Mass Multiplication of Entomopathogenic Fungi Using Agricultural Waste

*Swapan Kumar Ghosh, Sujoy Pal

Molecular Mycopathology Lab., Post Graduate Department of Botany,
Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata 700118, India
*Corresponding address: swapan.krghosh@yahoo.com

Abstract: Mass multiplication of entomopathogenic fungi is a crucial part of successful biological pest management programme. Agricultural wastes are the biodegradable low cost substrates from which mycoinsecticides are grown up in an adequate number very easily with commercial cost benefit. Rice straw, Rice husk, Rice bran, Sugarcane bagasse, Saw dust, Coconut coir, Corn cob and Vegetable waste were selected for mass multiplication solid substrate. Entomopathogenic fungi *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum* were selected for mass multiplication. In this experiment among the all substrates vegetable waste helps to produce maximum spore amount of all fungi strain; *Beauveria bassiana* produced 7.42 x 10^7 spores /g from it. *Metarhizium anisopliae* and *Trichoderma longibrachiatum* produced 8.80 x 10^7 and 7.96 x 10^7 Spores /g respectively only after 15 days of incubation. Rice straw, sugarcane baggase, coconut coir and corn cob are the second easy available mass production substrates and gave moderate amount of spores. Mass multiplication of the entomopathogenic fungi was least in the saw dust where *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum* sporulate 1.32 x 10^7, 2.50 x 10^7 and 1.41 x 10^7 spores/g respectively. Spore quality is one of the challenging parts for mass multiplication. Spore production and viability are depending on the duration of incubation; germination was reached up to 95% after 15 days of interval at 28^0C and gradually decline during 45 days preservation.
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1. Introduction

Green farming is internationally accepted standard methods to get a healthy crop with non polluted environment, and also nominated by the International Federation of Organic Agriculture Movements (IFOAM). Organic farming took a great attention from the last few decades and practiced by many nations. Biological control of pest thus took a legendary part in agricultural pest management and crop protection. Biological control agents such as entomopathogenic fungi can be used as a component of integrated pest management (IPM) of many insect pests and treated as "mycoinsecticide". Once a suitable isolate of entomopathogenic fungi has been identified from the bioassay procedure, the isolate then be tested on a larger scale, in the field. However, the amount of pathogen needed for replicated field trials can be hundreds or thousands of times greater than for small-scale work; at this stage, more efficient methods for mass production must be developed. Production of adequate quantities of good quality inoculums is an essential component of the biocontrol programme. Mass production helps to produce large scale of inoculums for niche markets and the immediate farming area. Entomopathogens can be mass produced using the diphasic liquid–solid fermentation technique. The liquid phase provides active growing mycelia and blastospores, while the solid phase provides support for development of the dry aerial conidia (Rousson *et al.,* 1983; Karanja *et al.*, 2010). *Beauveria bassiana* is one of the well known biocontrol agent has been mass produced on different solid substrates, including sugarcane wastes (Somasekhar *et al*., 1998), silkworm pupal powder (Chavan *et al*., 1998), and steamed rice (Feng *et al*., 1994, 2004). Solid mass production utilizing various domestic and agricultural waste of *Metarhizium anisopliae* was also reported (Khetan, 2001; Karanja etal 2010). Panahian (2012) reports that *Trichoderma* spp. propagules can be produce in liquid or solid fermentation. In both cases low cost of bedding materials with maximum spore yielding was chosen. Agricultural waste is one of the good sources of low cost material for mass multiplication of myco-insecticides and also helps in cost benefit for commercial development.

2. Material and Methods

2.1. Entomopathogenic fungal culture

Pure culture of entomopathogenic fungi; *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum* was inoculated in PDA medium and incubated in BOD at 28±2^0C for 7 days.

2.2. Preparation of solid substrate

Rice straw, Rice husk, Rice bran, Sugarcane bagasse, Saw dust, Coconut Coir, Corn cob and Vegetable waste were selected for mass multiplication solid substrate. They were washed by running tap

water to remove dust and soil. Rice straw, sugarcane bagasse, coconut coir and Vegetable rind and skin were cut into small pieces (approx 1 inch). 20g of solid raw matter was separately kept in 250ml conical flasks along with 10 ml of tap water and sterilized by autoclaving at 121^0C, 15 psi for 30 minutes, after sterilization flask were kept cool. The sterilization procedure was repeated on next day.

