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Mass production of Entomopathogenic fungi *Beauveria bassiana, Metarhizium anisopliae, Verticillum lecanii and Paecilomyces fumosoroseus* by using broken rice as substrate

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Abstract
The success of the biological control agents of insect pests depends not only on their experimentation and analysis, but also on the success of their mass production. The biological control strategy using entomopathogenic fungi like *Beauveria bassiana, Metarhizium anisopliae, Verticillum lecanii and Paecilomyces fumosoroseus* can only be successful if practical and economic methods of mass multiplication are available. Broken rice grains was evaluated for solid state multiplication of *B. bassiana, M. anisopliae, V. lecanii and P. fumosoroseus.* Mass multiplication of these bio-control agents were studied using different media to evaluate parameters like dry extraction of conidia, spore yield, spore count and germination. The influence of photoperiod and incubation time in the production of conidia were also evaluated. These studies indicate broken rice as the most productive substrate for conidial production of the four fungal genera, with a yield of $4.2x10^{10}$, 6.5x1010, 5.9×10^{9}, 7.8×10^{9} conidia respectively.

Keywords: beauveria bassiana, liriomyza trifolii, paecilomyces fumosoroseus, metarhizium anisopliae, verticillum lecanii

Introduction
The use of microbial control agents like Entomopathogenic fungi, an alternative to chemical control, as a component of integrated pest management (IPM) strategies is being widely explored for the management of wide range of insect pests. Entomopathogenic fungi is a soil fungus with a good potential for biological control of nematodes and also cause widespread epizootics in fruit flies (Jiji *et al*., 2006) [11], stink bugs (Rambadan *et al*., 2011) [23], reduviid bugs (Marti *et al*., 2006) [18], green house white flies (Gokce *et al*., 2005; Wraight *et al*., 2000) [7, 27] and mite pests (Fiedler and Sosnowska, 2007) [6]. The availability of the mycoinsecticides unlike chemical insecticides is a challenging factor in testing the pathogenicity of fungal pathogens against target insect hosts. For evaluation of the entomopathogenic fungus under field conditions, mass multiplication of the fungus on suitable substrate (Jagadeesh Babu *et al*., 2008) [8] is necessary. Lack of reliable substrates is a major constraint in the mass production and utilization of the mycoinsecticides (De Faria *et al* 2007) [5]. Hence studies were carried out to determine the most suitable and locally available solid substrate for the mass multiplication of the fungus.

Materials and Methods
The present study was carried out in Entomology Laboratory, Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad (India) in 2013. The studied were carried out by using broken rice as a solid substrate to evaluate mass production and economics of entomopathogenic fungi (EPFs).

Composition of different synthetic media used
1. *P. fumosoroseus*
Potato Dextrose Agar (PDA): (per liter) for
PDB: 24g
Agar: 15.0

2. *B. bassiana*
Sucrose: 4.5 g
Yeast extract: 1.5g
PH: 6
Distell water: 250g
Conical Flask: 500ml

3. *M. anisopliae*
Peptone: 2.5g
Glucose: 10g
D.water:250ml
Conical Flask: 500ml

4. *V. lecanii*
PDB: 24g
Agar: 15.0
D. water: 1lit

5. Sabouraud Dextrose Agar (SDA): per liter
Dextrose: 40.0
Peptone: 10.0
Agar: 15.0

Entomopathogenic fungi isolates and their original sources
Four fungal isolates were evaluated in these experiments.

Metarhizium anisopilae, Paecilomyces fumosoroseus were obtained from Division of Plant Pathology, CRIDA Hyderabad. (*B.bassiana, V. Lecanii)* were obtained from Chevella village, Rangareddy district, Telangana. The fungi were cultured on Potato Dextrose Agar (PDA) in slant and kept in fridge at 24ºC as a stock culture then grown on Potato Dextrose Agar (PDA) in Petri dishes and maintained at ambient temperature 27± 1^0C till usage. Fifteen to twenty-one days old-cultures were used as inoculums for mass culture in broken rice.

