

Effect of Leaf Litters and Soils on Viability of Entomopathogenic Fungi *Beauveria bassiana* (Bals.) Vuill

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Viability of *Beauveria bassiana* is extremely low due to toxic compounds in soils. This research was aimed to study the effect of four groups of media on viability of *B. bassiana* Bb-Pb2. The first group was leaf litters of onion, flowering white cabbage, cabbage, and chinese mustard, respectively; the second group was the soils containing decomposed residues of each plant of the first group; the third group was the mixtures of each media of both groups above (1:1), and the fourth group was natural top soil as a control. Each plastic bag filled with one kg of each medium was inoculated with ten ml of *B. bassiana* conidia (10⁶/ml of concentration) and incubated in open area for 8 weeks. The results showed that all leaf litters of those plants and their compost soils affected the fungal viability. The highest decreasing number of colony was found on onion's leaf litters, soil containing of decomposed onion, and the mixtures of both media. The treated *B. bassiana* showed significant reducing abilities of growth, conidia production and conidia germination on PDA media, except the one of control. It is suggested that the Bb-Pb2 isolate might not be effective as bioinsecticide in the soils containing either those leaf litters or composts.

Key words: *Beauveria bassiana*, viability, leaf litters, soils

INTRODUCTION

Onion (*Allium cepa*), flowering white cabbage (*Brassica rapa*), cabbage (*Brassica oleracea*), and chinese mustard (*Brassica juncea*) are horticulture commodities mostly consumed by people. However, plant pests i.e., *Spodoptera litura* and *Crociodomia pavonana* were a major constraint in their production (Negara 2003). Entomopathogenic fungi, *Beauveria bassiana* (Bals) Vuill (Deuteromycotina: Hyphomycetes) can be used to control this pests (Prayogo & Tengkan 2004a,b). However the infection ability of this fungi was depend on the viability and the persistence of conidia and mycelia in soils (Boot *et al.* 2004; Behle 2006).

High viable conidia have more chance to contact with hosts and therefore epizootic event has more chance to occurred (Klingen *et al.* 2002). Various factors such as light, soil types, the presence of plant residues, pesticides, soil-borne organisms, and secondary metabolite compounds of the plants, influence the viability of conidia (Gardner *et al.* 1977; Storey & Gardner 1988; Inglis *et al.* 1993; Quintela & McCoy 1998; Todorova *et al.* 1998; Batista *et al.* 2001; Klingen *et al.* 2002; Ekesi *et al.* 2003).

Farmers used plant wastes like leaves and stems as an organic fertilizer. In cabbage (Brassicaceae) plantation, fresh plant waste was buried directly, then high content of plant metabolite compounds was accumulated in the soils. The compound like isothiocyanates produced by Brassicaceae

clearly inhibit the growth of the entomopathogenic fungi (Inyang *et al.* 1999; Klingen *et al.* 2002), although they are produced by plants of the same family, the inhibition activities are different (Dawson *et al.* 1993; Vega *et al.* 1997; Inyang *et al.* 1999).

To control *C. pavonana*, conidia of *B. bassiana* were applied on cabbage leaves, therefore, no effect of the toxic compounds might be persistent in the soils influenced fungal growth. However, if *B. bassiana* is applied in soil to control soil pests, such as *Cyrtospora formicarius* which attacks sweet potatoes, we should consider either the presence of toxic compounds or the content of organic matters. *B. bassiana* was well adapted to the soils with a low organic matter content, approximately 1-2%, high pH (8.0-8.5) and a high clay content (10-20%) (Quesada-Moraga *et al.* 2007). Nutrient requirement for growth and sporulation was different for each fungal strain, as shown by two strains of entomopathogenic fungi *Paecilomyces lilacinus* M-14 and *P. lilacinus* IPC-P which need a C:N ratio of 10:1 and 20:1 respectively (Gao *et al.* 2007).

Basic information of Liliaceae and Brassicaceae families influence on entomopathogenic fungi viability is limited in Indonesia, the same goes for *B. bassiana* Bb-Pb2. This isolate would be grown on leaf litters of onion (Liliaceae), flowering white cabbage, cabbage, and chinese mustard (Brassicaceae), respectively and in the soils containing of those decomposed plants, and followed by observing its growth, conidia production and germination on potato dextrose agar (PDA) media in order to see further effect of those leaf litters and soils. Similar method was done by Tefera and Pringle (2003)

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who measured the number of germinated conidia as indication of fungal viability.

