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Original Article

Evaluation of Sucrose for *In Vitro* Germination and Growth of the Entomopathogenic Fungus *Beauveria bassiana* (BALSAMO) VUILLEMIN and *Paecilomyces* sp. (DEUTEROMYCETES, MONILIALES)

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Abstract

In order to examine some possible effects of granulated sugar and sucrose in *B. bassiana* conidia suspension, we examined the effects of sucrose on germination dan growth of *Beauveria bassiana* and *Paecilomyces* sp. *in vitro*. We hoped to provide some information relevant to mass production methods for *B. bassiana* and *Paecilomyces* sp. The conidia germination and growth of *B. bassiana* and *Paecilomyces* sp. were increased by addition of sucrose in medium. The addition sucrose may be a useful carbohydrate source for use in mass production and carrier ingredient into conidia suspension as bioinsecticide.

Keywords: *Beauveria bassiana*, *Paecilomyces* sp., conidia germination, mycelial growth, sucrose

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INTRODUCTION

Biological control with pathogenic fungi is a promising alternative to chemical control against many species of insect pest. Extensive use of synthetic insecticides can cause environmentalhazards and mortality of natural enemies. Biological control withpathogenic fungi might provide long-lasting insect control without damage to the environment or natural enemies organisms. The Deuteromycetes *Beauveria bassiana* are common entomopathogenic fungi that occur worldwide on many different habitats. Under field conditions, *B. bassiana* and *Paecilomyces* sp. are significantly more effective thanspeciesof another entomopathogenic fungi (Sabbour, Ragei, Abd-El Rahman, 2011).

The fungus of *B. bassiana* and *Paecilomyces* sp. can be applied in the form of conidia and mycelium suspension in the knapsack sprayer with sucroseaddition. The conidia suspension of entomopathogenic fungi, called bioinsecticide, is subsequently sprayed on the crop until insects pest in the field adhere to it. Critical factors that determines the infection process of the fungi is conidial germination and growth on insect surface. Conidial germination and fungal growth is a key indicator in the selection of *B. bassiana* (Altre *et al*., 1999) and *Paecilomyces* sp. virulent isolates as a biological control agent. According to Suganya and Selvanarayanan, (2010), germination rate of conidia on the surface of the host insect's body to determine the efficacy of insect pathogenic fungi. Although conidia are determine the efficacy of insect pathogenic fungi, the environmental factors governing the sporulation of entomopathogenic fungi have been poorly studied.

The previous research, showed that conidia germination and growth *B. bassiana* on synthethic media *in vitro* correlated with the success of germination and growth of *B. bassiana* the fungus on the insect surface which infects them (Sun, Fuxa, and Henderson, 2002). The increase conidial germination of *B. bassiana* on the insect surface of *D. ponderosae* probably is caused by nutrients which acquired the fungus in sufficient amounts (Hunt, Borden,

Rahe, Whitney, 1984). Therefore, lack of sufficient nutrients in synthethic media could reduce the success of the fungus to infect insects. Results of some research revealed that *B. bassiana* was able to grow and to utilize any carbohydrate added to the synthethic medium (Sabbour, Ragei, Abd-El Rahman, 2011). Nutritional requirements for germination and growth of the entomopathogenic fungus *B. bassiana* are not complex. For germination to occur, a utilizable source of carbon must be present (Smith and Grula, 1981). Carbohydrates as carbon source composed 18 to 42% of the mycelial dry weight of *B. bassiana* (Bidochka, Low, Khachatourians, 1990). Because of the nutritional versatility of *B. bassiana*, this fungus should be able to germinate and grow in several types of carbohydrate sources. For that, the source of carbohydrates in fungal synthethic media selected are cheaper and more effective as way to increase conidial germination and growth of *B. bassiana*. Sucrose was the best synthethic medium carbohydrate for supporting growth (Sabbour, Ragei, Abd-El Rahman, 2011) and sporulation of *B. bassiana* (Campbell, Barnes, Cartwright, Eikenbary, 1983). Large-scale availability of *B. bassiana* conidia is a primary requirement in the bio-control program. For a successful integrated pest management program, *B. bassiana* fungus should be amenable to easy and cheap mass multiplication. Sucrose ("gula pasir": bahasa Indonesia) contain sucrose 99% and this is cheaper and more easily obtained by farmers than pure sucrose, but the effects of sucrose on germination and growth have never been evaluated, so whether or not sucrose increased to *B. bassiana* remains uncertain.

