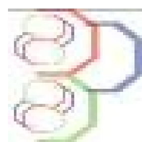

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Bioassay and Pilot Mass Production of Entomopathogenic Fungus, *Beauveria bassiana* for the Control of Coffee Berry Borer (*Hypothenemus hampei*: *Scolytidae*), Ferrari.

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ABSTRACT
Objective: The aim of this study is to evaluate the potential of entomopathogenic fungus (*Beauveria bassiana*) on coffee berry borer based on bioassay test and pilot mass production of conidia using diphasic fermentation.
Methodology and Results: The spore germination test is usually done before bioassay tests. The spore concentration was adjusted to $1x10^7$ spore/ml from the stock suspension using a Haemocytometer. The conidial viability was assessed. Ten coffee berry borers per treatment were dipped into 100 ml sterile beaker that contained 10 ml of *Beauveria* isolates spore suspension with $1x10^7$ spore ml^{-1} from the plate using sterile paintbrush. Spore production was determined by randomly selecting three beetles within each treatment for which, there was spore production. Mass production of *Beauveria* conidial on sorghum was done. Thirteen *Beauveria bassiana* isolates were screened for the biocontrol agent against of coffee berry borer. Four parameters (spore germination percent, 100% insect mortality, average survival time (LT_{50}), and spore production on the dead insect) were used for screening. 0nly 3 isolates scored $\geq$ 93% spore germination, all isolates showed 100% mortality, six isolates showed shorter time of LT_{50} mortality approximately ≤ 84 hrs (3.5) days and 4 isolates produced more than $1x10^7$ average mean spore production per beetle. There were significant differences on spore germination due to spore production of the isolates. Three isolates B7A, G2A and C3C merited for conidial mass production cultured on cooked sorghum using diphasic liquid-solid fermentation to produce spore powder. The mean spore concentration g^{-1} of spore, weight of harvested spore $kg^{-1}$of substrate, spore production kg^{-1} of substrate and the average mean spore germination potential (%) during conidia mass production using the 3 isolates were 4.80 $x10^{10}$, 8.26 ± 0.42, $4.01x10^{11} \pm 2.00 \times 10^{11}$ and 89.33 ± 5.01, respectively. There was a significance difference by the isolates on spore concentration and spore production by the isolates.
Conclusion and applications of results: These isolates that showed 100% mortality on coffee berry borers and also killed them within ≤ 3.5 days of LT_{50} indicated that *Beauveria bassiana* could control the insect pest. Production of spore per cadaver of coffee berry borer almost meets the demand of spore concentration required per hectare of coffee farm ($1x10^7$). The isolates of *B. bassiana* which are efficient in all criteria are considered to be good candidate on selecting biopesticides agents. Moisture content of the harvested spore is expected to be lower to maintain the viability and improved shelf life. The use of high quality standards for spores increases the likelihood of success when applied in the field. The shorter mean mortality time and high

germination potential for the spores are the main criteria of microbial biopesticides. A possible reason for having shorter time of mortality and higher spore production was that adequate viable spores could be there and therefore more promptly infected the beetles caused more rapid mortality during field application.
Key words: *Beauveria bassiana*, Bioassay, Coffee Berry borer, Mass production, Spore concentration

INTRODUCTION

Sustainable agriculture could be achieved not only through proper agricultural practices but also through continuous research and development of new technologies, particularly agricultural biotechnology, which is probably a very important investment to achieve greater competitiveness in the world market. Knowledge and continuous research is the key to assess the potential of biotechnology to increase agricultural productivity and to contribute for sustainability of agricultural systems. It is estimated that one third of global agricultural production, valued at several billion dollars is destroyed annually by over 20,000 species of field and storage pests Tipvadee (2002). Synthetic, broad-spectrum insecticide is a satisfactory and possible solution for pest control; however, the excessive use of chemical insecticides is a threat to human health, natural ecosystem and environment Mahmood *et al*. (2016). Society concerns over pesticides use have resulted in the development of new biologically based insect pests management strategies that are specific to a target pest offer an ecologically sound and effective solution to pest problems (Suman and Dikshit, 2010). In Ethiopia seventy percept of the coffee is produced as garden coffee by small farmers 25% is collected in forest and semi-forest coffee systems, and merely 5% is plantation coffee Woldemariam *et al*. (2008). Arabica coffee grows under very diverse environments including altitude (550 - 2600 m), annual rainfall (1000 - 2000 mm) Woldemariam *et al*. (2008). Major and medium growing districts contain an estimated 800,000 coffee farmers with approximately 520,000 of which 63.3 percent is in Oromiya, 35.9 percent in SNPP and 0.8 percent in Gambella. Smallholder producers are responsible for about 95 percent of production, while state-owned plantations account for 4.4 percent and private investor plantations 0.6 percent FDRE (2003). Among the major factors limiting increased coffee production worldwide are losses due to pests (disease, insects, nematodes and weeds), both indigenous and exotic. Insect pests are the most serious and the most numerous, with over 900 species having been recorded (Goettel e*t al*., 2005). Among which Antestia bug, *Antestiopsis intricate*, *A. facetoids* and coffee blotch miner, *Leucoptera caffeinia* are the major ones inflicting considerable damage. In addition to this Fekadu *et al*. (2016) observed that insect pests such as coffee berry borer (*Hypothenemus hampei*), coffee thrips, *Diarthrothrips coffeae*, green scale, Anthestia bug, white coffee borer, coffee blotch minor, coffee cushion scale, and *Stictococcus formicarius*, are potentially important pests in Ethiopia. Coffee berry borers (CBB) is the most prevalent and important insect pest of coffee in Ethiopia and a problem for the coffee industry (Girma *et al*., 2008). Among many biocontrol methods being explored for use against this insect, the fungal entomopathogen under phylum Ascomycota, class Sordariomycetes, Order Hypocreales family Cordycipitaceae, genus *Beauveria* and species *Beauveria bassiana* is one of them. Therefore, to this end pathogenicity test of the *Beauveria* isolates against the coffee berry borer (CBB) and pilot mass production of these biocontrol agent isolates against the insect pest were the main objective of this work. Biological control is defined as the action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence. Bioassay is defined as the use of a test organism to measure the relative infectivity of a pathogen or toxicity of a substance. This study includes screening of virulent *Beauveria* isolates based on bioassay test, the potent virulent potential of the *Beauveria bassiana* on coffee berry borer and pilot mass production of conidia using diphasic fermentation.

