Liquid Phase Media for Mass Production of *Metarhizium anisopliae* against Sorghum Chafer, *Pachnoda interrupta*, using Rice as Solid Substrate

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Abstract

Microbial biological pest control agents such as entomopathogenic fungi (EPF) can be an effective alternative for the control of sorghum chafer, *Pachnoda interrupta*, an important pest in Ethiopia. Production of inoculums of high quality with reasonable quantity on mass production substrates is important for its use as mycoinsecticides. Three liquid phase media *viz.* sucrose /waste brewer's yeast (SWBY), molasses/yeast extract (MYE) and lennoux broth base (LB) were evaluated for effects on mass production characteristics of three *M. anisopliae* isolates *viz.* PPRC51, PPRC2 and IC69 using rice as a solid substrate in the diphasic mass production technique. Effects of liquid media on shelf-life and virulence of the isolates against *P. interrupta* was also evaluated. Weight of spores/kg of rice substrate, number of spores/g of spore powder and viability of spores varied significantly across the three isolates ($P<0.0001$). There was no significant difference among liquid phase media and their interactions with isolates ($P > 0.05$). The highest weight of spores (119.72g/kg) was obtained from IC69 mass produced by using MYE as a liquid phase medium. PPRC2 produced the highest number of spores ($4.57x10^{10}$ spores/g) when using LB as liquid phase medium. Using MYE as liquid phase medium, the virulence of the three isolates of *M. anisopliae* did not vary significantly. Using either of the liquid media does not significantly affect all the quality variables for mass production, virulence and shelf life of the isolates selected for control of *P. interrupta*. Therefore any of the liquid phase media can be used for mass production of the selected isolates on rice without significantly affecting virulence and shelf life.

Keywords: Liquid-phase-media, mass production, *Pachnoda interrupta*, *Metarhizium anisopliae*

Introduction

Sorghum chafer, *Pachnoda interrupta*, is a major pest of sorghum and other cereal and horticultural crops. It causes up to 100% crop loss on sorghum in Ethiopia. As efficient and economical control measures are not available, control strategy heavily depends on use of chemical pesticides (Wolde-hawariat *et al.* 2007; Bengtsson *et al.* 2009). However, chemical pesticides are hazardous to the environment, pesticide applicators and the consumers. Use of microbial biological pest control agents such as insect pathogenic fungi, bacteria, virus and nematodes has been accepted as safe alternative crop protection tools to chemical pesticides in modern agriculture (Rao *et al.* 2006; Anand *et al.* 2009). Several entomopathogenic fungi including *M. anisopliae* and *Beauveria bassiana* have gained attention as microbial bio-control agents in the last 50 years (Jaronski and Jackson 2012) with over 170 commercially available products throughout the globe (Faria and Wraight 2007). Entomopathogenic fungi (EPF) based mycoinsecticides have been considered as safe alternative control strategy for several insect pests such as locusts and grass hoppers, white flies, termites, aphids and beetles (Lomer *et al.* 1997; Klein and Lacey 1999; Strasser *et al.* 2000; Wraight *et al.* 2001) and can also be used for the control of *P. interrupta* as a component of integrated pest management system.

Production of inoculums of high quality with reasonable quantity on mass production substrates is an important milestone for the use of EPF as mycoinsecticides. The diphasic technique of mass production of entomopathogenic fungi whereby blastospores are produced in a liquid medium under continuous shaking and used to inoculate solid substrates for production of aerial conidia is the most viable method of mass production of EPF (Burges and Hussey 1981; Lomer *et al.* 1997; Jaronski and Jackson 2012). Nutritional, cultural (artificial and natural media) and environmental conditions during fungal growth can influence the virulence, efficacy, longevity, thermal tolerance and ecological fitness of mass produced fungal propagules (Mc Clatchie *et al.* 1994; Jackson 1997; Ying and Feng 2006). Moreover, for each fungus strain, a convenient liquid medium needs to be developed (Jaronski and Jackson 2012).

The objective of this study was therefore to evaluate different liquid phase media for mass production of selected *M. anisopliae* isolates in order to develop mass production and preservation protocols and to determine their effects on virulence of the isolates to *P. interrupta* using rice as solid substrate.