This research aims to study the effect of the addition of sucrose on conidial germination and growth of fungi *B. bassiana* and *Paecilomyces* sp. *in vitro*.

2. MATERIALS AND METHODS

2.1 Experimental Method

***B. bassiana* Isolate**. This *B. bassiana* isolate was obtained from the Collection of Entomopathogenic Fungal Cultures (Mycology Laboratory, Department of Plant Disease and Pest, Agricultural Faculty, Brawijaya University Malang, Indonesia). This isolate was obtained originally from *Spodoptera litura* larvae [Lepidoptera: Noctuidae] in field, East Java, Indonesia. Cultures were rejuvenated on Sabouraud dextrose agar medium (SDA) and maintained in an environmental room at 25°C and 12 h/12 h (light/ dark) cycle. The isolate collection was selected as representative for testing of conidia germination and mycelial growth in vitro. Isolate collection in which more than 90% of the conidia germinated were used in the experiments

Basal Medium. In preliminary experiments, we found that a medium containing 2% agar supported good mycelial growth and sporulating of *B. bassiana*. Hence we used this medium as the basal medium, further called Water Agar 2% (WA).

Treatment Media. The treatment media that sucrose (5%) was tested using basal medium (WA 2%). The treatment media were incorporated into basal medium (WA 2%) and the basal medium was used as control. At least two different batches of cultures for sucrose were stored, one for each replication of the experiment.

Effects of sucrose on conidial germination

Conidia germination test. The germination test was done with two different media at a time with four replications for each medium. An aqueous conidial suspension (100Ul containing approximately 10^6 conidia/ml) was spread on a slide coated with a thin film of SDA medium. The inoculated slides were kept in Petri dishes (15 cm diameter) lined with blotting paper moistened with sterile water. Slides (one per dish) were rested a top glass rods placed on the blotting paper. The Petri dishes were placed in an environmental chambers set to the room temperature and 90% humidity.

Germination counts were made with a phase contrast microscope at room temperature (25 °C). Starting from 3 hours after inoculation, slides were observed at two hourly intervals to check for germination. Conidia were considered as germinated when the germination peg was at least twice the diameter of the conidium. At least 300 conidia were examined for germination counts on each slide.

Effects of sucrose on colony and mycelial growth

B. bassiana was cultured on SDA plate for one week. To make a conidial suspension, we cut a 5- mm diameter SDA plug together with the attached conidia from the plate and placed it into a test tube with 10 ml sterile 0,02% Tween 80. The conidial suspensions were sonicated using a vortex and the number of conidia was determined using haemocytometer. The suspensions were serially diluted to get a final suspension containing approximately 10^6 conidia/ml. A 0,2 ml aliquot of the final suspension was then plated out on each Petridish containing a different media that were basal medium or Water Agar (WA) 2%, and WA plus sucrose 5%. Five replicates were used for each treatment. All plates were incubated at 26 ^oC under a 12:12 light : dark regime was provided by two pairs of 40 watt fluorescent lights ca. 200 cm above plates.

The diameter of individual colonies were measured after eight days and used as the measurement of the mycelial growth. The diameter of each colony, or the largest diameter if not circular, was measured daily from the bottom of the dish. The colony growth rate was calculated by averaging the colony growth (mm/day) over eight days.

After 8 days, the culture was filtered on a Whatman No.1 paper. The culture (conidia and mycelia) were harvested under sterile conditions by flooding the plate with 2 ml sterile 0.05% Triton X-100 and then scraping the colony with forceps. To harvest all possible conidial residuals from the dishes, each dish was rinsed three times with 2 ml sterile 0.05% Triton X-100. The mycelial mass on the Whatman paper was washed using sterile distilled water, dried with blotting paper, placed in a Petri dish lined with additional blotting paper, and dried to a constant weight in a hot air oven at 80 °C

Statistical analysis

Experiments were set up in a completely randomized design with five replicates. All data, the colony growth rate, mycelial growth rate, and percentage conidial germination were subjected to analysis of variance and least significantly different (LSD) comparisons. A logarithmic transformation was applied to the percentage conidial germination data before statistical analysis.

