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EFFECT OF INOCULATION TECHNIQUE AND ADDITION OF LARVAL EXTRACT TO PDA MEDIUM ON THE VIRULENCE OF BEAUVERIA BASSIANA AGAINST SPODOPTERA LITURA (L.)

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ABSTRACT

Spodoptera litura Fabricius is an important pest that attacks crops and vegetables in Indonesia. *Beauveria bassiana* has the potential as a biological control agent. This research aimed to evaluate the effect of adding *S. litura* larvae extract (compared to control without larvae extract) in PDA medium combined with different types of inoculation techniques (leaf spray, larval spray, leaf dyeing, and larval dyeing) on fungal virulence *B. bassiana* against *S. litura* larvae. This research using 20 larvae of *S. litura* 2th instar for each treatment. The treatment includes eight combinations of inoculation techniques and the addition of larvae extract on a culture medium of *B. bassiana*. The highest *S. litura* mortality (71.67%) on PDA medium with larval extract and the larvae was dipped in a suspension of conidia. The lowest *S. litura* mortality (45.00%) on PDA medium without the addition of larval extracts and a suspension of conidial was sprayed onto the leaves. The shortest LT50 value (4.58 days) on PDA medium with larval extract and the larvae were dipped in a suspension of conidia. The longest LT50 value (8.09 days) on PDA medium without the addition of larval extracts and a suspension of conidial was sprayed onto the leaves as larvae diet.

Keywords: virulency, *beauveria bassiana*, entomophatogenic fungi, larvae extract.

INTRODUCTION

Spodoptera litura Fabricius is an important pest that attacks crops and vegetables in Indonesia [1]. *Spodoptera litura* is polyphagous which has a broad range of hosts [2]. *Spodoptera litura* is an important pest and can cause yield losses of up to 80% [3]. *Spodoptera litura* attack in the vegetative phase of plant leaves and attacks cause more than 20% damage in plants aged more than 20 days after planting [4]. Damage and yield loss due to *S. litura* pests are determined by insect populations, insect stages, plant stages, and plant vulnerability [5]. Management control of S. litura generally uses synthetic chemical insecticides and results in a negative impact on the environment and human health. Finding alternate pest control such as biological control agents is need to solve the above-mentioned problems [6]. Pest control that is safe for the environment by utilizing biological agents such as entomopathogenic fungi.

Entomopathogenic fungus *Beauveria bassiana* (Balsamo) has the potential as a biological control agent in controlling various pests. *Beauveria bassiana* causes illness and death in several larvae of the order Lepidoptera, Coleoptera, Hemiptera, and Hemiptera [7]. The virulence of insect pathogenic fungi is determined by the conidia produced. The conidia produced can influence insect pathogenic fungal infections in integument insects. The fungus *B. bassiana* has a high reproductive capacity is easily produced but in the production process often there is a decrease in virulence [8]. The virulence of *B. bassiana* fungi in *S. litura* larvae is low because the conidia are not virulent and the number of conidia attached to the larvae is also low. Low virulence can be influenced by the origin of isolates, production media, and inoculation techniques [7]. Virulence of *B. bassiana* can be increased by the addition of larval extracts in PDA (Potato Dextrose Agar) production medium and conidial suspension inoculation techniques that increase conidial contact in the insect integument. The inoculation technique consisted of leaf spray, larva spray, leaf dip, and larva dip. Virulence of *B. bassiana* by inoculation through *Hyphantria cunea* larvae was increased compared to leaf dyes as diet [9]. Virulence of B. bassiana by inoculation through spray *Chilo partellus* larvae increased compared to leaf spray as diet [10]. Inoculation techniques in larvae increase *B. bassiana* virulence rather than leaves as diet. The addition of larvae extract in the production medium SDAY (Sabouraud Dextrose Agar Yeast) increased the virulence of the fungus *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin against *S. litura* larvae [11]. The addition of larvae extract in production media resulted in an increase in virulence of *B. bassiana* fungi compared without the addition of larvae extract. The combination of the addition of larvae extract in PDA production media and conidial suspension inoculation techniques can increase the virulence of *B. bassiana* fungi against *S. litura* larvae. This research aimed to evaluate the effect of adding *S. litura* larvae extract (compared to control without larvae extract) in PDA medium combined with different types of inoculation techniques (leaf spray, larval spray, leaf dyeing and larval dyeing) on fungal virulence *B. bassiana* against *S. litura* larvae.

