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Selection of endophytic fungi from Shallot That Potential As Entomopathogens on *Tenebrio molitor* and *Spodoptera litura* larvae

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Abstract

Endophytic fungi are fungi that that live inside healthy plant tissues without causing diseases which can be used as biological control agents of shallot pests. The purpose of this study was to obtain types of endophytic fungi that are pathogenic to *Tenebrio molitor* and *Spodoptera litura* larvae (entomopathogens). Endophytic fungi were isolated from healthy plant (roots, bulbs, stems, and leaves) using Malt Extract Agar (MEA) medium. The pathogenicity test of the isolated endophytic fungus was carried out on the larvae of *Tenebrio molitor* and *Spodoptera litura*. The results showed that out of the 34 isolates tested; only eight isolates (23.52%) were entomopathogenic. Pathogenic nature is indicates the presence of mycosis symptoms in *T. molitor* larvae. The mortality of *T. molitor* larvae after the application of endophytic fungi ranged from 5.0 – 17.5%. These isolates are also pathogenic to *S. litura* larvae, but the mortality ranged from 8-56%. The endophytic fungus that is pathogenic to insect (entomopathogen) belong to *Aspergillus, Fusarium*, and *Trichoderma.*

Keywords: Biological agents, Pathogenicity, *Tenebrio molitor*, *Spodoptera litura*

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INTRODUCTION

Shallots are one of the leading vegetable commodities which intensively cultivated by farmers for a long time. This vegetable commodity belongs to the group of non-substituted spices that function as food seasonings as well as ingredients for traditional medicines. This commodity is also a source of income and employment opportunities that make a fairly high contribution to regional economic development. However, the demand and need for shallots that continue to increase every year cannot be followed by an increase in production (Ambarwati and Prapto, 2003). The main problem in onion farming is the high risk of crop failure caused by the limiting factor in shallot cultivation by *Spodoptera exigua*, *S. litura*, *Liriomyza* sp, *Gryllotalpa* spp (Udiarto *et al.,* 2005) attacks.

To overcome the problem of pests in shallots, conventional control is generally carried out, namely the intensive use of synthetic pesticides. The continuous use of pesticides will cause more serious problems, namely the killing of natural enemies, the occurrence of resurgence, secondary pest explosions, and environmental pollution (Rauf *et al.,* 2000). For this reason, it is necessary to find alternative controls such as Integrated pest control program (IPM) that can reduce the negative impact of these pesticides (Sastrosiswoyo and Oka, 1997). In an integrated pest control strategy (IPM), endophytic fungi is considred as an important components.

Endophytic fungi are fungi that live in plant tissues such as leaves, flowers, twigs, or plant roots without causing symptoms of disease in plants (Clay, 1988; Vega, 2008). Endophytic fungi can produce various functional compounds in the form of anticancer, antiviral, antibacterial, antifungal, and plant growth hormones (Noverita *et al*., 2009). The results of Vega *et al*. (2008) showed that there were 16 species of five genera of endophytic fungi that live in coffee plant tissues, namely Acremonium, Beauveria, Cladosporium, Clonostachys, and Paecilomyces. Beauveria and

Clonostachys are pathogenic to the coffee berry borer. Trizelia and Winarto (2016) reported that in cocoa plants found 3 genera of endophytic fungi namely Beauveria, Aspergillus, and Fusarium which are pathogenic to insects and have the potential to be used as bioinsecticides. Trizelia *et al* (2017, 2020) reported that two types of endophytic fungi were found in chili plants, namely *Beauveria bassiana* and *Aspergillus flavus* which are pathogenic to insects and can be used as bioinsecticides. Trizelia *et al*. (2017) reported that in wheat, two genera of endophytic fungi were found to be entomopathogenic, namely Beauveria and Aspergillus. A first step in the program for the utilization and development of endophytic fungi as biological control agents for shallot pests is to know the natural presence of these fungi in plants. According to Wang *et al*., (2019) the diversity of endophytic fungi is influenced by the type of plant and location. Irmawan (2007) reported that the abundance and diversity of endophytic fungi in rice as influenced by varieties and cultivation techniques. The purpose of this study was to obtain types of endophytic fungi that are pathogenic to insects (entomopathogens).