2.3. Incubation of fungi

Fungus inocula were scooped out from the growing culture plate with the help of cork borer (5mm) and placed in the sterilized solid medium aseptically. Each set was inoculated by the same amount of the fungus inoculum. All these procedures were done under laminar air flow chamber. They were incubated in BOD incubator at 28 ± 2^0C separately for 15, 30 and 45days. To avoid clumping, after 7 days of inoculation the flasks were shaken vigorously to separate the substrate and to break the mycelia mat. Three replica set was done for each experiment setup.

2.4. Harvesting of conidia

After incubation for desirable day conical flasks were shaken for homogenous mixture and 1g of culture was suspended in 10 ml of sterile distilled water to uptake the spore suspension. The concentration of spore after mass multiplication was counted with the help of Haemocytometer. Rest part of culture was keep for drying. Statistical data of spore formation was analyzed after Duncan's new multiple range tests (Duncan, 1955).

2.5. Quality control of spores

Spore viability in each substrate was also concluded after germination test with help of 2% sucrose solution in a concave groove slide. The whole setup was set in the moisture rich closed Petridish and incubated in BOD incubator at 28 ± 2^0C for 24 hr. Spore germination percentage was counted in the microscopic field at high power (10 x 45). Proportions of germinating and non-germinating spores were taken to determine percentage viability.

3. Results

In this experiment, several naturally available solid substrates were tested for mass multiplication of *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum*. They are generally easily available agricultural biodegradable waste. The result presented in the table-1 shows that spore production of *Beauveria bassiana* is highest in vegetable waste (7.42 x10^7 spores/g) after 15 days of incubation. During the same time rice straw gave 6.42 x 10^7spores/g; but spore production from this two substrate is statistically similar. Spore production of *Beauveria bassiana* is moderate in sugarcane bagasse (4.36 x 10^7spores/g) and corn cob (4.06 x 10^7 spores/g). Saw dust gave less support for spore production (1.32 x 10^7spores/g) in case of *Beauveria.* In general spore production was decreased after 15 days of incubation in all substrate except than sugarcane bagasse (5.56 x 10^7 spores /g) and coconut coir (2.83 x 10^7spores /g). In this experiment 6.86 pH supported best spore production after 15 days of experiment in *Beauveria bassiana.* After 45 days of incubation rice straw gave 5.45 x10^7spores /g which is higher than vegetable waste 4.30 x10^7 spores /g. general trends in case of other substrate spore yield is gradually decrease from 15 days to 45 days of incubation. According to the table 1 it shows that fungal growth rate is maximum in vegetable waste and took only 7 days to cover the substrate. In rice bran and saw dust growth rate is negligible and do not cover properly. Spore germination after 15 days harvest reached to more than 95% in case of vegetable waste, rice straw and corn cob and lowest germination (50.6%) was recorded in saw dust (table 1). Finally corn cob gave additional viability of spore germination (86%) after 45 days of incubation and that is the highest records among the all substrate.

Data represents in the table- 2 shows that *Metarhizium anisopliae* produced maximum spores in vegetable waste (8.80 x 10^7spores/g) during 15 days of incubation. In the same time of incubation sugarcane bagasse, corn cob and rice straw produced 5.90 x 10^7, 5.60 x 10^7, and 5.35 x 10^7spores/g respectively. Rice husk, rice bran, coconut coir produced moderate spores and saw dust gave lowest spore yield (2.12 x 10^7spores/g). After 30 days of incubation corn cob produced significantly different spores (6.66 x 10^7spores /g) in contrast with its previous day's yields. Spore production in coconut coir is also enhanced (3.90 x 10^7 spores/g) but statistically equal with its previous time production. In other substrate spore production became decreased. Final counting was done after 45 days of incubation; where spore yield was significantly decrease in all substrate. According to the table 2 data it is clear that spore yield from vegetable waste is significantly highest (6.35 x 10^7 spores/g) among the all substrate in case of *Metarhizium anisopliae.* Growth rate of *Metarhizium anisopliae* in vegetable waste and sugarcane bagasse is 7 days and in corn cob is 9 days. Growth rate in other substrate varied from 15 days to 30 days. Spore germination of *Metarhizium anisopliae* after 15 days was maximum reached to 95% in case of vegetable waste, sugarcane bagasse, rice straw and coconut coir; lowest germination was recorded from saw dust (60.6%). Spore germination rate in coconut coir is 97% during 30 days of incubation which is higher than its previous harvest time. Sugarcane bagasse and corn cob after 45 days of incubation gave maximum spore viability (86%) along with vegetable waste (85%).