MATERIALS AND METHODS

Research Design

This research was conducted at the Mycology and Nematology Laboratory of the Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya from April to September 2015. The experimental design was a completely randomized design with eight treatments and three replications, so there are 24 experimental units. The treatments included eight combinations of *B. bassiana* inoculation techniques and the addition of larval extracts and without larval extracts to the production media. This treatment was used by Potato Dextrose Agar (PDA) medium. Each treatment consisted of 20 larvae of *S. litura* 2th instar (Table-1).

Table-1. The combination of *B. bassiana* inoculation techniques and the addition of larval extracts to the production media.

No.	Treatment		
	Inoculation technique	**Addition of Larva Extract**	
1.	Spray leaves	+	-
2.	Dyed leaves	+	-
3.	Spray larvae	+	-
4.	Dyed larvae	+	-

Note: (-): Without the addition of larval extract and (+): With the addition of larval extract

Insect Rearing

Spodoptera litura larvae were used in this study obtained from soybean cropping. Then, the larvae were reared in the laboratory. The larvae were kept in plastic jars (d= 30 cm and h= 35 cm) and give fresh soybean leaves as diet. The larvae that will form pupae were placed separately on a plastic jar by giving soil (3 cm thick). After the pupa becomes Moth and placed in another plastic jar and fed with a 10% honey solution. Inside the jar wall, was hung gauze for Moth lay eggs. The eggs produced were transferred to a separate jar and kept until they become larvae. The larvae used in this study were 2th instar larvae and 5th instar larvae as extract larvae [12].

Beauveria Bassiana Isolate Preparation and Addition of *S. Litura* Larvae Extract

Beauveria bassiana isolates were grown on Potato Dextrose Agar (PDA) and incubated at room temperature for 21 days. Purification was carried out by taking part in the uncontaminated *B. bassiana* from the PDA medium. *Spodoptera litura* larvae extracts from 5th instar were obtained from the multiplication of larvae, weighed 10 grams and crushed and immersed in 0.89 gr of sodium chloride in 100 ml of sterile water. Then the extract was filtered using filter paper. The media used was the PDA medium. 100 ml of PDA media were given *S. litura* larvae extract with a concentration of 10% [11]. PDA medium with larvae extract according to the treatment were autoclaved at 121^{o}C at a pressure of 1 atm for 30 minutes. PDA medium was poured into the petri dish in LAFC. Then inoculated *B. bassiana* on PDA medium by taking culture using an *ose* needle, then incubated at room temperature.

Preparation of *B. Bassiana* Suspension

For application purposes, B. bassiana culture results from the addition of *S. litura* larvae extract on PDA medium. *Beauveria bassiana* culture (3 weeks age) was mixed with 10 ml of sterile water. Then added 0.01% of Polysorbate 80 and conidia was shaken slowly for 3 minutes and shed with L sticks. Then filtered using filter paper. A conidia suspension of *B. bassiana* was taken 1 ml with a sterile dropper pipette and then drops on the hemocytometer and covered with a glass cover. Then the conidia density was calculated under a binocular microscope at 400x magnification. Conidia density was calculated using the formula as follows [13]:

$$C = \frac{t}{(nx0.25)} \times 10^6$$

Where,

C = the conidia density per ml of solution,
t = the total number of conidia in the observed sample box,
n = the number of observed sample boxes (5 large boxes × 16 small boxes), and 0.25 is a correction factor for the use of small scale sample boxes on Haemocytometer.

Spore viability was determined by spore suspension incubated for 24 hours. After that one drop of the suspension was dripped on a glass preparation and covered with a glass cover. Then the number of conidia that germinate and not germinate was counted under a microscope at 400x magnification. Spore viability was calculated at 24 hours after incubation. Spore viability was calculated using the formula as follows [13]:

$$VG = \frac{v}{(g+u)} \; x \; 100\ \%$$

Where,

V = the spore germination (viability),
g = the number of spores that germinate, and
u = the number of spores that not germinate.