MATERIAL AND METHODS

Determination of spore germination: Spore germination test is usually done before bioassay tests (*Kreutz et al., 2004*). Assay of germination must be carried out within 24 hours as was also done by Bateman (1995). One month-old *Beauveria* isolate were sub cultured from Potato Dextrose Agar (PDA) to Sabouraud Dextrose Yeast Agar (SDAY) plate and incubated at 25 ± 2°C until sporulation. The sporulated fungi were scrapped using sterile spatula into a test tube. The spore concentration was adjusted to 1x10^{7} spore/ml from the stock suspension using Haemocytometer. The conidial viability was assessed according to Ali Alizadeh (2009). Consequently, 1ml of the final conidial suspension 1x10^{7} spore/ml of *Beauveria* isolates was inoculated into sterile mixture of 9ml of 1% water agar and 0.05% yeast extract. Then the suspension was vortexed and incubated at 25±5^{0}C for 24 hours. After incubation, 1ml of the spore culture was placed into haemocytometer and approximately 200 germinated and non-germinated conidia were counted to estimate percentage of germination under light microscope. Each count was done in triplicates to calculate the mean % of spore germination. Viability of the spores was confirmed and conidium was considered as germinated when a visible germ tube longer than half the diameter of the spore (Vega, 2008). Percentage of germination was calculated using the following formula.

$$\% \text{ of spore germinated} = \frac{\text{Number of spore germinated}}{\text{Total spore count}} \times 100$$

Study areas and CBB trapping: CBB were caught and assessed in three different localities of known coffee producing areas in the south western part of Ethiopia (Figure 1). These localities were selected as a representative sample of potentially coffee producing areas, namely Tepi coffee plantation area at "Baya II" (7^{0}10'36"N, 35^{0}24'50" E and 1,206 m.a.s.l.) on (20-30)-01-2013, Jimma zone, (Limu Goma II) (7^{0} 57'47"N, 36^{0}41'9"E and 1,409 m.a.s.l.) on (15-25)-03-2014 and Mizan-Aman (7^{0} 00'10" N, 35^{0}34'56" E and1325 m.a.s.l.) on (15-25)-11-2015 (Table 1).

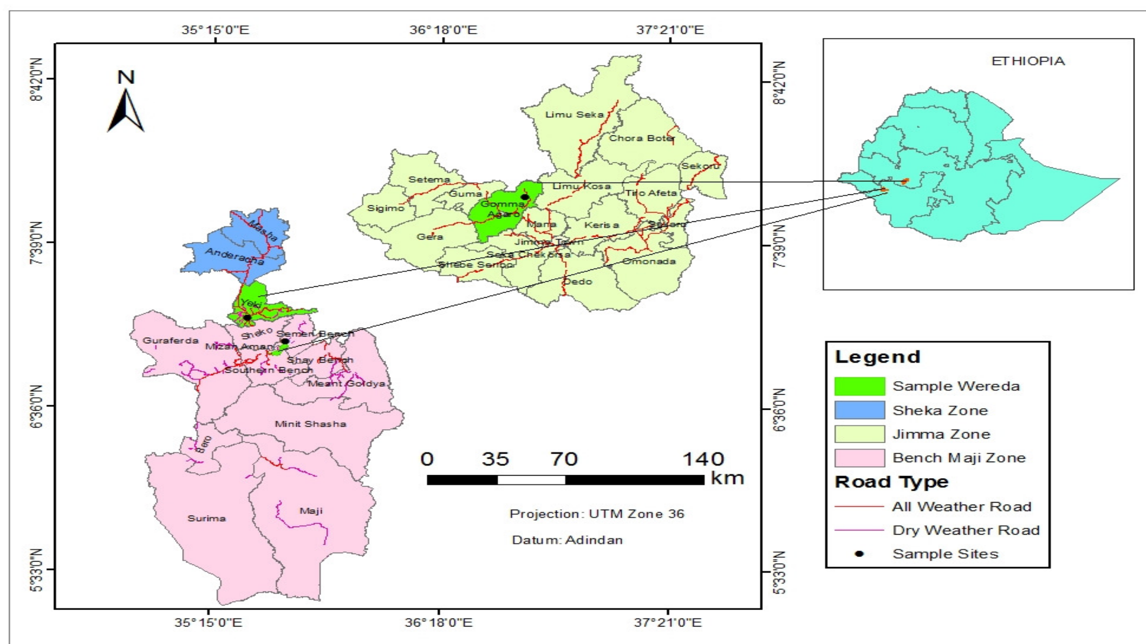

Figure 1: Map of coffee berry bore, (*Hypothenemus hampei*) mass trapping sites, districts and zone.

Table 1: Geographical location, altitudes and description of coffee berry borer (*Hypothenemus hampei*) trapped sites from the three coffee producing localities of Jimma and Benchi- Majii zones of South western of Ethiopia.

Study sites	Date data collection	Long/Latitude locations	Altitude	Description
Tepi- biy-II	Jan. 20 – Jan 30/2013	7^0 10'36"N,$35^0$24'50"E	1206	Flowering stage
Limu-Goma- II	Mar. 15 - 25/2014	$7^0$57'47"N, $36^0$41'9"E	1409	Dried condition
Mizan-Aman	Nov. 15 – 25/ 2015	$7^0$00'10" N, $35^0$34'56"E	1325	Harvesting, rain

The ecological and environmental conditions of these three localities are favourable for coffee production but different on climatic and weather conditions. Using locally made baiting trap the CBB was collected from these selected areas. The baiting traps lured with the mixture of 99% ethanol and 100% methanol undiluted in accordance with the methodology proposed by Mathieu *et al.* (1997). From each Ethanol: Methanol, E: M (1:1, 1:2 and 1:3) mixture 10 ml were injected into labelled vials. The female CBB entering the trap automatically fell into the container where they were drowned and collected in the sterile vial. Trapped CBB was collected within one-day intervals from the first day of trapping for ten days. The collected CBB were transported to the AAU laboratory for bioassay test using Beauveria bassiana as a biocontrol agent.

Coffee berry borer inoculation : The adult female beetles were identified using stereomicroscope with their size approx. ca. 1.7 mm. The colour of the young beetle when observed using stereomicroscope their abdomen appears a blackish-brown, with a lighter brown pronotum and darken to black at maturity (Russell, 2012). The identified female CBB were surface sterilized with 0.5% sodium hypochlorite solution and gently shaken for two minutes. Afterwards, they were rinsed three times in sterile distilled water and again sterilized with 70% ethanol for two minutes and rinsed with sterile distilled water three times and dried in a container lined with sterile towels paper (Posada and Vega, 2005). Surface sterilized CBB then separated into small groups to be assigned to the treatments, ten CBB were kept into a sterile plate in triplicate per treatment. Ten coffee berry borers per treatment were dipped into 100 ml sterile beaker that contained 10 ml of *Beauveria* isolates spore suspension with $1x10^7$ spore ml^{-1} from the plate using sterile paintbrush (Posada and Vega, 2005). For control 0.1% Triton X-100 without spore were used. The spore suspension and the insects were gently shaken for two minutes while dipped in their respective treatments. The suspension with CBB was empty into sterile 60mm plated gently. Using a sterile paintbrush the inoculated insects placed into sterile plate containing two pieces of sterile Whatman No.1 filter paper moistened with sterile distilled water. Five inoculated insects were kept per plate with reasonably separated one insect from the other in space. The experiment was conducted as a completely randomized design, with each isolate treatment replicated 3 times. Sterile distilled water was added to the filter paper as needed. All treatments were incubated in the dark at 25 $\pm$ 2^0 C in a growth chamber. Insect mortality was assessed with in 24hrs intervals. Mortality of the insects was checked based on stiffness of the cadavers and mycelia observed under microscope. Dead cadavers manifesting the mycelia growth on the surface were kept separately in the 5cm sterile plates containing sterile Whatman No.1 filter paper moistened with sterile distilled water until the fungi sporulated. Median of lethal time (LT_{50}) of each isolates was calculated by using probit analysis (Throne e*t al.*, 1995).

