using Group Randomized Design (RAL) with entomopathogenic treatment of *H. citriformis, B. Bassiana, and M.anisopliae,* were tested at concentrations of $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ conidia / ml, respectively. The test was done on the seedling seedling of citrus by putting / investing pest of *T.citricida,* CTV vector then sprayed entomopathogen with concentration according to treatment. Each treatment was repeated 4 times. Each replication consisted of 20 insects of *T. citricida*. Observations made daily to the mortality rate of pests to determine the effectiveness of control. The observed data were analyzed by Various (ANOVA) and continued test was performed by Duncan Multiple Duncan Test (DMRT).

*Test the effectiveness of single and double entomopathogenic fungi to control T. citricida in screen house.* The study was conducted at ICISFRI gauze house using JC citrus plant in pots of uniform type and age. Testing using was Completely Randomized Design (RAL) with 7 treatments: H.citriformis, B.bassiana, M. anisopliae, H. Citriformis + B. Bassiana, H. Citriformis + M.anisopliae, B. Bassiana + M.anisopliae and Unattended Control, each with 4 replications. Each repetition consists of 1 orange shoots invested with approximately 20 pests. Observations were made daily until approximately 2 weeks after treatment, or until symptoms of infection in the test pest. Observations were made on the percentage of deaths or mortality of test pests that died of infected entomopathogen or uninfected. The entomopathogen tested is effective if the test pest mortality is at least 30%.

*Physiology Characterization of entomopathogenic fungi.* Physiological characterization was performed on entomopathogenic fungi, ie temperature and humidity. The temperature treatments tested were 20 ° C, 25°C, 30°C, and 35°C and humidity: 60%, 70%, 80%. Each treatment using RAL with 3 replications. The variables observed include colony diameter, clear zone. The data were analyzed using anova and continued by LSD test of 95% significance level.

*Biochemical characterization of entomopathogenic fungi.* An antagonistic fungus colon that has been incubated on the PDA medium for 24 hours at 25°C, was taken one ose by using an ose needle and grown on the GDP medium in the test tube and incubated for 7 days until the fungus appeared. After that, the fungus that has grown on the test tube is shaken by using a 130 rpm incubator shaker for 48 hours. After completion, each medium was centrifuged at 2000 g (3800 rpm) at 4°C for 20 min. Supernatants are taken with a certain concentration using a spectrometer tool. Gelatin is used as a substrate to test the enzyme activity of this protease by following Hankin & Anagnostakis method (1975) Carboxy methyl cellulose (CMC) is used as a substrate for cellulase enzyme activity test following Andro et al method. (1984). The enzyme activity of chitinase from bacteria was tested using a method described by Lingappa and Lockwood (1962).

*Molecular characterization of entomopathogenic fungi.* Characterization of molecular microbial antagonists was initiated by DNA extraction of each ingredient, then centrifuged and the obtained pellets were washed with 500 µL of 70% ethanol, dried, then resuspended with 100 µl TE buffer. The DNA suspension is stored at -20°C or can be directly used for further processing. The next process is DNA amplification of the fungus using the primer pairs of ITS1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') and the primer pair FS1 (5'-GCAGGTATGGCTTTTTGGAA-3') and NS2 (5'-GGCTGCTGGCACCAGACTTGC-3'). The PCR reaction (volume 25 µl) consisted of 1 µL DNA samples with concentrations of 25-50 ng µl-1; 18.8 µl of nuclease free water; 2.5 µl 10x PCR buffer (10 mM KCl, 20 mM Tris HCl pH 8.8, 10 mM (NH4) 2SO4, 2 mM MgCl2, and 0.1% Triton X-100) (Fermentas, USA); 0.5 µl dNTP 10 mM (Fermentas, USA); Each 1 µl of primary ITS1 and ITS4 with concentration of 10 µM; 0.2 µl enzyme Taq DNA polymerase recombinant 5U µl-1 (Dream Taq DNA Polymerase Fermentas, USA). Amplification was preceded by the initial denaturation for 5 min at 94°C, followed by 35 cycles through three steps including denaturation for 1 min at 94°C, annealing for 1 minute at 55°C, synthesis for 2 min at 72°C C, at the final stage plus 10 minutes at 72°C. The amplified DNA analysis was performed by electrophoresis using 1% agarose gel in Tris Boric EDTA (TBE 0.5x) buffer and visualization using UV light.