Efficacy of *B. Bassiana* against *S. Litura* Larvae

Beauveria bassiana was grown on PDA medium and PDA medium was added with larval extracts were tested by spray inoculation on larvae and soybean leaves as *S. litura* diet. This spray inoculation technique was using hand sprayers. Inoculation spray-on larvae was used as many as 20 *S. litura* larvae (2th instar). Larvae were sprayed with 2 ml of *B. bassiana* inoculum with a density of 10^7 conidia/ml [11]. Then the *S. litura* larvae were carefully transferred using tweezers into a plastic glass covered with gauze. Each plastic cup filled with larvae

diet (fresh soybean leaves) and the diet was replaced every 24 hours. Spray inoculation on the leaves was used fresh soybean leaves. Fresh soybean leaves were sprayed with 2 ml of *B. bassiana* inoculum with a density of 10^7 conidia/ml [11]. Sprayed leaves were given to the larvae as diet. Twenty of *S. litura* larvae (2^{th} instar) were transferred to glass plastic using a soft brush. Plastic cups were covered with gauze cloth. Each plastic cup filled with larvae and soybean leaves as diet which had been sprayed with *B. bassiana* suspension. On the following day, the diet was replaced with fresh soybean leaves every 24 hours.

For dipping treatment, *B. bassiana* was grown on PDA medium and PDA medium was added by larval extract were tested by dipping the inoculation of larvae and soybean leaves as *S. litura* diet. Dip inoculation on larvae used 20 larvae of *S. litura* 2^{th} instar. Larvae was dipped in suspension *B. bassiana* with a density of 107 conidia/ml for 5 seconds and dried [11]. Then the *S. litura* larvae were carefully transferred using tweezers into a plastic glass covered with gauze. Each plastic cup filled with larvae and fresh soybean leaves as diet, the diet was changed every 24 hours. Dye inoculation on the leaves used fresh soybean leaves. Fresh soybean leaves were dipped in *B. bassiana* suspension with a density of 107 conidia/ml for 5 seconds and dried [11]. Dyed leaves were fed to the larvae as a diet. A total of 20 larvae of *S. litura* (2th instar) transferred to a plastic cup using a soft brush. Each plastic cup filled with larvae was fed soybean leaves which had been dipped in *B. bassiana* suspension. On the following day, the diet was replaced with fresh soybean leaves every 24 hours.

Mortality of *S. litura* larvae was observed every 24 hours for 8 days by recording the number of dead larvae. Observations were made by counting the number of larvae that died due to treatment and expressed in percent. The percentage of larval mortality was calculated using the formula according to are as follows [14]:

$$M = \frac{A}{D} \times 100\ \%$$

Where,
M = the percentage of larvae that died,
A = the number of larvae that died infected with fungi, and
D = the number of larvae tested.

Data Analysis

The percentage of viability was analyzed by t-test with a level of 5%. Data on the percentage of S. litura larvae mortality were analyzed by ANOVA with a level of 5%. If it shows a significant effect on the treatment, a follow-up test with Duncan test is carried out with an error level of 5%. To determine the Median Lethal Time (LT50) in S. litura larvae the probit analysis application was used [15].

RESULTS AND DISCUSSIONS

Mortality of *S. Litura* Larvae

The results showed that eight combinations of conidia inoculation techniques and the addition of larval extracts on PDA media were significantly affected the mortality of S. litura larvae. The highest mortality (71.67%) was produced by B. bassiana fungal infections from the media which added larvae extract and the larvae were dyed in conidia suspension (Table-2). The lowest mortality (45.00%) was produced by B. bassiana fungi without larval extract and conidia suspension was sprayed on the leaves as a larval diet (Table-2). The highest S. litura larvae mortality was also caused by a fungal infection of B. bassiana from the media added by extracts of larvae and larvae sprayed with conidial suspension (Table-2).

Table-2. The mortality of *S. litura* larvae with different inoculation technique and addition of larvae extract.

Treatments Perlakuan		Mortality (%)
Inoculation technique	Addition of larvae extract	
Sprayed leaves	-	45.00 a
Sprayed leaves	+	58.33 bc
Sprayed larvae	-	50.00 a
Sprayed larvae	+	66.67 de
Dyed leaves	-	46.67 a
Dyed leaves	+	63.33 cd
Dyed larvae	-	51.67 ab
Dyed larvae	+	71.67 e

Note: Numbers followed by different letters in the same column are significantly different in the Duncan test (α = 0.05), (-): Without the addition of larval extract and (+): With the addition of larval extract