Quantifying spore production on coffee berry borer : Spore production was determined by randomly selecting 3 beetles within each treatment for which, there was spore production. Each beetle was immersed with in 5 ml of sterile distilled water with 0.1% Triton X-100 and gently shaken for one minute and then diluted to 10ml (Posada and Vega, 2005). From a diluted spore suspension, 1ml was counted in triplicate per each sample in Haemocytometer. The data were analyzed using a one-way analysis of variance (ANOVA SPSS, 20). Statistical significance was checked at $P < 0.05$ and was considered significant. In this study, some of the parameters applied for bioassay test were used to select *Beauveria* isolates using a scoring system for developing the criteria of potential biocontrol agent. In this scoring system a "+" was assigned when the isolate fulfilled minimum values for each parameter as follows: insect mortality potential 100%; fungal spore germination > 85%; median of lethal time (LT_{50}) ≤ 84 hrs (3.5 days) and spore production ≥ $1x10^7$ spores per insect (Posada and Vega, 2005). Thus, the higher the number of "+" signs, the better the isolate with four "+" being optimal; three "+" being good; two "+" average; and below two "+" not satisfactory. Of the 13 *Beauveria* isolates only 3 showed some potential as biocontrol agents and were selected for pilot spore mass production (Figure 2).

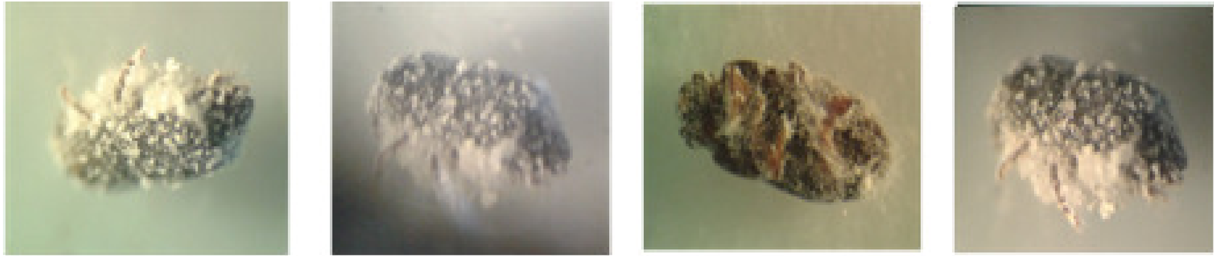

Figure 2: Mycelia and spore grown on the cadavers of CBB inoculated with spore $1x10^7$ spore ml^{-1} with different direction of the coffee berry borer cadaver. For each treatment 10 CBB were used in triplicates, adult CBB were dipped in each spore suspension, and dead CBB were picked and kept separately to produce spore on their surface. The above pictures illustrated mycelia growth and spore production on surface of CBB cadaver within 7-10 days after treatment.

Preparation of liquid state fermentation: Three isolates of *Beauveria*, B7A, C3C and G2A were selected based on the defined parameters for conidial mass production. One month old sporulated cultures of the selected isolates on PDA were inoculated into a sterilized 250ml of Sabouraud Dextrose Broth (SDB) in 500ml of flat bottom round flask. The inoculated cultures were kept on orbital shaker for a couple of weeks for the growth of blastospores for the inoculation of sorghum substrate for mass production (Figure 3A).

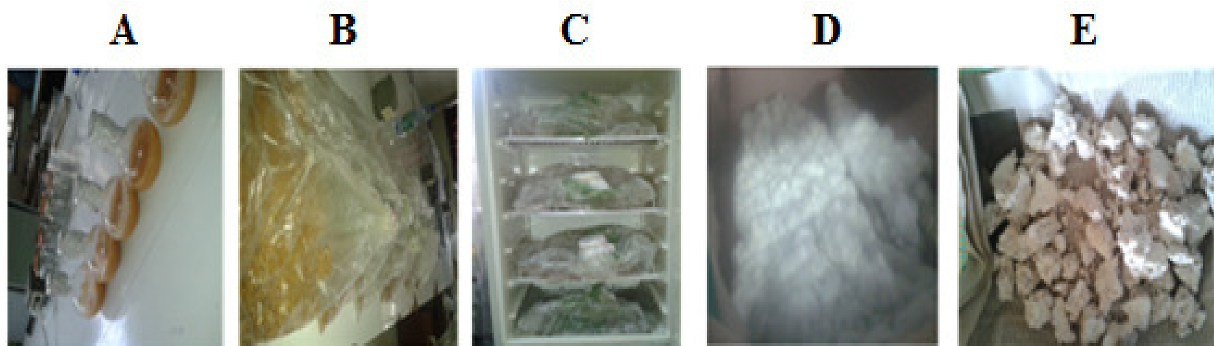

Figure 3: Mass production of Entomopathogenic fungus (*Beauveria* isolates) using diphasic fermentation. A= Blastospore in the liquid phase of fermentation, B= Inoculated sorghum substrate with liquid culture, C= Incubated inoculated substrate in the growth chamber, D= Complete mycelia coverage of the substrate and E= Air dried spore with sorghum substrate.

Spore mass production on solid substrate: Mass production of *Beauveria* conidial on solid substrate was done according to Gouli *et al.* (2005). One kg of sorghum was washed and dried into direct sun light. The dried sorghum substrate then mixed with water in the autoclaveable bags. The mixture then cooked in a water bath at 100^0C to boil for 2 hrs. The bags with the cooked sorghum substrate then autoclaved at 121^0C for 1 h. Mass production of each isolate was replicated 2 times (2 kg) of substrate per isolates were used. The bags containing the grain were sterilized at the previous day and remaining in the autoclave overnight to ensure their sterility is maintained. The autoclaved substrates were inoculated with grown *Beauveria* liquid culture inside a laminar flow hood. During inoculation the mouth of the bags were inserted in to a cylindrical autoclaveable PVC pipe and the mouth of the bags pulled out through the PVC pipe reasonably and wrapped on PVC pipe in such away to keep the mouth open. The mouth of the bags were covered with the sterile cheesecloth, tissue paper and aluminium foil cover from inside to outside respectively and held in place with a rubber band round the PVC pipe opening (Figure 3A&B). The inoculated bags were kept inside a growth chamber (Figure 3C). Open shelves were used to allows air to circulate freely around the bags, preventing localized heating of the substrate as the fungus grows. The inoculated substrate was distributed evenly inside the bag as thin a layer as possible to maximize the surface area for gas exchange and growth. Three to four days after inoculation, the substrate was gently mixed without opening the bags. The mixing process was repeated 1-2 more times, 3-4

days apart. After one week of inoculation aluminium cover were removed for the better supply of oxygen for fungal mycelia growth and sporulation. Within 15 days the fungal mycelia were completely covered the substrate and sporulated within 20 days.