Materials and Methods

Sources of fungal isolates and the experiments

Two of the isolates (PPRC51 and PPRC2) were obtained from the culture collections of Ambo Plant Protection Research Center (APPRC) in Ethiopia. The isolates were selected on the basis of their high virulence against P. *interrupta* under laboratory and field conditions (Belay 2016). The third isolate (IC-69) was obtained from the International Center of Insect Physiology and Ecology (ICIPE) and was used as a standard isolate.

Three experiments were conducted. The first one evaluated the effect of three

different liquid phase media on the quality of spores of selected isolates mass produced on rice solid substrate. The second experiment evaluated their virulence against *P. interrupta* while the third experiment evaluated the shelf life of the isolates. The first experiment was conducted at the International Center of Insect Physiology and Ecology (ICIPE) arthropod pathology unit laboratories in Nairobi, Kenya. The second and the third experiments (Bioassay against *P. interrupta* and shelf life tests) were conducted at Ambo Plant Protection Research Center (PPRC) laboratory, Ethiopia.

Preparation of fungal cultures

Isolates of three *M. anisopliae* (PPRC51, PPRC2 and ICIPE 69) were sub-cultured on SDA media and incubated for three weeks. After sporulation conidia were scraped using sterilized metal spatulas from the surfaces of the media and put into universal bottles containing 10ml of 0.05% Triton -X- 100 solution to prepare conidial suspensions. These suspensions served as stock for appropriate dilutions to obtain required conidial concentrations. The stock suspension of each of the isolates was used as soon as it was prepared.

Preparation and inoculation of liquid phase media and experimental design

Three liquid broth media *viz.* sucrose/waste brewer's yeast (SWBY), molasses/yeast extract (MYE), and lennoux broth base (LB) were used. SWBY, 20 g of sucrose and 20 g of dried yeast extract (from Kenya brewers) were mixed in 1 liter of distilled water, and brought to boil in the water bath for 15 minutes. Molasses yeast extract broth contained 20 ml of thick (viscose) sugarcane molasses and 20 g of yeast extract mixed in 1 liter of distilled water. Lennouex broth base was constituted by mixing 30 g of glucose (Panreac), 30 g of yeast extract (Fluka) and 10 g of peptone (HIMEDIA) in 1 liter of distilled water. All the prepared solutions were then separately steered for 10 minutes using a magnetic steer to homogenize the media. Seventy-five milliliters of each media were then poured to 250 ml flasks and sterilized in an autoclave for 20 minutes at 121 °C and 15 PSI. When the media cooled to approximately 50 °C, 25 µg/ml of chloramphenicol was aseptically added into each flask and hand shaken for a minute. Conidia of *M. anisopliae* were harvested from 3 weeks old cultures of each of the isolates and suspended in universal bottles containing 10 ml of 0.05 % Triton - X- 100. The media in each flask were finally inoculated with 1 ml of the suspension containing a 6 X 10^6/ml conidial concentration of the respective isolates. In addition to this, the prepared media were incubated in an incubator shaker at 25 °C and 100 RPM for 7 days for inoculation of solid substrate.

Each of the treatments (Table 1) was replicated three times and each flask served as a replicate. The experimental design was completely randomized design (CRD). The un-inoculated flasks from each medium with all the respective media contents except for conidia served as control treatments.

Table 1.Treatments used for evaluation of liquid media

Treatments	Isolates	Species	Liquid media* (broth)
T1	PPRC 2	*M. anisopliae*	MYE
T2	PPRC 2	*M. anisopliae*	SWBY
T3	PPRC 2	*M. anisopliae*	LB
T4	PPRC 51	*M. anisopliae*	MYE
T5	PPRC 51	*M. anisopliae*	SWBY
T6	PPRC 51	*M. anisopliae*	LB
T7	IC69	*M. anisopliae*	MYE
T8	IC69	*M. anisopliae*	SWBY
T9	IC69	*M. anisopliae*	LB
T10	Control	None	MYE
T11	Control	None	SWBY
T12	Control	None	LB

*MYE: Molasses and yeast extract, SWBY: Sucrose and waste brewer's yeast, LB: Lennoux broth

