IOP Conf. Series: Earth and Environmental Science **166** (2018) 012017 doi:10.1088/1755-1315/166/1/012017

Insecticidal activities of crude extact of *Metarhizium anisopliae* and conida suspension against *Crocidolomia pavonana* fabricius

Melanie[1], M Miranti[2], H Kasmara[2], S Hazar[3], and A Martina[3]

[1] Student of Environtmental Science Doctoral Programme, Faculity of Post-Graduated School, Padjadjaran University, Jl. Dipatiukur No. 35, Bandung, Jawa Barat 45363, Indonesia
[2]Departement of Biology, Faculty Mathematics and Natural Sciences, Padjadjaran University, Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Jawa Barat 45363, Indonesia
[3]Post Graduate students of Biology Departement, Faculty Mathematics and Natural Sciences, Padjadjaran University, Jawa Barat 45363, Indonesia

E-mail: melie_entobio@yahoo.com

Abstract. Pest resistance, resurgent and the residual effect of conventional insecticides are the basis to apply Insect Pest Management (IPM) strategies. *Metarhizium anisopliae* is an entomophatogenic fungus that has considerable promise for usage in IPM. The spores of fungus had great potential as insect pathogen, in addition the mycotoxins products having current interest as bioactive agent. *M. anisopliae* was exhibited to control pest insect populations of crops especially host plant of *Brassica oleracea* (Cruciferae), i.e. *Plutella xylostella*, *Spodoptera litura,* and *Crocidolomia pavonana*. The insecticidal activities of conidia suspensions and crude extract product containing mycotoxins were investigated, withthe parametersmortality and lethal time of *C. pavonana* 3rd instar larvae. The experimental design of crude extract and conidia suspensions of *M. anisopliae* on mortality effect used Completely Randomized Design (RAL). The bioassay of conidia suspensions mortality test used 6 level spore concentrations (0, 10^5, 10^6, 10^7, 10^8, and 10^9 spores/ml). Analysis of variance (ANAVA) at α 95% followed by Duncan multiple test at α 5% was carried out. The result of crude extract mortality test showed that *M. anisopliae* produced mycotoxins from the third day of fungal growth. The long growth of fungus is directly proportional with the produced mycotoxins. The mycotoxins was optimally produced in 7th days. The extract *M. anisopliae* at highest mortality effect on larvae was tested for 24 to 120 h. One hundred percent mortality of larvae were achieved at 120 h with an average lethal time were 3.6 days shorter than mortality caused by conidia infections. Thus, conidia infections showed that *M. anisopliae* at 10^5 - 10^9 spores/ml concentrations led to mortalities of 75%, 90%, 80%, 85%, and 95 %, respectively ($P<0.05$). The larvae lethal time had no different in all concentration levels, but the shorter lethal time (4.66 days) was showed in the infected larvae with 10^9 spores/ml concentration.

1. Introductions

Nowadays biological control is appreciated and regarded as a desirable technique for controlling pest insects, due to the low environmental impact and the reduce of resistance development [1]. Biocontrol application uses natural agents such as pathogens, parasites and predators, with the use of agrotoxins as a last resort [2]. *M. anisopliae,* most widely used entomopathogenic fungus in biological control, have been formulated and registered as mycoinsecticides [3]. *Metarhizium* grows vegetatively as hyphae producing mycelia and conidia that are infective propagules on insect cuticle [4]. Many researches on *M. anisopliae* have been carried out for use against a wide variety of pests such as insect pests of wood (termites), domestic pests, vector insects (mosquitoes) also insect pests of ornamental plants and cultivated plants (plantations, cereals and vegetables) [5]. *M. anisopliae* is known to be a potential biological agent for controlling *Spodoptera litura*, key pests of tobacco, soybeans and crop from Brassicaceae [6].