Spore drying and moisture content assessment: After 20 days the fungal biomass plus the substrate were transferred from the bags on to the trays which were wrapped with sterile news paper (Figure 3D). Two trays were generally used for each bag so that the substrate can be spread in a thin, to enhance hyphal maturation, sporulation and eventual drying (Figure 3E). Simultaneously, when the cultures exposed to open tray from each treatments 200g of subsample culture (sorghum plus conidia) were taken at random places in duplicates for moisture content assessment (four subsamples per treatment). To dry the sample cultures they were kept in a chamber with an average temperature of 12 ± 4^{0}C and an average relative humidity of 22 ± 6% for air dry. The moisture content of the culture and sorghum during air drying process were assessed in 24hrs intervals for ten days until the moisture content was stable. In order to monitor the efficiency of air drying, same amount of subsamples were taken from each treatment to oven drying for 24 hours at 105^{0}C (Rao *et al*., 2006). Percentage of the final moisture content of the harvested spore was calculated using the following formula.

$$\% \text{ of moisture} = \frac{MLod - MLad}{MLod} \times 100$$

Where: - MLod = Moisture Loss with oven drying
MLad = Moisture Loss with air drying

Harvesting of spore: Harvesting was done manually for 20 minutes. Any large clumps were first broken apart and the entire contents were gently mashed and then shaken. Spore powder separation from the dried substrate were done by placing the dried culture into a fine mesh bag size (25 x 50 cm), through a 35-mesh sieve (279 µm pore size) which was held inside a plastic bag (35 x 60 cm or larger) (Gouli, 2005). After that, aerial conidia were harvested by manually shaking the fungus colonized substrate. The separated conidia from the grains passed through the mesh was weighed and kept in to plastic bag (Figure 5).

Figure 5: Harvested powder spores of three *Beauveria* isolates using manual method from sorghum substrate after 20 days

Determination of spore concentration : After 10 days of air drying 1g of harvested spore was transferred into 10 ml sterilized distilled water containing 0.1% of triton-X-100 solution in 100 ml conical flasks. After adding into the flasks the suspension were shaken to mix for 10 min. The suspension was filtered through double layered muslin cloth. Counting of spores was made after the serial dilution of the suspension using haemocytometer for determining the number of spores (concentration) per gram.

Determining viability of spore: After spore powder were harvested the viability of the spore was assessed by sampling a 1g of the conidia and suspended in a small volume of 0.01% triton-X-100 and filtered through sterile cheese cloth and then adjusting the spore concentration to 1x10^7 spore/ml. Spore viability were determined as indicated the same method above.

RESULT

The mean spore germination (%) of 13 *Beauveria* isolates before bioassay was ranged from 76.33 ± 9.88 - 95.75 ± 1.77 and 9 (69.23%) isolates showed > 85% of spore germination at 24 hours. Three isolates, B7A, C3C and G2A were even scored the better spore germination potential (≥ 93%). (Table 2). There were significant differences in *Beauveria* isolates spore germination (%) due to isolates at (df = 12, F = 2.734 and P = 0.016).

Table 2: Average mean ± SD of spore germination (%), mortality (100%), average lethal time in hours (LT_{50}), spore production per coffee berry borer and scoring of bioassay test after dipping the insect into spore suspension of *Beauveria* isolates at 1 x10^7 ml^{-1} within 10 days.

Beauveria Isolates	Germination (%)	Mortality100%;	LT_{50}	Spore per CBB	Scoring
	A	B	C	E	F
B1D	82.17 ± 7.59^{ab}	100.00 ± 0.00	87.94	3.3 x 10^6 ± 1.1 x 10^{6} $^{a, b}$	-+-.-
B2J	88.50 ± 4.77^{ab}	100.00 ± 0.00	89.18	2.9 x 10^7 ± 2.3 x 10^{7} ^{b}	++-+
B5B	85.33 ± 7.59^{ab}	100.00 ± 0.00	93.32	1.5 x 10^7 ± 1.7 x 10^{7} $^{a, b}$	++-+
B6F	76.33 ± 9.88^{a}	100.00 ± 0.00	96.17	4.3 x 10^6 ± 2.0 x 10^{6} $^{a, b}$	-+--
B7A	93.83 ± 1.04^{b}	100.00 ± 0.00	66.68	8.3 x 10^7 ± 1.3 x 10^{7} ^{c}	++++
B7E	77.83 ± 9.70^{ab}	100.00 ± 0.00	94.57	5.0 x 10^5 ± 2.6 x 10^{5} ^{a}	-+--
B9D	88.17 ± 4.75^{ab}	100.00 ± 0.00	83.77	2.7 x 10^6 ± 9.9 x 10^{5} $^{a, b}$	+++-
C1C	83.17 ± 5.01^{ab}	100.00 ± 0.00	103.24	6.5 x 10^6 ± 8.0 x10^{5} $^{a, b}$	----
C3C	95.75 ± 1.77^{b}	100.00 ± 0.00	67.44	6.5 x 10^7 ± 2.1 x10^{6} ^{c}	++++
C5I	89.00 ± 6.38^{ab}	100.00 ± 0.00	85.26	5.5 x 10^6 ± 6.6 x10^{5} $^{a, b}$	++--
G2A	94.83 ± 0.58^{b}	100.00 ± 0.00	78.30	6.7 x 10^6 ± 3.8 x10^{6} $^{a, b}$	+++-
G3H	86.67 ± 2.84^{ab}	100.00 ± 0.00	80.58	3.4 x 10^6 ± 3.3 x10^{6} $^{a, b}$	+++-
G5I	87.00 ± 5.63^{ab}	100.00 ± 0.00	125.40	5.5 x 10^5 ± 3.3 x 10^{5} ^{a}	++--

Each treatment was replicates 3 times and ten CBB were subjected per each treatment then dead CBB were inspected and recorded with in 24hrs intervals. Mean ± SD, percentage of germination, mortality (%) and spore per CBB were calculated with SPSS 20. Different letters showed significant differences of spore germination test using ANOVA post hoc LSD Tukey's,. Lethal Time 50 (LT50) was determined using probit analysis. Scoring of each parameter was given based on; a "+" score was based on minimum values for each parameter as follows: mortality: 100 %; germination: > 85%; LT50 ≤ 84hrs (3.5 days) and spore production: ≥ 1x107 spores insect-1. The better the isolate, the higher the "+" score, with 4"+" being optimal, 3"+" good, 2"+" average and below 2"+", below average.*

