AGRIVITA VOLUME 34 No. 3 *OCTOBER – 2012* *ISSN : 0126-0537*

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF MORPHOLOGY IN FUNGUS *Beauveria bassiana* (Balsamo) Vuillemin COLONY NATURALLY FROM LEAF SURFACE, SOIL, AND INSECT AS HOST IN TOMATO PLANTATION

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[*)] **Received: June 12, 2012/ Accepted: September 24, 2012**

ABSTRACT

The research aimed to isolate entomo-pathogenic fungus *B. bassiana* and describe phenotypic characters in colony morphology as well as measure the growth rate from several natural habitats such as the leaf surface, soil and including larval insect *Spodoptera litura* and *Helicoverpa armigera*. The research was done in Kepanjen, Malang from July to October 2000. Purposive random sampling method was used for data gathering. The result showed that *B. bassiana* isolate managed to be isolated from the three natural habitats involving larva *S. litura* and *H. armigera*, the soil, and surface of tomato leaves. *B bassiana* isolate of insect habitat represented phenotype in colony morphology with curved character, while isolate of the leaves surface and soil indicated phenotype in colony morphology with raised and bowl-shaped character. Isolate of insect with curved colony character represented higher growth rate than that of the leaves surface or the soil with raised and bowl-shaped colony character. Thus, *B. bassiana* isolate with the curved colony of insect served as an isolate which was expected to be virulent as controlling agent to biological elements. This reseach approve that curve phenotype character in colony morphology can be used as virulence estimation in *B. bassiana*.

Keywords: *B. bassiana*, phenotypic characters in colony morphology, colony growth rate, habitat

INTRODUCTION

Entomopathogenic fungus *Beauveria bassiana* is one of cosmopolitan pathogens that recently become the primary candidate agent for biological control elements in term of influential crop pest. *B. bassiana* (Balsamo) Vuillemin (Deuteromycetes, Moniliales) was found by Agostino Bassi in 1835 and served as pathogen on insects. This parasite is not only transmitted via integument of insect, but transmitted also through food which is consumed. This type of fungus infects more than a hundred insects coming from different order and developmental phase and mites (Acarina). Thus, *B. bassiana* has been produced as biopesticide with various brand names in a number of countries. *B. bassiana* has been titled as an organism functioning as dual controlling agent to biological elements for insects and pathogen in crops (Ownley *et al*., 2004). Apart from serving as pathogen on insects, *B. bassiana* is also able to control pathogenic fungus *Pythium myriotylum* and pathogenic bacterium *Xanthomonas axonopodis* pv. *malvacearum*. On the surface of leaf, it is indicated that *B. bassiana* is capable of controlling *Phoenicococcus marlatti* Cockerell and disease caused by *Penicillium vermoesenii* Biourge in palm in laboratory (Asensio *et al*., 2007). In all types of habitats with tropical climate, all pathogenic fungi attacking insects can survive due to the availability of highly relative humidity (Wainwright, 1992). *B. bassiana* can be found in certain habitats such as on the leaf surface of yard-long bean (Daoust and Pereira, 1986) and in soil planted with strawberry (Sapieha-Waskewicz *et al*., 2005). Tropical climate causes *B. bassiana* survive naturally in all types of habitats throughout Indonesia.

Fungus isolation on the surface of leaves and in soil is common in pathogen on plants, but not in pathogen on insects. Pathogen

Accredited SK No.: 81/DIKTI/Kep/2011
http://dx.doi.org/10.17503/Agrivita-2012-34-3-p303-310

B. bassiana on insects is isolated from the leaf surface of brambly plants *Crataegus monogyna*, needle-like plants *Urtica dioica* and weed *Dactylis gomerata* and *Elytrigia repens* (Meyling and Eilenberg, 2006). As explained, *B. Bassiana* from the leaf surface is proven virulent to leaf insects, while isolate from soil is virulent to soil insects. Isolation *B. bassiana* in habitat of paddy was virulent to *Leptocorixa acuta* (Suharto *et al*., 1998). Characterization of isolate *B. bassiana* from soil is taken as the first step in order to produce effective isolate in controlling insects in soil, as well as isolate on leaf surface to insects on leaf.

Isolate *B. bassiana* isolated from larva *S. litura* will virulent to larva *S. litura*, including isolated from *H. armigera* will virulent to larva *H. armigera*. These two kinds of pest were taken because of their significant influence in tomatoes. Larva *S. litura* and *H. armigera* are proven difficult to be controlled with synthetic insecticide due to their resistance to all conventional active insecticide in all plant ecosystems. Isolation of *B. bassiana* from leaf surface of tomatoes and soil where the plant roots are housed is considered as advanced step in developing entomopathogenic fungi as biological agents.