Solid production of conidial spore

250g of broken rice was thoroughly dry cleaned and washed with water followed by air drying, (0.1%) of chitosan and 2-3ml of groundnut oil was added to it. followed by par boiling the rice. Par boiled rice was cooled and corn steep liquor (30g per 1 kg of rice), antibiotic (chlarophenical 25rpm) i.e. 25µg/1kg rice were added to it and autoclaved. 10% of the broth inoculum and incubate.

Spore harvesting and drying

The spores were harvested 3 to 12days after inoculation for solid media respectively, to evaluate spore yield. To harvest the spores as powder, it is necessary to dry the fungus to reduce moisture content and allow the spores to separate from the substrate. The spores which were harvested following this procedure can be preserved for a long time without loss of viability or pathogenicity (Bateman, 2007) [2]. To dry the cultures, the plastic bags were opened in a room with a temperature of 20± 5ºC and an average relative humidity of 50±5% and allowed to air dry. Harvesting was done manually for 20 minutes. The manual harvest consisted of back and forth movements of the sieve. The spore powder that was collected after sieving was weighed and kept in separate sterile vials for further assessments, such as moisture content and quality assessment.

Spore counting

That is 10 microliter of conidial suspension of *Metarhizium anisopliae, Verticillum lecanii, B. bassiana and P. fumosoroseus*, was placed on the hemocytometer. The cover glass was put over the grid carefully so that no air bubble entered between cover glass and slide. The conidia of Entomopathogenic fungi were counted under Olympus BX41 phase contrast microscope at higher resolution

Germination test

Spore suspension of (10^4/10^5 cfu/ml) was suspend in Tween 80. Media was smeared on slides and prepare an and then place it in a Petri dish. Then place 1-2 drops of spore suspension on the medium and spread over the slide. Then incubate for 18 to 36 h and observe under microscope (every 1 h) Count the germination spores (a) Non germinated spores (b). Then finally Count percentage a/a+b x 100.

$$\text{Spore count per ml} = \frac{\text{Total count of spores x Small squares x Conversion factor x Dilution factor}}{\text{Number of small squares calculated x Depth factor}}$$

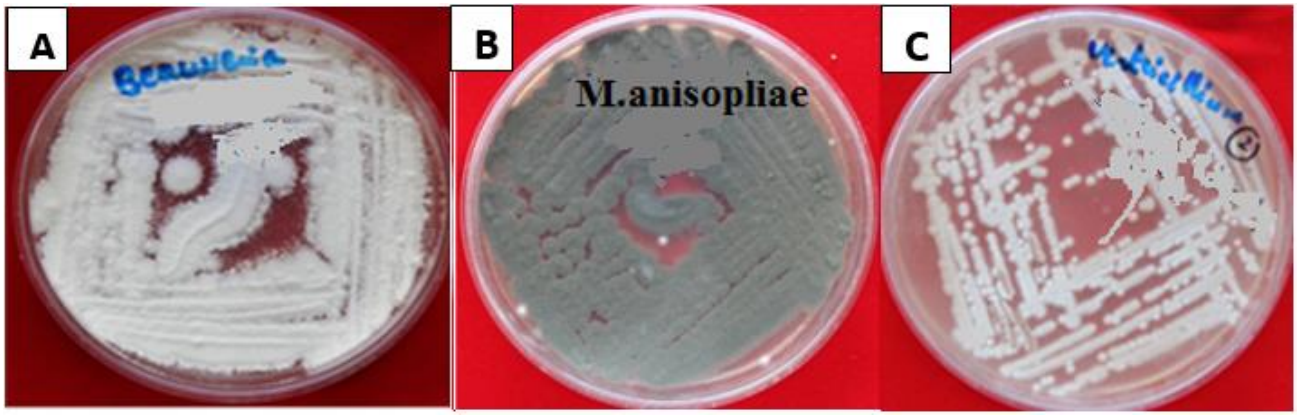

Fig 1: A=*Beauveria bassiana* B=*Metarhizium anisopliae* C=Verticillium isolates growing on Sabouraud Dextrose Agar and contamination tube lined with velvet material used to infect insects with spores (c).