C. pavonana is the important insect pest of Brassicaceae [7-9]. This diet can lead to the destruction of head cabbage, which cause attacks by *C. pavonana* as a very serious problem [7,10]. All stages of host plants are susceptible to attack by *C. pavonana* and major yield losses can occur if the pest population is not suppressed [7-8]. Management strategies to control *C. pavonana* beside of selective insecticides also used parasitoid and entomapatotoghenic fungus. *M. anisopliae* is known to be effective in controlling *C. pavonana*. The fungal spores are formulated in pellets and powders. The spores are infectious to *C. pavonana* larvae at spore concentration 10^7 spores/mL for 4 days post infection [11].

The secondary metabolites produced by the fungus are rich source of bioactive chemicals that increase the toxicity potential of entomophatogenic fungus [8]. *Metarhizium* are known to produce mycotoxins such as destruxins (dtxs), cytochalasins C and D, helvolic acid, myroridins, swainsonine, tyrosine betaine, serinocyclins A and B, aurovertins, hydroxyovalicin, viridoxins, fungerins, metacytofilin, hydroxyfungerins A and B, and 12-hydroxyovalicin and hydroxyfungerin, the 7- desmethyl analogs of fusarin C and (8Z)-fusarin C [12-18]. In fact, destruxins are most widespread mycotoxin of the secondary metabolites produced by *Metarhizium* [8]. However, strains of *Metarhizium* differ considerably in their metabolite profiles, with variation in destruxins production as a possible virulence or specificity factor [8].

However, entomopathogenic fungus has numerous benefits over other microbes for insecticide preparation. The development and formulation have used new technology that produce new insecticidal compound for larvicides or adulticides, i.e. in the bio-control of mosquitoes [11]. The fungal spores and metabolites have been significantly demonstrated to show efficacies against adults and its developmental stages of mosquitoes [11]. The weaknesses of using fungal suspensions is that it requires a longer time to reach insect pest mortality because the fungal spores need more time to germinate and penetrate into cuticles [4]. Thus, it was considered to be more feasible for large scale experiments and field implementation [11]. Metabolites of entomomopathogenic fungus were found significant pathogenic after filtration [19]. Efficacy of culture filtrates of five strains of *M. anisopliae* isolated from insects were evaluated against *An. stephensi* and *Cx. Quinquefasciatus* [20].

The extract of *M. anisopliae* has been characterized to determine the secondary metabolites. It contained a rich source of bioactive chemicals, responsible for insecticidal activity [12]. One of the important things to know about *M. anisopliae* extract characterization is the optimization of mycotoxins production. The purpose of this study was to determine the time of optimization of mycotoxins released by fungi products in rough extract which is showed by the effect mortality to *C. pavonana* larvae. In addition, the effectiveness of conidia suspension was compared with the rough extract of *M. anisopliae* on *C. pavonana* larvae.

2. Materials and Methods

2.1. Experimental Insect

The research object was crop caterpillar (*C. pavonana*). It is known as an important secondary pest in cabbage plantation, and other Brassicaceae plants. In this research, third instar of the larvae obtained from HPT Departement, Agriculture Faculty of UNPAD and BALITSA in Lembang were used. Feed bait and testing leaves are cabbage leaves (*B.olereace*) that is a typical food for *C.pavonana*. The larvae was cultured in a rearing box (40 × 25 × 25 cm^3). The ovitrap was used to collect the eggs at the bottom of the leaves. The group of eggs was reared in the separate rearing box (20 ×15 ×5 cm^3) with controlled humidity and temperature. Furthermore, the eggs were hatched and become caterpillar/larvae. The instar I-IV larvas were placed in different rearing boxes. The bioassay test used third instar larvae, the stage of active feeding.

2.2. Culture of M. anisopliae

The culture of *M. anisopliae* obtained from Department of Plantation Protection Centre, Bandung. Further prophylactic culture of *M. anisopliae* was carried out on Potato Dextrose agar medium (PDA) of fungal preparation and incubated at room temperature of 22-27^oC with pH between 3.3 to 8.5 [5]. It was observed and was made the growth curve, by using colony counter methode.

