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Evaluation of Different Media and Methods of Cultivation on the Production and Viability of Entomopathogenic Fungi, *Verticillium lecanii* (Zimm.) Viegas

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Abstract: For mass production of *V. lecanii*, three types of cultivation methods including liquid, solid and diphasic production systems were investigated. In the liquid state of production, six media were tested in stationary culture conditions. Among the six media tested, Molasses Yeast Broth (MYB) supported maximum sporulation (8.33×10^8 spores mL⁻¹) and biomass production (746 mg/100 mL). In the MYB, 4% molasses concentration was found to produce highest spore count (8.56×10^8 spores mL⁻¹) and biomass (776 mg/100 mL) followed by 5 and 6% molasses. Among the six solid substrates tested, rice grains supported highest spore production (1.14 g/100 g). In diphasic state of production, combination of MYB and rice grains produced the greatest amount of spores, (1.70 g/100 g). Results of this study indicated that diphasic method using MYB as liquid medium and rice as solid substrate are the best method and media for mass production of V1-7 isolate of *V. lecanii*.

Key words: *Verticillium lecanii*, mass production, media, cultivation method

INTRODUCTION

Verticillium lecanii has been recognized as an entomopathogen with high potential in biological control of aphids. Many isolates of this fungus demonstrate high pathogenicity to several species of aphids (Askary *et al.*, 1998; Derakhshan *et al.*, 2007; Kim *et al.*, 2007). A successful microbial insecticide should be able to produce high quantities of inoculums (Goettle and Roberts, 1992). The method of culturing largely depends on fungal species and the type of propagule required for formulation and method of application.

The type of growing medium affects conidial production of entomopathogenic fungi (El Damir, 2006; Pandey and Kanaujia, 2006). Variety of substrates have been used for mass production of *V. lecanii* viz., sorghum, pearl millet and maize (Lakshmi *et al.*, 2001), almond mesocarb (Lopez-Liorca and Carbonell, 1998), rice, rice bran, rice husk and the mixture of these components (Feng *et al.*, 2000), sugar beet molasses (Farsi *et al.*, 2005). The majority of industrial production systems of fungi utilize a two stages system in which fungal inoculum of mycelium or hyphal bodies is produced in liquid culture and transferred to a solid substrate for production of aerial conidia (Vimala Devi, 1994). Despite the many attempts to screen commercially available, low-cost

ingredients of industrialized biological pesticides, the research on nutritional requirements of fungal agents were overlooked and a systematic investigation of fungal nutrition utilization is much needed to improve mass production and accelerate commercialization (Sun and Liu, 2006).

In a previous study (Derakhshan *et al.*, 2007) a total of 25 isolates of fungal pathogens consisting of *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Paecilomyces fumosoroseus*, *Nomuraea rileyi* were tested on the cabbage aphid. Among the isolates, V.1-7 isolate of *V. lecanii* was selected for mass production, as it was found to be the most virulent isolate to *B. brassicae*. In this study we have evaluated different types of cultivation methods with different liquid and solid substrates for mass culturing of *V. lecanii*.

MATERIALS AND METHODS

This study was conducted at the Project Directorate of Biological Control (PDBC), Bangalore, India during 2005-2006. Isolate v1-7 of *V. lecanii* originally isolated from *Bemisia tabaci*, was obtained from microbial repository of PDBC. For mass production, three types of cultivation methods including liquid, solid and diphasic states were investigated.

Mass production on liquid media: In liquid state production, six liquid media viz., Molasses Yeast Broth (MYB), Potato Dextrose Broth (PDB), Potato Carrot Broth (PCB), Jaggery Yeast Broth (JYB), Sucrose Yeast Broth (SYB) and Potato Sucrose Broth (PSB) were tested. Conical flasks containing 100 mL sterilized liquid media were inoculated with 1 mL of spore suspension (1×10^7 spores mL^{-1}) with three replications and incubated in a growth chamber at $25 \pm 1^\circ\text{C}$, 90 ± 3 RH and 12:12 photoperiod for 14 days. To quantify the spore and biomass production, cultures were agitated vigorously and filtered through Whatman No. 2 filter paper. The fungal mat was oven-dried at $40-45^\circ\text{C}$ until constant weight was achieved. The filtrate was diluted in known quality of water and the total of spores mL^{-1} was assessed with the help of Naebauer improved haemocytometer. To determine spore germination, spore suspension (0.1 mL of 1×10^5 spores mL^{-1}) was spread on PDA Petri plates and incubated at $25 \pm 1^\circ\text{C}$, 90 ± 3 RH and 12:12 photoperiod. After 24 h, per cent germination was estimated by counting 100 spores for each plate using microscope at 400x magnification. A spore was considered germinated when the germ tube was at least equal to its width. Three plates were used per treatment and each plate served as a replicate.