Preparation and inoculation of solid substrates

Whole rice (local variety, Pishori, from Mwae, kiriyaga area, Kenya) was used as solid substrate. One kilogram of the substrate was washed with running water and pre-cooked by pouring boiled water (300 ml) into plastic containers containing the substrate, closing the containers and soaking for 30 minutes. Once the substrate was cool, 20 ml of soybean oil was added and thoroughly mixed with a clean fork to help reduce clamping. The well mixed substrate was sealed in Millner bags (polypropylene mushroom spawn autoclave bags) (60 cm long by 35 cm wide cut in to two) with gas-permeable vent patches and sterilized by autoclaving for 20 minutes at 121 °C and 15 PSI. The bags were allowed to cool overnight and inoculated with the respective media containing the blasto-spores of the *M. anisopliae* isolates. Control treatments were inoculated with the control flasks containing the media and an additional 75 ml of sterile water. For inoculation of the substrates, 75 ml of additional sterilized water was applied to each bag and bags were massaged to spread the inoculums and the water throughout the substrate. Samples were taken from the substrates to determine the moisture content immediately after inoculation of the bags with the respective isolate-liquid media combination. The bags were then sealed inside the laminar flow hood and transferred to shelves in a mass production room equipped with an automatic heater and dehumidifier at 24 °C. The mean moisture content of the rice and sorghum substrates was 56.18 % and 56.04 % respectively. The bags were checked after 24 hrs of incubation and mixed gently and tapped after one week and left for another two weeks. After full sporulation, the substrates were transferred to surface sterilized dry clean bowls and aerated to let dry and enhance sporulation for 5 days. Harvesting of spores was done using a 300 µm sieve to separate substrate and spores.

Determination of spore quality

The criteria used to determine the quality of the mass produced spores were based on the product quality control parameters elaborated by Lomer and Lomer (1999) in the Lubilosa insect pathology manual.

The quality of the mass produced spores was determined by taking measurements

on weight of spores/kg of substrate, number of spores/gm of spore powder, viability of spores, moisture content of spores and biological purity of spores. To measure weight of spores/kg of substrate, harvested spores were weighed on a Mettler balance. To determine the number of spores/gm of spore powder, 0.1 gm of harvested spores was added to universal bottles containing 10 ml of sterilized 0.01 % Tween 80 to make a stock suspension. Appropriate serial dilutions were done and finally spores were counted using an improved neubaur heamocytometer. For determination of viability harvested spores were added to universal bottles containing 10 ml of sterilized 0.01% Tween 80 to make a stock suspension. The concentration of the stock suspension was adjusted to 3 x 10^6 conidia /ml using an improved neubaur heamocytometer and 100 µl of the suspension was then spread plated on SDA media in 90 mm diameter Petri-dishes. Germination was stopped after 24 hrs of incubation at 25 ^{0}C by adding 1 ml of lacto-phenol blue solution. A sterile cover slip was then put on each Petri-dish and percentage germination was determined by counting at least 300 conidia under a compound microscope at x400 magnification. A conidium was declared germinated if it showed a growth as big as its size. Three replicate Petri-dishes were used for each isolate.

To determine the moisture content of spores at harvest, 1 gm of spore powder was added to pre weighed oven dried universal bottles and oven dried for 24 hrs. The universal bottles were then reweighed and moisture content was determined by using the formula (adapted from Lomer and Lomer 1999):

$$\frac{wet\ weight - dry\ weight}{wet\ weight} x100$$

Biological purity of mass produced spores was determined by serially diluting a 0.1 gm spore powder to a $1x10^8$ concentration and spreading plating 200 µl of the suspension on SDA media in three replications for each isolate. The plates were then incubated at 25 °C for 5 days. The number of pure colonies/ml and contaminant colonies/ml were then counted and multiplied by the respective dilutions and the percentage of contaminant colonies was calculated to get the percent purity (% of contamination) of the sample dry spore powder. All experiments were replicated three times and a completely randomized design (CRD) with a 3x3 factorial arrangement was used as the experimental design.

Shelf life of isolates

To test the shelf life of the selected isolates, 5 g of spores of the three *M. anisopliae* isolates mass produced on rice substrate were dried over night at 30 °C to remove excess moisture and sealed in polyethylene bags and covered with aluminum foil. The moisture content of spores was between 6-7 % before being sealed. The bags were then stored at 4 °C and at room temperature (22-24 °C) in the laboratory at PPRC. Completely randomized design was used. Three replicate bags were prepared for each isolate. Prior to sealing, the initial percentage germination of each replicate spore was tested on SDA media and recorded before storage. The germination of the spores was checked every month for six consecutive months.