The highest mortality was caused by the *B. bassiana* on PDA media which added larval extract. This shows that *B. bassiana* on medium containing larvae extracts was associated with the availability of certain nutrients. Dayakar and Subbarao [11] state that, the higher mortality of *S. litura* was produced by *B. bassiana* fungus on SDAY media with the addition of larval extracts rather than without larval extracts. Prasad [16] reports that higher *S. litura* mortality was produced by *B. bassiana* on carrot medium was added *S. litura* larvae extracts than without larvae extracts. The addition of larvae extract can increase the virulence of *B. bassiana* even though the production medium used is different. The mortality of *S. litura* larvae caused by the number of conidia that attach to the integument and enter the larval body. The highest mortality was found in a combination of dyed larvae inoculation techniques and the addition of larval extracts, due to the number of *B. bassiana* conidia which was more attached to the *S. litura* larvae integument. Zibaee *et al.* [9]

reported that inoculation of larvae dipped in a *B. bassiana* conidia suspension resulted in the highest mortality of *H. cunea* larvae. Trizelia *et al.* [17] also reported that inoculation of larvae sprayed with entomopathogenic fungi produced the highest mortality of *C. pavonana* larvae compared to sprayed leaf inoculation.

The highest percentage mortality was caused by *B. bassiana* from the medium which was added by extracts of larvae and larvae dipped in conidia suspension. This is due to the *B. bassiana* conidia was more attached to the *S. litura* larvae integument and have the highest conidia viability of 64.83% (Table-3). The results showed that the t-test on the conidia viability rate of *B. bassiana* from PDA medium and PDA medium was added larval extracts showed significantly different. The highest viability of conidia will accelerate and determine the success of the fungal infection process to the larval body. Prayogo [7] state that, if the viability of conidia is higher, the germination will be shorter (in the process of fungal infection of the larval body). The percentage increase in viability of *B. bassiana* conidia on PDA medium was added larval extract was 15.78% (Table-3). The kind of culture medium of *B. bassiana* determines the increased viability of conidia. Dayakar and Subbarao [11] reported that the increase in viability of *B. bassiana* conidia on SDAY media plus larvae extract was 4.17% compared with no addition of larvae extract. Herlinda *et al*. [8] also reported that the increase in viability in SDB media with the addition of clove flour by 9.82% compared without the addition of clove flour.

Table-3. The average viability of *B. bassiana* conidia from PDA medium with and without the addition of larval extract.

Kind of medium	Conidia viabilities (%)	Viability Increase (%)
PDA	49.05	0
PDA + larvae extract	64.83	15.78

Virulency of *S. Litura* Larvae

The results showed that, the shortest LT_{50} value (4.58 days) was produced from the media which added larvae extract and the larvae were dyed in conidial suspension (Table-4). The longest LT_{50} value (8.09 days) is produced by medium without larval extract and conidia suspension is sprayed on the leaves as larval diet (Table-4). This shows that the time was needed for *B. bassiana* from PDA medium was added larval extract with dyed larvae can kill 50% of *S. litura* larvae fastest than other combinations. This can be influenced by the presence of nutrients in the propagation media and conidia of *B. bassiana* more closely attached to the integument of S. liura larvae. According to Dayakar and Subbarao [11] stated that the decrease in LT_{50} values of *B. bassiana* from the culture medium added by larval extracts could be related to the presence of nutrients in the larval extracts. According to Tefera and Pringle [10], larvae dipped in conidia of *B. bassiana* increase conidial germination in the integument and folds of larvae cuticles *C. partellus,* so that LT_{50} values tend to be short.

Table-4. LT_{50} values from B. bassiana medium with and without larval extract and different inoculation techniques against S. litura larvae.

Treatments Perlakuan		LT_{50} (days)
Inoculation technique	Addition of larvae extract	
Sprayed leaves	-	8.09
Sprayed leaves	+	6.56
Sprayed larvae	-	8.06
Sprayed larvae	+	5.04
Dyed leaves	-	7.06
Dyed leaves	+	6.32
Dyed larvae	-	7.92
Dyed larvae	+	4.58

Note: (-): Without the addition of larval extract and (+): With the addition of larval extract

CONCLUSIONS

The highest virulence of *B. bassiana* fungi based on the highest mortality and the shortest LT_{50} value was caused by added larvae extracts on PDA medium and larvae dyed in conidial suspension. Beauveria bassiana infections from the medium were added by larvae extracts and larvae sprayed with conidia suspension also produce the highest mortality. In contrast, the lowest virulence of *B. bassiana* based on the lowest mortality observation variable and the longest LT_{50} value was caused by *B. bassiana* and the leaves were sprayed with conidia suspension. Larvae extract added to the PDA medium caused conidia viability to be higher, mortality increased, and LT_{50} value was shorter and can increase *B. bassiana* virulence against *S. litura* larvae.

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