## RESULTS AND DISCUSSION

From the re-isolation of 3 entomopathogen isolates as a source of inoculum treatment which has been passed into inoculation of aphid, obtained pure isolate of each H. citriformis, dark gray color with rapid dust development colony structure, B. Bassiana colonies show growth of white fungus, older age Culture on the mycelium formed spores jamu wara greenish. M. Anisopliae colonies are attractive and regularly grown, greenish, at the beginning of a whitish growth and on the edges of growth identical to the bright white-colored culture so that it contrasts with the color of the colony in the middle. (Figure 1).

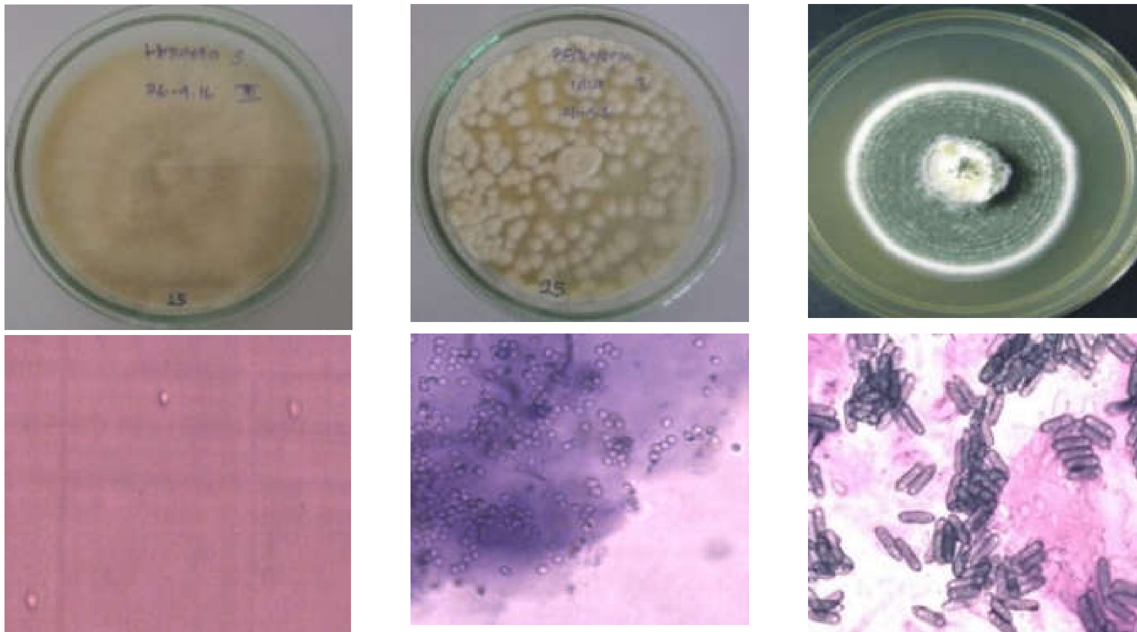

Figure 1 – a. Colonies of *H. citriformis, b B. Bassiana, c. M. anisopliae* at 6 days after isolation, d. Spores of *H.citriformis, e. B. Bassiana, f. M anisopliae*

Figure 2 shows the spores of each entomopathogen having different characteristics, the relatively small density of *H. citriformis* spores compared to the other, glossy shiny hialin (figure 1a, d). The spores observed are quite large, the shape tends to be round with dark colors (figure 1c, e) and smaller size of *H.citriformis*. Spores *M. anisopliae* oval shape, larger size. Hyaline color (figure 1 c,f).

*Effectiveness Test 3 entomopathogenic fungi in several concentrations to control T. citricida in the laboratory*. The results showed that in general the three entomopathogenes tested against aphid pests in the laboratory separately with various concentrations were able to control the aphid population. The ability to control aphids can be seen from first day to 13th day (Table 1). The three types of entomopathogen test show that the lethal velocity shows the differences between treatments. The result of statistical analysis showed that B. basiana percentage of death was 50% at the fastest on day 7 at treatment with concentration $10^6$, and 100% mortality was obtained on day 13 at concentration $10^2$, $10^3$, $10^5$, $10^7$, $10^8$ and in the treatment with entomopathogen M. Anisopliae showed the 50% percentage of deaths as fastest on the 9th day on almost all but the treatment with concentrations of $10^4$ and $10^8$. Whereas 100% mortality was obtained at day 13 at concentrations of $10^3$, $10^4$, $10^5$, $10^6$ (table 2). And the percentage of deaths of H. citriformis, 50% fastest citriformis at 9th day on treatment with concentrations of $10^4$, $10^6$ and $10^8$, while 100% mortality was obtained on day 13 at concentrations $10^2$, $10^4$, $10^6$, (table 3). From the separate test results each entomopathogen chosen for subsequent test is the best concentration of entomopatogen B bassiana at concentration $10^6$, M. anisopliae at concentration $10^5$ or $10^6$, while H.citriformis also at concentration $10^6$.