MATERIALS AND METHODS

Preparation of Treated and Control Media. Four groups of media were tested in this study. The first group was leaf litters of onion, flowering white cabbage, cabbage, and chinese mustard, respectively. The leaf litters including their stems were chopped for about 2 cm in length and allowed to dry at room temperature for seven days. The second group was soils containing decomposed residues of each plant of the first group which were taken from 15 cm in depth of top soils where farmers buried and let the plant wastes decomposed for approximately two weeks. Both medium groups were obtained from vegetable fields at Ciloto, West Java. The third group was mixed media of first and second group at the ratio 1:1. The fourth group was the natural top soil as control. One kg of each medium was put in plastic bag and placed in open area for one week before inoculation of conidia. The experiments were conducted from April to June 2007 and arranged in a complete random design with each treatment consisted of three replicates.

Preparation of Fungal Inoculum. *Beauveria bassiana* Bb-Pb2 from culture collection of Indonesian Legumes and Tuber Crops Institute (ILETRI), Malang, East Java, was originally isolated from death *Riptortus linearis* insect found at corn field, in Probolinggo (East Java), in 2005. The isolate had been subcultured five times on PDA media.

Bb-Pb2 isolate was grown on PDA media in Petri dish of 5.5 cm in diameter and incubated at room temperature (27-30 °C) for 30 days. Each culture was then added with ten ml of sterile water. The conidia were scrapped with a paint soft brush and the suspension was transferred into test tubes. The conidia were counted using a haemocytometer. The suspensions were diluted in order to get a conidial concentration of 10^6 /ml.

Inoculation of Conidia. Inoculation was carried out one week after the media were placed at open area, by spreading ten ml of conidial suspension on the surface of each media in plastic bag. The media were then further left at open area for eight weeks.

Determination of Colony Number. The number of fungal colonies in each media was counted every week up to eight weeks. For this purpose, 1.0 g of sample was taken from four sampling points of the media surface layer. The samples were mixed thoroughly and 1.0 g was diluted with 9.0 ml sterile water in a test tube and shaken vigorously using vortex for 30 seconds. One milliliter of the suspension was poured into Petri dish and added with 10 ml of PDA containing 2.0 g/l of chloramphenicol. The Petri dish was swirled gently with a rotary motion in order to get the homogeneous mixture. All media were incubated at 27 °C for three days and the colony forming-units (cfu) were counted under a binocular microscope of 40x magnification.

The Growth of Colonies and Conidia Production on PDA Media. All mycelial colonies obtained from the previous work, were then subcultured on PDA media and incubated at room

temperature. These experiments were repeated four times. Fourteen days after incubation, the diameter of each colony was measured. Afterward the colonies were incubated for another 16 days. One gram of these colonies was cut out and put into test tube filled with 10 ml of sterile water, stirred using vortex for 60 seconds, and then filtered using sterile gauze. The number of conidia in these suspensions was counted using a haemocytometer.

Germination of Conidia. The percentage of conidial germination was determined by incubating the suspensions at room temperature (27-30 °C) for 10 hours. The counting was done at one smallest square of haemocytometer and repeated for 9 other smallest squares. Percentage of germinated conidia was calculated using the equation: $Pgc = (a/b) \times 100\%$ whereas Pgc, a, and b indicated the percentage of germinated conidia, number of germinated conidia, and total number of conidia for one smallest square, respectively.

Data Analysis. Data were firstly transformed to $\arcsin \sqrt{x}$ before analyzing with MINITAB 14 program. Variance analyses (ANOVA) were performed and followed by the Duncan's Multiple Range Test (DMRT) at $\alpha = 0.05$.

RESULTS

Colony Number of *B. bassiana* from Treated and Control Media. The results of fungal colony number indicated that the use of the first (leaf litters), second (the soils containing of each decomposed plant of the first group), and third (the mixtures of each media of both groups) groups of media significantly affected the viability of *B. bassiana* Bb-Pb2. These results were based on an average values of the fungal colony number of each group. It was clear that the tree former groups produced less colony number than that of control media (Table 1).

