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Growth parameters of some isolates of entomofungal pathogens and production of dust-free spores on rice medium

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ABSTRACT: **Four isolates in each of *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metschinikoff) Sorokin and *Verticillium lecanii* (Zimmerman) Viégas, pathogenic to key pests of crops like, coffee berry borer, coconut rhinoceros beetle, cashew stem borer, citrus and coffee green scale etc. were assessed for the colony growth on Potato Dextrose Agar (PDA), biomass and spore production in Potato Dextrose Broth (PDB) and dry spore production in rice grown through di-phasic production system. The isolates Bb5a (*B. bassiana*), Ma4 (*M. anisopliae*) and Vl5 (*V. lecanii*) were found fast growing with the colony diameter of 6.3, 8.1 and 5.7cm, respectively on PDA after 15 days of incubation. Biomass production in PDB was comparatively higher in shake cultures than in stationary cultures for all isolates except Bb4, Bb5a, and Vl3a. Among the *B. bassiana* isolates, biomass production in stationary cultures was highest in Bb4 isolate (0.78 g/100ml) and in shake culture in Bb3 isolate (1.02g/100ml). Ma2 in stationary culture (0.8g/100ml) and Ma3 isolate in shake cultures produced maximum biomass (1.09g/100ml). Vl 2a isolate among *V. lecanii* isolates produced maximum biomass in stationary culture and shake culture (0.77 and 1.03g/ 100ml, respectively). *M. anisopliae* isolates formed mycelial pellets of various sizes under shake culture condition in PDB. In two-stage system of mass production, the maximum spore production on rice was observed in Bb5a, Ma4 and Vl5 isolates (96.0, 49.8 and 17.5 x 10^8 spores g^{-1}) and maximum spores per gram of rice were harvested in Bb5a, Ma2 and Vl2a isolates (28.00, 45.50 and 15.60 mg g^{-1}). Maximum viable spores per gram of spore dust were recorded in Bb5a, Ma4 and Vl5 isolates (4.7 x10^{10}, 2.5 x10^{10} and 1.7 x10^9g^{-1}). Taking into consideration of quantity of spore dust production and viable spores in the spore dust, Bb5a, Ma2 and Vl2a were identified as potential isolates for large-scale production of dry conidial powder.**

KEY WORDS: *Beauveria bassiana*, biomass, *Metarhizium anisopliae*, radial growth, spore production, *Verticillium lecanii*

INTRODUCTION

Entomofungal pathogens are well known biocontrol agents that kill their host insects through invasion and profuse mycelial growth. The most convenient and durable stage of the dusty hyphomycetes fungi especially, *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae*

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(Metschnikoff) Sorokin and *Verticillium lecanii* (Zimmerman)Viégas for application and storage is conidium, which is a natural distributive stage (Burges, 1998). A dose of 10^{13}–10^{14} spores/ ha was recommended by Bartlett and Jaronski (1988) for the management of insects under field conditions. Production of spores can easily be done on defined or semi-defined agar-based medium since they are facultative pathogens (Goettel and Inglis, 1997). The method of culturing largely depends on fungal species, type of propagule required, formulation and method of application. Such mass production techniques for entomopathogenic fungi on different media were developed and reviewed by Goettel and Inglis (1997), Bartlett and Jaronski (1988), Feng *et al.* (1994) and Burges (1998). In this study, the growth parameters of four isolates in each of *B. bassiana, M. anisopliae* and *V. lecanii* were assessed and production of dust-free spores through two-stage system for the development of oil-based formulation was demonstrated.

MATERIALS AND METHODS

The twelve fungal isolates (Table 1) maintained at the Project Directorate of Biological control, Bangalore, were sub-cultured in minimum of ten slants on Potato Dextrose Agar medium (PDA). Incubation was done in a growth chamber at 25°C, 90±2 per cent relative humidity and 12-12h photoperiod for 10-15 days based on the growth rate of isolates and stored in a refrigerator at 8-10°C.

