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Productivity of different species of entomopathogenic fungi based on one type of technology

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Abstract One type of technology based on millet grain was estimated for the mass production of different species and strains of entomopathogenic fungi including *Beauveria bassiana*, *Metarhizium anisopliae*, *Mariannaea* sp. and *Tilachlidium* sp. The unified technology previously detailed and developed for the entomopathogenic fungus *B. bassiana*, based on millet grain, was acceptable for the production of fungal aerial conidia for all species and strains used in the experiments. All fungi actively colonized the grain substratum, formed mycelial biomass and produced conidia within 14 days at 22-24^{O}C. The yield of conidia fluctuated from $0.4x10^9$ to $6.5x10^9$ conidia/g of dry substratum depending on species and strain of fungus.

Key words: entomopathogenic fungi, production, biphasic technology, conidia

Introduction

Entomopathogenic fungi within the genera *Beauveria*, *Metarhizium*, *Mariannaea* and *Tilachlidium* contain ubiquitous species usually existing in nature as soil semisaprophytes with entomophilous and entomopathogenic properties. Several species and strains of these fungi have been commercialized as microbial insecticides in different countries. But fungal entomopathogens have additional properties which can be used for plant protection from phytopathogenic microorganisms and to support plant productivity. Over the past two decades, different unexpected roles have been reported for fungal entomopathogens including the possibility to suppress phytopathogenic microorganisms by the association of these fungi with plant roots and their colonization into in plant tissues as endophytes, resulting in beneficial effects on the plants (Vega, 2008; Ownley *et al*., 2010; Gao *et al*., 2010). The

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discovery of the new properties of entomopathogenic fungi gives an additional impetus to comprehensively study this group of insect pathogens and to study the mass-production of conidia for laboratory and field experiments.

The development of a simple unifying technology for the mass-production of entomopathogenic fungi remains the most pressing problem. One of the most important considerations for the practical use of entomopathogenic and antagonistic fungi is the production of stable fungal propagules using technology that is economically effective. Numerous methods exist for the production of both fungal conidia and blastospores, which are relatively resistant to different environmental factors. Researchers have used the traditional methods of the microbial industry for the mass production of entomopathogenic fungi including submerged, superficial and combinative culturing technologies (Taborsky, 1990; Feng *et al*., 1994; Lacey and Goettel, 1995; Jenkins, 1995, Jenkins *et al.,* 1998; Kassa *et al.,* 2008; Wraight and Carruthers, 1999; Gouli *et al*., 2006, 2009, 2011; Gandarilla-Pacheco *et al*. 2012). The existing technologies for the production of mycopesticides were developed based on traditional microbial industry methods using bioreactors as the primary unit of production for the creation of the microbial biomass. This is unacceptable for the cultivation of filamentous fungi because this group of microorganisms demands solid substrata and air space for the sporulation of the product of value, which are the conidia. In actuality, the commercial industrial production of mycopesticides used a combined technology including two principal stages. The first stage is the submerged cultivation of fungi to obtain fungal biomass as the sowing material. The second stage includes the inoculation of solid substrata by the fungal biomass obtained from the first stage. This technology provides the optimal aeration for the fungus and results in the greatest yield of stable and active conidia. Different types of grain or other solid substrata could be used to produce of entomopathogenic fungi including rice, wheat, barley, millet, corn, different agro-industrial residues and any hard material containing nutrient materials that provide for fungal growth and maturity (Nelson *et al.*, 1996; Posada-Flores, 2008; Santa *et al*., 2005). The production units used for the mass-production of fungi are varying from glass containers to plastic bags with different sizes and forms. The development of a unified technology appropriate for the mass-production of different species of fungi can be useful for the production of different fungal pathogens for scientific and applied research. The principal purpose of this work is to demonstrate the possibility of the use of a biphasic technology initially elaborated for *Beauveria bassiana,* for the mass production of different entomopathogenic fungi.

Materials and methods

Fungi

Several entomopathogenic fungi including *Beauveria bassiana* (*Bb*), *Metarhizium anisopliae (Ma*), *Mariannaea* sp (*M*sp.), and *Tilachlidium* sp. (*T*sp) were used for the experiments (Table 1). Fungi were obtained from the Worldwide Collection of Entomopathogenic Fungi at the Entomological Research Laboratory (University of Vermont). All fungal cultures, with the exception of the Ethiopian strain of *Ma* PPRC-29, were deposited to the ARS USDA Collection of Entomopathogenic Fungal Cultures. Fungi were kept at -80˚C. The initial production of all fungi was conducted using potato dextrose agar for a period of 14 days at 22-24˚C. Mature conidia were collected from the surface of the solid medium and a water conidial suspension was prepared and adjusted to $1x10^8$ conidia/ml to obtain the inoculum for the submerged cultivation.