The availability of *B. bassiana* virulence as myco-insecticide or pathogen which naturally survives in the field is the first step in development of controlling agents to biological elements. Virulence isolate can be obtained through selection using one to three characters such as biological, molecular, or phenotypic in colony morphology. Selection of fungus isolate according to phenotypic character in colony morphology is easier and more affordable compared with that of biological and molecular characters. Various isolates of *B. bassiana* indicate that phenotypic character in colony morphology is correlated to virulence isolates (Padmavathi *et al*., 2003; Cheng-Shu *et al*., 2002).

This research aimed to: 1) isolate entomopathogenic fungus *B. bassiana* from several natural habitats such as the leaf surface, soil where the roots grow, larva *S. litura* and *H. armigera* in tomato plantation, 2). Describe phenotypic characters in colony morphology of isolate *B. bassiana* in different habitats, 3). Measure growth rate of fungus colony. Moreover, the development of isolate *B. bassiana* from every habitat as controlling agent to biological elements was discussed according to the results obtained from this research.

MATERIALS AND METHODS

Tomato Plantation Site

Three native habitats consisting of leaf surface, soil in which the roots grew, and insects as host, where isolate *B. bassiana* was taken were selected by conducting a survey according to survey method for isolation of fungus pathogen on insects in cabbage (Alam, 1992; Riethmacher *et al*., 1992) and orange (Subandiyah *et al*., 2000). Selection of habitat for the fungus was initiated by visiting several tomato plantation sites located in suburbs and regency areas. The plantation sites surveyed consisted of Pendaringan village, Kepanjen sub-district, Malang regency which was 18 km from the west part of Malang. From the plantation sites surveyed, it was observed that they were in humid condition, and the population of larvae *S. litura* and *H. armigera* was evenly scattered in the plantation.

The tomatoes surveyed in the sites were planted during rainy season from July to October 2000. Survey was conducted every other week for five times initiated when the plants were 72 days old and the last survey was done at 107 day after planting. The sites surveyed were cultivated by applying fertilizer, cleaning the weed and irrigating the land as done by the local farmers. Pesticide was sprayed by farmers twice or thrice every season depending on the intensity of attack by pest in tomato.

Collecting Insects as Host, Leaves and Soil as Native Habitats for Isolates from The Plantation Sites

Collection was carried out once a week, and it ceased when isolate *B. bassiana* was obtained. In each collecting stage, 20 tomato plants were purposively selected for sampling. From those 20 plants were the dead and living larvae *S. litura* and *H. armigera*, leaf and soil samples taken.

The dead and living larvae were separately placed in plastic boxes. Twenty leaves were taken from 20 plants in every collecting stage. The leaves taken were supposed to be fresh and green. Leaf picking was done by cutting the stem with a knife. All 20

cut leaves were then moved into polyethylene plastic bags, and the bags were tightened with sellotape. Soil sample was taken from the site in the depth of 20 cm by using special tool for sampling (Sapieha-Waskiewicz *et al*., 2005), then the soil was moved into plastic bag. Those three plastic bags were moved into an air-tight box at 15-20^0 C. Those native habitats for isolates consisting of collected leaves, soil and larvae were, furthermore, taken to laboratory for incubation and isolation of fungus isolate.

Isolate *B. bassiana* from Insect, Leaf Surface and Soil in Tomato

Insects as Host

Parasitic insects as host in the plantation were *S. litura* and *H. armigera*. In laboratory, the living larvae were placed separately in plastic glass, where the glass was 10 cm in height with the diameter of 5 cm in the base. Some chopped leaves of *Ricinus communis* were put into the glass for feeding. The chopped leaves were washed before incubated to inhibit contaminant in the leaves. The process from larva to pupa was observed, while the dead larvae were incubated in Petri dish. The larvae bearing fungal disease were also incubated in Petri dish layered with wet tissue paper. During incubation, the Petri dish was covered in order to increase relative humidity in the dish and encourage sporulation indicated by the emergence of white mycelium *B. bassiana* on the integument of larvae. Incubation was maintained at room temperature (20-23^o C) in laboratory. The isolation of fungus from larvae *S. litura* and *H. armigera* was conducted on larvae infected in the plantation and those infected after reared in laboratory. The isolation of fungus was performed by applying method by Poinar and Thomas (1982). Larvae *S. litura* and *H. armigera*indicating any symptoms of *B. bassiana* infection were sterilized in alcohol (70%) for 30 seconds, and those infected were sterilized using aquadest and dried. Larvae were then cut by using sterilized scalpel into some parts with 5 mm in width and length of each part. The chopped parts were planted in PDA medium in Petri dish with diameter of 9 cm. the colony growing in PDA medium was then neutralised by means of sub-culture. The culture of larvae was kept in refrigerator at 5^0 C (Townsend *et al*., 1995). Three colonies for every isolate were selected randomly and the available type of fungus was indentified with the help of taxonomy literature (Asensio et al., 2007; Barnett and Hunter, 1998). Furthermore, the culture of isolate *B. bassiana* was used as experimental material in conidium germination.

