Potential of endophytic fungi from chilli as bioinsecticides against *Tenebrio molitor* Lin. and *Spodoptera litura* Fab. larvae

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ABSTRACT

Endophytic fungus is a fungus that is associated with healthy host tissues without causing disease symptoms. This fungus can be developed as the biological control agent of chilli pests. This study is aimed at isolating, selecting and evaluating the potency of endophytic fungi from chilli crops (*Capsicum annuum* L.) as bioinsecticides. Endophytic fungi were isolated from leaves, trunk, branches and roots of chilli. Screening endophytic fungal isolates pathogenic to insect were conducted using fifth instar larvae of *Tenebrio molitor*. The isolates with the highest mortality were further assayed against *Spodoptera litura* larvae. The results of the research showed that of the 46 isolates of endophytic fungi that were successfully isolated from chilli, 22 isolates were pathogenic (47.82%) and 24 isolates not pathogenic in insects. Mortality of *T. molitor* larvae ranged from 15-30% and the percentage of sporulation ranged from 11.11- 100%. Endophytic fungi were also able to infect *S. litura* larvae. Mortality of second instar *S. litura* larvae ranged from 23.34-64.99%, depending up on the conidial concentrations. Endophytic fungi from chilli were pathogenic in insect and can be developed as bioinsecticides identified as *Aspergillus flavus*.

MS History: 19.12.2016 (Received)-31.03.2017 (Revised)- 06.04.2017 (Accepted)

Key words: Endophytic fungi, entomopathogenic, chilli, bioinsecticides.

Citation: Trizelia, Haliatur Rahma and Martinius. 2017. Potential of endophytic fungi from chilli as bioinsecticides against *Tenebrio molitor* Linnaeus, and *Spodoptera litura* Fabricius, larvae. *Journal of Biopesticides*, **10**(1): 10-16.

INTRODUCTION

Chilli (*Capsicum annum* L.) is one of the most important vegetable crops in Indonesia. This crop has been used for domestic consumption and marketed afresh or after being processed. Chili is usually used as a spice in cooking, raw material of various industries of food, beverages and pharmaceuticals. Productivity of chilli in Indonesia is low, only 8 ton/acre lower than the potential yield that can be achieved, namely 12 ton/acre. One of the main obstacles limiting yield increase in chilli is pests.

The most important control strategy for reducing population of the pests is through application of synthetic insecticides. Intensive pesticide applications aimed at reducing pest population in chilli have caused the development of resistance in insect pests to some products and increased residues in fruits. Safety and environmental issues regarding the use of chemical insecticides have led to the development of alternative insect control measures, including endophytic fungi.

Fungal endophytes are extremely common and highly diverse microorganisms that live within plant tissues, but they usually remain asymptomatic. Traditionally endophytes have been considered plant mutualists, mainly by reducing herbivory via production of mycotoxins, such as alkaloids (Faeth and Fagan, 2002). The capability of colonizing internal host tissues has made endophytes valuable for agriculture as a tool to improve crop performance (Azevado *et al*., 2000).

Senthilkumar *et al*. (2014) reported that several endophytic microbes are known to

have anti-insect properties. Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. Endophytic fungi, *A. flavus* and *N. sphaerica* isolated from teak leaves produce phytochemicals such as Duroquinone, Adamantine derivative, Dodecanoic acid, tetradecanoic acid, pentadecanoic acid and Myristic acid which are reported to have insecticidal activity. Vega *et al*. (2008) studied fungal endophyte mediated plant defence as a novel biological control mechanism against the coffee berry borer, the most devastating pest of coffee throughout the world. A survey of fungal endophytes in coffee plants from Hawaii, Colombia, Mexico and Puerto Rico has revealed the presence of various genera of fungal entomopathogens including *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys*, and *Paecilomyces*. Two of these *B. bassiana* and *Clonostachys rosea* were tested against the coffee berry borer and were shown to be pathogenic

However, up to date, study on the role of endophytic fungi as bioinsecticides has been conducted mostly in grasses and trees, whereas in chilli it is very limited. Hernawati *et al.* (2011) reported that the species diversity of fungal endophyte from leaves of chilli was low. Only five species found i.e. *Aspergillus flavus*, *Nigrospora* sp., *Coniothyrium* sp and sterile hypha 1 (SH1), and sterile hypha 2 (SH2). *Nigrospora* sp., SH1 and SH2 reduce population growth of *Aphis gossypii*. Glover, 1877, with SH2 provides the highest suppression but *Aspergillus* sp. and *Coniothyrium* sp. have minor effect on population growth of *A. gossypii*. The objective of the research was to isolate and evaluate the potency of endophytic fungi from chilli crops (*Capsicum annuum* L.) as bioinsecticides.