Mortality of coffee berry borers dipped into spore suspensions of 1x10^7 ml^{-1} showed high mortality with 13 of the isolates reaching 100% mortality even though there was discrepancies on number the mortality of the coffee berry borers at different hours (days) intervals. Two (15.38%) isolates (B7A and C3C) showed LT_{50} mortality less than 72 hrs (3 days), 3 (23.08) isolates (B9D, G2A and G3H) cause mortality within less than 84 hrs (3.5 days), 4 (30.77%) isolates (B1D, B2J, B5B and B7E) less than 96 hrs (4 days) and 2 (15.38%) isolates (C1C and G5I) showed between 96 and 120 hrs (4 and 5.2 days). The overall mortality tested 5 (38.46%) isolates B7A B9D, C3C, G2A and G3H) showed LT_{50} mortality approximately ≤ 3.5 days (Table 2). There was no mortality observed in the controls within 5 days and LT 50 was 125.4hrs (5.23 days). From the average mean spore production 4 (30.77%) isolates B2J, B5B, B7A and C3C produced more than 1x10^7 spores per beetle (Table 1). seven (53.85%) isolates (B1D, B6F, B9D, C1C, C5I, G2A and G3H produced between 1x10^6 and 1x10^7 spores per beetle and two isolates, B7E and G5I produced between 1 x 10^5 and 1x10^6 spores per beetle. There were significant differences in spore production on coffee berry borer cadavers at (F = 25.525, df = 12, P = 0.000) (Table 2). Scoring of each parameter tested for screening potential *Beauveria* isolates that fulfil the minimum requirements were scored as "+' sign. Two isolates which qualify the minimum values for each parameter with 4"+"were B7A and C3C while 5 isolates (B2J, B5B, B9D,G2A and G3H) scored 3+ and the rest 6 isolates scored 2"+" and below (Table 2). The moisture content fell rapidly during the first 4 days of assessment when the substrate exposed from the bag onto the tray and after wards the moisture loss was relatively small and remained steady at around 10th days (Figure 4). All the cultures of the three isolates showed almost the same pattern of moisture loss.

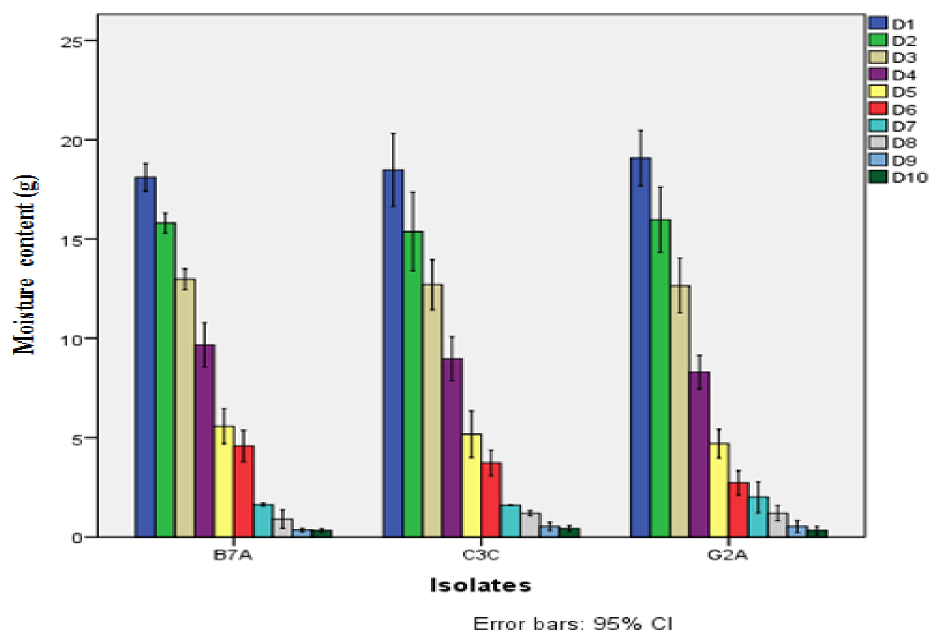

Figure 4: Loss of moisture content during air drying of *Beauveria bassiana* B7A, C3C and G2A cultured on sorghum. From each treatment four subsamples (200g) were taken for air drying moisture loss assessment and mean ± Sd of moisture loss were calculated using SPSS 20 and Bars represent confidence intervals for means (P = 0.05).

The mean weight of air dried and oven dried of the culture and the substrate of the three *Beauveria* isolates B7A, C3C and G2A were (143.87 ± 0.98, 145.13 ± 2.78 and 145.14 ± 2.65) and (136.85 ±1.59, 137.16 ±1.22 and 137.05 ±1.15) respectively while the mean percentage of moisture content of the three isolates B7A, C3C and G2A were 11.09 ± 2.39, 12.62 ± 5.66 and 12.86 ± 3.75 respectively (Table 3).

Table 3: Mean ± Sd (g) of weight of culture after 10 days air drying, oven drying and percentage of moisture attained.

Isolates	Air drying	Oven drying	% of moisture
B7A	143.87 ± 0.98	136.85 ±1.59	11.09 ± 2.39
C3C	145.13 ± 2.78	137.16 ±1.22	12.62 ± 5.66
G2A	145.14 ± 2.65	137.05 ±1.15	12.86 ± 3.75

From each treatment, 200g of four subsamples were taken for assessment. Air and oven drying moisture loss from each were recorded with in 24 hrs (1 day) interval for ten days. The mean ± Sd were calculated using SPSS 20.

After 10 days of air dried and harvested the average mean spore concentration per gram were 4.80×10^{10}, 4.07×10^9 and 3.33×10^9 and weight of powder spore per 1kg of the substrate were 8.48 ± 0.71, 7.32 ± 0.56 and 8.26 ± 0.42 by the isolates of B7A, C3C and G2A respectively.

The spore production and the average mean spore germination potential (%) of the B7A, C3C and G2A were ($4.01 \times 10^{11} \pm 2.00 \times 10^{11}$, $3.06 \times 10^{10} \pm 1.90 \times 10^{10}$ and $2.71 \times 10^{10} \pm 1.45 \times 10^9$) and (89.33 ± 5.01, 86.50 ± 6.61 and 88.50 ± 3.77) respectively (Table 4).

Table 4: Average mean ± SD of spore concentration, spore powder (g), spore production and spore germination (%) of entomopathogenic fungi, *Beauveria bassiana* per kg of sorghum substrates

Isolates	Spore Concentration	Spore powder	Spore Production	Germination (%)
A	B	C	D	E
B7A	$4.80 \times 10^{10} \pm 2.66 \times 10^{10}$B	8.48 ± 0.71^{A}	$4.01 \times 10^{11} \pm 2.00 \times 10^{11A}$	89.33 ± 5.01^{A}
C3C	$4.07 \times 10^{9} \pm 2.24 \times 10^{9}$A	7.32 ± 0.56^{A}	$3.06 \times 10^{10} \pm 1.90 \times 10^{10B}$	86.50 ± 6.61^{A}
G2A	$3.33 \times 10^{9} \pm 1.85 \times 10^{9}$A	8.26 ± 0.42^{A}	$2.71 \times 10^{10} \pm 1.45 \times 10^{9B}$	88.50 ± 3.77^{A}

The above table indicated the spore concentration, spore powder, spore production and germination potential of B7A, C3C and G2A Beauveria isolate after 20 days of incubation and 10 days of drying. A = Isolates, B = The mean concentration of spores per gram of harvested spore; C = The mean powder spore harvested per kg of sorghum and D = B x C; spores produced / kg sorghum. Each trial replicate 3 times and average mean ± SD were calculated using SPSS 20. Different letters indicates the significance difference at LSD of Tukey's.