Tomato Leaf

Isolation method of *B. bassiana* from tomato leaf surface applied dilution and casting procedure (Vandenberg, 1996). A sheet of leaf picked from the plantation was cut in 2x2 cm^2 square as much as 1 gr. the cut leaf was inserted into test tube already filled with 10 ml of sterilized aquadest. Moreover, at one end of the test tube was covered with aluminum foil, and the conidia suspension was spun by using vortex for 20-30 seconds to obtain homogenous suspension. To obtain the culture, sub-culture technique was applied in PDA medium. Conidia suspension in the tube was diluted up to 10^6 conidia/ml where 1 ml was taken and poured into Petri dish and added with 10 ml liquid PDA at 45^0 C. Petri dish was slightly shook to make conidium suspension in PDA homogenous, and then incubated for 8 days until the emergence of colony at room temperature was visible. Three colonies for each isolate were randomly selected and the available fungus was identified with the help of taxonomy literature. (Asensio et al., 2007; Barnett and Hunter, 1998). The culture of isolate *B. bassiana* from the tomato leaf surface was then used as experimental material in conidia germination.

Soil

The method used to facilitate the isolation of conidia from the soil where the roots grew was the procedure by Mietkiewski *et al*. (1997). One gram of soil was taken from the soil observed in the laboratory and was refined. To obtain the culture, sub-culture technique was applied in PDA medium as it was for leaf surface. Three colonies for each isolate were selected randomly and the available fungus was identified by means of taxonomy literature (Asensio et al., 2007; Barnett and Hunter, 1998). The culture of *B. bassiana* from the soil in PDA medium was then used as material for experiment in conidium germination.

RESULTS AND DISCUSSION

RESULTS

Phenotypic Characters in Colony Morphology of Isolate

Isolate *B. bassiana* on insect, leaf surface and soil in tomato plantation indicating phenotypic characters in Colony Morphology was presented in Table 1.

Isolate *B. bassiana* from soil in the plantation (Bb2)

Isolation of fungus on insect from the soil was performed by means of dilution and casting technique in PDA medium. This technique was modified based on the method applied by Sapieha-Waskiewicz (2005) to isolate *B. bassiana* in the soil of plantation area. *B. bassiana* was identified according to the conidium morphology. Conidium *B. bassiana* was single-celled, round to oval, colourless or hyaline, with diameter of 2-3 µm. *B. bassiana* had single and in-group conidia, conidium base was bulgy with ovoid shape (Barnet and Hunter, 1998). The zigzag ramification of conidiophores represented the characteristic of Beauveria (Utomo *et al*., 1988). The identified isolate *B. bassiana* from soil (Bb2) manifested phenotypic character of colony where it was round, intact-edged, raised or flat on the surface, and yellowish white (Table 1). These phenotypic characters in colony morphology were in line with the result of the previous research by Suharto *et al*. (1998). Colony *B. bassiana* showed yellowish white or opaquely white colour. The hypha shaped like floss in cotton where it characterized *B. bassiana*which is categorised in class of Hyphomycetes. The shape of colony *B. bassiana* was round which was correlated with the apical growth of colony where the colony grew in all direction.

Isolate *B. bassiana* from Larvae *S. litura* (Bb3)

Infection of *B. bassiana* in larvae *S. litura* was characterised by symptoms like fungus with a colony of white hyphae growing on the integument of larvae. The phenotypic character of the colony growth was apical. This similar type of growth was caused by the isolates which were reared in the same PDA media. The growth type of colony *B. bassiana* was apical, where it grew in all direction. Like colony of isolate from the soil, colony of isolate from larvae *S. litura* (Bb3) is in line with Suharto *et al*. (1998), where the colony *B. bassiana* was opaquely white. The phenotypic characters in colony of isolate Bb3 were qualified by round shape, yellowish white colour, curved surface, and intact edge. The isolate *B. bassiana* from larvae *S. litura*(Bb3) revealed the white colony with hyphae resembling floss in cotton, which characterizes class of Hyphomycetes.