From the results in table 1 above, it can be seen that on the 7th day the percentage of mortality already exists above 50%. The best result of mortality percentage was obtained by *B.bassiana* $10^6$: 70.45% followed by *H.citriformis* $10^6$ of 63,85% and *M. anisopliae* concentration $10^6$ of 58,95%. There are 2 more treatments that show good result that is *H.citriformis* concentration $10^3$ and $10^4$ each equal to 51,85% and 56,3%. While the other treatment is still below 50% of death rate. Over time the death of aphid more and on the 9th day some of the treatment has shown percentage of death 100% that is treatment of *B.bassiana* $10^{-6}$, *M. Anisopliae* $10^{-3}$ being treatment of *H.citriformis* no one died 100% on day 9. Best result of each Entomopathogen is used for the second test (b). Insects infected with entomopathogenic fungi are characterized by the growth of white hyphae on the surface of the body's cuticle, and enters the hemocoel. Insects infected with entomopathogenic fungi

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Tabel 1 – Percentage of *T. citricida* mortality infected with 3 entomopathogen at 7 concentrations

| Treatment | consentration | Presentage (%) of Mortality of aphid... day after enthomophatogen infected. | | | | |
|---|---|---|---|---|---|---|
| | | 5 | 7 | 9 | 11 | 13 |
| *B. bassiana* | $10^2$ | 25,19ab | 38,56 ab | 67,02a | 92,40a | 100,00a |
| | $10^3$ | 12,10bc | 24,72b | 63,52a | 80,82a | 100,00a |
| | $10^4$ | 14,55bc | 17,19b | 33,23a | 80,19a | 95,05a |
| | $10^5$ | 29,06ab | 34,95ab | 45,14a | 80,90a | 100,00a |
| | $10^6$ | 37,00ab | 59,20ab | 70,50a | 89,18a | 98,68a |
| | $10^7$ | 23,95ab | 42,70ab | 73,95a | 90,86a | 100,00a |
| | $10^8$ | 13,08abc | 32,90ab | 55,45a | 86,25a | 100,00a |
| *M. anisopliae* | $10^2$ | 18,42a | 32,92a | 67,65a | 81,62ab | 96,97a |
| | $10^3$ | 18,53a | 44,73a | 70,00a | 98,75a | 100,00a |
| | $10^4$ | 24,38a | 35,31a | 49,06a | 84,69ab | 100,00a |
| | $10^5$ | 26,25a | 48,75a | 65,00a | 82,50ab | 100,00a |
| | $10^6$ | 14,64a | 27,50a | 51,61a | 97,50a | 100,00a |
| | $10^7$ | 23,85a | 41,67a | 54,90a | 78,65ab | 88,85a |
| | $10^8$ | 26,81a | 40,62a | 49,74a | 63,45b | 85,89a |
| *H.citriformis* | $10^2$ | 20,91ab | 34,43a | 48,75a | 73,75b | 100,00a |
| | $10^3$ | 15,69ab | 32,58a | 46,12a | 89,05ab | 98,81a |
| | $10^4$ | 14,44ab | 30,68a | 65,47a | 100,00a | 100,00a |
| | $10^5$ | 15,25ab | 30,50a | 45,50a | 85,75ab | 97,00a |
| | $10^6$ | 24,17a | 45,97a | 63,61a | 86,81ab | 100,00a |
| | $10^7$ | 15,35ab | 34,13a | 47,66a | 90,42ab | 98,33a |
| | $10^8$ | 28,84a | 38,10a | 58,21a | 69,97b | 94,20a |
| Water control | 0,00c | 0,00c | 0,00c | 2,75a | 15,75a | 17,25a |

*Mean follows by the same letters on the same coloums is not significantly according Duncan test 5%.*

*Test the effectiveness of single and double entomopathogenic fungi to control T. citricida in screen house.* From the results of the first stage of testing, the selected concentration is used as an ingredient in the testing of the following stages, namely to know the effectiveness of entomopathogen singly and double. The treatments are *H. citriformis, B. bassiana, and M.anisopliae, H. citriformis + B. Bassiana, H. citriformis + M.anisopliae, B. Bassiana + M.anisopliae* and 1 water control. The type of aphid used is the dominant type found in the field during the test. To obtain the uniformity of test pests is done mass multiplication in the home screen using citrus plants that sprout. The test results showed that the highest and fastest percentage mortality of *T.citricide* was obtained in *B. bassiana and B. bassiana + H. citriformis* treatment at 100% at 5 days after investing with *T.citricida* compared to other treatments (table 2).