There was a significance difference by the isolates on spore concentration per gram and spore production by the isolates at (df = 2, F= 8.208 and P = 0.019) and at (df = 2, F = 10.174 and P = 0.012) respectively. However, there was not statistical significance difference from harvested spore powder and spore germination (%) potential at (df = 2, F = 3.548, P = 0.096) and at (df = 2, F = 0.230, P = 0.801) respectively.

DISCUSSION

The coffee berry borer is a serious insect pest of coffee production in Ethiopia and strategies attempted to overcome this pest have not yet been designed. Other countries use chemical pesticides such as endosulfan but problems such as resistance to chemical insecticides and environmental issues forced to ban these chemicals from use (Brun *et al*., 1989). In the present study, laboratory trails were done to initiate the utilization of *Beauveria bassiana* as a biopesticide, which is an environmentally friendly pest management method in Ethiopia to control or maintain the coffee berry borer under the economic injury level. The use of highly pathogenic fungi such as *Beauveria bassiana* has proved to be economically and biologically effective to control coffee berry borer in the field. The current results could be the initial attempt in the development of biopesticides in Ethiopia that could be very important for insect control in the coffee production system. The low prices paid to coffee growers and unstable market prices in Ethiopia have resulted in a reduction of coffee pest management inputs FAO (2014). Moreover, coffee berry borer is an extremely difficult pest to control, mostly due to its cryptic nature and lack of the knowledge of the control strategy of this devastating coffee pest will aggravate the damage of the coffee production (Fernando *et al*., 2009**).** The development of sustainable pest management strategies using entomopathogenic fungal isolates that provide a fast kill and produce a high number of spores in the insect cadaver can play an important role in causing natural epizootics, thus reducing coffee berry borer populations and leading to a more sustainable agricultural system (Lacey *et al*., 2001). Any investment in fungal entomopathogen production in coffee producing countries aimed at the coffee berry borer should be based on use of a strain with the highest potential for causing high mortality and for being recycled in coffee fields. If the coffee is growing under shade like most coffee production in Ethiopia, have high humidity conditions favourable for *B. bassiana* (Staver *et al*., 2001). In the field *Beauveria bassiana,* exclusively attacks adult *H. hampei* and infection can be detected by a blob of white, cotton wool like mycelium, growing away from the body of the insect, appearing at the entrance of the tunnel (perforation) bored into the coffee berry (Damon, 2000). *Beauveria bassiana* is a broad host range insect pathogen that has been approved by EPA (Environmental Protection Agency, December 22, 2006), for use as an insect pest biological control agent and is available from various commercial companies world-wide (Goettel *et al.*, 2005). Today, the fungus is considered a natural controller of the pest because it is found infecting the insect in all the coffee producing areas where coffee berry borer has been reported. The time of application of *Beauveria bassiana* can be best and mostly effective when female *H. hampei* are just starting to penetrate the berries. Usually effective control coincides with the beginning of the rainy season and later during harvesting when the pest migrate to new berries (Esayas *et al*., 2004). The effect of *Beauveria bassiana* on the target pest may be on different ways like, insects infected with an entomopathogenic fungus may alter their behaviour during mating and oviposition (Goettel *et al*., 2005)

decreasing their progeny. In the case of coffee berry borer, the infection can cause physiological damage to insects in such a way that they cannot mate inside berries, or eggs do not develop after mating (Roy *et al.,* 2006). Fungal infection can also induce aberrant behaviour that can decrease the fitness of the insect. These behaviours include male copulating more with infected females, or infected females not copulating, which are behaviours previously reported in other species of insects (Roy *et al.,* 2006). An assessment of insect mortality and average survival time presents only a partial view of pathogenicity and does not provide adequate information to select the best fungal isolates Posada and Vega (2005). In this present study, a method to evaluate candidate fungal entomopathogens based on four parameters: spore germination (%), insect mortality (%), lethal time LT_{50}, and spore production per insect. This information provides a better picture to determine which fungal isolates should be considered for subsequent mass production, formulation and possible commercialization. Attempts were carried out to develop a screening method that can be applied in future studies aimed at assessing the potential use of fungal entomopathogens in insect pest management strategies (IPM). The mean germination percentage of the 13 *Beauveria* isolates before bioassay was ranged from 76.33 ± 9.88 - 95.75 ± 1.77 and 9 (69.23%) isolates showed > 85% of spore germination at 24 hours. Posada and Vega (2005) obtained 0-95%, from fifty *Beauveria* isolates at 24 hrs. Unlike Posada and Vega (2005), none of this study isolates showed 0% of germination rather the lowest germination percentage were 76.33%. These 9 isolates that exhibited higher spore germination potential and others 4 (30.77%) isolates were used for bioassay test against the CBB. Proper evaluation of germination is an important part of determining the potential of spores for field application as well. The germination value obtained at this time was a parameter that needs to be considered the spore survival following their application in the field. Isolates also should keep their potential of germination under different circumstances such as after conidial mass production. It is evidence that the isolates, B7A, C3C and G2A, which were best in spore germination during bioassay screening, showed reduction of germination potential on average by 6.69% after mass production of spore viability test but still they showed more than 88.11% of spore germination on average after mass production and harvested. *Beauveria* isolates with the highest potential for causing high mortality and for being recycled in coffee fields, which, if growing under shade, have high humidity conditions favourable for *B. bassiana* (Staver *et al*., 2001). The average mean spore germination potential (%) of the isolates, B7A, C3C and G2A after harvested the spores from the substrate during mass production were 89.33 ± 5.01, 86.50 ± 6.61 and 88.50 ± 3.77 respectively. There was no statistical significance difference among the isolates spore germination after mass production at (df = 2, f = 0.230 p = 0.801). The spore germination potential of Beauveria isolates at 24 hrs 4 (30.77%) of isolates scored < 85% of germination but all of them showed 100% mortality on CBB pathogenicity test. Posada and Vega (2005) reported that isolates exhibiting no germination with in 24 hrs still causing 100% mortality. The delay of spores from germination may be caused by damage of the spore by environmental factor such as temperature and low humidity, which are the potential cause for loss of spore viability. Most of the isolates from this result showed > 85% spores germinate within 24hrs implies that they are less likely affected by potentially adverse conditions that might reduce their viability in the field application. The germination potential and pathogenicity of the spores are the most important quality control parameters in rapid mortality of coffee berry borer. To assess pathogenicity potential of the isolates dipping method was preferred than spraying when conducting bioassay on coffee berry borer with fungal entomopathogens (Marin *et al*., 2000). The first reason was the uniform spore concentrations and thus pathogenicity can be easily compared across isolates and the second one was dipping provides a massive spore concentration to the insect and results in faster mortality than spraying (Posada *et al.,* 2002). Dipping method also has a chance of contacting every insect's body uniformly. Marin *et al.* (2000) have shown that coffee berry borer mortality caused by *B. bassiana* is just over three days using the dipping method while spraying and exposing the insects to the droplet deposit can take more than 10 days (Posada *et al*., 2002). Even though, dipping methodology does not mimic field application methods it is a valuable tool for assessing what strains should be studied in more detail when screening of fungal isolates. From these results the mortality of coffee berry borers dipped into $1x10^7$ spore ml^{-1} of showed high mortality with 13 of the isolates reaching 100% mortality even though there were some discrepancies on number the mortality of the coffee berry borers at different hours (days) intervals (Table 2). Posada and Vega (2005) have also obtained high coffee berry borer mortality with 47 of the fungal isolates reaching 100% mortality, two isolates causing 97.5% mortality and one causing 67.5% mortality with $1x10^7$ spores suspensions ml^{-1}. The average survival time (LT_{50})