Fig 2: Solid substrate (broken rice) with well-sporulated *Beauveria bassiana. Paecilomyces fumosoroseus, Metarhizium anisopliae, Verticillum lecanii*

Fig 3: Culture bags along with control bags

Fig 4: Small-scale plastic bag-based mass production of *B. bassiana and M. anisopliae.*

Result and Discussion

The spore was counted by using the Heamocytometer. The spore count of *Beauveria bassiana Paecilomyces fumororoseus, Verticillum Lecanii and Metrahizium anisophilae is* 7.8×10^9, 5.9×10^9, 4.2×10^{10} and 6.5×10^{10} spore/ml respectively. The germination observed for *Beauveria bassiaana was* at 12h and maximum germination was after 20 hrs for *Paecilomyces fumoroseus* it was after 18hrs and maximum germination at 24hrs, *Verticillum Lecanii showed germination* at 18hrs and maximum germination after 36 hr *Metrahizium anisophilae* germinated after 12h and showed a maximum germination at 36h. This production can be done in larger scale and can be used for further field as well as laboratory study.

Various fermentation containers such as conical flasks, Petri's plates, tubes, trays, and plastic bags can be used for the mass production of entomopathogenic fungi (Wraight *et al*., 2001; Jaronski, 2014) [28, 10]. One of the advantages of using bags for solid multiplication is that there is a possibility of breaking the subtract clumps formed and in some cases the light is used for optimally for sporulation (Jaronski, 2014) [10]. In our study, mass production potentials of *B. bassiana, M. anisopliae, V. lecanii and P. fumosoroseus* were assessed (fig 1) conidial production among different strains of the entomopathogenic fungi (*B. bassiana, M. anisopliae, V. lecanii and P. fumosoroseus) showed* statistically *insignificant* differences. However, strains of *P. fumosoroseus* showed higher production than the strains of *M. anisopliae B. bassaina and V. lecanii* in the respective order. The result indicated that the sporulation of these fungi differed significantly among different subtracts. Highest sporulation was recorded after four weeks of incubation on broken rice for all fungi are 7.8×10^9 5.9×10^9 4.2×10^{10} 6.5×10^{10} respectively.

Values comparable to the present study were reported by Sahayaraj and Namasivayam (2008) [24] with production of a value close to 1.1×10^7 conidia g1 of subtract. They also proposed that rice grains were the most suitable subtract for the mass multiplication of *B bassiana* Latifian *et al*. (2013) [13] evaluated the solid state multiplication of *B. bassiana* on different plant materials, including sugarcane, corn, barley, rice, millet, and sorghum. They found that a selected strain of B. bassiana (IRAN441c) recorded a maximum production of 6.24 x 10^4 conidia g-1 on rice. Nonetheless, better spore production has been reviewed and commented by Bradley *et al*. (1992) [3] and Bradley *et al*. (2002) [4] on different substrates, e.g., barley, where selected *Beauveria* strains were produced in the order of 2.6 x 10^{10} conidia g-1 on culture reactors.

Babu *et al*. (2008) [1] reported that conidial production of the fungus *M. anisopliae* on rice (amended with yeast extract) was significantly greater than other solid plant substrates, with a mean value of 1.1 x 10^9 conidia g-1 of substrate. When *M. anisopliae* were multiplied on rice Latifian *et al*. (2014) [12] it recorded maximum conidial production of 2.8 x 10^6 conidia g-1. Loera *et al*. (2016) [14] used rice grains as the only substrate for the production of conidia with a selected strain of *M. anisopliae* in plastic bags and managed to obtain about 1 x 10^9 conidia g-1 of substrate. Some authors mentioned that the structure of the substrate is as important as the availability of nutrients and that an ideal substrate should provide a large surface area to favor aeration and formation of conidia (Lomer & Lomer, 2008; Machado *et al*., 2010; Mascarin *et al*., 2010) [15, 16, 19].

Rice hull is a by-product of the rice industry, which has more surface area per gram than the rice grain. However, in the present work, any of the rice hulls combinations when used as a solid mass multiplication substrate produced fewer conidia per gram of substrate compared to the rice grain for the fungal strains evaluated. This could be due to the fact that rice hulls have fewer nutrients or the availability of the same for the fungal strains is less. So, even if rice hull is a by-product of rice milling latter cheaper than the broken rice, the proportion of nutrient in broken rice is higher, making the latter a better option for mass multiplication of biocontrol fungi. Also, different published protocols of mass multiplication use additives such as Torula yeast extract or sugarcane molasses to bypass the need of nutrients of some agricultural substrates and increase the production of conidia (Prakash *et al*., 2008; Sene *et al*., 2010; Jaronski, 2014, Mishra *et al*., 2016) [22, 25 10, 20]. Thus the use of additives could be one possible option to optimize the production the conidia of these entomopathogenic fungal strains in further studies.