Table 1. Mass production of *Beauveria bassiana* spores in different substrates

Sl no.	Substrate	pH of medium	Spores /g			Time taken for full cover	Cumulative % of spore germination after harvest *		
			15 days of incubation	30 days of incubation	45 days of incubation		15 days	30 days	45 days
1.	Rice straw	7.26	6.42 x 10^7 a	5.93 x 10^7 a	5.45 x 10^7 B	15 days	95.2 (77.34)	72 .0 (58.05)	45 .0 (42.13)
2.	Rice husk	6.90	2.60 x 10^7 c	2.51 x 10^7 c	1.95 x 10^7 C	12 days	91.4 (72.95)	84.2 (66.58)	80.0 (63.44)
3.	Rice bran	7.20	2.93 x 10^7 c	2.48 x 10^7 c	2.41 x 10^7 C	Partial	65.9 (54.27)	60.1 (50.83)	50.0 (45.00)
4.	Sugarcane bagasse	6.54	4.36 x 10^7 b	5.56 x 10^7 a	2.45 x 10^7 B	15 days	95 .0 (77.08)	95.3 (77.48)	82.0 (65.05)
5.	Saw dust	6.00	1.32 x 10^7 c	1.21 x 10^7 c	0.95 x 10^7 C	Not cover	50.6 (45.34)	40.6 (39.58)	35.0 (36.27)
6.	Coconut coir	5.54	2.39 x 10^7 c	2.83 x 10^7 c	2.16 x 10^7 C	30 days	87 .0 (68.87)	95 .0 (77.08)	73 .0 (58.69)
7.	Corn cob	6.65	4.06 x 10^7 b	4.37 x 10^7 b	3.64 x 10^7 B	16 days	95.0 (77.08)	92.5 (74.11)	86.0 (68.03)
8.	Vegetable waste	6.86	7.42 x 10^7 a	5.65 x 10^7 a	4.30 x 10^7 A	7 days	98.2 (82.29)	90.1 (71.66)	75.0 (60.00)

Mean values followed by a different letter indicates significant difference (P=0.05), according to Duncan's multiple range test.

*Data in the parenthesis are angular transformation value of percentage.

Table 2. Mass production of *Metarhizium anisopliae* spores in different substrates

Sl no.	Substrate	pH of medium	Spores /g			Time taken for full cover	Cumulative % of spore germination after harvest *		
			15 days of incubation	30 days of incubation	45 days of incubation		15 days	30 days	45 days
1.	Rice straw	7.26	5.35 x 10^7 b	5.56 x 10^7 a	4.80 x 10^7 b	15 days	94.2 (76.02)	62 .0 (51.94)	45 .0 (42.13)
2.	Rice husk	6.90	3.00 x 10^7 c	3.25 x 10^7 c	3.25 x 10^7 c	20 days	81.4 (64.45)	84.2 (66.58)	80.0 (36.44)
3.	Rice bran	7.20	3.10 x 10^7 c	2.94 x 10^7 c	2.05 x 10^7 c	25 days	75.5 (60.33)	60.1 (50.83)	60.0 (50.77)
4.	Sugarcane bagasse	6.54	5.90 x 10^7 a	4.50 x 10^7 b	4.01 x 10^7 b	7 days	97 .0 (80.02)	95.3 (77.48)	86.0 (68.03)
5.	Saw dust	6.00	2.12 x 10^7 c	2.50 x 10^7 c	1.95 x 10^7 c	30 days	60.6 (51.12)	50.6 (45.34)	45.0 (42.13)
6.	Coconut coir	5.54	3.86 x 10^7 b	3.90 x 10^7 b	3.40 x 10^7 b	25 days	86 .0 (68.03)	97 .0 (80.02)	73 .0 (58.69)
7.	Corn cob	6.65	5.60 x 10^7 a	6.66 x 10^7 a	5.35 x 10^7 b	9 days	95.0 (77.08)	92.5 (74.11)	86.0 (68.03)
8.	Vegetable waste	6.86	8.80 x 10^7 a	8.25 x 10^7 a	6.35 x 10^7 a	7 days	98.5 (82.31)	95.1 (77.24)	85.0 (67.21)