of the 13 isolates two showed < 72 hrs (3 days), 4 isolates 72 - 84 hrs (3-3.5 days), 4 isolates 84 - 96 hrs (3.5 - 4 days) and 2 isolates showed between 96 - 120 hrs (4 - 5.2 days). With the same method of treatment Posada and Vega (2005) using fifty isolates obtained 3.2 - 9.6 days of average survival time (LT_{50}) and from thirty-one isolates caused 100% coffee berry borer mortality within 3.2 and 4.0 days, while 17 isolates took between 4 - 6 days; two isolates took longer than 6 days to kill the insects. From the over, all of this bioassay experiment 3 of the isolates caused 100% mortality within ≤ 78 hrs (3 days). There was no mortality in the controls due to fungal growth. The average survival time for the controls was 129.5 hrs (5.39) days. The highest average mean spore production per beetle was observed by isolates of B2J, B5B, B7A and C3C and produce more than $1x10^7$ spore/beetle (Table 2). These isolates produce spore above the minimum requirement of spore production (>1 x 10^7) which is the baseline concentration used to evaluate *B. bassiana* against coffee berry borers at the National Coffee Research Center (Cenicafé) in Colombia (Marin *et al*., 2000). Thus, *B. bassiana* production on the cadaver could provide a fresh source of fungal inoculum directly in the coffee agro-ecosystem if the sprayed spores colonize the beetle. Posada and Vega (2005) proposed interesting assumption and estimation from two *B. bassiana* isolates applied on coffee berry borer treatment and produce spore per beetle was $1x10^7$ per plant and 5000 plants per hectare would contribute (2 beetles x 5000 plants/hector x10^7 spore/beetle = $1x10^{11}$) spores per ha, which can be equal to what is applied in a commercial application. Four *Beauveria* isolates that produce more than $1x10^7$ spore per beetle from this experiment could be a potential source of coffee ecosystem inoculum and can persist their life cycle especially these coffee that grow under the shade. Fungal entomopathogen isolates that provide a fast kill and produce a high number of spores in the insect cadaver can play an important role in causing natural epizootics, thus reducing coffee berry borer populations and leading to a more sustainable agricultural system.

Based on the applied screening parameters the scoring system to rate the potential of the *Beauveria* isolates used in the bioassays test were showed from 0 - 4 "+". For scoring system a "+" was assigned when the isolate fulfilled minimum values (according to this context) for each of the parameter. Among the 13 isolates evaluated based on the parameters only 2 isolates (B7A and C3C) scored 4 "+" and 5 isolates (B5B, B2J, B9D, G2A and G3H) scored 3 "+" as a good potential biocontrol agents. Thus, the criteria that were sated can be used to narrow down the number of strains to be assessed bioassay studies. However, by incorporating isolate G2A, only three isolates, B7A, C3C and G2A were selected for the next biopesticide conidial mass production. Isolate G2A showed best spore germination potential (94.83 ± 0.58) % and less $LT_{50,}$ (78.30 hrs or 3.25 days) than the rest isolates (B5B, B2J and B9D) that showed better spore production/beetle. The success of microbial control of insect pests depends not only on the isolation, characterization and pathogenicity, but also on the successful mass production of the microbial agents in the laboratory. Large-scale availability of the biocontrol agent is a primary requirement in the biocontrol program. For a successful integrated pest management program, the agents like the entomopathogenic fungi should be amenable to easy and cheap mass multiplication. Several naturally available substrates were tested for mass multiplication of *B. bassiana.* Masoud *et al.* (2013) used solid substrates; sugar cane, corn, barley, rice, millet and sorghum and liquid; potatoes, wheat flour, rice flour, corn flour and sugar cane molasses for mass production of *Beauveria bassiana*. Moisture loss is a result of the metabolic activity of the fungus, transpiration and diffusion while the cultures are developing. Metabolic and added water moisture in the production process should be very low and be acceptable level. To reduce the moisture content of the *Beauveria* isolates culture and sorghum in to each treatment were dried for ten days at which until the moisture content was stable (Figure 4). During assessment the moisture content was fell rapidly during the first four days of the assessment when the substrate exposed from the bag on to open air tray and then after the moisture loss was relatively small and remained steady at around 10th day (Figure 2). The corresponding dry weight at each day attended was inversely proportional to the moisture loss in the first four days and the weight reduction became very small and finally remains stable at the 10 day. Posada (2008) achieved the lowest moisture content from harvested spore powder was around 10% for the 15 days harvesting period and this moisture content was, obtained under controlled conditions of air drying. It was still considered as too high to store the spores and to preserve their viability. Ideally, a moisture content of the spore would have been preferable to be 5% (Bateman,1995). However, the mean percentage moisture content that was achieved from the culture and sorghum during air drying process was not satisfactory (Table 3). The mean percentage of moisture content of these cultures was ranged from 11.09 ± 2.39 - 12.86 ± 3.75. The mount of moisture content that was achieved at 10 days as compared to Posada (2008) was