2.3. M. anisopliae Extraction

The culture of *M. anisopliae* sporas were harvested from growth mediums and collected into the macerator. It was soaked for 24 h using 95% ethanol solvent. Ethanol is a polar solvent and can attract polar compounds including mycotoxins compounds that polar protein molecules. The macerate was concentrated by a vacuum rotary evaporator to evaporate the solvent with a low pressure (40^0C) to obtain a viscous extract in the form of a paste [21].

2.4. Prepared Conidia suspensions of and extract solutions

One g of conidia (containing > 10^9 spore/ml) was dissolved into 5 ml aquadest and homogenized by $Tween^{80}$, then mixed with vortex. Further, the suspension was diluted into 10^9, 10^8, 10^7, 10^6, 10^5 spore/ml of level concentrations. *M. anisopliae* extract was made into a concentration of 1000 ppm (1 mg/1000 mL). Then, the cabbage leaf in Ø 5 cm was dipped into 0.05 ml of extract and the surface was dried for 1 minute.

2.5. Bioassay test

The bioassay of conidia suspensions was performed by topical method (suspension was infected into pro-thorax of larvae) and the bioassay test of extract *M. anisopliae* used leaf dipped method [22]. The *C. pavonana* third instar larvae was used in both tests. Each treatment used 5 larvas with 5 replications. Observations were made daily with parameter of larval mortality and lethal time of larvae.

2.6. Data analysis

The results were analyzed by ANOVA and if the results were significantly different (α 5%), continued with Duncan Multiple Range Test.

3. Result and Discussions

3.1. Mortality effect of crude extract M. anisopliae to the C. pavonana larvae

The growth patterns of *M .anisopliae* cultivated in PDA medium was showed in growth curve (**Figure 1**). By counted the colony of fungal spores , there was an exponential phase on the 1st day to 6th day of growth in PDA. The conidia of *M. anisopliae* declined on the 7th day. It was the phase of fungal death marked by the decreasing population until 10th day.

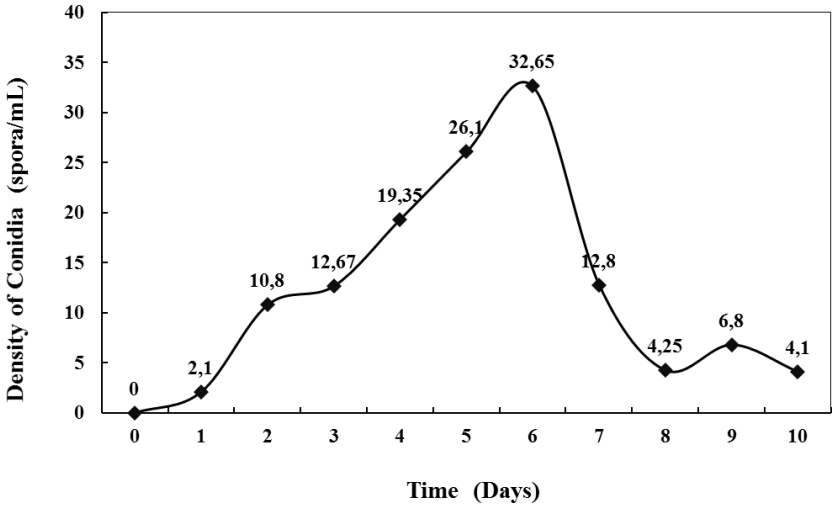

Figure 1. The growth curve of *M. anisopliae* in PDA Culture

M. anisopliae crude extract contained secondary metabolites in the form of mycotoxins known to cause the mortality of *C. pavonana* larvae. Furthermore, the mycotoxins produced from *M. anisopliae* exctract grown on the 1st day until the 7th day, were tested for their mortality effect, in order to determine the most optimal fungal growth time. Finally, mycotoxin with the highest insecticidal effect of *C. pavonana* larvae was then resulted (**Figure 2**).