Mean values followed by a different letter indicates significant difference (P=0.05), according to Duncan's multiple range test.

*Data in the parenthesis are angular transformation value of percentage

In case of *Trichoderma longibrachiatum* 15 days spore production was highest in vegetable waste (7.96 x10^7spores /g) and minimum in saw dust (1.02 x 10^7spores/g), after 30 days of incubation except corn cob and vegetable waste all substrate produced more spores than their previous time harvest. Table 3 represents that highest spore production obtained from of rice straw (7.65 x 10^7spores/g). Lowest spore yield was obtained from saw dust (0.98 x 10^7spores/g) after 45 days harvest. *Trichoderma longibrachiatum* growth rate was highest in vegetable waste (6 days) and for other substrate it takes 10-20 days, but in coconut coir and saw dust fungi never covered the substrate.

Spore germination rate was highest in corn cob (97%) during 15 days interval. Spore germination gets enhanced of that substrate which produced more spore after 30 days. Rice straw and sugarcane bagasse shows 96 % germination after 30 days of incubation. Highest spore germination after 45 days was found in Corn cob (87.1%) and lowest in saw dust (35 %).

Table 3. Mass production of *Trichoderma longibrachiatum* spores in different substrates

Sl no.	Substrate	pH of medium	Spores/g			Time taken for full cover	Cumulative % of spore germination after harvest *		
			15 days of incubation	30 days of incubation	45 days of incubation		15 days	30 days	45 days
1.	Rice straw	7.26	6.16 x 10^7 a	7.65 x 10^7 a	6.12x 10^7 A	15 days	72 .0 (58.05)	96.2 (78.79)	65 .0 (53073)
2.	Rice husk	6.90	2.95 x 10^7 c	3.27 x 10^7 c	3.25 x 10^7 C	20 days	90.4 (71.95)	85.2 (67.37)	80.0 (63.44)
3.	Rice bran	7.20	2.10 x 10^7 c	2.64 x 10^7 c	2.05 x 10^7 C	20 days	65.9 (54.27)	60.1 (50.83)	55.0 (47.87)
4.	Sugarcane bagasse	6.54	6.10 x 10^7 a	6.23 x 10^7 a	6.02 x 10^7 A	10 days	95.3 (77.48)	96 .0 (78.48)	86.6 (68.53)
5.	Saw dust	6.00	1.02 x 10^7 c	1.41 x 10^7 c	0.98 x 10^7 C	Not cover	40.6 (39.58)	50.6 (45.34)	35.0 (36.27)
6.	Coconut coir	5.54	1.25 x 10^7 c	1.65 x 10^7 c	1.10 x 10^7 C	Partial	61 .0 (51.35)	65 .0 (53.73)	56 .0 (48.45)
7.	Corn cob	6.65	5.61 x 10^7 a	5.50 x 10^7 b	4.65 x 10^7 B	16 days	97.0 (80.02)	93.6 (75.35)	87.1 (68.95)
8.	Vegetable waste	6.86	7.96 x 10^7 a	6.85 x 10^7 a	5.55 x 10^7 B	6 days	94.2 (76.06)	90.5 (72.05)	85.0 (67.21)

Mean values followed by a different letter indicates significant difference (P=0.05), according to Duncan's multiple range test.
*Data in the parenthesis are angular transformation value of percentage