Table 1. Fungi using for experiments

Species of fungi	UVM Collection identification numbers	USDA ARS Collection numbers	Source, place and year of isolation	Authors
Beauveria bassiana	ERL-1170	9587	Soil, avocado plantation, CA, USA, 2002	S. Gouli V. Gouli
Beauveria bassiana	ERL-1637	9337	Soil, forest, VT, USA, 2008	S. Gouli V. Gouli
Beauveria bassiana	Commercial strain, GHA, using for production of BotaniGard mycoinsecticide			
Mariannaea sp.	ERL-1882	10204	Pear thrips, *Taeniothrips inconsequens* (Thysanoptera; Thripidae), NH, USA, 2010	S. Gouli V. Gouli
Metarhizium anisopliae	PPRC-29	n/a	Sorghum chafer, *Pachnoda interrupta* (Coleoptera, Scarabaeidae), Ethiopia, 2003	S. Aysheshim
Metarhizium anisopliae	ERL-1171	9593	Soil, avocado plantation, CA USA, 2002	M. Brownbridge S. Gouli
Tilachlidium sp.	ERL-1790	10132	Leaf beetle (Chrysomelidae; Coleoptera), 2010, FL, USA	W. Reid S. Gouli

Sowing material

Material for inoculation of millet grain was prepared based on submerged cultivation of fungi. Erlenmeyer's flasks (500 ml) with 150 ml potato dextrose medium were inoculated using 10 ml of a1x10^8 conidia/ml suspension. All fungi were cultivated in an incubator shaker (Model EXCELLA E24, New Brunswick Scientific, USA) for a period of 72 hours at 22-24^{O}C. The fungal biomass of each fungus was separated from the mycelial fraction by filtration through a double layer of gauze, and the number of fungal propagules including conidia and blastospores was determined. The titer of all fungal suspensions was adjusted to 1 x 10^8 conidia/ml using distilled sterile water, and this material was used for the inoculation of the millet grain.

Preparation of substratum

Millet grain was dispensed into thermoresistant plastic bags (60 x 30 cm) which were used as the production units. Each plastic bag was filled with 500 g of grain and 250 ml of distilled water acidified with ascorbic acid at a rate of 0.5 ml/L of stock (stock: 50 g ascorbic acid/100 ml water). The plastic bags with the soaked millet grain were placed in a water bath for a preliminary thermal treatment at 90-95^{O}C for one hour until the full volume of the water in the plastic bags was absorbed by the grain. The thermally-treated material was then sterilized at 121^{O} C for one hour and cooled to 20-25^{O}C, at which point the sterilized grain was ready for fungal inoculation. The maintenance of sterility is the most important part of the mass production technology and the forming of a mouth for the plastic bag is critical for the prevention of contamination during the period of grain inoculation and incubation. Our special stopper construction was found to be suitable for all of the manipulation steps needed for the inoculation and incubation of the grain substratum (Fig. 1a, b).


a.

b.

Fig. 1. a, b. Construction of plastic bag mouth: a – the first position; b – second position.

Inoculation of grain in the cultivation units

50 ml of fungal suspension was used to inoculate the sterilized millet grain within the plastic bags. After inoculation, the contents of the bags were carefully mixed to assure even distribution of the fungal propagules among the nutrient substratum. Each production units was then closed using a special assembly comprised of rings having a diameter 10 cm and length of 6 cm, which formed the ventilation mouth (Fig. 1). The mouth of the bag was closed by a special stopper consisting of three layers including paper, then fabric and aluminum foil, which faced to the exterior of the bag. The first two layers provided protection to the nutrient substratum from contamination and the third layer prevented premature drying of the cultivated material for the first four days.

Cultivation of fungi

The bags with inoculated grain were placed on the lattice shelves at 22-24^{O}C. The grain must be evenly distributed on the bag surface and the surface of grain layer must not come into contact with upper side of bag for the better aeration. After four days of cultivation, the content of bags was carefully mixed and the upper parts of the aluminum foil covers were removed to improving air circulation. The bag stoppers were opened after 10 days in order to accelerate fungal sporulation and to aid in the drying of the biomass. The cultivation material was kept in the open bags for a period of four days.