The longer *B. bassiana* was incubated in all treated media, the less number of colonies could be isolated. At first week of incubation, the colony numbers of first group were 6.00-37.67 x 10⁵ cfu/ml with an average (Av.) of 17.92 x 10⁵ cfu/ml; for second group 7.00-28.67 x 10⁵ cfu/ml (an Av. of 14.08 x 10⁵ cfu/ml), for third group 6.00-42.00 x 10⁵ cfu/ml (an Av. of 20.08 x 10⁵ cfu/ml); and for fourth group reached an Av. of 67.00 x 10⁵ cfu/ml. At eight weeks of incubation, the colony number greatly decreased by as much as 0.00-1.00 x 10⁵ cfu/ml (an Av. of 0.40 x 10⁵ cfu/ml) for first group, but no colonies for second and third groups were obtained since the seventh week. In contrast, the colony number of control media was still high for up to 38.00 x 10⁵ cfu/ml at eight weeks of incubation (Table 1).

At first group, the highest colony number was found on flowering white cabbage's leaf litters (FWCLL), followed by chinese mustard's leaf litters (CMLL), cabbage's leaf litters (CLL), and the lowest colony number was found on onion's leaf litters (OLL). At eighth week, the fungal colonies were still obtained from all leaf litters except from OLL which had no colony growth after three weeks of incubation (Table 1). Similar results were shown by the second group, but the fungal colonies had not been obtained any more from soil containing decomposed flowering white cabbage (DFWC),

soil containing decomposed chinese mustard (DCM), soil containing decomposed onion (DO) since the sixth week. For soil containing decomposed cabbage (DC), the fungal colonies had not been obtained any more since the seventh week (Table 1).

At third group, the highest colony number was found on the mixture media of chinese mustard's leaf litters with their composts (CMLL + DCM), followed together by the mixture media of either flowering white cabbage's leaf litters or cabbage's leaf litters with their composts (FWCLL + DFWC; CLL + DC, respectively). The lowest colony number was the mixture media of onion's leaf litters with their composts (OLL + DO) similar to OLL and DO. The fungal colonies had not been found any more from all media since the sixth week except

from the mixture media of flowering white cabbage's leaf litters with their composts (FWCLL + DFWC) which had no more colonies since the seventh week (Table 1).

Based on the fungal colony number of all media, the highest one was found in the mixture media of chinese mustard's leaf litters with their composts (CMLL + DCM) and the longest persistence time of *B. bassiana* (8 weeks) was found at CLL, FWCLL, and CMLL media.

The Growth of Fungi on PDA Media. The results showed that growth abilities of all fungal isolates obtained from two weeks' old treated media were decrease when they were grown on PDA media. The average diameter of colonies were in a range of 4.22-4.45 cm, but the ones obtained from the control media could reach 7.83 cm (Figure 1).

Table 1. The colony number of *Beauveria bassiana* from four groups of media counted every week during incubation

Treatments	Average colony number ($\times 10^5$) cfu/ml at (n) week*							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th
OLL	6.00e	0.67e	0.00d	0.00d	0.00c	0.00b	0.00b	0.00b
FWCLL	37.67bc	20.67bc	14.33bc	9.00bcd	6.67bc	0.67b	0.34b	0.30b
CLL	8.33de	8.00cde	7.00cd	6.67cd	4.00bc	1.34b	1.34b	1.00b
CMLL	19.67cde	18.00bcd	6.34cd	3.00cd	2.67c	0.34b	0.34b	0.30b
Average	17.92	11.83	6.92	4.67	3.33	0.59	0.51	0.40
DO	7.00e	4.00de	3.67cd	1.4cd	0.34c	0.00b	0.00b	0.00b
DFWC	28.67bcd	22.33bc	13.34bc	8.00bcd	3.34c	0.00b	0.00b	0.00b
DC	11.00de	9.00cde	5.67cd	4.67cd	3.34c	0.34b	0.00b	0.00b
DCM	9.67de	9.34cde	6.34cd	4.67cd	4.34bc	0.00b	0.00b	0.00b
Av.	14.08	11.17	7.25	4.67	2.84	0.09	0.00	0.00
OLL + DO	6.00e	3.33de	0.34d	0.34d	0.34c	0.00b	0.00b	0.00b
FWCLL + DFWC	17.66cde	14.00bcde	13.00bc	12.00bc	8.67bc	0.67b	0.00b	0.00b
CLL + DC	14.67de	14.33bcde	13.34bc	11.67bc	10.00bc	0.00b	0.00b	0.00b
CMLL + DCM	42.00b	25.67b	23.00b	18.67b	14.67b	0.00b	0.00b	0.00b
Average	20.08	14.33	12.42	10.67	8.42	0.17	0.00	0.00
CS	67.00a	62.00a	67.67a	60.34a	59.00a	52.00a	47.67a	38.00a
DC (%)	23.96	29.21	24.21	29.98	30.99	26.15	15.90	24.21
DMRT 0.05	18.56	13.23	10.62	9.53	9.77	1.84	1.68	3.55