relatively high. Prolonged period exposing the spore for drying will affect the spore viability (Gouli *et al.,* 2005). This may be the challenge of mass production and commercialization of biopesticide producers especially for small-scale producers that do not use technologies for this purpose. One way to decrease the moisture content would be to keep the spore mix with a desiccant such as silica gel, which would help to achieve the objective of spore storage with low moisture content (Moore and Caudwell, 1997). The use of silica gel to maintain the spore moisture at a low level would not pose a technical problem even for larger scale production. Moisture content can influence the weight of the spores harvested. The duration of the drying process depends on the ambient temperature and humidity, air circulation and ventilation. Drying of the fungal spore too rapidly or slowly can be harmful to conidia, so it is critical to determine viability after drying is complete. If the conidia are exposed to open air for a long period of time the spore will lose their viability and if they are stored with high moisture they will germinate and loss their viability. Therefore, biopesticide mass production the two main challenges are attending the lowest acceptable moisture content and keeping viability of the spore. Mass production of spore from 1kg of sorghum substrate at 20 days showed significantly higher spore production using liquid-solid phase fermentation. The average mean spore concentration of B7A, C3C and G2A *Beauveria* isolates were 4.80 x10^{10}, 4.07 x10^{9} and 3.33 x10^{9} spore g^{-1} respectively. Beauveria isolate of B7A showed statistically significant difference in spore count g^{-1} from other isolates. Masoud *et al.* (2013) obtained (6.24 x10^{4} spores/ml) on rice and (4.34 x10^{4} spores/ml) on sorghum seeds from150g of solid wastes substrate. Interestingly Avishek *et al.* (2014) obtained 8.3 x 10^{9} spores' g^{-1} with rice water and flour using the new technique of polyurethane foams medium in first 10 days, which is almost nineteen times higher than the conventional solid-state fermentation after 20 days incubation. Herta *et al.* (2005) using agricultural potatoes, coffee husks and sugar-cane bagasse obtained maximum production (1.07 x 10^{10} spores g^{-1}) at the 10th day of fermentation. From the spore concentration of *Beauveria,* isolates inoculated on sorghum substrate produced relatively better spore g^{-1} than others authors that use different or same substrates using solid phase fermentation. The average mean spore concentration per gram was significantly higher by the isolates of *Beauveria*. In the case of isolate B7A the spore concentration per gram of the substrate was relatively higher (4.80 x 10^{10}) as compared to other isolates and also showed statistically significance difference at ($p < 0.05$, P = 0.019). The weight of spore powder harvested (g) per 1kg of the substrate of each isolates of B7A, C3C and G2A were 8.48 ± 0.71, 7.32 ±0.57 and 8.26 ± 0.42, respectively. Sahayaraj and Namasivayam (2008) from 100g of agricultural products obtained the biomass (g) from Rice 0.11, Wheat 0.19, Sorghum 0.41, Pearl millet 0.47, Raghi 0.64 and Maize 0.56. The biomass products obtained from this from the sorghum substrate showed relatively more comparing with Sahayaraj and Namasivayam (2008) with different or same substrate. Harvested spore biomass from all isolates did not show statistically significant different at $P < 0.05$, P = 0.096). The harvested spore powder and spore production from *Beauveria* isolates on sorghum were the result of the mixture of sorghum bran and spores. On this work, manual shaking method was used to harvest the spore from the substrate and this could be stronger than the mechanical method allowing more sorghum bran will mix and pass through the sieve mesh thereby adding more weight to the spore powder. Posada and Vega (2005) compared mechanical and manual method of spore harvesting and they found out that the spore concentration was highly variable for harvest methods and for isolates. The spore concentration was higher for the mechanical method and this result was the opposite for the spore powder weight where a higher yield was obtained using the manual method. The advantage of mechanical method over the manual method is that the same frequency will be generated during the time the cultures being sieved, while in the manual method the rhythm of shaking changed more often because the operator became tired. The mechanical and manual harvesting methodologies had low efficiency to extract the spores from the grains and this resulted in low spore productivity and requires looking for better methods of *B. bassiana* mass production and spore harvesting if the fungus is going to be used as a mycoinsecticide (Bateman, 2007). If industrial production of entomopathogenic fungal spores as a mycoinsecticide it is necessary to adapt or change production methods to increase spore production and harvest with greater efficiency (Ye *et al.* 2006). In the case of *B. bassiana* products based on gains, it will be necessary to improve the extraction method to remove the spores that are retained from the substrate. Posada and Vega (2005) have produced spore based on rice substrate using manual and mechanical extraction method the rice retained which were twice the spores produced. The spore production per kg sorghum substrate were significantly higher from B7A, C3C and G2A isolate at 15 days and produced 4.01x10^{11} ± 2.00 x 10^{11A}, 3.06 x10^{10} ±

1.90 x 10^{10B} and 2.71x10^{10} ± 1.45 x 10^{9B} spore/kg respectively. Tincilley *et al.* (2003) also harvested significantly higher spore production of *B. bassiana* in chopped carrots (2.08 x 10^{10} spores/100 g), tapioca (1.74 x 10^{10} spores/100 g) and potato (1.67 x 10^{10} spores/100 g) compared to PDA medium (1.45 x 10^{10} spores/100 ml). Sahayaraj and Namasivayam (2008) also using a variety of agro-industrial residue from 100g of substrate obtained spores count (x10^{8}) from Rice 11.24,Wheat 11.76, Sorghum 10.24, Pearl millet 9.78, Raghi 10.72 and Maize 9.44. Tincilley *et al.* (2003) using 6% molasses obtained (1.53 x 10^{10} spores/100 ml), 4% (1.25 x 10^{10} spores/100 ml) and 5% (1.25 x 10^{10} ml) and 3% (1.08 x 10^{10} spores/100 ml). Spore production by B7A isolates showed almost similar amount with Tincilley *et al.* (2003) but higher than Sahayaraj and Namasivayam (2008) while others isolates C3C and G2A produces almost slightly more biomass even using same substrate (sorghum).

CONCLUSION

Screening and mass production of entomopathogenic fungi, *Beauveria bassiana* for biopesticides against coffee berry borer are very critical steps. The pathogenicity potential of thirteen of these isolates was checked with their spore germination potential. Pathogenicity, 100% mortality, average survival time (LT_{50}) and spore production of each isolates on insects cadaver were the criteria and seven isolates scored the minimum requirements and they were considered as the potential biocontrol agents. It is clearly indicated that the two isolates B7A and C3C scoring and fulfilling all the 4 criteria to be selected as the better isolates for biocontrol agents. This information provides a better picture to determine which isolates should be considered for subsequent mass production, formulation and possible commercialization. The use of high quality standards for spores increases the likelihood of success when applied in the field. The shorter mean mortality time and high germination potential for the isolates that selected for mass production were the main criteria. A possible reason was that the higher proportion of viable spores and therefore they more promptly infected the beetles and caused more rapid mortality. The spore concentration per gram, powder spore harvested and spore produced per kg of the sorghum substrate were promising. Three of the *Beauveria* isolates were good at spore production using the sorghum substrate. However, the moisture content that was achieved after culture and substrate are air dried was not satisfactory as compared to the recommended moisture content to store and keeping the viability the spore. Although the total spore production of these isolates were higher the spores with the highest weight may be due to high moisture content, this did not mean that they have high spore concentrations. From this study, it is clearly observed that the production of high amount of spores may also due to the use of sorghum bran substrates during the harvesting time of this experiment. These substances will block the nozzles of equipments during application of the spores *Beauveria* isolates. These characteristics need to be evaluated if fungal production is to be scaled up to avoid application problems in the field situations.

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