Growth parameters

a. Radial growth

The growth rate of mycelia in terms of diameter of fungal mat (cm) was assessed on solid media (PDA). A fungal disc measuring 0.5cm of the respective isolates before sporulation was inoculated at the center of the plate and incubated at 25°C. The colony diameter was recorded on 5th, 10th and 15th days after inoculation.

b. Biomass and spore production

Conical flasks containing potato dextrose broth medium @100ml were inoculated with one cm discs of the fungal isolates from 12 day-old culture and incubated in a growth chamber at 25±1°C for 10 days. To assess the growth and spore production under shake culture condition, the inoculated flasks were incubated in a shaker at 120

Table 1. List of entomopathogenic fungal isolates used in the study

Fungal pathogen	Host insect	Place of collection
B. bassiana - Bb 3	*Neochetina eichhorniae* Warner	Bangalore
B. bassiana - Bb 4	*Spodoptera litura* Fabr.	Bangalore
B. bassiana - Bb 5a	*Hypothenemus hampei* (Ferrari)	Madikeri
B. bassiana - Bb 6	Tree hopper	Bangalore
M. anisopliae - Ma 2	*Amsacta albistriga* Walk.	Davangere
M. anisopliae - Ma 3	*Oryctes rhinoceros* Linn.	Kasargod
M. anisopliae - Ma 4	*Plocaederus ferrugineus* Linn.	Puttur
M. anisopliae - Ma 5	*Holotrichia serrata* Fabr.	Coimbatore
V. lecanii -Vl 1	*Spodoptera litura* Fabr.	Bangalore
V. lecanii -Vl 2a	*Lepidosaphes beckii* (Newman)	Madikeri
V. lecanii -Vl 3a	*Coccus viridis* (Green)	Madikeri
V. lecanii -Vl	*Meconellicoccus hirsutus* (Green)	Pune

rpm and 25±1°C for 10 days. To quantify the biomass, cultures in both conditions were filtered through Whatman No. 2 filter paper on 10th day. The fungal mat was oven-dried at 45-50°C until constant weight was achieved. To estimate the spore production, the fungal mat was macerated with pestle and mortar using 0.02 percent Tween-80 as an emulsifier to get uniform spore suspension. Spores were further extracted by passing the suspension through a muslin cloth. The filtrate was diluted in known quantity of water and the total number of spores/ml was assessed with the help of a Neubauer's improved haemocytometer.

Mass production of fungal isolates on rice medium

a. Preparation of inoculum

As detailed earlier in case of biomass and spore production, each isolate was grown in PDB in a shaker maintained at 120rpm and 25±1°C for 3 days. Three-day-old shake cultures containing blastospores and mycelial bits were used as inoculum for the inoculation on rice. Before inoculation, the colony forming units (CFU) were assessed by Naubauer's haemocytometer.

b. Preparation of rice media

Broken raw rice @ 150g/autoclavable polypropylene bag (20x28cm) was soaked overnight with tap water and the excess water was drained. To that, 3g of calcium carbonate and 3g of calcium sulphate were added and mixed thoroughly to get uniform coating of salts over rice. This process helped in neutralizing the pH of the medium, preventing the rice particles sticking together, and provided more surface area for the fungus to grow and sporulate. The bags were then sterilized by autoclaving twice at 121°C for 20 minutes. The rice medium was cooled and inoculated with 25ml of respective inoculum (10^8spores/ ml) and one ml of 100ppm streptomycin sulphate under aseptic condition. The bags were once again sealed manually and incubated for 10-14 days at 25°C, 90±2 per cent relative humidity and 12-12h photoperiod for the production of aerial conidia.

After incubation, rice grains with the growth of each isolate were dried at 30°C under aseptic conditions until the moisture content was reduced to 8 per cent. After drying, the spore production of each isolate on rice was estimated by Naubauer's haemocytometer. Then, the rice 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 105m sieve to get a fine spore dust (conidial powder). The quantity of spore dust obtained per gram of rice medium in each isolate was estimated. The CFU count in the spore dust was assessed by serial dilution plate technique. The data were subjected to analysis of variance.