MATERIALS AND METHOD

Isolation and purification of endophytic fungi

Healthy chilli crops were chosen for sampling. Chilli crops were collected from agriculture land at Parabek, West Sumatera. The crops material was brought to the laboratory in sterile bags and processed for isolation of endophytic fungi. The crops material were rinsed in running water. After washing, the root, stem, branches and leaves were cut into small pieces (1 cm in length). Isolation of endophytes from cilli samples was carried out as described by Hazalin *et al.* (2009). Plant samples, which included leaves, stems, roots, and branch were washed under running tap water for 10 min followed by immersion in 70% ethanol for 1 min and in NaOCl (2.5%-5.25%) for 3 min, drained and immersed in 70% EtOH again for 30 sec. Finally, the samples were rinsed with sterile H_2O. Each plant sample was cut aseptically into 1 cm long segments. The cut surfaces of the segments were placed on Petri dishes containing potato dextrose agar (PDA) supplemented with chloramfenicol (250 µg/L). Hyphal tips from the growing colonies were transferred to fresh PDA containing Petri plates with the addition of antibiotics to obtain pure cultures for bioassay and identification. The fungi were identified on the basis of their morphological and cultural characteristics. Fungal samples were placed on the slides, stained with lactophenol cotton blue and observed under compound microscope. Microscopic characteristics were noted and identification was done.

Screening of endophytic fungi

Screening of endophytic fungi isolates having potential as bioinsecticides was done towards the fifth instar larvae of *Tenebrio molitor*. For the first screening, a plate assay using conidia on a plate was done as follows: Ten larvae *T. molitor* put on a conidia-developing plate for 24-hrs which then contacted the conidia. The plate without fungi was used as a control. After 24-hrs, the larvae were transferred into another petri dish and provided fish pellet. The number of dead *T. molitor* was checked every 24-hrs. When the insects were dead, they were removed. The cadavers were transferred

to humid Petri dishes lined with moistened filter paper and incubated and observed. They were examined for external sporulation of fungus to confirm that death was due to mycosis.

Pathogenicity of endophytic fungi against *Spodoptera litura* larvae

One isolate of endophytic fungi that is highly pathogenic on *T. molitor* larvae, was than assayed on the second instar larvae of *S. litura*. The fungi were cultured on PDA for 14 days under 27°C. Ten ml sterile water containing 0.03% Tween 80 was added to petri plate and gently rubbed with sterile spatula to harvest conidia. Conidial suspension was filtered throught three layers of muslin and adjusted to 1x 10^7, 1x10^8 and 1x10^9 conidia mL^{-1} using Neubauer haemocytometer. Fifteen of second instar larvae of *S. litura* were placed in a Petri dishes (90 x 15 mm) with a filter paper at the bottom. Conidial suspensions was sprayed on the dorsal of larval by using hand sprayer. In the control, larvae were treated with sterile distilled water containing 0.03% Tween 80. After that, the larvae were fed with fresh spinach leaf. This experiment was repeated four times and each experiment unit consisted of 15 larvae. The number of dead *S. litura* larvae were checked every 24 h; the results of the 7^{th} day were subjected to probit analysis and LT_{50} values were calculated. This experiment was arranged in the Completely Randomized Design (CRD) and mortality data were analysed by means of the analysis of variance (ANOVA) and Duncan's Multiple Range Test (DNMRT) (P=0.05%)

RESULT AND DISCUSSION

Screening of Isolates

We showed that not all the tested endophytic fungal isolates can infect the larvae. The results of the research indicated that of the 46 isolates of endophytic fungi that were successfully isolated and tested 22 isolates are pathogenic in insects and 24 isolates are not pathogenic. Mortality of *Tenebrio molitor* larvae after application of endophytic fungi varies depending on the isolates. Endophytic fungal isolates tested induced cumulative mortalities between 15 and 30% in fifth instar larvae of *T. molitor* (Table 1). ANOVA of mortality data showed significant difference between endophytic fungal isolates ($df_{22,69}$=4.05; P< 0.001).