2.2 Review Method

The integrative review method is the only approach that allows for the combination of diverse methodologies (for example, experimental and non-experimental research) (Whittemore and Knafl. 2005).

There were some journals used to evaluate the effect of sucrose for *in vitro* germination and growth of the entomopathogenic fungus especially *Paecilomyces* sp.

RESULT AND DISCUSSION

Effects of sucrose on conidial germination

The percentage of germination varied significantly according to the media used. These germination rates were significantly different on two different media. The highest germination rate on this test was obtained from Water Agar plus Sucrose medium (83.50%) and significantly higher than those of the control medium (43%) at 10 days incubation (Table 1). Its germination rate relative to control showed that Water Agar plus Sucrose medium increased the germination rate (42.3%).

Table 1. Effects of sucrose on conidia germination (%) of *Beauveria bassiana* on Water Agar as basal medium after 10days incubation (modified from Agustiawati, 2011)

Media	Germination Rate (%)		Increasing of germination rate relative to control (%)
	Range	Mean	
Water Agar 2% (WA/control)	20-75	43 a (*)	0
WA plus Sucrose 5%	82-88	83.50 b	42.3

(*) Each data represents the average. Measurements were made on 10 days incubation at room temperature. Each point represents the mean of five replicates. Values in the same column followed by the different letters were significant different at the $p < 0.05$ level according to least significantly different (LSD) comparisons tests.

*Highly significant difference ($p < 0.05$).

Two different carbohydrate sources signicantly increased *B. bassiana* sporulation relative to control. Results of previous research, revealed that *B. bassiana* was able to grow and to utilize any carbohydrate added to the growth medium. Sucrose was the best substrates for supporting sporulation. Naturally occurring entomopathogenic fungi, *B. bassiana* was sporulated best on d-sucrose, d-trehalose, and d-glucose (Campbell, Barnes,Cartwright, Eikenbary, 1983). Therefore, it can be argued that sucrose was the best substrate tested and have the same potency to increase conidial germination.

In the medium as shown in Fig. 1, a high level of mycelial growth was obtained sucrose. In the sucrose medium, the highest mycelial growth (12, 6 g/1) were obtained from the carbon sources tested (Kim, *et al*., 2002).

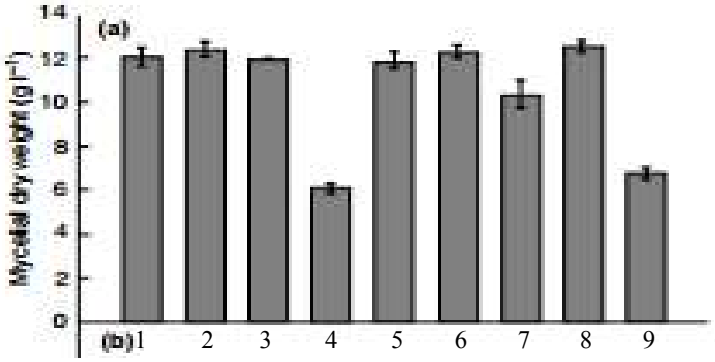

Fig. 1. Effect of carbon sources on mycelial dry weight by *Paecilomyces sinclairii* in shake flask cultures. (1-9 respectively: Cellobiose, Dextrose, Fructose, Lactose, Maltose, Mannitol, sorbitol, Sucrose, and Xylose). (Source: Kim, *et al*., 2002).

Addition of 1 per cent sucrose increased the growth and sporulation of *B. bassiana* in sorghum (6.65×10^9 cfu) and barnyard millet (6.24×10^9 cfu). However, population of *B. bassiana* was significantly reduced in sorghum and barnyard millet grains when amended with 2 per cent sucrose (Prasad, 2013).

Effects of sucrose on colony radial and mycelial growth

The colony radial growth was highly affected by two different media in tests of *B. bassiana*. The mycelial growth rate revealed the highest radial growth rate not significantly different between Water Agar medium (3.57 mm) and Water agar plus sucrose (4.63 mm) (Table 2).