The results showed that crude extract of *M. anisopliae* in PDA produced mycotoxin starting on the 3rd of fungal growth. The fungal growth was directionally proportional with the production of mycotoxins. The most optimum time of mycotoxin production was up to day 7th, and it was observed from the highest mortality effect on larvae (24 to 120 h): 53.3% mortality in 24 h and 100% mortality of larvae at a 120 h, respectively (**Figure 2**).

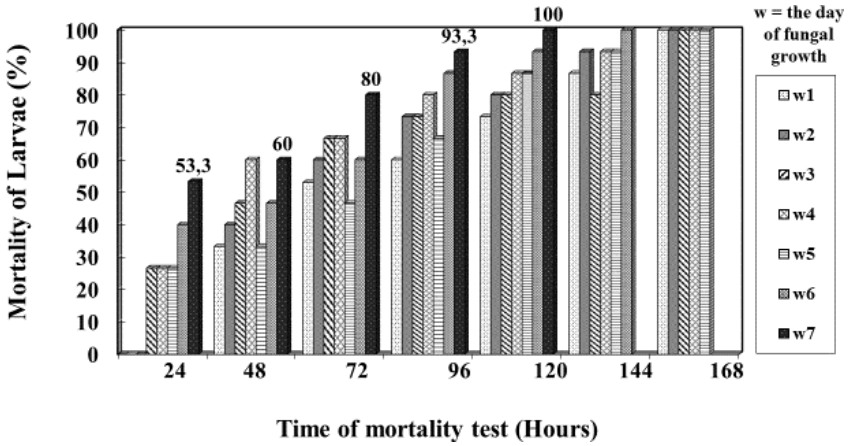

Figure 2. The histogram of mortality effect of *M. anisopliae* extract to *C. pavonana* larvae

The time of fungal growth had an effect on the mycotoxin product observed by lethal time of *C. pavonana* larvae (P <0.05). The mycotoxin product on the 7th day showed significantly different shortened the lethal time of *C. pavonana* larvae for an average of 3.6 days (**Figure 3**). Thus, the optimum time of mycotoxins from *M. anisopliae* extract on PDA media was produced on day 7th.

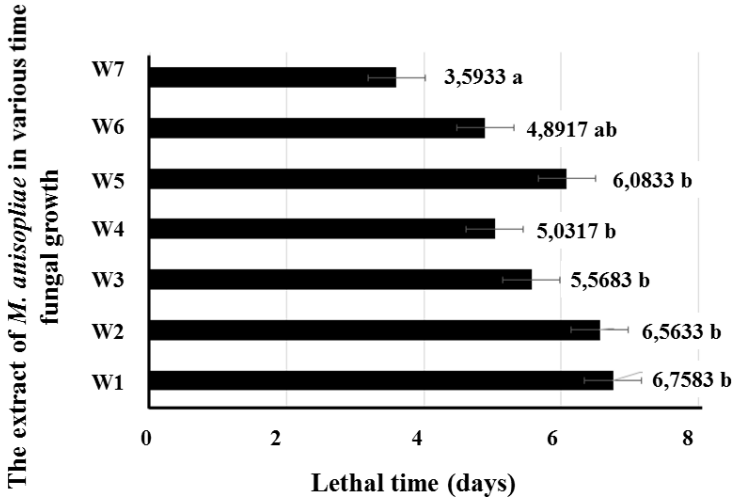

Figure 3. The histogram of the extract of *M. anisopliae* in various time fungal growth effect to lethal time of C. *pavonana* larvae

3.2. Mortality effect of M. anisopliae conidia suspensions to the C. pavonana larvae

The infection of *M. anisopliae* to the larvae showed that conidia suspension at concentration from 10^5 to 10^9 spores/ml had a mortality effect on larvae (P <0.05) with 75%-95% mortality rate, but the difference concentration factor did not significantly effect to the mortality rate of *C. pavonana* larvae (**Figure 4**)