*Data were first transformed to $\arcsin \sqrt{x}$ before examination. The averages on the columns that followed by the same letters are not significant based on Duncan Multiple Range Test (DMRT) at α value = 0.05. OLL: onion's leaf litters, FWCLL: flowering white cabbage's leaf litters, CLL: cabbage's leaf litters, CMLL: chinese mustard's leaf litters, DO: decomposed onion, DFWC: decomposed flowering white cabbage, DC: decomposed cabbage, DCM: decomposed chinese mustard, CS: control soil, DC: diversity coefficient.

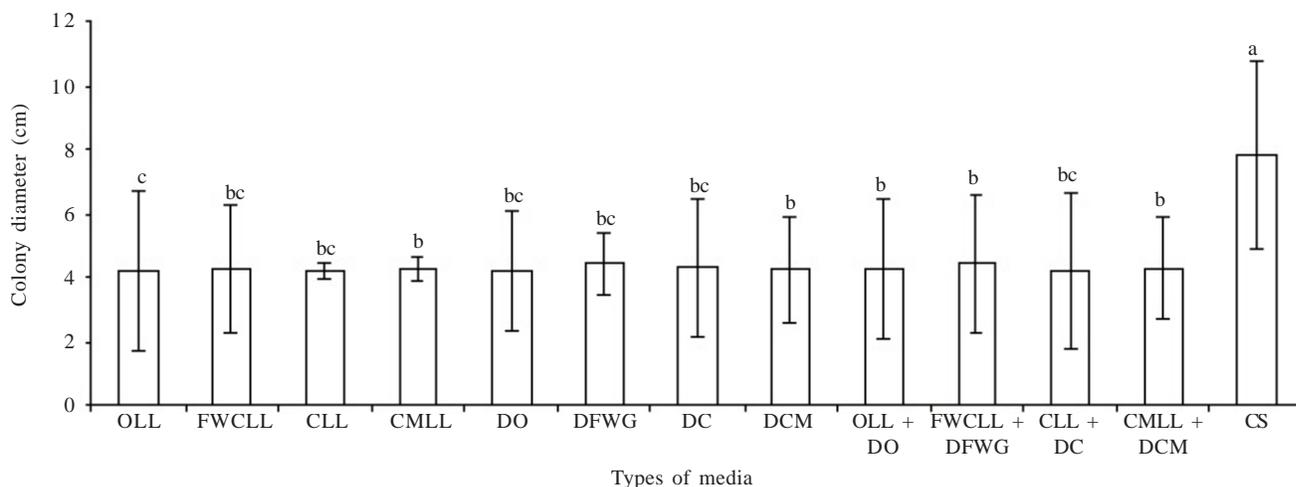


Figure 1. The growth of *B. bassiana* on PDA media after their growth in several types of media. OLL: onion's leaf litters, FWCLL: flowering white cabbage's leaf litters, CLL: cabbage's leaf litters, CMLL: chinese mustard's leaf litters, DO: decomposed onion, DFWC: decomposed flowering white cabbage, DC: decomposed cabbage, DCM: decomposed chinese mustard, CS: control soil. The same letters on bars indicate statistical insignificance based on Duncan Multiple Range Test (DMRT) at α value = 0.05. Values are means of four independent replicates \pm standard deviation (vertical bars).

