DOI: http://doi.org/10.4038/jfa.v6i1-2.5185

Possibility of Use of Agricultural Byproducts for Mass Production of *Beauveria bassiana* (Balsamo) Vuillemin to Control Coffee Berry Borer (*Hypothenemus hampi* (Ferrari))

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ABSTRACT

Coffee Berry Borer (*Hypothenemus hampei* (Ferrari)) is the most frequently occurring and destructive pest of coffee (*Coffea* spp.) in Sri Lanka. *Beauveria bassiana* (Balsamo) Vuellemin is an entomopathogenic fungus that has a great potential as a biological control agent for the control of coffee berry borer. An experiment was carried out to investigate the effect of different agricultural byproducts including five solid substrates i.e. coir dust, saw dust, refused tea, disposable parts of maize cob (pith + chaff + woody ring) and oil cake, and two liquid substrates i.e. coconut water and molasses. Mass production was done under two temperature levels, at room temperature (30 ± 2 ^{0}C) under incubator temperature (25 ± 2^{0}C). Fungus was inoculated into sterilized solid and liquid substrates in equal concentrations. Inoculated substrates were placed in the incubator and under room temperature and experiment continued for 8 weeks. The highest number of spores were observed in molasses (6.9475x10^{13} spores/ml) among all the tested substrates. There was no significant difference ($p > 0.05$) with respect to the spore production in all substrates at room and incubator temperatures. Optimum spore production was achieved 42 days after inoculation of the fungus in all substrates irrespective of the temperature.

Keywords: *Beauveria bassiana*, Coffee berry borer, Entomopathogenic fungi, Mass production, Substrates

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INTRODUCTION

Biopesticides based on bacteria, viruses, entomopathogenic fungi and nematodes have often demonstrated considerable scope as plant protection agents against several insects (Patel *et al*., 1990). Use of entomopathogenic fungi as biological control agents for insect species has increased the global attention during the last few decades. The mycoinsecticides based on *Beauveria bassiana* (Balsamo), *Paecilomyces fumosoroseus* (Wize) and *Verticillium lecanii* (Zimm.) have been used to control various insect pests (Sahayaraj and Namasivayan, 2008).

Coffee is one of the main beverage crops grown in Sri Lanka next to Tea and total annual production is about 2,974 Mn Kg grown in 5,959 ha of coffee lands in Sri Lanka (Anon, 2011). The coffee berry borer (CBB) (*Hypothenemus hampi (Ferrari))* emerged as the key pest and causes 50% yield reduction in coffee (Perera, 1983).

Entomopathogenic fungi can be used as a component of integrated pest management (IPM) of many insect pests. Under natural conditions, these pathogens are a frequent and often cause natural mortalities of insect populations. The main drivers behind the push for myco-insecticides are the need for more specific agents as components of IPM programmes due to concerns over chemical residues on human health and the environment (Karanja *et al.*, 2008).

Considering the biosafety of the environment, effective management of the pest and the cost effectiveness, *Beauveria bassiana* is highly promising in controlling CBB (Posada and Fransisco, 2008) and

there is a great potential of using *B. bassiana* for the control of CBB (Yapa *et al.,* 2007; Dharmadasa *et al.,* 2010). This fungus belongs to the family Cordycipitaceae under phylum Ascomycota. The fungus has been shown to caused high mortality in coffee berry borer in various countries (Mantilla *et al.,* 2008) and more than 90% of mortality percentage of CBB could be achieved in the laboratory by using 1 x 10^7 *Beauveria bassiana* spores / ml concentrations (Yapa *et al.,* 2007).

Beauveria bassiana kills the pest as a result of the insect coming into contact with fungal spores. An insect can come into contact with the fungal spores in several ways: by having the spray droplets land on its body, by moving on a treated surface or by consuming plant tissue treated with the fungus. It is being used to control a number of pests such as termites, thrips, whiteflies, aphids and different beetles (Anon, 2013) (Table 1). Mostly, the production of *B. bassiana* spores for coffee berry borer biocontrol is done using a simple sterilization technique based on cooked rice (Posada and Fransisco, 2008). However, substrates that yield high spore production, high pathogenicity and adequate shelf life remain a challenge for mass production and field application.