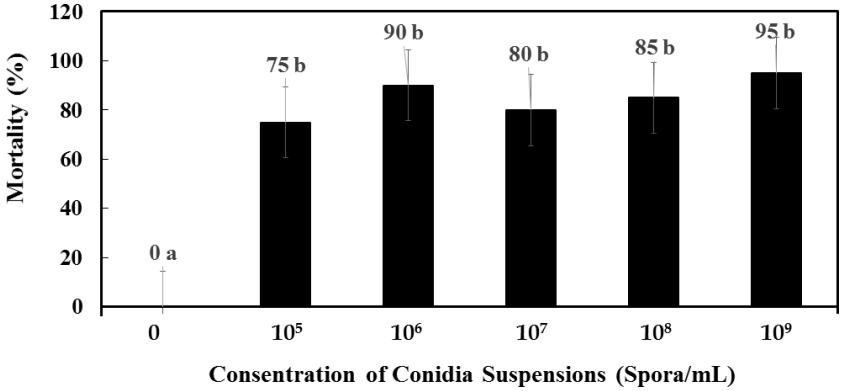

Figure 4. The histogram of *C. pavonana* larvae mortality due to infection of *M. anisopliae* conidia suspensions at various concentration levels

The conidia suspensions of *M. anisopliae* infected *C. pavonana* larvae and significantly affected to lethal time (P <0.05), with there was no different among of level concentrations. The shortest average lethal time of *C. pavonana* larvae was 4.66 days using 10^9 spore/mL conidia suspensions, and the longest was 6.3 days using 10^5 spore/mL of conidia suspensions (**Figure 5**).

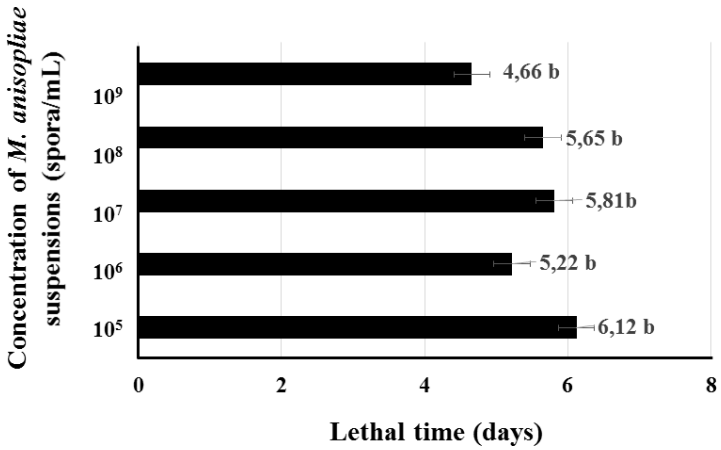

Figure 5. The histogram of the *M. anisopliae* conidia suspensions in various concentrations effect to lethal time of C. *pavonana* larvae

Potato dextrose agar (PDA) media were found most favoured for soil fungal [23], such as *M. anisopliae* that naturally spread in soil [4, 24]. The composition of PDA consists of 20% potato extract and 4% glucose, and low pH (4.5-5.6). It was the ideal conditions for optimal fungal growth in a culture medium containing nutrients with appropriate osmotic pressure and pH [25]. In this study, *M.anisopliae* showed the optimum growth phase in 48-72 hours. Culture media influenced the germination of conidia, appressorial development and mycelial growth of *M. anisopliae* [25]. The 72- hour incubation period is a phase with lest active dividing cells. In this phase, fungal cells obtain nutrient intake and perform metabolic reaction, such as secondary metabolite productions [26]. The product of secondary metabolite was invested by the cells, because it was no longer needed by cell [26]. Determination in incubation period is very important for harvest timing, quantity and quality of the fungal secondary metabolic product, especially for industrial scale process. Penicillin production uses *Penicillium chrysogenum* generally last for 72 hours (about 3-7 days). However, it is necessary to extend the incubation period to 8-10 days to reach the stationary phase for optimize penicillin production. The medium enricedh with a mixture of leachate and sucrose can improve the penicillin product [27]. However, from this study, the first mortality of larva *C. pavonana* (25%) was observed using the extract of 3rd days fungal growth. The highest mortality effect on larvae (100%) was observed using the extract from the 7th days fungal growth.