Bioassay for virulence against *P. interrupta*

The virulence of mass produced spores of three *M. anisopliae* isolates was evaluated. The isolates were selected on the basis of their amenability to mass production on rice substrate for final bio-assay against *P. interrupta*. Two of the isolates (PPRC51 and PPRC2) were isolated from *P. interrupta* in Ethiopia and had high virulence to *P. interrupta* and the third isolate was a commercialized isolate IC69 from ICIPE (isolated from soil in D.R. Congo) which was included as a standard for comparison. Adult beetles were collected during the mating season of 2014 from breeding areas around Rassa and kept in plastic baskets containing moistened sterile soil collected from the same area. The baskets had side openings for aeration and the tops were covered with muslin cloth to prevent beetles from escaping. Collected beetles were fed with slices of ripe banana and observed for any natural infection for 10 days before being used for bio-assays.

Ten beetles were put in sterile 300 ml plastic tubs with perforated lid. A 1 mg spore of each of the isolates was added on top of the beetles in each of the plastic tubs and the beetles were allowed to move in the tubs for 30 minutes. The beetles were then transferred to 120 mm diameter plastic Petri-dishes containing moist filter paper and incubated at room temperature for 10 days. During incubation, beetles were fed with slices of ripe banana changed every other day. To provide adequate moisture, 1 ml of sterile distilled water was added to the Petri-dishes every day. Mortality was assessed every day and dead beetles were removed and surface sterilized with 70 % ethanol and rinsed three times in sterile distilled water. The surface sterilized beetles were then transferred to sterile Petri-dishes containing moist filter paper and incubated at 25 ^{0}C to check for sporulation and to confirm death due to fungal infection. A CRD with four replications was used and controls of each isolate were treated with the respective spores killed at 80 ^{0}C in an oven for 48 hrs. Mean percentage mortality of *P. interrupta* was used as measure of virulence.

Statistical analysis

All percentage data were arcsine transformed before analysis to stabilize the variance and normalize the data. Where there were no interaction effects, data were analyzed as in a single factor experiment to show mean separation among the main effects. Means were separated using LSD. In the case of bioassay for virulence of *P. interrupta*, percent mortality data were corrected for control mortality using Abbot's formula (Abbot 1925). All data were analyzed using the ANOVA procedure of SAS statistical soft ware version 9.2.

Results

Effects of liquid phase media on quality of mass produced spores

Weight of spores/kg of rice substrate varied highly significantly across the three isolates ($P = 0.0001$, $F = 582.66$, $df = 2, 18$). There was no significant difference among blastospore media ($P=0.09$, $F = 2.63$, $df = 2, 18$) and their interactions with isolates ($P = 0.18$, $F = 1.75$, $df = 2, 18$).

The highest weight of spores (119.72 g/kg) was obtained from IC69 mass produced by using MYE as a liquid phase

medium (Figure 1). The isolate also produced spores of 110.25 g/kg and 109.37 g/km of rice with LB and SWBY liquid media, respectively. PPRC51 and PPRC2 generally produced less than 48 g of spore/kg of rice substrate when produced using all liquid phase media as source of blastospores with the lowest (36.78 g/kg) produced from PPRC2 with SWBY medium.