Small and medium-scale conidia production vary with different key parameters like substrate used, pH, temperature, moisture, light, aeration (structure of the substrate), different additives, and others. Optimal conditions and every particular strain must be evaluated for each entomopathogenic fungal species (Mascarin et. al, 2010 Mar & Lumyong, 2012; Taylor *et al*., 2013 Muniz-Paredes *et al* 2017) [19, 17, 26, 10,]. Further studies with our fungal strains could be deepened in the assessment of mass production on different rice structures or conformations like

the grain size or parboiled rice.

Table 1: Net weight of spore

S. No	Strain	Quantity	Net weight of spores
1	*Beauveria bassiana*	250g	4.6g
2	*Paecilomyces fumosoroseus*	250g	6.9g
3	*Verticillum Lecanii*	250g	3.5g
4	*Metrahizium anisophilae*	250g	3.7g

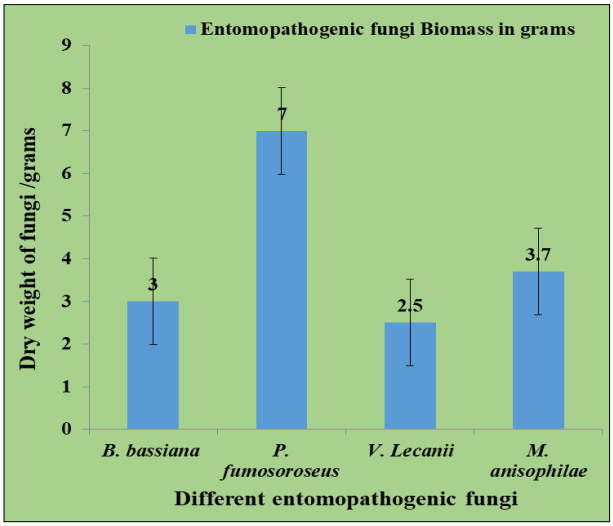

Fig 5: Mass production of Entomopahogenic fungi on broken rice (net weight of spore)

Table 2: Percent of growth at different time intervals

S. No	Strain	% of Growth at different time (hours)			
		25%	50%	75%	100%
1	*Beauveria bassiana*	10.00	12.00	15.00	20.00
2	*Paecilomyces fumosoroseus*	12.00	12.00	18.00	24.00
3	*Verticillum Lecanii*	12.00	18.00	32.00	36.00
4	*Metrahizium anisophilae*	12.00	12.00	18.00	36.00

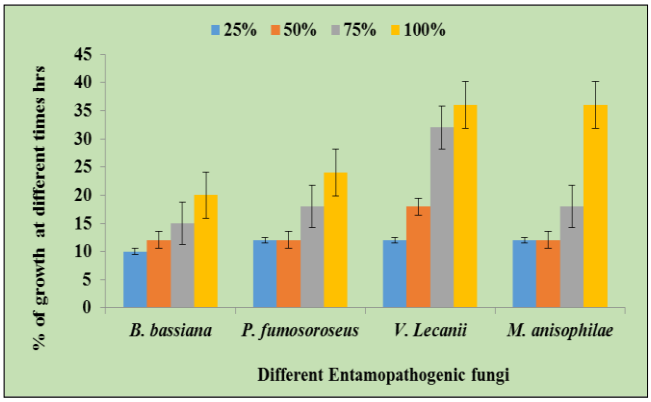

Fig 6: Mass production of Entomopahogenic fungi Percent of growth at different time intervals

Table 3: Different Entomopathogenic fungi strains spore count

S. NO	Strain	Spore/ML
1	*Beauveria bassiana*	7.8×10^{9}
2	*Paecilomyces fumosoroseus*	5.9×10^{9}
3	*Verticillum Lecanii*	4.2×10^{10}
4	*Metrahizium anisophilae*	6.5×10^{10}