Table 1. Phenotypic Characters in Morphology of colony *B. bassiana* at 21 days after inoculation in *Potato Dextrose Agar* (PDA)

No	Isolate			Phenotypic characters of colony			
	Code	Habitat	Colony Growth Rate (mg/day)	Shape	Edge	Surface	Colour
1	Bb2	Soil	0.83	Round	Intact	Raised, flat	White
2	Bb3	Larva *S. litura*	2.02	Round	Intact	Curved	Yellowish white
3	Bb6	Tomato leaf surface	3.57	Round	Intact	Raised, bowl shape	Yellowish white
4	Bb16	Larva *H. armigera*	0.88	Round	Intact	Curved	White

Isolate *B. bassiana* from tomato leaf surface (Bb6)

Isolation technique of isolate from the tomato leaf surface was executed by means of dilution and casting into PDA medium. Such a technique was modified according to a method by Meyling and Eilenberg (2006) to isolate *B. bassiana* on the leaf surface. Isolate *B. bassiana* from the tomato leaf surface (Bb6) brought phenotypic characters of colony qualified by round shape, intact edge, raised and bowl-shaped surface, and yellowish white colour (Table 1). The growth type of colony was apical.

Isolate *B. bassiana* from Larvae *H. armigera* (Bb16)

Larvae *H. armigera* infected by fungus produced mycosis in laboratory as soon as the larvae were incubated in Petri-dish layered with damp paper. Identification of conidia and conidiophores revealed that isolate on larvae *H. armigera* (Bb16) was *B. bassiana*. Isolate larvae *H. armigera* showed phenotypic characters in colony qualified by white colour, curved surface, and intact edge in PDA medium with 21-day period of rearing (Table 1). These different isolates indicated that different habitats from which isolates were taken had relatively similar colony morphology except for the surface of colony in PDA medium. Relatively similar colony morphology was triggered by all isolates reared in the same medium of PDA.

DISCUSSION

Morphological Characters of Isolate Colony *B. bassiana*

The four different isolates (Bb2, Bb3, Bb6, and Bb16) generated colony growth with similar phenotypic characters in PDA medium, but not for the surface of colony. The similar phenotypic characters in colony comprised round shape, intact edge, and white or yellowish white colour. Phenotypic characters in different colony surfaces covered isolate Bb3 and Bb16 isolates with curved shape; Bb6 and Bb2 which were raised, flat or bowl-shaped. Phenotypic characters in colony morphology of isolate *B. bassiana* were classified into two colonies: dusty and chalky colony (Padmavathi *et al*., 2003).

Dusty colony represented higher growth rate of colony (mm/day) than the chalky one (Padmavathi *et al*., 2003). The curved character colony of isolate (Bb3 and Bb16) had higher growth rate of colony than raised and bowl-shaped character (isolate Bb2). Thus, it was hypothesised that dusty character was the same as the curved one, while chalky character was the same as the raised and bowl-shaped character in *B. bassiana*. The increase of colony growth rate in *B. bassiana* was caused by high potency of conidium sprouts (Cheng-Shu *et al*., 2002).

In this research, isolate Bb3 and Bb16 of habitat for insect was potential to be virulent isolate. This resulted from high growth rate of isolate or high potency of sprouts which could increase virulence in isolate Bb3 and Bb16. Furthermore, isolate Bb6 showed the highest growth rate of all isolates. Isolate Bb6 from the leaf surface let this isolate gain direct exposure to sunlight. The adaptation of isolate Bb6 on the leaf surface triggered the highest growth rate and virulence of Bb6. Isolate *B. basiana* taken from a habitat with hot and dry temperature was more virulent than commercial isolate (Mycotrol) to *Lygus hesperus* (McGuire *et al*., 2005).

Development of Isolates as Controlling Agents to Biological Elements

Isolation of *B. bassiana* from the tomato leaf surface (isolate Bb6) explains that saprophytic *B. bassiana*is capable of living in its natural habitat. Conidium *B. bassiana* on the leaf surface was likely to be caused by inoculum from the soil and air via wind current and the movement of the infected insect. Proliferation of conidium *B. bassiana* from soil or from the dead insect as host (cadaver) onto the leaf surface of *Urtica dioica* was by means of the movement of aphids *Microlophium carnosum* and their predator *Anthocoris nemorum* in the laboratory (Meyling and Eilenberg, 2006). Alam (1992) agreed that the proliferation of conidium *B. bassiana* in cabbage plantation was possibly caused by wind current.