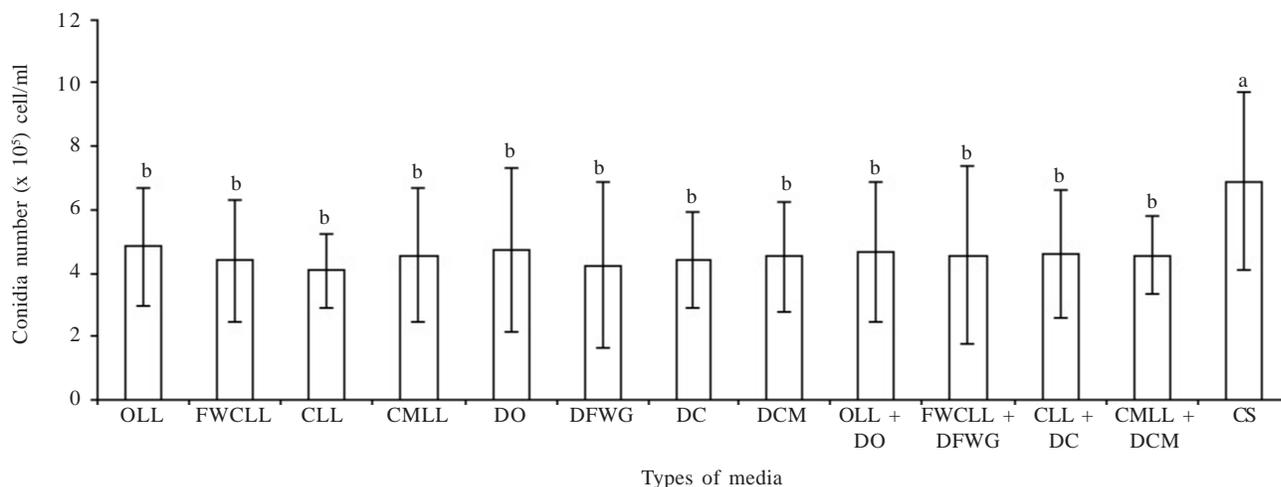


Figure 2. Conidia Number of *B. bassiana* produced on PDA media after their growth in several types of media. OLL: onion's leaf litters, FWCLL: flowering white cabbage's leaf litters, CLL: cabbage's leaf litters, CMLL: chinese mustard's leaf litters, DO: decomposed onion, DFWC: decomposed flowering white cabbage, DC: decomposed cabbage, DCM: decomposed chinese mustard, CS: control soil. The same letters on bars indicate statistical insignificance based on Duncan Multiple Range Test (DMRT) at α value = 0.05. Values are means of four independent replicates \pm standart deviation (vertical bars).