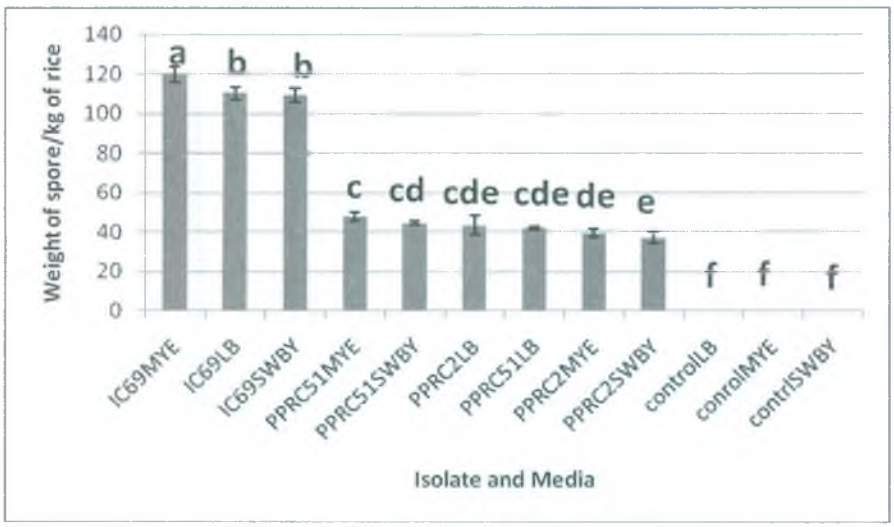

Figure 1.Mean weight of spores ± SE produced per kg of rice substrate by three *M. anisopliae* isolates using three different liquid phase media. Bars containing similar letters are not significantly different (α = 0.05), LSD = 7.43, P < 0.0001. MYE: Molasses and yeast extract, SWBY: Sucrose and waste brewer's yeast, LB: Lennoux broth

The number of spores/g of spore powder also varied significantly (P = 0.0018, F = 9.21, df = 2, 18) among the isolates. However, the liquid phase media (P = 0.37, F = 1.03, df = 2, 18) and interaction between isolates and liquid phase media (P = 0.55, F = 0.79, df = 4, 18) did not show any significant variations.

PPRC2 produced the highest number of spores ($4.57x10^{10}$ spores/g) when using LB as liquid phase medium (Figure 2). The lowest number of spores was obtained from PPRC51 ($1.24x10^{10}$ spores/g) using MYE liquid phase medium.

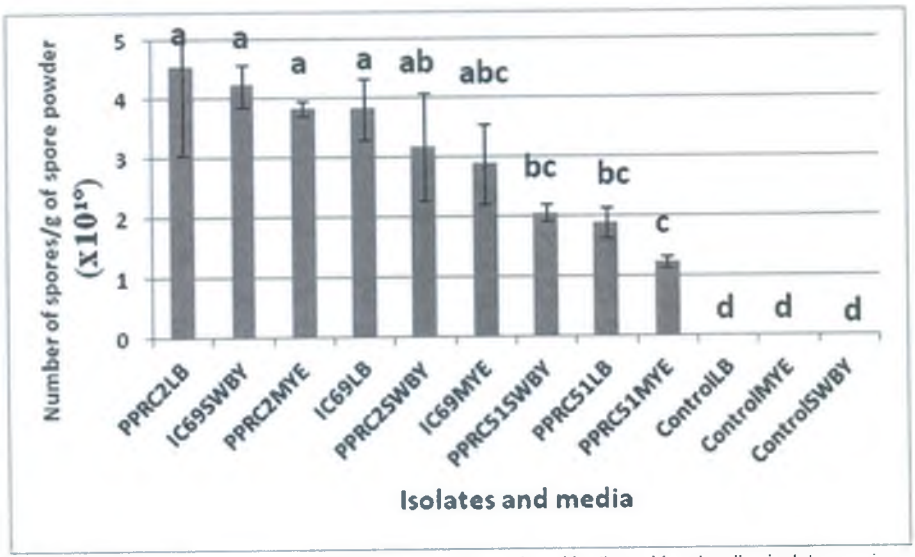

Figure 2.Mean number of spores per gm ± SE of spore powder produced by three *M. anisopliae* isolates on rice substrate using three different liquid phase media. Bars containing similar letters are not significantly different (α = 0.05), LSD = 1.69 x10^{10}, P <0.0001. MYE: Molasses and yeast extract, SWBY: Sucrose and waste brewer's yeast, LB: Lennoux broth.

The viability of spores after harvest also varied significantly among the isolates (P = 0.0093, F = 6.13, df = 2, 18). There was no significant variation in affecting viability of spores among the different liquid phase media (P = 0.14, F = 2.25, df = 2,18) and their interaction with the isolates (P = 0.36, F = 1.16, df = 4, 18). All the isolates showed over 90 % viability regardless of the source of the liquid phase media. Highest viability was recorded from IC69 with the use of MYE liquid phase medium (Figure 3).

The moisture content of spores at harvest did not show any significant variation among isolates when using the different liquid phase media as source of blastospores (P = 0.17, F = 1.93, df =2, 18 and P = 0.26, F = 1.45, df = 2, 18 respectively). On the other hand, the interaction between isolates and liquid phase media was significant (P = 0.03, F=3.33, df = 4, 18). PPRC2 produced with MYE as a liquid medium contained the highest moisture (55.33 %) compared to IC69 and PPRC51 which contained 48.67 % and 36.67 % moisture at harvest, respectively, using the same liquid phase medium. The lowest moisture content (36 %) was also recorded from PPRC2 produced with SWBY as liquid phase medium (Table 4).