Effect of molasses concentration on biomass and spore production of *V. lecanii*: As MYB medium produced maximum biomass and spores, the effect of different concentrations of molasses viz., 1, 2, 3, 4, 5, 6 and 7% were evaluated to determine the optimum concentration of molasses in MYB. Experiment conditions were the same as mentioned earlier.

Mass production on solid substrates: For solid state of production, five cereal grains viz., rice, wheat, maize, Sorghum and ragi were evaluated. Raw grains were taken in autoclavable polypropylene bags ($20 \times 28 \text{ cm}$) at 100 g/bag and were soaked with tap water for 12 h and the excess water was drained completely. To each of these bags, 2 g of calcium carbonate and 2 g of calcium sulphate were added and mixed thoroughly to get uniform coating of salts over grains. This process helped in preventing the grain particles sticking together and thereby providing more surface area for the growth of fungus. The bags were then sterilized by autoclaving twice at 121°C for 20 min. The grain media was inoculated with 1 mL of fungal suspension (1×10^7 spores mL^{-1}) under aseptic condition. The bags were once again sealed manually and incubated for 14 days at $25 \pm 1^\circ\text{C}$, $90 \pm 3\%$ RH and 12-12 photoperiod for the production of aerial conidia. Three replications were used for each substrate. After 14 days of

incubation, grains with fungal growth were dried under aseptic conditions at 30°C until the moisture content was reduced to 8%. After drying, the spore production of each grain was estimated by Naubauer's improved haemocytometer. Then, the grains with fungal growth were sieved through a sterile coffee filter with vigorous agitation and the coarse dust thus collected was further sieved through a sterile 105μ sieve to get fine spore dust. The quantity of spores obtained per 100 g of each substrate was estimated.

Mass production in diphasic system: For diphasic state of production, MYB, PDB and PSB as liquid media and broken rice, broken corn and broken sorghum as solid substrates were tested. One hundred milliliter of each above mentioned broths prepared in 250 mL conical flask and inoculated with 1 mL of fungal suspension (1×10^7 spores mL^{-1}) in a shaker maintained at 120 rpm for 3 days. Fifteen milliliter of 3 day-old shake cultures inoculated to each of the bag containing 100 g grain and mixed well with the grains. The inoculated bags incubated as mentioned earlier. Production of spore, spore dust and viable spore count (cfu g^{-1}) for each treatment were estimated.

All data were subjected to analysis variance (ANOVA) and the means were separated by using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Liquid media: Biomass and spore production and viable spore count are significantly affected by type of media (Table 1). Among six liquid media tested, MYB produced maximum biomass (746 mg/100) followed by PSB and PCB, 573 and 563 mg/100 mL of medium respectively. Also MYB supported maximum sporulation (8.33×10^8 spores mL^{-1}) followed by PCB and PSB, 60.50 and 6.30×10^8 spores mL^{-1} , respectively. The results indicated that MYB medium is a suitable liquid medium as it produced maximum biomass and spores. Sugarcane molasses concentrations in MYB medium had significant

Table 1: Effect of different liquid media on biomass, spore production and spore germination

Media	Dry weight of biomass (mg/100 mL)	Spore production ($\times 10^8$ spores mL^{-1})	Spore germination (%)*
PCB	563 ^b	6.50 ^b	91.50
PDB	469 ^d	3.95 ^c	90.33
PSB	573 ^b	6.30 ^b	91.00
JYB	505 ^c	2.45 ^d	90.50
SYB	453 ^d	2.50 ^d	89.50
MYB	746 ^a	8.33 ^a	91.50

Means followed by the similar letter(s) in the columns are not significantly different at 5% by Duncan's Multiple Range Test (DMRT), *: Non significant

effect on biomass and spore production (Table 2). Of the different molasses concentrations tested, 4% molasses supported higher biomass and spore production followed by 5 and 6% molasses.