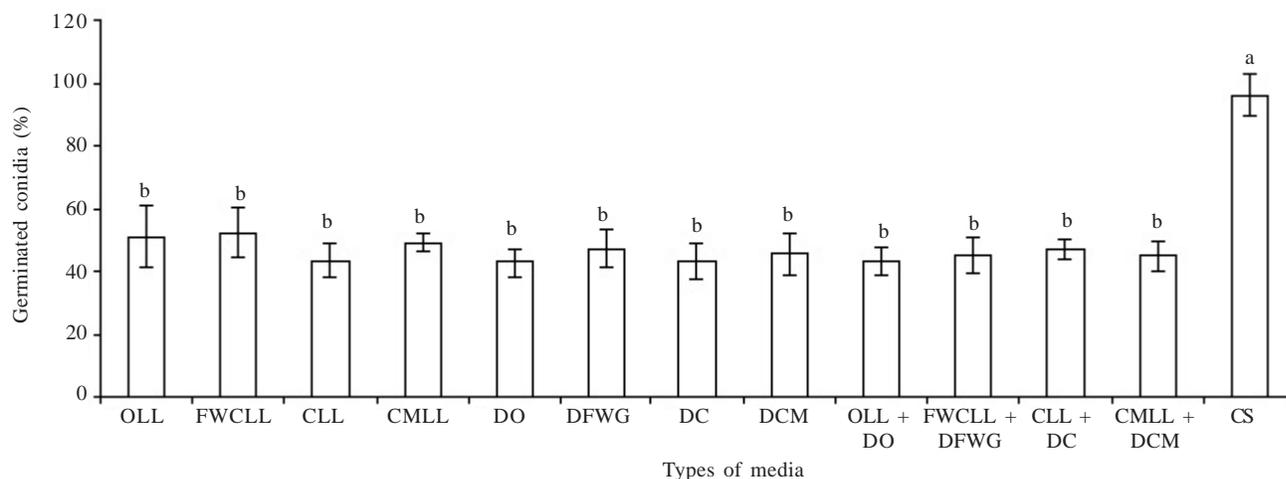


Figure 3. Germinated conidia of *B. bassiana* after their growth in several types of media. OLL: onion's leaf litters, FWCLL: flowering white cabbage's leaf litters, CLL: cabbage's leaf litters, CMLL: chinese mustard's leaf litters, DO: decomposed onion, DFWC: decomposed flowering white cabbage, DC: decomposed cabbage, DCM: decomposed chinese mustard, CS: control soil. The same letters on bars indicate statistical insignificance based on Duncan Multiple Range Test (DMRT) at α value = 0.05. Values are means of ten independent replicates \pm standart deviation (vertical bars).

The Production of Conidia on PDA Media. The results showed that the abilities of conidia production of all fungal isolates obtained from two weeks'old treated media were decrease when they were grown at PDA media. They produced conidia in a range of 4.10 - 4.86×10^5 cells/ml but the ones of control media produced 6.89×10^5 of conidia (Figure 2).

Germination of Conidia. The results showed that the germination abilities of conidia of all fungal isolates obtained from two weeks'old treated media were decrease. The percentage of germinated conidia was significantly lower in a range 43-52% than the ones of control media that could reach 95% (Figure 3).

DISCUSSION

Based on the colony number of *B. bassiana* obtained from all treated media, the lower ones were found in OLL, DO, and OLL + DO. According to Robinson (1995) when this plant is crushed into pieces, they give off sulfoxides, a sulfur compounds which are an antibacterial and antifungal compounds. In addition, onion plant produces also gallic acid which is toxic against *Paecilomyces fumosoroseus* (Vega et al. 1997). The toxicity of these metabolite compounds rapidly decreased soon after onion's leaf litters decomposed in the soil as shown by DO and OLL + DO media. In these media, *B. bassiana* had persisted until the fifth week of incubation but

for OLL media it had persisted only until the second week of incubation.

The conidia number of *B. bassiana* in other treated media containing flowering white cabbage, cabbage, and chinese mustard, respectively were less than that of control media. These plants are Brassicaceae and according to Daxenbichler *et al.* (1991) the plants of this family contain glucoerucin. This compound consisted of 4 methylthiobutyl isothiocyanate which is toxic against most of Hyphomycetes (Manici *et al.* 1997), and its highest concentration is on root parts (Birch *et al.* 1992). Further, Dawson (1993) and Manici *et al.* (1997) reported that isothiocyanate compound produced by different species, but in the same genus, shows different effects on inhibition of growth and development of fungi. As shown by this research, flowering white cabbage, cabbage, and chinese mustard belong to Brassicaceae with similar genus varied in their effects on *B. bassiana* population. Inyang *et al.* (1999) revealed that 2-hidroxy-2-phenylethyl isothiocyanate derived from *Barbarea vulgaris* is slightly toxic against *Fusarium culmorum*, but 2-phenylethyl isothiocyanate hidrolized from 2-phenylethyl glucosinolate is highly toxic against *Metarhizium anisopliae*. Polygonaceae produces high amount of gallic acid (7,000 ppm) which is highly toxic against entomopathogenic fungi, particularly *P. fumosoroseus* (Vega *et al.* 1997). These results indicate that plant wastes of Liliaceae and Brassicaceae when incorporated into the soil as organic fertilizer have negative effects on growth, development, and viability of *B. bassiana*.