Conidia can be said as effective agents in controlling biological elements only when they are virulent and persistent during and among tomato planting seasons. The development of isolate Bb6 from the leaf surface was addressed to the insight into the ecology of fungi by observing the persistence in tomato plantation. Isolation of *B. bassiana* was initiated from the tomato leaf surface, and this research is also applicable to other horticultural plants. Consequently, it requires continual observation on

persistence. Isolation of *B. bassiana* was once done on hedgerows (Meyling and Eilenberg, 2006). To increase persistency of inoculum *B. bassiana* on tomato leaf surface, inoculum needs to be placed under canopy in order to reduce the radiation diffused by sunlight. The technique was applied by spraying conidium suspension as inoculum into the inside of the canopy when the level of solar radiation was quite low at 06.00 pm. From this application, the cadavers of *S. litura* infected by *B. bassiana* were found inside the canopy of the plant. The persistence of *B. bassiana* on the leaf surface of yard-long beans (Daoust and Pereira, 1986) was determined by the intensity of solar radiation. When isolate indicated the persistence, then it was eligible to be brought to the development of virulent isolate.

On tomato leaf surface, compounds available in plant affected *B. bassiana* virulence. Alkaloid tomatine from tomato leaves affected the pathogenicity of *B. bassiana* on larvae of *S. litura* (Suganya and Selvanarayanan, 2009); nympha of whitefly (*Trialeurodes vaporariorum*) (Westwood (Poprawski *et al*., 2000). The development aimed to understand the correlation between isolate *B. bassiana* and compounds in tomato leaves and increase the pathogenecity of *B. bassiana* on larvae *S. litura* and *H. armigera*, where, further, the cadavers could be used as inoculum. Isolate *B. bassiana* (CA) resulted in the formation of cadavers as inocula, where, then, it was selected as controlling agent for *H. coagulata* (Dara *et al*., 2008). Inoculum of isolate Bb3 and Bb16 could be sprayed before the second population of those two kinds of pest multiplied in number and caused economic damage in tomato plantation. Larvae *S. litura*, which consume leaves, had a close contact with conidia on the leaf surface which resulted in the death of the larvae and became cadavers. Insects as host which had frequent contact were infected by *B. bassiana*, died and became cadavers (Meyling and Eilenberg, 2006; Reay *et al*., 2008). Infection by *B. bassiana* and the death of larva (cadavers) were correlated with the humidity in tomato plantation during the survey conducted in rainy season (Alam, 1992). Strategy of using cadavers as inoculum was spread at the beginning of planting season before the population reached its critical economic condition. In cabbage, population of entomo-pathogenic fungi *Pandora bunckii* and *Zoophthora radicans* didn't manage to infect *Plutella xylostella* before the population of the pests caused serious damage on the plant (Riethmacher *et al*., 1992). In such a case, the application of inoculum *B. bassiana* by spraying needed to be implemented before the population of *Agrilus panipennis* (Coleoptera: Buprestidae) was high (Castrillo *et al*., 2010).

Isolate Bb2 from the soil of plantation showed good result which is linear with Dara *et al*., (2008) explaining that *B. bassiana* was a fungus in soil and could survive in such a habitat from which infection infecting insects as hosts came. Isolate *B. bassiana* could be isolated from the soil of strawberry plantation where synthetic pesticide was not applied. Among the following types of entomophatogenic fungi: *M. flavoviridae* (Metsch.) Gams and Rozsypal, *P. farinosus*, and *Conidiobolus major* Brefeld, *B. bassiana* can live the longest life in plantation soil in which the pesticide is continually applied for 12-19 years (Mietkiewski *et al*., 1997; Sapieha-Waszkiewicz *et al*., 2005). Based on those two previous studies, it can be drawn that isolate Bb2 can survive in soil of tomato plantation regardless continual application of synthetic pesticides of all types. In cabbage, entomopathogenic *Pandora blunckii* and *Zoophthora radicans* infects *P. xylostella* after its population and the damage caused was quite high (Riethmacher *et al*.,1992). Spraying inoculum *B. bassiana* could be employed as controlling strategy before the pest population of *Agrilus planipennis* (Coleoptera: Buprestidae) doubled in number (Castrillo *et al*., 2010).

CONCLUSIONS

B. bassiana can be identified based on four different habitats: larva *S. litura*, larva *H. armigera*, leaf surface, and soil in plantation area. Except for the colony surface, colony morphology of isolate *B. bassiana* was shown the same characters. Based on the phenotypic surface of the colony, two characters of colony were found in two shapes: 1) curved and 2) raised/bowl-shaped. Isolate with curved character indicates higher growth rate (mm/day) than that with raised/bowl-shaped character. Scrutinizing the potency of sprouts and isolate virulence from every different habitat would clarify the correlation between phenotypic characters of colony and isolate potential as controlling agents.

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