4. Discussion

Mass production of entomopathogenic fungi and testing of germination are important steps in successful utilization of biocontrol agents. The substrates used in this experiment were relatively cheap, easily available and acted as nutritive solid media for mass production of the entomopathogenic fungi; this will help for cost beneficial green farming. Various agricultural wastes showed good results for mass multiplication of entomopathogenic fungi; and also commercially cost benefited solid substrates (Feng *et al*., 1994, 2004; Chavan *et al*., 1998; Somasekhar *et al*., 1998). In this experiment among the all substrate vegetable waste produced maximum amount of spore of all fungi strain; *Beauveria bassiana* produced 7.42 x 10^7 spores /g from it. *Metarhizium anisopliae* and *Trichoderma longibrachiatum* produced 8.80 x 10^7 and 7.96 x 10^7 Spores /g respectively only after 15 days of incubation for their good nutrient sources and physical supports. Sharma eta al (2002) reported that vegetable waste, rice straw and grain shows best result for *B. bassiana* and *M. anisopliae* Sahayaraj and Namasivayam (2008) recorded the maximum spore production in vegetables and rice husk (10.76 x 10^8 spores/100 g) for *B. bassiana.* Puzari *et al*. (1997) reported that rice husk supplemented with 2% dextrose solution recorded more sporulation of *M. anisopliae*.

In this present research rice straw, sugarcane baggase and corn cob is the second easy available mass production substrate and gave moderate amount of spores. Mass multiplication of the entomopathogenic fungi was least in the saw dust where *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum* sporulate 1.32 x 10^7, 2.50 x 10^7 and 1.41 x 10^7 spores/g respectively. Coconut coir, Rice husk and rice bran are semi important medium for their moderate amount of spore production. Only *Metarhizium anisopliae* can produced 3.90 x 10^7spores/g in coconut coir after one month of incubation, but other two fungi cannot grows and produced aliquot amount of spores for mass multiplication. This may be caused for the nutrition lack. Spore production of fungi depends on their growth rate and substrate type; the structure of the substrate is more important than the nutrients supplied. An ideal substrate will provide a high surface area of volume ratio to provide inter-particle spaces for aeration and formation of conidia. Saw dust and rice bran thus gave lowest product.

Large-scale availability of the pathogen is a primary requirement in the bio-control programme. For a successful integrated pest management entomopathogenic fungi should be amenable to easy and cheap mass multiplication. Spore quality is one of the challenging parts for mass multiplication; only viable spore can effective for the management programme. Posada-Flórez (2008) reports that spore germination at 24 hours was over 75% in case of *Beauveria.* Spore germination rate of all fungi during the first month is near to 95% and gradually loss the ability of germination. Spore germination beyond 56th day in storage at 28^0C dropped significantly was reported by Karanja *et al*. (2010). The rate of spore production of all substrate was gradually decreased during the time of incubation. It may be that they germinate and become mycelia, thereby, causing the loss of biomass. Lack of moisture is also responsible for spore quality, Moore *et al*. (2000) reported that moisture loss was a result of the metabolic activity of the fungus, transpiration and diffusion while the cultures were developing and fungal spores are living

organisms and their viability diminishes with time depending on environmental conditions.

Alternatively cultures may begin to degrade because there are not enough resources to continue growing. In all case vegetable waste, sugarcane bagasse, rice straw and corn cob shows the best germination viability during the first month of harvest. Posada-Flórez (2008) reports that to avoid a loss of spore production the harvest needs to be done early. Further experiments around 10–20 day intervals should be carried out to determine the optimum harvest period. In this study showed that the best harvest time is 15 days.

5. Conclusion

From this experiment this can be concluded that among the all kind of agricultural wastes vegetable waste had the highest potency to enhance maximum spore production for all kind of entomopathogenic fungi within 15 days of incubation. *Beauveria bassiana, Metarhizium anisopliae* and *Trichoderma longibrachiatum* sporulate $7.42X10^7$ spores/ g, $8.80X10^7$ spores/ g and $7.96\ X10^7$ spores/ g respectably. pH of the medium became slightly acidic (6.86) which may helps for the sporulation. Spore germination rate after harvest was maximum in 15 days i.e nearly 95% for all fungus type.

Therefore, the best mass multiplication agricultural waste is vegetable waste and optimum harvesting time is 15 days after incubation.

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