The study showed that there are no difference at the mortality effect of conidia suspension in all levels concentration. The pathogenic spores were germinated and penetrated to cuticle, as soon as possible, to attack the immune system [28]. The death of the larvae caused by the pathogenicity of the opportunistic fungus and possibly due to the characteristic of spore virulence, the rapid growth of opportunistic fungus, which infects, injures or weakens the insect's immunity [1]. So it depends not only on the number of infected spores.

The fungal spores *M. anisopliae* have virulence levels to kill the infected larvae. The shortest average of lethal time of *C. pavonana* larvae was 4.66 days using 10^9 spore/mL conidia suspensions, and the longest was 6.3 days using 10^5 spore/mL of conidia suspensions. A higher concentration is representative with the possibility of a number of infected spores, possibly increasing contact between host with pathogen and shorter at time of death larvae.. Deaths of larvae are also caused by damage to the intestines due to mycotoxins released by fungi [29]. From this study, the effect of *M. anisopliae* extract succeeded in shortening the time of death of *C. pavonana* lar larvae. The rough extract of *M. anisopliae* from the growth of the 7th day fungus shortened the

deadly time of *C. pavonana* larvae to
3.6 days. The time of death of the larva with infected conidia suspension takes more time for the germination spores to occur on the insecticular cuticle followed by penetration of growing insects in the hemolymph that kill mosquitoes within 7-14 days, depending on dosage, formulation and fungal strain [29].

Penetrate hyphae produces a number of enzymes such as lipase, protease and chitinase enzymes capable of degradation of the cuticle. Furthermore, the spores will develop in the hemocoel by absorbing hemolymph and producing destrucsin which can lead to the death of the larvae. A few days after the larvae dies, the body of the larvae begins to harden and stiffen. This is because the whole body of the larvae is enveloped by mycelium [30].

M. anisopliae secretes large amounts of trehalase acid, a type of enzyme that reduces trehalose in hemolimfa of insects. Trehalose is the main sugar found in hemolimfa insects. The presence of a trehalase enzyme in the body of a fungus infected insect will lead to a decrease in adaptation of insects in the environment [31]. *M. anisopliae* also produces destrucsin cyclic peptide poison. Some of the destrucin, especially destrucin A, B and E show insecticidal activity [4,36].Mycotoxins of *M. anisopliae* inhibits the feeding and mobility of insect larvae [4]. Destrucsin is synthesized via multifunctional peptide synthetic [4], [36] and injection of destrucsin in pest insects causes muscle paralysis [32]. Insect paralysis results in a direct link between insect damage and mortality.Although some studies of *M. anisopliae* are less prospective, one of the fungal application constraints is slowly killing target insects. Furthermore, through this research we found that the crude extract of *M. anisopliae* is more effective than conidia suspension to control *C. pavonana* larvae primarily to slow the death time of the larvae. However, in further research we would like to know the mycotoxin production opportunities in growth media, and the increasing factor of growth medium of mycotoxin production. In the following decade, better knowledge of *M. anisopliae* applications and increased public concern about the effects of reducing pesticide use by using entomophatogenic products for a sustainable environment, especially in controlling *C. pavonana* in vegetable plantations is expected to change.

4. Conclusions

The results of mortality test of crude extract showed that *M. anisopliae* had produced mycotoxin from the 3rd day of fungal growth. The longer the fungal growth, the higher the resulting mycotoxin was obtained. Mycotoxins was optimized on the 7th days. This was demonstrated by the extract of *M. anisopliae* having the highest mortality effect on larvae tested for 24 to 120 hours. One hundred percentage of mortality of larvae was achieved at 120 hours with an average time of 2.5 days shorter rather than mortality caused by conidia infection. The average lethal time of 10^9 spores/mL suspension was 4.66 days. The extraction of *M. anisopliae* proved to be more effective in controlling *S. litura*, observed from the lethal time was shorter than the conidial suspension.