It was found that there is no significant difference in spore germination of the spores produced in different media although PCB and MYB medium were higher than other media (Table 1). The findings are in accordance with those of Farsi *et al.* (2005) who reported that among different media for mass production of *V. lecanii*, sugar beet molasses had the highest rates of sporulation, Tincilley *et al.* (2004) evaluated different concentration of sugar cane molasses for mass production of *Nomuraea rileyi* and found that maximum biomass and spore production was observed at 6% molasses.

Solid media: The spore count recorded on rice, sorghum and corn were on par and significantly higher than ragi and wheat. Maximum spore dust was harvested from rice grain followed by corn. It was found that viable spore counts in different grains are not significantly different (Table 3). These finding are in agreement with Nelson *et al.* (1996) who found that for spore production of *Beauveria* and *Metarhizium*, these fungi produced more spores on rice over other growing substrates. Lakshmi *et al.* (2001) studying the mass culturing of *V. lecanii* on three grain media found that broken sorghum grain with a spore load of 1.5×10^9 spores g^{-1} was the best followed by pearl millet broken grain medium with 1.3×10^9 spores g^{-1} .

Diphasic system: Combinations of MYB + rice and PSB + rice supported significantly higher spore production than other treatments yielding 2.28 and 2.21×10^9 spores g^{-1} , respectively. Other combinations were on par. Results indicated that MYB + rice produced highest amount of spore dust and MYB + corn and PCB + sorghum and PCB + corn were on par and poorest media to produce spore dust. Results on viable spore count showed that there were no significant differences among treatments (Table 4). These findings are in accordance with those of Nirmala *et al.* (2006) who reported that spore production of four isolates of *V. lecanii* in diphasic system (PDB and rice) after 10 days is ranges from 0.23 to 1.75×10^9 spores mL^{-1} .

Mass production is an important component of a successful microbial insecticide program. Present results of mass production of *V. lecanii* revealed that among liquid media, MYB medium with 4% sugar cane molasses and among solid substrates, rice grains and in diphasic system, combination of MYB+rice supported highest

Table 2: Effect of different concentrations of molasses on spore and biomass production

Molasses concentration (%)	Dry weight of biomass (mg/100 mL)	Spore production ($\times 10^9$ spores mL^{-1})
1	706 ^d	6.39 ^e
2	733 ^c	7.51 ^d
3	753 ^b	8.35 ^b
4	776 ^a	8.56 ^a
5	763 ^b	8.42 ^{ab}
6	756 ^b	8.38 ^{ab}
7	753 ^b	8.07 ^c

Means followed by the similar letter(s) in the columns are not significantly different at 5% by DMRT

Table 3: Effect of different solid media on spore production and spore viability

Media	Spore count ($\times 10^9$ g^{-1})	Spore dust (g/100 g)	Viable spore count ($\times 10^9$ cfu g^{-1})*
Rice	1.97 ^a	1.25 ^a	1.80
Sorghum	1.90 ^a	0.88 ^b	1.78
Ragi	1.66 ^b	0.71 ^c	1.56
Wheat	1.65 ^b	0.87 ^b	1.50
Corn	1.84 ^a	1.10 ^a	1.67

Means followed by the similar letter(s) in the columns are not significantly different at 5% by DMRT. *: Non significant

Table 4: Effect of media on spore production and spore viability in diphasic system of mass production

Liquid media	Solid media	Spore count ($\times 10^9$ g^{-1})	Spore dust (g/100 g)	Viable spore count ($\times 10^9$)*
MYB	Rice	2.28 ^a	1.70 ^a	2.10
	Sorghum	2.10 ^b	1.27 ^{bc}	1.93
	Corn	2.07 ^b	1.21 ^c	1.82
PCB	Rice	2.12 ^b	1.32 ^{bc}	1.89
	Sorghum	2.09 ^b	1.19 ^c	1.91
	Corn	2.12 ^b	1.24 ^c	1.98
PSB	Rice	2.21 ^a	1.62 ^{ab}	2.03
	Sorghum	2.08 ^b	1.54 ^{abc}	1.82
	Corn	2.09 ^b	1.47 ^{abc}	1.88

Means followed by the similar letter(s) in the columns are not significantly different at 5% by DMRT. *: Non significant

spore production and resulted in 2.43 and 1.16 times more spores than MYB and rice in liquid and solid states respectively. Such differences can have significant effects on the economical production of *V. lecanii* for field application.