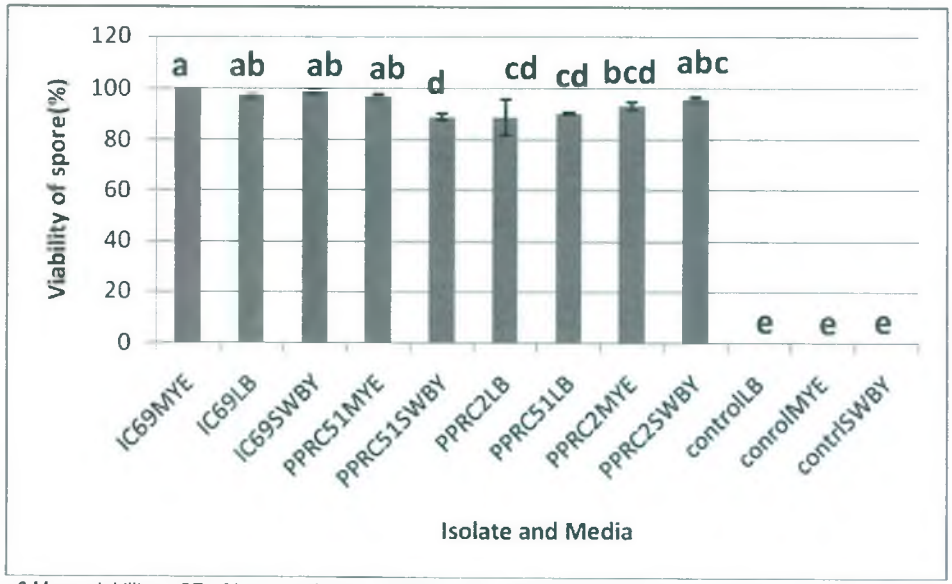

Figure 3.Mean viability ± SE of harvested spores of three *M. anisopliae* isolates mass produced on rice substrate using three liquid phase media. Bars containing similar letters are not significantly different (α = 0.05), LSD = 6.34, P < 0.0001. MYE: Molasses and yeast extract, SWBY: Sucrose and waste brewer's yeast, LB: Lennoux broth.

Table 4. Mean moisture content (%) at harvest of three *M. anisopliae* isolates mass produced on rice substrate using three different liquid phase media

ISOLATE	MEDEA			LSD
	LB	MYE	SWBY	
IC69	45.33*	48.67*	48*	ns
PPRC2	38*	55.33*	36*	ns
PPRC51	40.67*	36.67*	45.33*	ns
control	0	0	0	ns
LSD	ns	ns	ns	

*Isolate x Media interaction significant (P=0.03)

Biological purity did not show significant variation among the isolates (P = 0.41, F = 0.95, df = 2, 18) and the liquid phase media (P = 0.42, F = 0.91, df = 2,18). However, the interaction of isolates and liquid phase media was highly significant (P = 0.0001, F = 86.64, df = 4, 18) (Table 5).

Table 5. Biological purity of three *M. anisopliae* isolates mass produced on rice using three different liquid phase media.

	MEDIA			
ISOLATE	LB	MYE	SWBY	LSD
IC69	98.54*	99.53*	99.76*	ns
PPRC2	99.93*	97.86*	99.34*	ns
PPRC51	99.2*	100*	97.5*	ns
Control	0	0	0	ns
LSD	ns	ns	ns	

*Isolate x Media interaction significant (P = 0.0001)

PPRC51 produced using MYE as liquid phase medium produced spores with 100 % purity while producing the lowest purity (97.5 %) on SWBY medium compared to other media. Generally the biological purity of the spores produced using all the three liquid phase media was greater than 97%.

Shelf life of isolates

The shelf life of the spores of the isolates stored at 4 °C over six months did not show any significant variations (P>0.05) (Table 6). However, the percent germination of the spores declined from the maximum initial of 87.2 % to 68.67 % for isolate IC69. Maximum viability was observed on isolate PPRC51 (75 %) while isolate IC69 showed minimum viability (68.67 %) at the end of six months. Spores stored at room temperature (22-24 °C) showed significant variations starting from the first month through the third month (P = 0.04, F = 5.66), (P = 0.01, F = 9.69) and (P = 0.01, F = 10.47) for months 1-3, respectively. There was no significant variation in viability among the three isolates after the third month (months 4-6) (P>0.05). The maximum viability after six months of storage at room temperature was recorded from the isolate PPRC51 (64.37 %) while the minimum viability was recorded from the isolate PPRC2 (62.17 %).