Table 2 – Mortality of *T. citricida* entomopathogenic parasited

| Treatment | Presentage of mortality of *T. citricida (%)* on... day | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| *H. citriformis* | 12,50 | 41,25 | 61,25 | 83,33 | 86,67 | 100,00 |
| *B. bassiana* | 45,95 | 70,27 | 95,95 | 100,00 | 100,00 | 100,00 |
| *M. anisopliae* | 2,50 | 2,50 | 2,50 | 13,75 | 27,50 | 38,75 |
| B. *bassiana + H. citriformis* | 39,7 | 76,9 | 84,6 | 98,7 | 100,0 | 100,00 |
| *M. anisopliae + H.citriformis* | 9,59 | 35,62 | 47,95 | 57,53 | 76,71 | 93,15 |
| *B. bassiana + M. anisopliae* | 3,75 | 6,25 | 13,75 | 25,00 | 30,00 | 43,75 |
| B. *bassiana + M. anisopliae + H. citriformis* | 3,75 | 8,75 | 18,75 | 30,00 | 42,50 | 68,75 |
| control | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |

Then followed by a single *H. citriformis* treatment with a 100% mortality on day 6. The infection of *M. anisopliae + H.citriformis* simultaneously reached 100% mortality on day 7 after investment and triple treatment of infection with 3 entomopathogen *B. Bassiana + M. anisopliae + H. citriformis* reached 100% mortality on the 8th day. According to Robert & Yendol (1971), one of the factors achieving mortality in harang influencing the successful use

of entomopathogenic fungi is its emitting power, viability, virulence and entomopathogenic spores Which is sprayed. Single infant treatment with Hirsutella alone was still lower than *B. bassiana and B. bassiana* + *H. citriformis.* It is possible that both can decrease pathogenicity.

Figure 2 – Imago *T. citricida* dies infected with entomopathogen a. *H.citriformis, b. B.bassiana, c. M.anisopliae* results of treatment

The entomopathogenic *B. bassiana* conidia germ combinations in combination with *M. anisopliae* increased compared with the bifurcation of the *B. bassiana* and *M. anisopliae* fungi singly. This means there is a mutually beneficial relationship between *B. bassiana* and *M. anisopliae* when combined. This relationship can be categorized as synergism. Definition of synergism is 2 species when living together mutually beneficial than when living alone.

Table 3 – Percentage ability of entomopathogenic fungus sprouts with single, double and triple infection

| Treatment | Ave |
|---|---|
| Single infection *H. citriformis* | 75 |
| Single infection *B. bassiana* | 53,33 |
| Single infection *M.anisopliae* | 42 |
| Double infection *B. bassiana* + *H. citriformis* | 90,67 |
| Double infection *M. anisopliae* + *H. citriformis* | 62,33 |
| Double infection *B. bassiana* + *M. anisopliae* | 72,67 |
| Triple infection *B. bassiana* + *M. anisopliae* + *H. citriformis* | 65,33 |

From the results of observation and data analysis on the number of spores attached to the aphid after treatment, it is known that between the treatment of double infection and triple infection more than the single treatment of infection (table 3). This is probably due to the spores in single infection treatment already entered, penetrating into the insect's integrity earlier than the double infection. In addition, the amount of spores sprayed on the treatment of double and triple infections are also more. According to Robert & Yendol (1981), *M. anisopliae* fungus was able to develop and penetrate into insect integrins 12-24 hours after contact, while according to Steinhaus (1999), *B. bassiana and Hirsutella* fungi were able to germinate at 24-48 hours after contact When the environment is humid. Aphid mortality due to the treatment is suspected due to direct contact of bioinsecticide on its body and the presence of different media content and active ingredients of each treatment. Surtikanti & Yasin (2009) states that at the time of contact, the spores form a sprout tube and secrete enzymes to soften the larval cuticle so that the spores can penetrate into the body of the larvae. According to Herlinda (2010), the mortality difference of host insects by entomopathogenic fungi due to differences in viability and conidia virulence. Temperature and humidity factors may also affect the development of *B. bassiana* and *M. anisopliae* fungi. The average temperature of 28.36$^{o}$C and relative air relative humidity 85.35% in the study room supports the life and development of the *B.bassiana* and *M. anisopliae* fungi. Sheroze *et al*. (2003) states that a temperature of 30$^{o}$C and a relative humidity of 80% is a good condition for the growth of *B. bassiana and M. anisopliae* fungi. Meanwhile, according to Bukhari *et al.* (2010), insect mortality caused by entomopathogenic fungi is due to factors such as larval characteristics of species, age and density of larvae, type and concentration of entomopathogenic fungi and environmental influences.