Therefore, suggesting of cheap and more spore productive substrates is a timely requirement for commercial production of *B. bassiana*. It has been mass produced on different solid substrates, including sugarcane wastes, silkworm pupal powder, agar medium and steamed rice (Karanja, 2008). Agricultural byproducts such as coir dust, coconut water, saw dust, oil cake, and refuse tea are abundant in Sri Lanka and are good source of organic compounds. These surplus need to be ecologically disposed after maximum utilization of their nutrients. Fungi require a supply of organic compounds for their energy production and growth and they are universally present where organic matter is found (Heritage *et al.,* 1996). However, the most viable mass production technologies include making use of a diphasic strategy in which the fungal inoculum is produced in liquid culture, which is further utilized for inoculating the solid substrate(s) for conidia production (Latifian *et al*., 2013). The present study was undertaken to evaluate five solid substrates *viz* coir dust, saw dust, refuse tea, oil cake and disposable part of maize cob (pith + chaff + woody ring) and two liquid substrate such as coconut water and molasses for the mass production of *B. bassiana* under room (30 ± 2 ^{0}C) and incubator temperatures (25±2 ^{0}C).

MATERIALS AND METHOD

Location and Collection of Fungus

The experiment was carried out in the laboratory of the Department of Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP) from January to April 2013. The fungus, *Beauveria bassiana* was isolated from the CBB infected coffee berries (Plate 1) found in the fields of Central Research Station, Export Agriculture Research Institute, Matale, Sri Lanka.

Isolation and Culturing of Fungus B. bassiana

Culture medium was prepared by mixing Potato Dextrose Agar (PDA) and distilled water in a conical flask (15.8 g of PDA for 400 ml distilled water) and medium was homogenized by placing the flask in a hot plate stirrer for 10 to 15 minutes until entire medium is dissolved without any lumps of PDA in the flask.

Table 1: Host range of *Beauveria bassiana*

Host Species	Order:Family	Reference
Hypothenemus hampei	Arthropoda: Curculionidae	(Posada *and* Fransisco, 2008)
Agrilus species	Coleoptera: Buprestidae	(Rupesh and Sardul, 2010)
Lymantria species	Lepidoptera: Lymantriidae	
Eutectona machaeralis	Lepidoptera: Hyblaeidae	
Melolontha species	Coleoptera: Scarabaeidae	
Hyblaea puera	Lepidoptera: Hyblaeidae	
Rhynchophorus ferruginens	Arthropoda: Curculionidae	(Gabarty *et al.*, 2013)
Heilipodus erythropus	Coleoptera: Curculionidae	(Toledo *et al.*, 2008)
Cratomorphus diaphanus	Coleoptera: Lampyridae	
Diabrotica speciosa	Coleoptera: Chrysomelidae	
Cycloneda sanguinea	Coleoptera: Coccinellidae	
Plagiodera erythroptera	Coleoptera: Chrysomelidae	
Doru lineare	Dermaptera: Forficulidae	
Euchistus sp.	Hemiptera: Pentatomidae	
Balacha melanocephala	Hemiptera: Cicadellidae	

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Plate 1. Coffee berries with *Beauveria bassiana* infected *Hypothenemus hampi* (White coloured fungal mycelia near the apex of the berry)

This medium was sterilized by autoclaving for 45 minutes at 121 ^{0}C and 15 psi. After sterilization, the medium was poured into several Petri dishes in the Laminar Flow Cabinet and they were kept for few minutes to solidify. Fungus was extracted from CBB infected berries and inoculated into the PDA plates in the Laminar Flow Cabinet. Fungus inoculated Petri dishes were sealed using "parafilm" and placed in the incubator at 25$^{\pm}$2 ^{0}C. Subculturing was done 14 days after inoculation for purification.

Preparation and Inoculation of Different Substrates

Molasses and coconut water were used as liquid substrates and saw dust, coir dust, refuse tea, coconut oil cake and disposable part of maize cob (pith, woody ring and chaff) were used as solid substrates for this experiment.

Plate 2. *B.bassiana* grown on maize cob and saw dust

Hundred grams of solid substrates and 100 mL of liquid substrates were measured and adequate distilled water was added to moisten the solid substrates.

Samples were put in poly propylene bags and sealed with cotton plug and aluminum foil. Sixteen bags from each substrate were prepared and sterilized by autoclaving at 121^0 C and 15 psi for 45 minutes. Fungus was scraped from stock culture grown on PDA and it was suspended in sterile water containing 0.05% Tween 80 surfactant to prepare a fungal suspension containing approximately 1.2 x 10^8 conidia per ml. Sterile bags were inoculated with 10 ml of prepared fungal suspension and the bag was massaged from outside to evenly distribute the inoculum. One set of bags consisting eight sealed bags from each substrate was incubated at 25 ± 2 ^{0}C in an incubator whilst same set of bags was placed in a container at room temperature (30 ± 2 ^{0}C).