Drying of biomass

After two weeks of cultivation, the fungal biomass was transferred to flat trays and distributed into a layer no more than 1 cm deep. The drying process was conducted in a special facility fitted with a dehumidifier (Roebuck&Company, IL, USA). The estimation of the moisture of the biomass was made using the Moisture Analyzer (Omnimark Instrument Corporation, Switzerland).

Productivity estimation

The productivity was estimated based on 1 g of inoculated millet grain. All procedures included the following steps. After drying, 1 g of biomass was put in a 20 ml volume tube and 10 ml of a water solution containing 0.1% Silwet-L77 (Loveland Industries) was added. The material was shaken with glass beads for 1 min using a vortex-genie (Scientific Industries, Inc., Bohemia,

New York, USA). The material was then put into a mixer and distilled water was added to a final volume of 1 L. The material was then intensively mixed for a period of 30 sec. The calculation of the number of conidia was done using haemocytometer (Goettel and Inglis, 1997), and the titer of conidia was recalculated in accordance with the dilutions.

Viability evaluation

The conidia viability was estimated after drying the sporulated fungal biomass. Suspensions of conidia in water were adjusted to 10^6 conidia /ml and distributed on the surface of PDA medium in an 8.5 cm-diameter Petri dish. The inoculated material was incubated for a period of 20 hours at 20 ± 2^0C, after which, the area necessary for the calculation of the percentage of germinated conidia was fixed using lactophenol stain and covered by a glass coverslip. The viability was evaluated by calculating the number of germinated conidia out of 100 for four different areas of the Petri dish for each production unit.

Statistical analyses

The number of viable conidia for the different fungal strains was compared using a Kruskall Wallace test In the presence of a significant difference and in order to determine which treatments were different, a Tukey-Kramer test was done on the ranked average value (SAS Institute 2011).

Results and discussions

The technology detailed for the production of the entomopathogenic fungus *B. bassiana* based on the millet grain was acceptable for the production of fungal aerial conidia of different fungal species and strains. All fungi colonized the millet grain substratum, formed mycelial biomass and produced conidia within14 days at 22-24^{O}C. The best yield of conidia was obtained for the *B. bassiana* strain ARSF-9337. The number of conidia was three times greater than the two other *B. bassiana* strains used in the experiments including *B. bassiana* ARSEF-9587 and the commercial GHA strain (Fig. 2). The productivity yield of *B. bassiana* strains based on millet grain was close to the results obtained by different authors using the biphasic liquid-solid fermentation technology (Jenkins, 1995). In particular, the production of conidia for the South-American strains of *B. bassiana* based on cooked rice was less than $1x10^{10}$ conidia/g substratum (Posada-Flores, 2008). The yield of another strain *B. bassiana*, LPB 01, maintained in the Collection of Laboratory of Biotechnological Processes, State University of Parana (Brazil), based on

solid fermentation technology, ranged from less than 10^9 to a maximum 3.4 x 10^9 conidia/g of substratum in case of production on refused potatoes, coffee husks and sugar-cane bagasse. The maximum harvest was obtained when the sugar-cane bagasse was used (Santa *et al*., 2005). The effect of solid media including rice, wheat and barley with additives (glucose and yeast extract), temperature and length of incubation on the conidial production of *B. bassiana* strains were also investigated in New Zealand. The maximum conidial yield achieved with rice was 4.38 x 10^9 conidia/g of substratum (Nelson *et al.*, 1996).