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REFERENCES

- Askary, H., Y. Carriere, R.R. Belanger and J. Brodeur, 1998. Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. *Biocontrol Sci. Technol.*, 8 (1): 23-32.

- Derakhshan, A., R.J. Rabindra and B. Ramanujam, 2007. Efficacy of different isolates of entomopathogenic fungi against *Brevicoryne brassicae* (L.) at different temperatures and humidities. *J. Biol. Control*, 21 (1): 65-72.
- Duncan, D.B., 1955. Multiple ranges and multiple F tests. *Biometrics*, 11: 1-42.
- El Damir, M., 2006. Effect of growing media and water volume on conidial production of *Beauveria bassiana* and *Metarhizium anisopliae*. *J. Biol. Sci.*, 6 (2): 269-274.
- Farsi, M.J., H. Askari, K.T. Jahromi and A.K. Pakdel, 2005. Effect of important ecological factors on blastospore production of *Verticillium lecanii* DAOM 198499 in liquid medium. *Iranian J. Agric. Sci.*, 36 (1): 109-119.
- Feng, K.C., B.L. Liu and Y.M. Tzeng, 2000. *Verticillium lecanii* spore production in solid-state and liquid-state fermentations. *Bioprocess Eng.*, 23 (1): 25-29.
- Goettle, M.S. and D.W. Roberts, 1992. Mass Production, Formulation and Field Application of Entomopathogenic Fungi. In: *Biological Control of Locusts and Grasshoppers*, Lomer, C.J. and C. Prior (Eds.). Wallingford, Oxon, CAB International, UK., pp: 230-238.
- Kim, J.J., M.S. Goettel and D.R. Gillespie, 2007. Potential of *Lecanicillium* species for dual microbial control of aphids and cucumber powdery mildew fungus, *Sphaerotheca fuliginea*. *Biol. Control*, 40 (3): 327-332.
- Lakshmi, S.M., P. Alagammai and S. Jayaraj, 2001. Studies on Mass Culturing of the Entomopathogenic White Halo Fungus, *Verticillium lecanii* on Three Grain Media and its Bioefficacy on *Helicoverpa armigera* (Hubner) on Pigeon Pea. In: *Microbials in Insect Pest Management*, Ignacimuthu, S. and A. Sen (Eds.). Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, pp: 111-115.
- Lopez-Liorca, L.V. and T. Carbonell, 1998. Use of almond mesocarp for production of the entomopathogenic fungus *Verticillium lecanii*. *Can. J. Microbiol.*, 44 (9): 886-895.
- Nelson, T.L., L. Low and T.R. Glore, 1996. Large scale production of New Zealand strains of *Beauveria* and *Metarhizium*. In: *Proceeding of the 49th The New Zealand Plant Protection Conference*. The New Zealand Plant Protection Society Incorporated, pp: 257-261.
- Nirmala, R., B. Ramanujam, R.J. Rabindra and N.S. Rao, 2006. Growth parameters of some isolates of entomofungal pathogens and production of dust-free spores on rice medium. *J. Biol. Control*, 19 (21): 129-133.
- Pandey, A.K. and K.R. Kanaujia, 2006. Effect of different grain media on sporulation, germination and virulence of *Beauveria bassiana* (Balsamo) Vuillemin against *Spodoptera litura* Fabricius larvae. *J. Biol. Control*, 19 (1): 29-133.
- Sun, M.H. and X.Z. Liu, 2006. Carbon requirements of some nematophagous, entomopathogenic and mycoparasitic Hyphomycetes as fungal biocontrol agents. *Mycopathologia*, 161 (2): 295-305.
- Tincilley, A., S. Easwaramoorthy and G. Santhalakshmi, 2004. Attempts on mass production of *Nomuraea rileyi* on various agricultural products and byproducts. *J. Biol. Control*, 18 (1): 35-40.
- Vimala Devi, P.S., 1994. Conidia production of the entomopathogenic fungus, *Nomuraea rileyi* and its evaluation for control of *Spodoptera litura* (Fab.) on *Ricinus communis*. *J. Invertebr. Pathol.*, 63 (2): 145-150.