RESULTS AND DISCUSSION

a. Radial growth on PDA

The measurements on colony diameter of each of the isolate on 5, 10 and 15 days after incubation are presented in Table 2. Among the four isolates of *B. bassiana* tested, Bb 5a showed the maximum colony diameter on 15th day of observation (6.3cm), which was on par with colony diameters of Bb 3 (6.13cm) and Bb4 isolates (6.13cm). Bb6 isolate recorded significantly lower radial growth (4.05cm) on 15th day. Incase of *M. anisopliae*, Ma4 isolate showed significantly higher colony diameter (8.10cm) than Ma2, Ma3 and Ma5 isolates on 15th day (6.38, 6.35 and 3.17cm, respectively). Among the four *V. lecanii* isolates, V15 recorded maximum colony diameter on 15th day of observation (5.70cm), which is significantly higher than the colony diameters of Vl 3a, Vl 2a, and Vl 1 isolates (3.82, 3.75 and 3.07cm, respectively). The results also indicate that isolates of *M. anisopliae* had higher mean colony diameter (6 cm) compared to that of *B. bassiana* (5.65cm) and *V. lecanii* (4.09cm) isolates. Kulat *et al.* (2002) observed the highest radial growth (4.07cm) of *M. anisopliae* cultured on Sabouraud's dextrose agar with yeast (SDAY) medium for 10 days of incubation. The isolates of *M. anisopliae* in the present study showed a mean radial growth rate of 4.55cm on 10th day on PDA. Generally, *V. lecanii* isolates were found slow growing with low colony diameter throughout the period of observation. It indicates the innate property of *V. lecanii* as described by

Table 2. Radial growth of fungal isolates on PDA medium

Fungal isolate	Diameter (cm) of colony on day		
	5	10	15
B. bassiana -Bb 3	2.68	5.93	6.13
B. bassiana -Bb 4	2.25	5.40	6.13
B. bassiana -Bb 5a	3.00	5.45	6.30
B. bassiana -Bb 6	2.35	3.55	4.05
Mean	2.57	5.00	5.65
M. anisopliae -Ma 2	2.78	4.70	6.38
M. anisopliae -Ma 3	2.75	5.05	6.35
M. anisopliae -Ma 4	3.50	5.85	8.10
M. anisopliae -Ma 5	1.58	2.60	3.17
Mean	2.65	4.55	6.00
V. lecanii -Vl 1	1.80	2.35	3.07
V. lecanii -Vl 2a	1.80	2.73	3.75
V. lecanii -Vl 3a	1.58	2.58	3.82
V. lecanii -Vl 5	2.65	5.52	5.70
Mean	1.96	3.30	4.09
SEM±	0.07	0.11	0.06
CD (P=0.05)	0.22	0.34	0.20

Hall (1981) that the colony diameter in *V. lecanii* would be 18-22 mm after 10 days of incubation on agar medium.

a. Biomass production in Potato Dextrose Broth (PDB)

The biomass production of the twelve isolates under stationary and shake culture conditions in PDB in terms of mycelial dry weight (g) per 100ml medium is presented in Table 3. Biomass production was invariably high in shake cultures compared to stationary cultures in all isolates except Bb 4, Bb 5a and Vl 3a. Among the *B. bassiana* isolates, maximum biomass production was observed with Bb 4 and Bb 6 isolates in stationary cultures (0.78 and 0.76g/100ml, respectively) and Bb 3 in shake culture (1.02g/100ml). In the four *M. anisopliae* isolates tested, Ma2 in stationary culture (0.80g/100ml) and Ma2, Ma 3 and Ma 4 isolates in shake cultures produced maximum biomass (1.03, 1.09 and 1.07g/100ml, respectively). Biomass production of Vl 2a, Vl 3a and Vl 1 isolates of *V. lecanii* in stationary culture was statistically on par with each other (0.77, 0.74 and 0.73g/100ml, respectively). However, in shake cultures, Vl 2a produced significantly higher biomass production (1.03g/100ml) compared to other isolates.