Results given in Table 1 indicate the mortality percentage of various isolates on *T. molitor* larvae. C123 isolates imposed higher mortality on larvae in comparison with other isolates; but D227 showed the lowest mortality within the tested population of *T. molitor* larvae ($df_{22,69}$ = 4.05; P < 0.001). Mortality on larvae could be caused not only through direct physical invasion of the hyphae, but also due to some enzymatic mechanisms or toxic metabolites produced by the fungus. Zimmermann (2008) stated that the pathogenicity of the fungi is primarily mediated by entry through the external larval integument. Conidia attach and germinate on cuticle and penetrate into insect body. Upon entry into the hemocoel, the mycelia grow and spread throughout the whole body and then form hyphae and produce blastospores. Host death often occurs due to a combination of fungal toxins, physical obstruction of blood circulation, nutrient depletion and organ invasion.

It can be inferred from our data that there were considerable variations in fungal isolates' virulence towards *T. molitor* larvae. According to Hajek and St. Leger (1994), aggressiveness of entomopathogenic fungus is related to proteolytic, lipolytic and chitinolytic mechanisms that can act after conidial adhesion on the larval cuticle or after invasion of the gut. Infected larvae stop feeding, become lethargic, and die soon due to toxin produced by the fungus in the host. Aerial hyphae appear a day after death and later conidia cover the body giving green colour.

Based on morphological characterization of fungal isolates that are pathogenic in insects, it is found that to belong to one species namely *Aspergillus flavus*. St. Leger *et al.* (2000) reported that *Aspergillus* spp. cause disease in a broad range of organisms, but it is unknown if strains are specialized for particular hosts. Conidia of *A. flavus* were not virulent when applied to the surface of healthy caterpillars.

Table 1. Mortality of fifth instar larvae of *Tenebrio molitor* 7 days post inoculation of the endophytic fungi

Isolates	Mortality (%)	Mycosis (%)
C123	30.00 ± 0.00 a	100.00
B421	27.50 ± 9.57 ab	36.36
C111	25.00 ± 5.77 abc	90.00
C211	25.00 ± 5.77 abc	60.00
C212	25.00 ± 5.77 abc	60.00
D112	25.00 ± 5.77 abc	100.00
C222	22.50 ± 5.00 abc	55.56
D321	22.50 ± 5.00 abc	55.56
A224	20.00 ± 0.00 abc	25.00
C113	20.00 ± 0.00 abc	62.50
D121	20.00 ± 8.16 abc	87.50
D221	20.00 ± 8.16 abc	75.00
B413	17.50 ± 5.00 bc	57.14
C112	17.50 ± 5.00 bc	100.00
C221	17.50 ± 9.57 bc	11.11
D113	17.50 ± 9.57 bc	85.71
D114	17.50 ± 5.00 bc	100.00
D124	17.50 ± 5.00 bc	71.40
C121	15.00 ± 5.77 c	100.00
C321	15.00 ± 5.77 c	16.16
D223	15.00 ± 5.77 c	50.00
D227	15.00 ± 5.77 c	16.67
K	0.00 ±d	0.00

Means followed by same letters within a column are not statistically different at $P < 0.05$ level (ANOVA followed by Duncan test)

However, conidia from all strains were virulent (100% mortality within 48 h) when injected (3,000 spores per larvae). Strains of *A. flavus* did not affect nonwounded bean leaves, corn kernels, or insects at 22°C, but they killed insects following hemocoelic challenge and caused symptoms ranging from moderate to severe in corn kernels and bean leaves injured during inoculation. Carrión and Bonet (2004) reported that there were 13 fungus species associated with the coffee berry borer and its galleries. These fungi were divided into four functional groups: 1) saprobic fungi that the borer uses as food: *Fusarium heterosporum* Nees ex Fries, *Cladosporium* sp., *Cladosporium oxysporum* Berkeley & Curtis, and *Penicillium echinulatum* Fassatiová; 2) saprobic fungi that degrade borer feces: *Aspergillus flavus* Link, *Aspergillus niger* Tieghem, *Mucor luteus* Linnemann, *Penicillium* sp. 1, *Humicola grisea* Traaen, and *Gliocladium penicilloides* Corda; 3) fungi that are coffee parasites: *Fusarium oxysporum* Schlechetend and *Fusarium solani* (Martius) Saccardo; and 4) the entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin.