MATERIALS AND METHODS

Isolation of endophytic fungi

Five healthy shallot plants were collected from agricultural land in the Sungai Pua area, West Sumatera, Indonesia. Isolation of endophytic fungi was carried out by taking all parts of the shallot plant (leaves, stems, tubers, and roots). The shallot plants are washed in running water to clean the dirt. The plant parts were cut to a size of 1 cm, then sterilized by soaking in a 70% alcohol solution for 2 minutes, then transferred to a 3% NaOCl solution for 2 minutes, and washed three times with sterile distilled water. The plant parts were dried with sterile filter paper. After drying, the plant parts were grown in Malt Extract Agar medium (MEA) amended with chloramphenicol (250mg/L) and incubated for one week at room temperature. Fungi growing out from the plant tissues were transferred on to fresh PDA medium. Purification was carried out by transferring the endophytic fungi growing on MEA in a petri dish to a petri dish containing PDA. Endophytic fungi were identified based on cultural characteristics and morphology of fruiting bodies and spores

Screening Endophytic fungus

The first screening of endophytic fungi that are pathogenic to insects was carried out on the fifth instar larvae of *Tenebrio molitor*. The test was carried out by placing 10 *T. molitor* larvae into PDA media containing fungal cultures. The larvae were left in the culture medium for 24 hours to allow contact between the fungal conidia and insects. For control, the larvae were placed in media without endophytic fungi culture. After one day the larvae were transferred to a plastic petri dish with a diameter of 9 cm and provided fish pellets. In this experiment, 40 larvae of *T. molitor* larvae were used for each endophytic fungus isolates. The mortality of the *T. molitor* larvae was observed daily until seven days after inoculation. The cadavers were transferred to Petri dishes lined with moistened filter paper and incubated to confirm that death was due to mycosis. The surface of cadavers with mycosis confirmed the presence of fungal infection, and the fungal isolates were further subjected to the second round of pathogenicity screening to confirm their virulence to *Spodoptera litura* larvae

The fungus that was able to kill *T. molitor* larvae was then tested on *S. litura* larvae. Tests were carried out on second instar *S. litura* larvae. The fungi were cultured on PDA for 14 days under 27°C. Fungal conidia suspension was prepared by adding 10 mL of sterile distilled water and 0.05% Tween 80 to the fungal culture in a petri dish. Conidia were removed from culture using a soft brush and conidial suspension was filtered through three layers of muslin and adjusted to 10^8 conidia/mL using Neubauer hemocytometer. Ten-second instar larvae of *S. litura* were placed in Petri dishes and sprayed with fungal suspension by using a hand sprayer. In the control, larvae were treated with sterile distilled water containing 0.05% Tween 80. Next, the larvae were fed with fresh shallot leaf. This experiment was repeated five times and each experiment unit consisted of 10 larvae. The number of dead *S. litura* larvae was checked every 24 hrs for seven days.

Statistical analysis

Frequency of colonization of endophytes was calculated as total number of segments yielding given fungus divided by total number of segments incubated. The data regarding mortalityof larvae were subjected to analysis of variance (ANOVA). Means were separated using Duncan's Multiple Range Testat 5% significance level to evaluate the impact of treatments on mortality.

RESULTS AND DISCUSSION

Endophytic fungal colonization

The results of observations of endophytic fungal colonization frequency on shallot showed that the ability of fungi to colonize shallot differed between plant parts. The frequency of Colonization of endophytic fungi was higher in the stem compared to the roots, leaves, and bulbs. The lowest colonization of endophytic fungi recorded on bulbs. The frequency of Colonization of endophytic fungi on parts of the shallot plant can be seen in Figure 1.

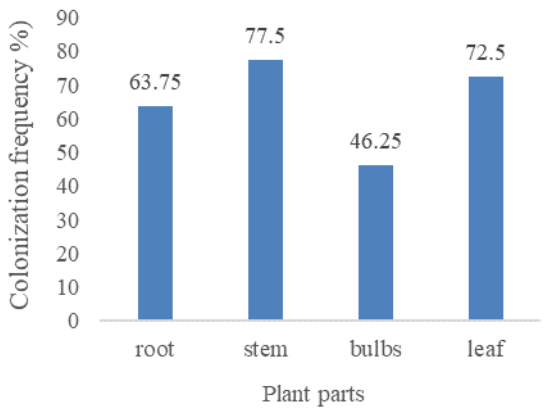

Figure 1. Colonization rate of endophytic fungi on leaf, stem, bulbs and root samples of shallot