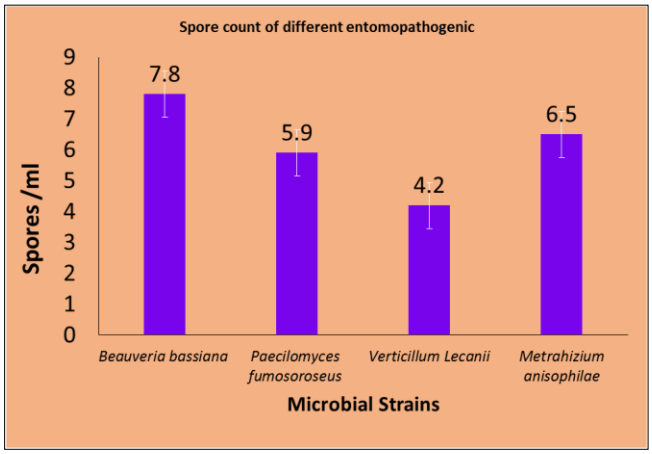

Fig 7: Different Entomopathogenic fungi strains spore count

Conclusion

The data of this study showed that using broken rice as substrate and incubating with 24 h of light were better for mass production of aerial conidia of different strains of *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillum lecanii and Paecilomyces fumosoroseus*. The substrates and parameters evaluated in this study will be a promising strategy even for medium-scale production of conidia for mycoinsecticides with low costs. For all the above, the results of the present work confirm that each fungal strain has optimal conditions for mass multiplication. In addition, the results obtained provide information for a better understanding of key nutritional requirements and culture conditions that can improve the mass production of *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillum lecanii and Paecilomyces fumosoroseus*. This information can be useful even to small-scale farmers with basic infrastructure to culture these biocontrol fungi easily.

References

1. Babu J, Venkatachalapathy CM, Anitha CN. Evaluation of locally available substrates for mass multiplication of entomopathogenic fungi, Metarhizium anisopliae (Metch.) Sorokin. Journal of Invertebrate Pathology. 2008; 46:335-336.
2. Bateman R. Production Processes for Anamorphic Fungi. IPARC, Silwood Park, Ascot, Berks, 2007. http://www.dropdata.net/mycoharvester/Mass_production. PDF (accessed 24.11.12.).
3. Bradley CA, Black WE, Kearns R, Wood P. Role of production technology in mycoinsecticide development. In: Frontiers in Industrial Microbiology. Springer, Boston, MA, 1992, 160-173.
4. Bradley CA, Wood P, Black W, Kearns R, Britton J. United States Patent Application Publication, 2002. Publication number: US 2002/0006650 A1.
5. De Faria M, Wraight S. Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control. 2007; 43:237–256.
6. Fiedler Z, Sosnowska D. Nematophagous fungus *Pacecilomyces Lilacinus (*Thom) Samson is also a biological agent for control of greenhouse insect and mite pests. Biological Control. 2007; 52:547-558.
7. Gokce A, Kubilay ER. Pathogenicity of Paecilomyces spp. to the Glasshouse Whitefly, Trialeurodes vaporariorum, with some observations on the Fungal