The results of pathogenicity test presented all isolates of endophytic fungus capable of causing mortality and also forming conidia on *T. molitor* larvae (Table 1) ($df_{22,69} = 4.05$; $P < 0.001$). Sporulation (mycosis) varied among the isolates. Only 5 isolate showed signs mycosis at 100%. C123 isolates also caused high mycosis values on *T. molitor* larvae. Sporulation of entomopathogenic fungi in insect cadavers has been shown to be influenced by many environmental variables such as light, relative humidity (Luz and Fargues, 1998) or saprophytic bacteria or fungi (Chouvenc *et al*., 2012).

Mortality of *S. litura* larvae

Larval mortality of *S. litura* after application on endophytic fungus (C123 isolates) differed

significantly at different conidial concentrations. Mortality of larvae was dose dependent and increased with increasing concentration of conidia. Mortality was 23.33%, 50.00%, and 64.99% at 1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}, respectively ($df_{3,12} = 42.36$; $P < 0.001$) (Table 2). The highest mortality rate (64.99%) was observed at 1×10^9 conidia mL^{-1}

Table 2. Mortality of second instar larvae of *S. litura* 7 days after treatments of different concentrations of endophytic fungus isolates and their median lethal time (LT_{50})

Concentration (conidia mL^{-1})	Mortality (%)	LT_{50} (day)
1×10^9	64.99 ± 9.99 a	6.24 (5.61-7.14)
1×10^8	50.00 ± 8.61 b	8.69 (7.62-10.66)
1×10^7	23.33 ± 8.61 c	13.13 (10.21-23.84)
Control	0.00 ± 0.00 d	-

Means followed by same letters within a column are not statistically different at $P < 0.05$ level (ANOVA followed by Duncan test)

Concentration of conidia influenced positively the mortality of *S. litura* larvae. Higher mortality rate of *S. litura* was observed with increasing conidial concentration of endophytic fungus. This means that the higher number of conidia, giving better opportunities for conidia to attachment, germinate and penetrate into the body of the target pest insects. Conidial concentration was found to be an important factor in the expression of virulence of the fungal isolates. Roberts and Yendol (1971) suggested that one factor for infection of the entomopathogenic fungi in insects is the amount of inoculums. A minimum number of infected units (spores or conidia) must contact a host before diseases induced and exposure to lower numbers will permit escape

Effect of conidial concentration on mortality larvae has been reported by many researchers. Asi *et al.* (2009) reported that mortality of the aphids after application entomopathogenic fungus increased with increase in spore concentration and exposure time. Trizelia and Nurdin (2010) also reported that mortality of second instar *Crocidolomia pavonana* larvae was dependent on the fungal isolates and conidial concentration. Generally with increasing conidial concentrations, mortality also increased.

Baidoo and Ackuaku (2011) also demonstrated that *Aspergillus flavus* could infect *Eldana saccharina* larvae and that its pathogenicity is influenced by dose. The least spore concentration recorded the least larval mortality, whilst the largest concentration recorded the highest mortality.

The median lethal time (LT_{50}), time taken for the death of 50% of *S. litura* due to different concentration ($1x10^7$ to $1x10^9$conidia/ml) were found to be different. The median lethal time (LT_{50}) of *S. litura* larvae at high concentration $1x\ 10^9$ conidia mL^{-1} was shorter as 6.24 days compared to 13.13 at low concentration 1×10^7 conidia mL^{-1}. The median lethal times decreased with increase in conidial concentration (Table 2).

Also according to Sileshi *et a.* (2013), lethal time (LT_{50}) depends on the spore suspension and with the increase in spore suspension there is a decrease in time taken. Moorehouse *et al.* (1994) also demonstrated that LT_{50} was correlated with spore dose. The mode of infection and the type of toxin from endophytic fungus that are pathogenic in insect need further investigation. In the laboratory evaluation, the endophytic fungi were observed to be potent in controlling insect pest of chilli and could be used as bioinsecticides.

ACKNOWLEDGMENT

This research was financially supported by the research grant (020/SP2H/LT/DRPM/II/2016) of the Directorate General of Higher Education, Ministry of Research, Technology and Higher Education, Indonesia.

REFERENCE

Asi, M. R., Bashir, M. H., Afzal, M. and Imran, S. 2009. Effect of Conidial Concentration of Entomopathogenic Fungi on Mortality of Cabbage Aphid, *Brevicoryne brassicae* L. *Pakistan Journal of Life Social Science*, **2**: 175-180.