Spore Harvesting

The spores were harvested at four different time intervals *viz*, 14, 28, 42 and 56 days after inoculation. Harvesting was done manually. Two bags from each substrate kept under room temperature and incubator were extracted at a time. Each solid substrate was dissolved in 1L of 0.05% tween 80 solution, and suspension was filtered through a fine cloth to extract the spores. Same procedure was also carried out for liquid substrates.

Spore Counting

Counting of spores were made after a serial dilution of the suspension using double ruled Neubauer haemocytometer to determine the number of spores in the prepared substrate.

Statistical Analysis

The experiment was set up as a completely randomized design with a factorial arrangement. Significance of temperatures, spore harvesting time and different substrates on spore production were analyzed using SAS version 6.0 software computer package and means were compared using LSD test at 95% level of confidence.

RESULTS AND DISCUSSION

Large scale availability of the biological agent is a primary requirement for the biocontrol program and integrated pest management. For industrial production of *B. bassiana* spores, it is necessary to change production methods in innovative ways to increase the spore production with greater efficiency. Solid and liquid substrates, spore harvesting time and temperature level were the factors affecting on spore production of *B. bassiana*.

Spore Production in Different Substrates

There were significant differences ($P < 0.05$) in mean spore production of *B. bassiana* grown on different substrates. Among all seven substrates tested, molasses showed significantly higher spore production for all harvested time periods compared to the other substrates irrespective to the temperature (Table 2). Molasses is a byproduct in raw sugar processing factories. From a nutritional point of view, it is primarily a source of energy, due to its high sugar content (60-70% dry matter) and it contains considerable amounts of sucrose (approximately 32 to 42%) and acts as a best nutritive media. This may support to increase fungal growth and sporulation than the other substrates.

Oil cake was found to be the next best substrate and gave little higher spore

production than other five substrates (0.7793 x1 0^{13} spors/ml). Oil cake is the residue left during the production of coconut oil and it is also high in proteins and minerals. Spore production of coir dust, saw dust, refuse tea, oil cake, disposable part of maize cob (pith + chaff + woody ring) and coconut water were not significantly different with each other in relation to spore production. Molasses is cheap, freely available, more nutritive and it acts as the best liquid substrate for mass production of *B. bassiana* when compared to the substrates tested in the present study (Table 2).

Table 2. Mean spore production in *B.bassiana* in different substrates

Substrates	Mean Spore production ($x10^{13}$)
Molasses	$6.9475^{a} \pm 4.87$
Coconut water	$0.0957^{b} \pm 0.21$
Maize Cob	$0.0133^{b} \pm 0.01$
Refuse tea	$0.0174^{b} \pm 0.01$
Coir dust	$0.0131^{b} \pm 0.00$
Saw dust	$0.0215^{b} \pm 0.01$
Oil cake	$0.7793^{b} \pm 0.49$

Means with same letter are not significantly different at p<0.05

Effect of Temperature on Spore Production

There was no significant difference (P>0.05) in number of spores produced at room temperature and incubator temperature for all substrates. According to Moore *et al.*. (2000), fungal spores are living organisms and their viability diminishes with time depending on environmental conditions, especially storage temperature. The optimal growth temperature for most strains of *Beauveria bassiana* is 27–28°C and requires a relative humidity of at least 92% to germinate (Ferron, 1981). This study showed that *B. bassiana* was able to grow at room temperature with slightly higher spore production than that at incubator temperature, but statistically it was insignificant (Table 3). Use of incubators for large-scale production is impracticable due to high cost and low storage capacity. Therefore, room temperature appeared to be the ideal temperature for cheap and easy mass multiplication as there was no significant difference in rate of spore production among all the substrates between these two temperature levels.

Variation in Spore Production with Time

Significant different spore production rate could be observed in all tested substrates with harvesting time irrespective of the temperature ($P < 0.05$). Production of spores gradually increased from the inoculation up to 42^{nd} day in which, all substrates gave significantly higher number of spores than previous two harvests (Table 4 and 5). Beyond 42^{nd} day, spore production was declined significantly in all substrates and this pattern was clearly shown in molasses that was kept under room temperature (Plate 3).