The persistence of fungal colonies was also vary among the treated media. Based on the present results, persistence of *B. bassiana* was slightly longer at the first group consisted only of leaf litters than the other two media groups containing decomposed plants, except at onion's leaf litters. These result was supported by Hall (1980) who found that most of entomopathogenic fungi are soilborne fungi which are able to live as saprobe on dead organic matters for several months. In addition, the occurrence of this fungi was frequently associated with a large organic matter content in soils which provide various substances such as carbohydrates, amino acids, organic acids, and vitamins which are basically needed for the fungal growth (Rovira 1979; Quesada-Moraga *et al.* 2007). Soils with greater organic matter also have greater diversity and density of arthropod hosts in which fungi can multiply (Klingen & Haukeland 2006). However, *B. bassiana* was more abundant in low organic matter soils, which may relate to the fungistatic compounds found in organic matter that have been shown to affect *B. bassiana* than *M. anisopliae* (Lockwood 1977).

Decreasing of *B. bassiana* colony number had been noticed since the first week of inoculation. This may be due to the influence of carbon to nitrogen (C:N) ratio. Gao *et al.* (2007) found that C:N ratio significantly affected fungal growth and sporulation of the entomopathogenic fungi, therefore, consideration for nutrient requirements is essential for improving yields of fungal biocontrol agents. Besides, the less number of colonies due to the inaccurate sampling method that the soil samples were just taken from the surface of media.

Conidia might be in deeper layer of the soil due to the heavy rain during the experiment. Another cause might be due to the presence of residual fungicides such as benomyl, triadimefon, dithane M45, and macozeb, used by farmer in the field where we took the samples. Environmental factors such as temperature and humidity in the soils, as well as UV light influenced the fungal growth and sporulation (Furlong & Pell 1996; Ekesi *et al.* 2003; Rangel *et al.* 2004). Therefore, it is necessary to concern those factors which influenced the optimal performance of fungi in pest control.

According to Leland (2001), entomopathogenic fungi will suffer from growth inhibition if there are fungicidal effects around. This inhibition will continue although the fungi had been grown on artificial media. Similar to our results that the effects of treated media were not only on fungal population, but also on the ability of the isolate of two week's old media to grow and to produce conidia at PDA media. The treated media influenced conidia to germinate as well. These results were based on the colony diameter of *B. bassiana* at PDA media in which it was only 4.0 cm due to the effect of treated media, whereas it was 7.83 cm (almost twice) due to the control soils. Similar result with Trizelia (2005) who reported that normal diameter of the *B. bassiana* colonies was 7.90 cm.

Based on this phenomenon, it is suggested that the entomopathogenic isolates obtained from the soils rich with litters need to be grown first on host insects in order to minimize soil fungicidal effects, although Klingen *et al.* (2002) found that the entomopathogenic fungi affected by fungistatic soil, were still capable to kill insects.

Conidia are one of critical organ which are needed to infect host insect. Vega *et al.* (1997), Inyang *et al.* (1999), and Rask *et al.* (2000) showed that isothiocyanates of Brassicaceae inhibited the conidia germination of *M. anisopliae* and *P. fumosoroseus*. The more concentration of allelochemicals such as salisylic acid, gallic acid, tannic acid, chlorogenic acid, sinigrin, saponin, and catechol, the less conidia of *P. fumosoroseus* geminated (Vega *et al.* 1997). According to Kassa (2003) the effective bioinsecticides should be having a germination ability higher than 80%, but in the present results, due to the influence of leaf litters of onion, flowering white cabbage, cabbage, and chinese mustard and their composts, the germination ability of *B. bassiana* varied between 43-52%. It is suggested that *B. bassiana* Bb-Pb2 might not be effective as bioinsecticide at the soils containing either those leaf litters or their composts.

Future studies should be undertaken to determine the conditions which reduced the viability of *B. bassiana* Bb-Pb2 by evaluating the effects of organic matter content, carbon concentration, C:N ratio, soil pH, clay content, and concentration of plant toxic compounds in the field in order to make clear the time and suitable condition to release *B. bassiana* in the field. Physical factors such as temperature, UV light, soil texture, geographical location, and habitat types such as cultivated habitats, forest, field are also need to be considered before entomopathogenic fungi could be recommended for pest control.

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