Table 6. Mean percent germination of spores of different isolates of *M. anisopliae* stored for various lengths at 4 °C and room temperature (22-24 °C) at Ambo PPRC

				4 °C			
ISOLATE	Initial	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
PPRC2	85.27a*	82.73a	81.9a	79.5a	78.5a	75.93a	71.4ab
PPRC51	86.53a	85.13a	83.73a	81.23a	79.94a	77.47a	75a
IC69	87.2a	85.67a	83.03a	78.87a	77.27a	72.83a	68.67b
LSD (P-value)	ns (P>0.05)	ns (P>0.05)	ns (P>0.05)	ns (P>0.05)	ns (P>0.05)	ns (P>0.05)	ns (P=0.09)
				22-24 °C			
ISOLATE	Initial	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
PPRC2	85.27a	77.9ab	74.6a	71b	69.5a	65.63a	62.17a
PPRC51	86.53a	81.33ab	79.97a	76b	72.4a	68.73a	64.37a
IC69	87.2a	83a	80.33a	74.93a	71.8a	68.73a	63.7a
LSD (P-value)	ns (P>0.05)	3.78 (P=0.04)	3.57 (P=0.013)	2.82 (P=0.011	ns (P>0.05)	ns (P>0.05)	ns (P>0.05)

*Similar letters within a column are not significantly different

Effects of liquid phase media on virulence of mass produced spores

Because of the low number of beetles collected from the field in July 2014, only spores produced using MYE as liquid phase medium were used for the bioassay against *P. interrupta* adults. The virulence of the three isolates of *M. anisopliae* did not vary significantly (P = 0.34, F = 1.28, df = 2, 6). PPRC2 caused the highest mortality (80.92 %) followed by PPRC51 and IC69 which caused 77.75 % and

57.14 % mortality, respectively (Figure 4). The mean number of conidia per gram of each of the isolates used for the bioassay was $2.67x10^8$ (PPRC2), $9.2x10^7$(PPRC51) and $5.07x10^7$(IC69).

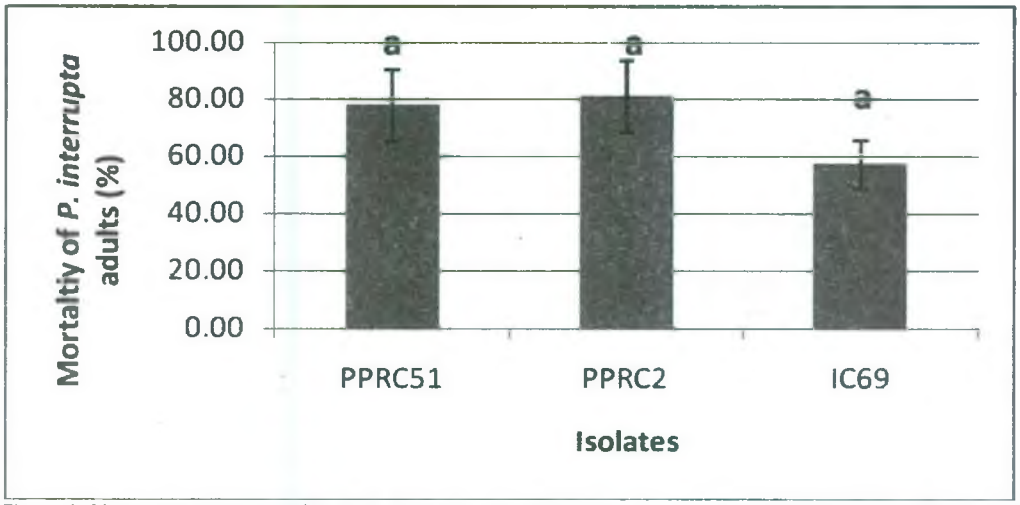

Figure 4. Mean percentage mortality of *P. interrupta* caused by three *M. anisopliae* isolates mass produced on rice substrate using MYE as source of liquid phase medium.