Acknowledgement

We are grateful to Rector & DRPMI Padjadjaran University, that was supported this research with “Hibah Internal Unpad (HIU)” in scheme of Fundamental Research Programme 2017 grant.

5. References

[1] Abd El- Ghany T M, El-Sheikh H H , Abd El-Rahman G A and Abd El-Nasser A M 2012 Biodiversity of entomopathogenic fungi in new cultivated soil with their using to control of

Galleria mellonella Int J Cur.Res. Rev. 04 17-31
[2] Patricia V T, Neiva T O, Elza Á L and Alves L 2014 Biological insect control using Metarhizium anisopliae: morphological, molecular, and ecological aspects Ciência Rural Santa Maria 44 644
[3] Faria M R and Wraight S P 2007 Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types Biol.Control 43 237- 256
[4] Augusto S and Marilene H 2010 Metarhizium anisopliae enzymes and toxins Toxicon 56 1268
[5] Irna M, Suzzana F S and Irda S 2017 Potensi jamur entomopatogen untuk mengendalikan ulat grayak Spodoptera litura F. pada tanaman tembakau invivo J. Agroekoteknologi FP USU 5 484
[6] Nuraida dan A Lubis 2016 Pengaruh formulasi dan lama penyimpanan pada viabilitas, bioaktivitas dan persistensi cendawan Metarhizium anisopliae terhadap Crocidolomia pavonana Fabricius J.HPT Tropika 16 196-2012
[7] Aleni U, Peter M R , Richard S, Yu-rong H, Gurion A, Myron P Z and Michael J F 2014 Trichogramma chilonis Ishii: a potential biological control agent of Crocidolomia pavonana in Samoa Biol. Control 73 31-32. [1]
[8] Morallo R B and Navasero W R 2003 Biology of Crocidolomia pavonana (F.) and population inventions against this recurrent pest of crucifers in the Philippines Philippines Entomologist 17 141-168
[9] Karungi J, Lubanga U K, Kyamanywa S and Ekbom B 2010 Oviposition preference and offspring performance of Crocidolomia pavonana (Lepidoptera: Pyralidae) on different host plants J.Appl.Entomol. 134 704-713
[10] Takeuchi H, Zalucki M P and Furlong M J 2009 Crocidolomia pavonana larval foraging behaviour and feeding site preferences on cabbage Brassica oleracea Entomol. Exp. Appl. 133 154- 164
[11] Gavendra S and Soam P 2014 New prospective on fungal pathogens for mosquitoes and vectors control technology J.Mos. Res. 4 36-52
[12] Lozano T, Garrido J, F. Lafont and Quesada M. 2015. Insecticidal activity of a destruxin-containing extract of Metarhizium brunneum against Ceratitis capitata (Diptera: Tephritidae) J. Econ. Entomol. 108 463
[13] Fuji Y, Tani H, Ichinoe M and Nakajima H 2000 Zygosporin D and two new cytochalasins produced by the fungus Metarrhizium anisopliae J. Nat. Prod. 63 132–135
[14] Ryuji U, Rie I, Yuichi Y, Rokuro M, Kazuro S, Hiroshi T and Satoshi O 2005 New insecticidal antibiotics, hydroxyfungerins A and B, produced by Metarhizium sp. FKI-1079 J. Antibiotics 58 804- 809
[15] Krasnoff S B, Sommers C H, Moon Y S, Donzelli B G, Vandenberg J D, Churchill A C and Gibson D M 2006 Production of mutagenic metabolites by Metarhizium anisopliae J. Agric. Food Chem. 54 7083-7088
[16] Zimmermann G 2007 Review on safety of the entomopathogenic fungus Metarhizium anisopliae Biocontrol Sci. Technol. 17 879-920
[17] Azumi M, Kei-ichi I, Hiroshi K, Takuya N, Fumio I, Tsuyoshi F and Yasuhiro I 2008 Aurovertins F–H from the entomopathogenic fungus Metarhizium anisopliae J. Nat. Prod. 71 278-280
[18] Carlos A C, Ana L C, Letıcia A S, Thais G, Donald W R, Norberto P L and Gilberto U L B 2010 Fungal Biol. 114 473-480
[19] Vyas N, Dua K K and Prakash S 2015 Larvicidal activity of metabolites of Metarhizium anisopliae against Aedes and Culex mosquitoes J Comm Disea. 38 (2) 1-3
[20] Mohanty S S and Prakash S 2008 Laboratory and field evaluation of the fungus Chrysosporium lobatum against the larvae of mosquito Culex quinquefasciatus Parasitol Res.