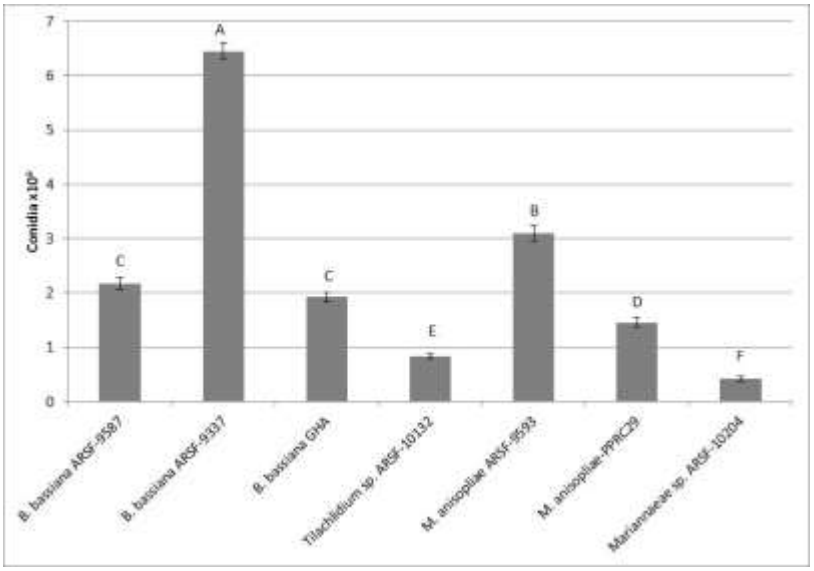

Fig. 2. Number of conidia produced for the different fungal strains.

The production of conidia for the fungus *M. anisopliae* strain PPRC-29 was 1.5 x 10^9 conidia/g of millet grain. The second strain of *M. anisopliae*, ARSEF-9593, was the most productive with a conidia harvest reaching 3.1 x 10^9 (Fig. 2). These results were close to the results obtained for isolates from New Zealand which produced a maximum of 1.42 x 10^9 conidia/g of dry substrate based on rice (Nelson *et al*., 1996).

The entomopathogenic fungi *Tilachlidium* sp., strain ARSF-10132, and *Mariannaea* sp., strain ARSF-10204, gave a conidia yield of 0.8 x 10^9 and 0.4 x 10^9 conidia/g respectively (Fig. 2). The viability of conidia after drying was as following: *B. bassiana* strains ARSEF-9587 – 87.8±3.2%, ARSEF-9337 – 91.3±2.1%, GHA - 92.4±1.1%; *M. anisopliae* strains ARSEF-9593 – 82.3±3.8%, and PPRC-29 – 76.3±3.2%; strain *Tilachlidium* sp. – ARSF-10204 - 76.3±4.2%, and strain *Mariannaea* sp. - ARSF-10132 -86.7±2.7%.

Productivity is an individual property of each species and strain of fungus, but there are several important technological practices which have universal significance. The key procedure is the optimal satiation of the grain by water at 90-95˚C followed by sterilization. The second essential demand is to guarantee sterility during all steps of the mass-production of the fungus. This task is a matter of some difficulty because the plastic bags using as the basic cultivation unit do not have stable mouth opening. In our experiments, we developed a simple and reliable method for the bag mouth construction s (Figure 1a, b). And finally, the best possible aeration has to be ensured using a special mouth stopper based on paper and fabric; and finally, the inoculated bags have to have maximal possible spherical form to ensure that there is sufficient air space under the substratum (Fig. 3). The neck of bags provides the support for the optimal ventilation of the substratum (Fig.4). These fine points have the principal significance in the mass-production technology of filamentous fungi.

Fig. 3. Form of inoculated cultivation unit.

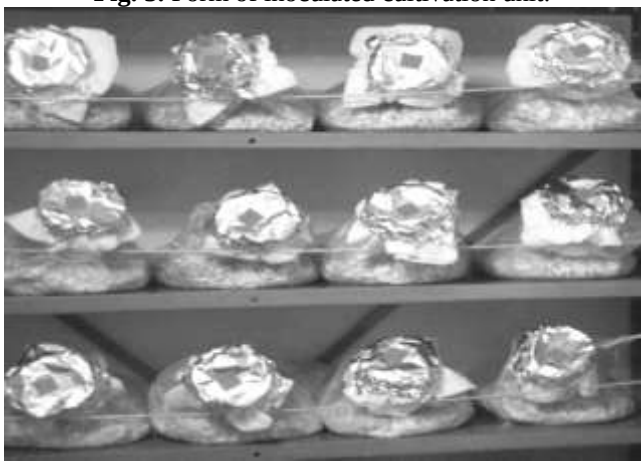

Fig. 4. Optimal disposition of inoculated cultivation units.

Based on the conducted experiments we conclude that millet can be utilized as substratum for the mass-production of different species of fungi using a single unifying technology. The harvest of conidia from the millet grain exceeded productivity when compared to other grain substrata including wheat, barley, corn and other solid nutrient materials (Nelson *et al.*, 1996; Santa *et* al., 2005; Soundarapandian and Chandra, 2007). The presented universal technology for the mass-production of different species of entomopathogenic fungi can be used for the preparation of the quantities of fungal material necessary for laboratory and especially field experiments.

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