Infection Process. Turkish Journal of Agriculture. 2005; 29:331-339.
8. Jagadeesh Babu CS, Venkatachalapathy CM, Anitha CN. Evaluation of locally available substrates for mass multiplication of entomopathogenic fungi, Metarhizium anisopliae (Metch.) Sorokin. Journal of Bio Pesticides. 2008; 1(2):146–147.
9. Jaronski ST, Mascarin GM. Mass Production of Fungal Entomopathogens. In: Microbial Control of Insect and Mite Pests. 2017; 9:141-155.
10. Jaronski ST. Mass production of entomopathogenic fungi: state of the art. In: Mass production of beneficial organism's invertebrates and entomopathogens, 2014, 357-413.
11. Jiji T, Praveena R, Babu K, Naseema A. Occurence of Paecilomyces lilacinus on melon fly Bactrocera cucurbitae Coq. and its cross infectivity on B. dorsalis (Hendel) and Bhindi leaf roller Sylepta derogate Fb. Abst. Colloq. on Nanoscale Sci. and Arthropod Bio resources, Thiruvananthapuram, 2006, 15.
12. Latifian M, Rad B, Amani M. Mass production of entomopathogenic fungi Metarhizium anisopliae by using agricultural products based on liquid-solid diphasic method for date palm pest control. International Journal of Farming and Allied Sciences. 2014; 3(4):368-372.
13. Latifian M, Rad B, Amani M, Rahkhodaei E. Mass production of entomopathogenic fungi Beauveria bassiana (Balsamo) by using agricultural products based on liquid-solid diphasic method for date palm pest control. International Journal of Agriculture and Crop Sciences. 2013; 5(19):23-37.
14. Loera O, Porcayo J, Loza R, Montesinos M, Favela E. Production of Conidia by the Fungus Metarhizium anisopliae Using Solid-State Fermentation. Methods in Molecular Biology. 2016; 1477:61-69.
15. Lomer CH, Lomer CJ. Mass production of fungal pathogens for insect control: insect pathology manual. Section VII. CABI Bioscience Publication, 2008, 20.
16. Machado ACR, Monteiro AC, Almeida AMBD, Martins MIEG. Production technology for entomopathogenic fungus using a biphasic culture system. Pesquisa. 2010; 45(10):1157-1163.
17. Mar TT, Lumyong S. Conidial production of entomopathogenic fungi in solid state fermentation. Asia-Pacific Journal of Science and Technology. 2012; 17(5):762-768.
18. Marti GA, Lastra CC, Pelizza SA, García JJ. Isolation of Paecilomyces lilacinus (Thom) Samson (Ascomycota: Hypocreales) from the Chagas disease vector, Triatoma infestans Klug (Hemiptera: Reduviidae) in an endemic area in Argentina. Mycopathologia. 2006; 162(5):369-372.
19. Mascarin GM, Alves SB, Lopes RB. Culture media selection for mass production of *Isaria fumosorosea* and *Isaria farinosa*. Brazilian Archives of Biology and Technology. 2010; 53(4):753-761.
20. Mishra S, Kumar P, Malik A. Suitability of agricultural by-products as production medium for spore production by Beauveria bassiana HQ917687. International Journal Recycling of Organic Waste in Agriculture. 2016; 5:179-184.
21. Muniz-Paredes F, Miranda-F, Loera O. Production of conidia by entomopathogenic fungi: from inoculants to final quality tests. World Journal of Microbiology and Biotechnology, 2017, 33-57. doi: 10.1007/s11274-017-2229-2
22. Prakash GB, Padmaja V, Kiran RS. Statistical optimization of process variables for the large-scale production of Metarhizium anisopliae conidiophores in solid-state fermentation. Bio Resource Technology. 2008; 99(6):1530-1537.
23. Rambadan S, Jugmohan H, Ayub Khan. Pathogenicity and haemolymph protein changes in Edessa meditabunda F. (Hemiptera: Pentatomidae) infected by *Paecilomyces lilacinus*. Journal of Bio pesticides. 2011; 4(2):169-175.
24. Sahayaraj K, Karthick S, Raja Namasivayam. Mass production of entomopathogenic fungi using agricultural products and by products African Journal of Biotechnology. 2008; 7(12):1907-1910.
25. Sene L, Alves LFA, Lobrigatte MFP, Thomazoni D. Production of conidia of *Metarhizium anisopliae* in solid media based on agro industrial residues. Arquivos do Institute Biologico (Sao Paulo). 2010; 77:449-456.
26. Taylor B, Edgington S, Luke B, Moore D. Yield and germination of the entomopathogenic fungus Beauveria bassiana when grown on different rice preparations. Journal of stored products research. 2013; 53:23-26.
27. Wraight SP, Carruthers RI, Jaronski ST, Bradley CA, Garza CJ, Galaini-Wraight S, *et al.* Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silver leaf whitefly, *Bemisia argentifolii*. Biological Control. 2000; 17(3):203-217.
28. Wraight SP, Jackson MA, de Kock SL. Production, Stabilization and Formulation of Fungal Biocontrol Agents. In: Fungi as biocontrol agents, 2001, 253-288.