102 (5) 881-886
[21] Keith B M, Yew-Min T, Johnny M, Liu J, Chun W 2009 Probing inhibitory effects of destruxins from Metarhizium anisopliae using insect cell based impedance spectroscopy: inhibition vs chemical structure Analyst Journal 134 1447-1425
[22] Desak M M, Madihah, Melanie, Hikmat K, Rani M, Fakhrur R and Wawan H 2016 Antifeedant activitiy of ethanol extracts of five selected plants against larvae of taro caterpillar (Spodoptera litura fabricius, 1775) Proc. Int. Conf. 6th ISSH (Bogor) vol 1 (Humanosphere Science School-Biomaterial LIPI) pp 38-48.
[23] Mukesh S, Meenakshi S 2011 Influence of culture media on mycelial growth and sporulation of some soil dermatophytes compared to their clinical isolates J.Microbiol.Anti M.3(8)196-200
[24] Ibrahim L, Butt T M and Jenkinson P 2002 Effect of artificial culture media on germination, growth, virulence, and surface properties of the entomopathogenic fungus hyphomycete Metarhizium anisopliae Mycological Res. 106 705-715
[25] Soundarapandian P and R. Chandra 2007 Mass production of endomopathogenic fungus Metarhizium anisopliae (Deuteromycota; Hyphomycetes) in the laboratory J.Microbiol. 2 (9) 690-695
[26] Barbara C E, Suraia S, Sergio de A and Monica T P 2006 The influence of culture conditions on the biosynthesis of secondary metabolites by Penicillium verrucosum Dierck Microbiol. Res. 161 273-280
[27] Rajan S 2017 Basic of Fermentation Technology (Dehradun & Uttaranchal Institute of Management, India : MedCrave Group LLC) p 54
[28] Prayogo Y, Tengkano W 2004 Pengaruh konsentrasi dan frekuensi aplikasi Metarhizium anisopliae isolat Kendal terhadap tingkat kematian Spodoptera litura J. Ilmiah Sainteks XI 3 233-243
[29] Scholte E J, Bart G J K and Willem T An entomophatogenic fungus (Metarhizium anisopliae) for control of the adult African malaria vector Anopheles gambiae 2008 Entomol. Ber. 68 (1) 21-26
[30] Prayogo Y 2005 Potensi, kendala dan upaya mempertahankan keefektifan cendawan entomopatogen untuk mengendalikan hama tanaman pangan J. Buletin Palawija 10 53-65
[31] Zhao H, Charnley A K, Wang Z, Yin Y, Cao Y Y, Peng G and Xia Y 2006 Identification of an extracellular acid trehalase and its gene involved in fungal pathogenesis of Metarizium anisopliae J. Biochem. 140 319-327
[32] Hu Q B, Ren S X, Chang J H, and Musa J M 2006 Investigation of destruxin A and B from 80 Metarhizium strains in China, and the optimization of cultural conditions for the strain MaQ10 Toxicon 48 491-498