*Physiology Characterization of entomopathogenic fungi.* Characterization of physiology represented by the character of temperature and humidity is done on entomopathogenic fungi to be produced secondary metabolite formula. Temporary results of testing the effect of temperature on entomopathogenic growth are presented in graphical form as follows. From the observed growth of entomopathogen at 4 different temperatures, it was found that *H.citriformis* was able to grow better than the other 2 entomopathogen (*B.bassiana and M anisopliae*). Under conditions ranging from 20°C to 35°C *H.citriformis* grow optimally, with a colony diameter of 9 cm to day 4 at temperatures of 20°C and 25°C, and up to days 7 and 8 at temperatures of 30°C and 35°C. The lowest growing and growing ability is obtained from entomopathogen M anisopliae. The highest growth only reached 4 - 4.52 cm in storage temperature of 25°C and 30°C. Medium at temperatures 20 and 35°C growth is very low (figure 3). *B. Bassiana* able to grow optimal at temperature 20 and 25°C starting day 8 is in previous condition slow growth.

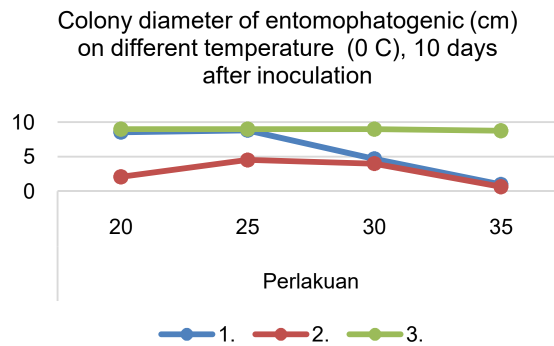


Figure 3 – Effects of 3 entomopathogenic fungi grown at different temperatures

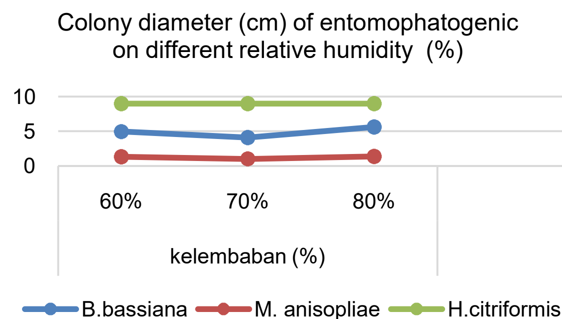


Figure 4 – Influence of entomopathogenic fungi grown on different relative humidity

The physiological characteristics of the three entomoptogenes tested against different relative humidity showed different growth outcomes. *H.citriformis* grown at 60%, 70% and 80% moisture is able to grow optimally until it reaches the diameter of 9 cm from 5th to 7th day. *B.bassiana* is able to grow in 3 different but not very optimal humidity. Its growth is still lower than *H.citriformis.* The physiological characteristics of M. anisopliae in different humidity are very low, even lower than the other 2 entomopathogens. Its growth capability is only 1.02 - 1.38 cm only (figure 4). The optimum temperature for *M. anisopliae* growth ranges from 22°C-27° C. Spore will form sprouts over moisture above 90% but will germinate well and its pathogenicity increases when the air humidity is very high up to 100%. However, its pathogenicity will decrease when air humidity is below 86% (Prayogo *et al.,* 2005). Temperatures above 35°C will inhibit the growth of entomopathogenic fungi, the fungus can survive but will be difficult to develop. Conidia fungus will die at 40°C for 15 minutes but can tolerate a wide range of hydrogen ion conenters between pH 5-10 and optimum pH of about

7 (McCoy *et al.,* 2005). The effectiveness of entomopathogenic fungi is also determined by environmental conditions, such as rainfall and sunlight, especially ultraviolet rays that can damage conidia fungi. Konidia is one of the organs.