Azevedo, J. L., Maccheroni Jr, W., Pereira, J. O., and Luiz de Araújo, W. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electronic Journal of Biotechnology*, **3**: 40-65.

Baidoo, P. K. and Ackuaku, S. K. 2011. The effects of spore concentrations of entomogenous fungi on larval mortality and development of the maize stem borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae). *Journal of Applied Bioscience,* **47**: 3221– 3229.

Carrión, G. and Bonet, A. 2004. Mycobiota associated with the coffee berry borer (Coleoptera: Scolytidae) and its galleries in fruit. *Annals of the Entomological Society of America*, **97**: 492-499.

Chouvenc, T., Efstathion, C. A., Elliott, M. L., and Su, N.Y. 2012. Resource competition between two fungal parasites in subterranean termites. *Naturwissenschaften*, **99**: 949-958.

Faeth, S. H. and Fagan, W. F. 2002. Fungal Endophytes: Common host plant symbionts but uncommon mutualist. *Integrative and Comparative Biology*, **42**: 360-368.

Hajek A. E. and St. Leger, R. J. 1994. Interaction between fungal pathogens and insect hosts. *Annual Review of Entomology,* **39**: 293–322.

Hazalin N. A. M. N., Ramasamy, K., Lim, S. M, Wahab, I. A, Cole, A. L and Majeed, A. B. A. 2009. Cytotoxic and antibacterial activities of endophytic fungi isolated from plants at the National Park, Pahang, Malaysia. *BMC Complementary and Alternative Medicine*, **9**:46 doi:10.1186/1472-6882-9-46

Hernawati, H., Wiyono, S. and Santoso, S. 2011. Leaf endophytic fungi of chili (*Capsicum annuum*) and their role in the protection against *Aphis gossypii* (Homoptera: Aphididae). *Biodiversitas*, **12**: 187-191.

Luz, C. and Fargues, J. 1998. Factors affecting conidial production of *Beauveria bassiana* from fungus killed cadavers of *Rhodnius prolixus. Journal of Invertebrate Pathology*, **72**: 97-103.

Moorhouse, E. R., Gillespie, A. T. and Charnley, A. K. 1994. The influence of temperature on susceptibility of vine weevil, *Otiorhychus sulcatus* (Fabricius) (Coleoptera: Curculionidae), larvae *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Annals of Applied Biology*, **124**: 185-193.

Roberts, D. W. and Yendol, W. G. 1971. Use of fungi for microbial control. In: *Microbial Control of Insects and Mites* (Burges, H. D and Hussey, N.W. ed.), Academic Press, New York, 125-149 **PP**.

Senthilkumar,N, Murugesan, S., and Babu, D.S. 2014. Metabolite Profiling of the Extracts of endophytic fungi of entomopathogenic significance, *Aspergillus flavus* and *Nigrospora sphaerica* isolated from tropical tree species of India, *Tectona grandis* L. *Journal of Agriculture and Life Sciences*, **1**: 108-114.

Sileshi, A., Sori, W. and Dawd, M. 2013. Laboratory evaluation of entomopathogenic fungi *Metarhizium anisophilae* and *Beauveria bassiana* against termite, *Macrotermes* (Isoptera: Termitidae)**.** *Asian Journal of Plant Science,* **12***:* 1-10.

St. Leger, R. J., Screen, S. E. and Syams-Pirzadeh, B. 2000. Lack of host specialization in *Aspergillus flavus*. *Applied Environmental Microbiology,* **66**: 320-324.

Trizelia and Nurdin, F. 2010. Virulence of entomopathogenic fungus *Beauveria bassiana* isolates to *Crocidolomia pavonana* Fab. (Lepidoptera: Crambidae). *Agrivita*, **32**: 254-261.

Vega, F.E., Posada, F., Aime, M. C., Pava-Ripoll, M., Infante, F., and Rehner, S. A. 2008. Entomopathogenic fungal endophytes. *Biological Control*, **46**: 72-82.

Zimmermann, G. 2008. The entomopathogenic fungi *Isaria farinosa* (formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* species complex (formerly *Paecilomyces fumosoroseus*): biology, ecology and use in biological control. *Biocontrol Science and Technology*, **18**: 865–901.

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