The difference in the colonization ability of endophytic fungi on the shallot plant is thought to be due to the specificity of the tissue required by endophytic fungi and their adaptability to plant physiological conditions. Yadav *et al.* (2016) stated that tissue type has a prominent effect on species diversity and colonization frequency of endophytic fungal community on Eugenia jambolana. Muvea *et al.* (2014) reported that the fungal colonization and distribution in onion tissues depended on the inoculation technique, the endophyte selection by the host. tissue morphology and physiology, microbiome interactions. This result is in agreement with reports by Nalini *et al*. (2014) also reported that the colonization of endophytic fungi in medicinal plants was higher in stems compared to roots. The results of this study are different from those reported by several previous researchers. Muvea *et al*. (2014) reported that endophytic fungal colonization in onion plants was higher in roots than in stems and leaves. The results of the study by Kharwar *et al*. (2011) reported that the percentage of endophytic fungus colonization on parts of the *Adenocalymma alliaceum* plant was higher in leaves compared to stems and petiole. Tong *et al*. (2011) revealed that the leaf part is the most commonly penetrated by endophytic fungi in addition to the large leaf surface area and a thin cuticle layer that can provide more surface area for endophytic fungi penetration and colonization. In addition, the role of leaves as a place for plant photosynthesis can affect the increase in the abundance of endophytic fungi.

Screening isolates

The results of the pathogenicity test of the endophytic fungus of shallots against *Tenebrio molitor* larvae showed that of the 34 isolates tested, only eight isolates were entomopathogenic. The mortality of *T. molitor* larvae after application of the endophytic fungus varied among isolates. The results of the analysis of variance showed that the mortality between isolates was significantly different ($F = 3.32$; $df = 34.105$, $P < 0.0001$. The mortality of *T. molitor* larvae ranged from 5-17.5% (Table 1).

Table 1. Mortality of *T. molitor* larvae 7 days after inoculation of the endophytic fungus from shallot

Isolates	Larval mortality (%) ± SE	Mycosis (%)
S1A41	17.5±7.5 a	50.0
S2D23	17.5 ±10.31 a	41.67
S1A22	15± 2.89ab	100.0
S1B33	12.5±4.79abc	75.0
S2D25	10± 7.07abcd	50.0
S1A23	7.5±4.79bcd	50.0
S2D11	5 ±2.89cd	50.0
S1B44	5±5 cd	25.0
Control	0.00 d	0

Means followed by same letters within a column are not significantly different, according to Duncan's Multiple Range Test. ($P \leq 0.05$)

In this experiment, some fungal endophytes from shallot can be pathogenic by causing diseases to the insect. The occurrence of death in *T. molitor* larvae is thought to be caused by physical damage to the larval body due to fungal infection and the presence of toxic compounds produced by the fungus. The host is killed by one or more factors, including nutritional deficiency, tissue destruction, or disruption of normal biological functions through clogging of the vessels with blastospores, or by toxic substances from the fungus that are released into the insect (Tanada and Kaya, 1993; Goettel *et al*., 2000).

Based on the results in Table 1, it was seen that there were significant differences in mortality of *T. molitor* larvae between isolates. Isolates S1A41 isolated from the roots of the shallot plant and S2D23 isolated from leaves produced the highest mortality of 17.5%. The isolates S1B44 isolated from stems and S2D11 isolated from leaves resulted in the lowest mortality of 5%. Based on the pathogenicity screening with *T. molitor* larvae, these fungal isolates showed lower virulence against *T. molitor* larvae (<50% mortality rate). The difference in virulence of endophytic fungi isolates in killing *T. molitor* larvae was thought to be due to differences in physiological characteristics between isolates such as conidial germination, the number of toxins, and enzymes produced. Tanada and Kaya (1993) stated that the difference in pathogenicity of each fungal isolate was caused by differences in the ability to produce enzymes and mycotoxins during the process of infection in insects such as when in contact with the cuticle and in the hemocoel. The virulent isolates had higher enzymes than the avirulent isolates. Bugti *et al*. (2020) stated that virulence and pathogenicity of fungi are greatly affected by biotic factors such as fungal species Shahriari *et al*. (2021) reported that the difference in mortality of *Chilo suppressalis* larvae after the application of entomopathogenic fungi was due to differences in conidial germination, sporulation rates and activities of the extracellular enzymes. Trizelia *et al*. (2017) reported that 46 isolates of endophytic fungi were isolated from chili plants, only 22 isolates were pathogenic to insects larvae of *T. molitor* that are infected by entomopathogenic fungi undergo mummification and the larval body becomes hard. Within one day following death, fungal hyphae began emerging through the cuticle, particularly at the intersegmental regions where the cuticle is thinnest. Within 72 h of host death, cadavers were covered by conidia and mycelia. Based on macroscopic and microscopic identification, endophytic fungi from shallot plants that are pathogenic to insects belong to the genus Fusarium, Aspergillus, and Trichoderma. *T. molitor* larvae infected by endophytic entomopathogenic fungi will have symptoms according to the colony color of the fungus. The larvae of *T. molitor* infected with the fungus Fusarium are covered by white hyphae or mycelia and the larvae of *T. molitor* infected with the fungus Aspergillus are covered by hyphae or mycelia that are light green while the larvae of *T. molitor* infected with the fungus Trichoderma are shown by colored hyphae or mycelium dark green.