Table 3. Mean spore production in *B. bassiana* under two storage conditions

Temperature	Mean spore production ($x10^{13}$)
Room temperature	$1.3177^{a} \pm 3.58$
Incubator temperature	$0.9359^{a} \pm 2.29$

Means with same letter are not significantly different at p<0.05

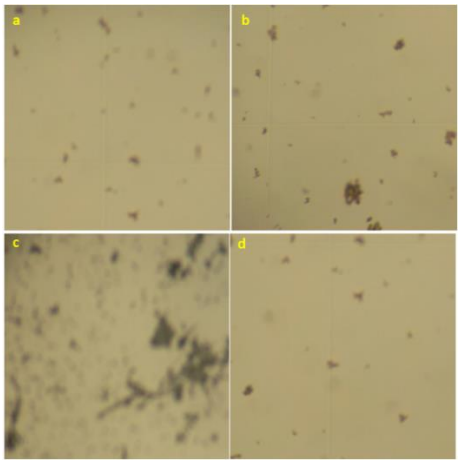

Plate 3. Variation in the number of spores in molasses kept under room temperature; a) 14, b) 28, c) 42, d) 56 days after inoculation

The pattern of variation in spore production can be attributed with the reduction of the available nutrient level in substrates with the time as fungus continuous to utilize the nutrients. Alternatively substrates may begin to degrade and the resources would not be sufficient for continuous growth and production of spores in the fungus. Therefore, to avoid loss of spore production, harvesting of spores should be done 42 days after inoculation of fungus. However, spore production pattern in maize cob in incubator was not significantly different with time though it showed similar pattern of spore production in studied substrates (Table 4).

CONCLUSION

The study revealed that all substrates supported growth of *Beauveria bassiana*. Molasses was the best substrate among all tested substrates with respect to the spore production of *Beauveria bassiana.* There was no significant effect of two tested incubation temperature on spore production of the fungus. To obtain optimum harvest from the substrate, harvesting should be done 42 days after inoculation of the fungus. The findings of this research could be utilized for the mass production of *Beauveria bassiana* (Balsamo) Vuillemin.

Table 4. The variation in number of spores produced with time under the incubator (25 ± 2^0C)

Substrate	Number of Spores (x 10^{13})			
	14 DAI	28 DAI	42 DAI	56 DAI
Molasses	6.000 ^{b} ± 0.000	6.840 ^{ab} ± 0.170	8.980 ^{a} ± 1.103	1.540 ^{c} ± 0.198
coconut water	0.010 ^{a} ± 0.000	0.011 ^{a} ± 0.000	0.020 ^{a} ± 0.000	0.013 ^{a} ± 0.002
Maize cob	0.009 ^{a} ± 0.001	0.009 ^{a} ± 0.001	0.015 ^{a} ± 0.004	0.010 ^{a} ± 0.003
Refuse tea	0.017 ^{b} ± 0.005	0.020 ^{b} ± 0.000	0.034 ^{a} ± 0.001	0.020 ^{b} ± 0.002
coir dust	0.012 ^{ab} ± 0.002	0.014 ^{ab} ± 0.003	0.016 ^{a} ± 0.000	0.009 ^{b} ± 0.001
saw dust	0.016 ^{a} ± 0.002	0.014 ^{a} ± 0.003	0.019 ^{a} ± 0.004	0.022 ^{a} ± 0.001
Oil-cake	0.306 ^{b} ± 0.246	1.200 ^{a} ± 0.113	0.960 ^{a} ± 0.113	0.068 ^{b} ± 0.006

Means with same letter under each substrate are not significantly differ at p<0.05, DAI: Days after inoculation

Table 5. The variation in number of spores produced with time under the room temperature

Substrate	Number of Spores (x 10^{13})			
	14 DAI	28 DAI	42 DAI	56 DAI
Molasses	3.360 ^{b} ± 0.000	6.000 ^{b} ± 0.000	17.940 ^{a} ± 2.630	4.920 ^{b} ± 1.188
coconut water	0.008 ^{b} ± 0.000	0.010 ^{b} ± 0.002	0.624 ^{a} ± 0.102	0.068 ^{b} ± 0.002
Maize cob	0.011 ^{b} ± 0.000	0.014 ^{b} ± 0.001	0.027 ^{a} ± 0.002	0.011 ^{b} ± 0.001
Refuse tea	0.011 ^{ab} ± 0.001	0.014 ^{a} ± 0.000	0.015 ^{a} ± 0.000	0.008 ^{b} ± 0.002
coir dust	0.010 ^{b} ± 0.001	0.015 ^{a} ± 0.001	0.017 ^{a} ± 0.001	0.010 ^{b} ± 0.001
saw dust	0.018 ^{c} ± 0.003	0.025 ^{b} ± 0.000	0.027 ^{ab} ± 0.001	0.032 ^{a} ± 0.001
Oil-cake	0.500 ^{b} ± 0.085	0.660 ^{b} ± 0.085	1.640 ^{a} ± 0.170	0.900 ^{b} ± 0.141

Means with same letter under each substrate are not significantly different at $p<0.05$, DAI: Days after inoculation

ACKNOWLEDGEMENT

Authors wish to express their gratitude to all the staff members of the laboratory of Plantation Management, Wayamba University of Sri Lanka for supporting the study.

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