*Biochemical characterization of entomopathogenic fungi.* In entomopatogen *H. citriformis* no chitinase content, cellulase, protease muapupun phosphat.Pada *B.bassiana* from jember found enzyme cellulase content and phosphate solvent, but *B.bassiana* other isolates contain only cellulase only. While on *M anisopliae* has solubilized phosphate content only. Microorganisms are considered more potent as stable biological controls if they contain lysozyme, protease, H2O2 or organic acid (Isnansetyo 2005).

Table 4 – Enzyme content in potential entomopathogen

| Isolate | Enzim Dalam Entomopatogen | | | |
|---|---|---|---|---|
| | Khitinase | Selulase | Protease | Phosphate solvent |
| *H citriformis* | - | - | - | - |
| *B.bassiana Jb* | - | + | - | + |
| *B.bassiana Lab* | - | + | - | - |
| *M anisopliae* | - | - | - | + |

Table 5 – Length of primary and secondary roots

| Isolate | Panjang akar primer | Panjang akar sekunder |
|---|---|---|
| *H citriformis* | - | - |
| *B.bassiana Jb* | - | + |
| *B.bassiana Lab* | - | + |
| *M anisopliae Jb* | - | - |
| *Kontrol* | - | - |

Seeds that were dipped with *B.bassiana* solution, its have a longer secondary root length than other tratments (Table 5).

*Molecular characterization of entomopathogenic fungi.* Characterization of molecular microbial antagonists using PCR by DNA amplification of fungi using primer pair ITS1 (5 'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5 'TCCTCCGCTTATTGATATGC 3') (figure 5a).

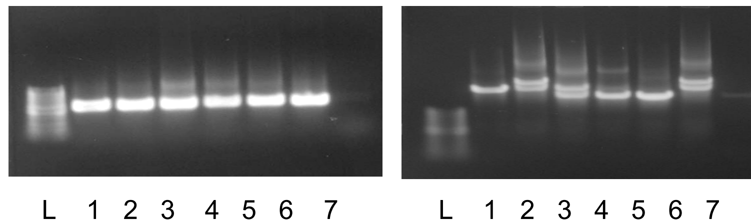


Figure 5 – Visualization of DNA detection of entomopathogenic insect pest insect CTV (a). Amplification of DNA with primary ITS 4 and ITS 5, (b). DNA amplification with primary FS1 and NS2. L = 1 kb leader, 1. = B.bassiana Jb, 2 = M anisopliae Jb, 3. = H.citriformis, 4 = B.bassiana Jbr, 5 = B.bassiana Pur, 6 = M anisopliae Pur, 7 = Phacelomyces Sp. Jbr

From the final observation with elektroforeses showed that 7 entomopathogen isolates B, bassiana, H, citriformis, M anisopliae and Phacelomyces sp. It shows no difference not very real locus at position 800 kb, even Phacelomyces sp. Not out of band band, this means that ITS1 and ITS4 primers can not detect the entomopathogen species. In the next period DNA amplification is done using the primary pair FS1 and NS2.

## CONCLUSION

The best entomopathogen suspension concentrations in controlling CTV vector pests in the laboratory were *B. bassiana* $10^{-6}$, *H.citriformis* concentrations $10^{-6}$ by 63.85% and *M. anisopliae* concentrations $10^{-6}$. In the entomopatogenic Effectivity Test against the CTV vector pest at screen house, the best results were in single treatment of *B. bassiana and H. citriformis* with 70.11% mortality. Entomopatogen *H.citriformis* has the best physiological

character than the other 2 entomopatogens (*B.bassiana and M anisopliae*) at 4 temperatures tested and at 60%, 70% and 80% moisture. *B.bassiana* is able to grow at 3 different moisture but not very optimal and *M. anisopliae* on different humidity very low growth. The biochemical character of *H. citriformis* did not find the content of khitinase, cellulase, protease and phosphate solvent. *B.bassiana* from jember found content of cellulase enzyme and phosphate solvent, but *B.bassiana* other isolates contain only cellulase. *M anisopliae* has solubilized phosphate solvent only. The molecular character of entomopathogenic fungi with primer ITS1 and ITS4 did not show different diversity, but with the primary FS1 and NS2 there was diversity, *H.citriformis* had two bands, as well as *M anisopliae* each at different loci. Medium *B bassiana* has only one band only

## ACKNOWLEDGMENTS

Appreciation and gratitude are presented to the Ministry of Technology and Higher Education which has funded the research through the INSINAS 2016 Grant. Thank you also to Unun Triasih SP and Dina Agustina, S.Si. who has been technically helpful in this research.

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