Table 2. Effects of sucrose on colony radial growth (mm) of *Beauveria bassiana* on Water Agar as basal medium after 14 days incubation (modified from Agustiawati, 2011)

Media	Colony diameter (mm)		Increasing of growth relative to control (%)
	Range	Mean	
Water Agar 2% (WA/control)	3.3-3.9	3.57 a(*)	0
WA plus Sucrose 5%	4.1-5.5	4.63 b	29.91

(*)Each data represents the average. Measurements were made on 14 days incubation at room temperature. Each point represents the mean of five replicates. Values in the same column followed by the different letters were significant different at the $p < 0.05$ level according to Least significantly different (LSD) comparisons tests.
*Highly significant difference ($p < 0.05$).

Based on Table 2, it can be argued that the sucrose effectively increased mycelial growth of *B. bassiana* fungus. Nutritional requirements for germination and growth of the entomopathogenic fungus *B. bassiana* are not complex (Smith and Grula, 1981). There was an increase in the *B. bassiana* biomass production and the contribution of carbohydrate to mycelial dry weight (Bidochka, Low, Khachatourians, 1990). Carbohydrates composed 18 to 42% of the mycelial dry weight, and this value was lowest in unsupplemented medium and highest in medium supplemented with different carbohydrate sources. Results revealed that the isolate of *B. bassiana* was able to grow and to utilize any carbohydrate added to the growth medium. Sucrose was the best substrate for supporting growth and followed by glucose (Sahab and Sabbour, 2011).

Based on Table 1 and 2, sucrose can increase for growth and germination of conidia of *B. bassiana*.

Effects of sucrose on mycelial growth

Colony dry weight of mycelial growth was highly dependent on the medium used. These colonies dry weight were significantly different on two different media. The highest mean colony dry weight (5.7 mg) was obtained from medium Water agar plus sucrose than Water agar (2.3 mg).

Table 3. Effects of sucrose on mycelial growth of *Beauveria bassiana* on Water Agar as basal medium after 14 days incubation (modified from Agustiawati, 2011)

Media	Colony dry weight (mg)		Increasing of colony dry weight relative to control (%)
	Range	Mean	
Water Agar 2% (WA/control)	5.3- 3.3	2.3 a(*)	0
WA plus Sucrose 5%	9,3- 8,9	5,7 b	31,93

(*)Each data represents the average. Measurements were made on 14 days incubation at room temperature. Each point represents the mean of five replicates. Values in the same column followed by the different letters were significant different at the $p < 0.05$ level according to Least significantly different (LSD) comparisons tests.
*Highly significant difference ($p < 0.05$).

The sucrose medium was better for germination of conidia (Table 1) than water agar medium. *B. bassiana* grew best on d-melizitose but sporulated best on d-sucrose, d-trehalose, and d-glucose (Campbell, Barnes, Cartwright, Eikenbary, 1983).

Discussion

The sucrose medium can be improved by adding larvae extracts of insect pest as host to increase the pathogenicity of *B. bassiana*. The virulence of *B. bassiana* fungal isolates increased when grown on SDA medium supplemented with larval extract (Dayakar, Subbara, 2011). Further, the another research would be suggested that beside granulated sugar, some of nutrients effected on germination and growth of *B. bassiana*. Amino acids, salts, and vitamins were combined with dextrose to test their effect on growth and sporulation of *Entomophthora virulenta* in liquid shake culture. The addition of a vitamin solution to the tested media did not enhance growth or sporulation (Perry and Latgé, 1980).

In one study, sucrose added to *Beauveria bassiana* (Botanigard) increased mortality Western flower thrips (WFT) *Frankliniella occidentalis* by 20 percent. It is believed that the sucrose is an insect feeding stimulant. By stimulating feeding, the thrips had more contact with infected spores on treated leafs, resulting in better control (Anonym, 2013). Therefore, the addition of sucrose into the conidial suspension in the spray tank was suggested before they are implemented on the crop in field. Then, the sucrose was added on media of the conidia mass production. More research is needed in this area, related with variety and complexity of factors which are interrelated in this field.

CONCLUSION

The sucrose as a carbon source of carbohydrate increased conidia germination and mycelial growth of *B. bassiana* and *Paecilomyces* sp. *in vitro*. For mass production *B. bassiana* and *Paecilomyces* sp., sucrose may be worth investigating as carbon source and as carrier ingredient in the conidia suspensions as bioinsecticide which would increase their pathogenicity.

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