c. Spore production in PDB

Spore production in different isolates in PDB in stationary and shake cultures are presented in Table3. Bb 5a among *B. bassiana* isolates produced maximum sporulation in stationary and shake cultures (62.5×10^8 spores ml^{-1} and 36.2×10^8 blastospores ml^{-1} respectively). This corroborates with the findings of Samsinakova *et al.* (1981) who

Table 3. Biomass and spore production of different isolates in PDB medium (after 10 days)

Fungal isolate	Stationary culture		Shake culture	
	Dry weight of mycelia (g/100ml)	Spore count (x 10^8spores/ml)	Dry weight of mycelia (g/100 ml)	Blastospore count (x10^6/ml)
B. bassiana -Bb 3	0.62	0.68	1.02	97.60
B. bassiana -Bb 4	0.78	24.52	0.77	111.50
B. bassiana -Bb 5a	0.72	62.50	0.66	3620.00
B. bassiana -Bb 6	0.76	26.32	0.85	1.00
Mean	0.72	28.51	0.83	979.80
M. anisopliae -Ma 2	0.80	28.28	1.03	4.00
M. anisopliae -Ma 3	0.69	12.51	1.09	1.50
M. anisopliae -Ma 4	0.67	28.00	1.07	1.50
M. anisopliae -Ma 5	0.64	14.22	0.83	1.00
Mean	0.70	20.75	1.01	2.00
V. lecanii -Vl 1	0.73	0.95	0.90	8.50
V. lecanii -Vl 2a	0.77	7.28	1.03	17.00
V. lecanii -Vl 3a	0.74	3.98	0.60	2.30
V. lecanii -Vl 5	0.47	2.95	0.86	23.00
Mean	0.68	3.79	0.85	12.70
SEM±	0.01	3.96	0.05	0.17
CD (P=0.05)	0.05	12.31	0.14	0.54

obtained 10^8 conidia of *B. bassiana* in the medium composed of peptone 0.8 per cent and sorbitol one per cent and Rombach (1989) recorded 7.4x 10^8 of blastospores ml^{-1} in *B. bassiana* using the media containing sucrose (2.5%) and yeast extract (2.5%). Ma 2 and Ma 4 isolates of *M. anisopliae* showed higher spore production in stationary culture (28.28 and 28.00x10^8spores ml^{-1} resectively). All isolates of *M. anisopliae* recorded low levels of blastospore production in shake cultures that ranged between 0.1x 10^7 ml^{-1}in Ma5 to 0.4 x 10^7 ml^{-1} in Ma 2. Kulat *et al.* (2002) observed the highest spore count of 9.43 x 10^6 spores ml^{-1} with *M. anisopliae* in Barner's medium followed by Emerson YPSS medium (8.29 x 10^6) and SDA +Y medium (7.16 x 10^6) after 10 days of incubation. The results of this study revealed clearly that all the isolates of *M. anisopliae* produced more number of spores on PDB medium indicating their innate property of heavy sporulation. It was interesting that all isolates of *M. anisopliae* invariably produced various sizes of mycelial pellets (1 m to 4 mm diam) in shake cultures. Kleespies and Zimmermann (1992) also noticed the formation of discrete mycelial pellets with blastospores in some strains of *M. anisopliae*. Mycelial pellets are the mass of mycelia that are also considered as reproductive propagules like blastospores and spores. In case of *V. lecanii* isolates, higher spore production in stationary culture was observed in Vl 2a, Vl 3a and Vl 5 (7.28,

3.98 and 2.95x10^8 spores ml^{-1} resectively). In shake cultures, the spore production in the four isolates of *V. lecanii* did not differ significantly from each other (0.23 to 2.30x10^8 spores ml^{-1}).