Discussion

An important characteristic of a selected strain of EPF (particularly *M. anisopliae* and *B. bassiana* strains) is its ability to conidiate *in-vitro* on solid substrates (Jaronski and Jackson 2012). Investigating the nutritional requirements of EPF is important to improve inoculums supply through mass production and facilitate commercialization of biopesticides (Sun and Lui 2006). Although there are a range of varieties of grains investigated for use as solid substrates (Sahayaraj and Namachivayam 2008), the most commonly used substrates are rice (*Oryza sativa*) and flaked barley (*Hardeum vulgare*) (Jaronski and Jackson 2012). Sahayaraj and Namasivayam (2008) found $1.12x10^9$ conidia per 100 g of rice substrate for *B. bassiana* by directly inoculating the substrate with conidial suspension without using liquid media. In this study, direct inoculation was not used for obvious advantages of the liquid phase media. Direct inoculation is not preferable to using liquid media because it does not allow detection of contaminants and may expose the substrate to be overwhelmed by bacteria which use the lag phase of the fungi as an advantage to proliferate (Jaronski and Jackson 2012). In addition to avoiding contamination, use of liquid media in the diphasic mass production system for EPF has been reported to increase spore yield from solid substrates. Inoculating rice with conidia of *B. bassiana* produced in liquid substrate composed of crystalloid flour, potato and dextrose. Santoro *et al.* (2005) found spore yield of $2.7x10^{12}$/g of substrate increasing productivity by up to 1000 fold than produced on solid substrate without the liquid media.

Typically, a hypocrealean fungus can produce 20-150 g conidia/kg of substrate, containing 1×10^{10} - 2×10^{11} conidia/g of solid substrate (depending on the fungus)

(Jaronski and Jackson 2012). The findings of this study also confer to this fact. For example PPRC2 produced the highest number of spores ($4.57x10^{10}$spores/gm) when using LB as liquid phase medium and $3.85x10^{10}$ when using MYE liquid medium which were not significantly different from each other. Spore yield per kg of rice substrate also showed a similar trend with the highest weight of spores (119.72 g/kg) obtained from IC69 mass produced by using MYE as a liquid phase medium and the lowest (36.78 g/kg) produced from PPRC2 with SWBY medium. Use of any of the liquid media did not affect the number of spores/g of spore powder and the weight of spores/kg of substrate in this study. It is to be noted that the commercialized isolate IC69 produced more than twice as high as the Ethiopian isolates (PPRC51 and PPRC2) indicating that strain selection is more important than liquid media selection for higher spore yield.

Santoro *et al.* (2005) suggested that sporulation was influenced by the source of nutrition in the liquid medium and caused reduction in production time while increasing productivity of *B. bassiana*. The source of liquid medium has also been reported to affect spore production of entomopathogenic fungi on solid substrates (El-Damir 2006). In this study however, spore productivity appeared to be affected more by the strain than by the liquid media used. One of the reasons for this variability might be the fact that the experiments were conducted on different species of fungi and different types of liquid media. This can be regarded as an indication for the importance of developing specific mass production protocols for each specific species or strain of fungi intended to be used as biopesticides. The viability, moisture content at harvest and biological purity of spores of the tested isolates also did not vary significantly due to media except for slight and high interaction effects in moisture content and biological purity, respectively.

Mortality of *P. interrupta* caused by spores of mass produced isolates also did not differ very much from the mortality caused by spores produced on artificial media. Although statistical comparison was not done to avoid biases and erroneous conclusions, simple comparisons between the mortalities caused by spores of the isolates harvested from artificial media and solid substrates are not extremely different. PPRC2 caused mortality of 80.92 % and 77.14 % when harvested from rice substrate and SDA media respectively. Similarly, PPRC51 caused 77.75 % and 82.40 % mortality when harvested from rice substrate and SDA media respectively.

In conclusion, the current study has demonstrated that using either of the liquid media does not significantly affect the virulence, shelf life and all the quality parameters for mass production of the *M. anisopliae* isolates selected for control of *P. interrupta*. The study has also shown that liquid media supplemented by sugarcane molasses can enhance blastospore and can be used to produce higher number of blastospores. In view of these findings, molasses and waste brewer's yeast which are readily available from sugar industries and breweries in Ethiopia can be used to constitute liquid medium for successful mass production of EPF on rice substrate as a cheap source of liquid phase medium for these isolates without significantly affecting their virulence and shelf life.

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