The genus Fusarium is one of the most important plant pathogens. However, it has been reported that several types of Fusarium can be pathogenic to insects. Santos *et al*. (2020) reported that at least 30 species and 273 isolates of Fusarium were reported as pathogenic to at least one species of insect. Ten complexes of Fusarium species harbor entomopathogenic fungi, of which *F. incarnatum-equiseti*, *F. fujikuroi*, *F. oxysporum,* and *F. solani*. Mortality varies (5 to 100%) between different isolates against different insects. Aspergillus spp. cause disease in a broad range of organisms, but it is unknown if strains are specialized for particular hosts. Aspergillus spp. are generally regarded as opportunistic pathogens that require wounds or otherwise weakened hosts for colonization. Conidia of *A. flavus* were not virulent when applied to the surface of healthy caterpillars. However, conidia from all strains were virulent when injected 3,000 spores per larvae. Strains of *A. flavus* did not affect nonwounded insects at 22°C, but they killed insects following

hemocoel challenge (St. Leger *et al*. 2000) Trizelia *et al*. (2017) reported that *Aspergillus flavus* isolated from chilli is pathogenic to *T. molitor* larvae, mortality varies from 15-30%. Trichoderma is a genus of filamentous fungi widely used as a biocontrol agent on pathogenic fungi. Trichoderma is also capable of controlling insect pests directly through parasitism and the production of insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites; and indirectly through the activation of systemic plant defensive responses, the attraction of natural enemies, or the parasitism of insect-symbiotic microorganisms (Poveda, 2021). Nasution *et al.* (2018) reported that Trichoderma can infect *Oryctes rhinoceros* larvae. Mortality of larvae range from 60 up to 86.67% at 10 days after application. Endophytic fungi isolates that are entomopathogenic are indicated by the presence of mycosis symptoms in the body of *T. molitor* larvae (Table 1). The percentage of mycoses varied between isolates. Isolate S1A22 produced the highest percentage of mycoses, namely 100%, while isolate S1B44 produced the lowest percentage of mycoses, namely 25%. Wakil *et al*. (2015) reported that the percent mycosis in the cadavers of *Sitophilus oryzae* previously treated with *Metarhizium anisopliae* was affected by fungal concentrations.

Mortality of *S. litura* larvae

The results showed that endophytic fungi isolates had significantly different pathogenicity against second instar *Spodoptera litura* larvae ($F = 18.09$; $df = 8.36$, $P < 0.0001$. S1A41 isolate was the most virulent isolate with the highest average mortality of *S. litura* larvae). ie 56.00% on seven days after inoculation. S1A22 isolate was an isolate that had a low virulence category with a mortality of 8.00% (Table 2).

Previous studies have shown that morphological and physiological characteristics of fungi such as hyphal growth rate, conidial viability, conidia production, conidia size, enzyme secretion affected virulence of entomopathogenic fungus. Chang *et al*. (2021) reported that the amounts of cuticle penetration-related enzymes among different isolates of the same species or different species of entomopathogenic fungi showed a positive correlation with the pathogenesis of the entomopathogenic fungi.

Table 2. Mortality of *S. litura* larvae 7 days after inoculation of the endophytic fungus from shallot

Isolates	Larval mortality (%) ± SE
S1A41	56.00± 5.10 a
S2D11	52.00 ±3.74 a
S2D25	42.00± 5.83 ab
S1B44	42.00±3.74 ab
S1B33	34.00± 5.10 b
S1A23	18±5.83 c
S2D23	12.00 ±5.83 cd
S1A22	8.00±4.89 cd
Control	0.00±0.00 d

Means followed by same letters within a column are not significantly different, according to Duncan's Multiple Range Test. ($P \leq 0.05$)

Different entomopathogenic fungi isolates or species showed variations in the activities of cuticle penetration-related enzymes, which might influence the pathogenesis of entomopathogenic fungi. The different times required to kill insect hosts might also be correlated with the timing or level of cuticle penetration-related enzyme expression. A total of 12 fungal isolates showing pathogenicity and rapid insect-killing activity against *T. molitor* larvae were selected for further pathogenicity screening against *S. litura* larvae. Seven of the selected fungal isolates showed 100% mortality, and the other five isolates showed 80% mortality to *S. litura* larvae after 8-days of treatment. Although the seven fungal isolates showed 100% mortality against *S. litura* larvae at 8 dpi, the length of time required to reach 100% mortality was different, suggesting that different biological characteristics of fungal isolates might influence the infection process. All fungi use a combination of enzymes and mechanical force to penetrate the host cuticle (Butt, 2002).

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