Mass production on rice

Spore production on rice, quantity and per cent harvest of spores, and viable spore count in the spore dust are presented in Table 4. The CFU of inoculum of all isolates ranged between 0.04 x 10^4 (Vl 5) and 2.2 x 10^6 (Bb 4).

a. Spore production in rice

In the four isolates of *B. bassiana*, the highest spore production of 96 x 10^8 spores g^{-1} of rice was observed in Bb 5a isolate. Sreedharan *et al.* (2001) recorded yields of 4 x 10^{11} spores/ 50g bottle of rice media with a strain of *B. bassiana* isolated from coffee berry borer. Puzari *et al.* (1997) mass cultured *B. bassiana* in a solid media composed of rice hull, saw dust and rice bran (75:25:100) and harvested 39.33 x 10^7 conidia/ml. Among the isolates of *M. anisopliae*, Ma 4 recorded the highest production

Table 4. Mass Production of different isolates of the fungi on rice medium

Fungus-Isolate	Moisture content of sporulated rice (%)	Spore count on rice before extraction (x10^{8}spores/g rice)	Quantity of spore dust (mg/g^{-1}rice)	Per cent spores extracted	Viable spore count (x10^{8}cfu/g^{-1}spore dust)	Viable produced (x10^{6}cfu/g^{-1} spores rice)
		(A)	(B)	(C)	(D)	(E)
B. bassiana-Bb 3	39.39	22.25	22.40	22.70	15.00	33.60
B. bassiana -Bb 4	39.31	36.80	6.00	8.00	162.50	97.50
B. bassiana -Bb 5a	40.19	96.00	28.00	21.40	470.00	1316.00
B. bassiana -Bb 6	41.32	21.20	10.70	13.30	62.00	66.30
Mean	40.05	44.06	16.78	16.35	177.38	378.35
M. anisopliae-Ma 2	31.11	24.80	45.50	30.60	130.00	591.50
M. anisopliae-Ma 3	39.56	29.80	9.20	7.40	121.00	111.30
M. anisopliae-Ma 4	38.23	49.80	13.60	12.80	250.00	340.00
M. anisopliae-Ma 5	49.82	12.40	5.80	2.40	17.00	9.86
Mean	39.68	29.20	18.53	13.30	129.50	263.10
V. lecanii -Vl 1	35.84	1.55	9.10	0.60	0.10	0.09
V. lecanii -Vl 2a	36.05	1.68	15.60	3.70	1.85	2.89
V. lecanii -Vl 3a	35.32	0.23	5.00	1.70	0.15	0.08
V. lecanii -Vl 5	36.76	17.50	9.20	8.80	17.00	1.56
Mean	35.99	5.24	9.73	3.70	4.77	1.16
SEM±	1.24	2.06	-	-	71.55	
CD (P=0.05)	3.85	6.40	-	-	222.68	-

Values of column 'E' are calculated based on the values of columns 'B' and 'D'

of 49.8 x 10^8 spores g^{-1} of rice media. Jenkins *et al.* (1988) also reported a consistent yield of 5 x 10^{12} conidia kg^{-1} of solid media with *M. anisopliae*, which is similar to the yield obtained in the present experiment. The highest number of spores produced in *V. lecanii* isolates was by Vl5 isolate (17.5 x 10^8 spores g^{-1} of rice). Among the three species of fungal pathogens tested, *B. bassiana* produced the highest mean yield of 4.41 x $10^9 g^{-1}$ of rice medium followed by *M. anisopliae* (2.92 x $10^9 g^{-1}$) and *V. lecanii* (0.52 x $10^9 g^{-1}$).

a. Spore Quantity

The quantities of spore dust (conidial powder) obtained among the isolates of *B. bassiana* were 28.0 mg g^{-1} of rice media in Bb 5a, 22.4mg in B b 3, 10.7mg in Bb 6 and 6.0mg in Bb 4. Whereas in *M. anisopliae*, it ranged between 5.8 mg (Ma5) and 45.5 mg (Ma2). Cherry *et al.* (1999) harvested dry conidial powder with an average of 31.1mg g^{-1} of rice substrate in two-stage system of mass production of *M. anisopliae* var. *acridum* strain IMI330154. In the isolates of *V. lecanii*, spore dust yield varied from 5.0mg (Vl 3a) to 15.6mg (Vl 2a). The highest mean quantity of 18.53 mg spore dust g^{-1} of rice media was obtained with *M. anisopliae* isolates followed by 16.78 mg (*B. bassiana)* and 9.73 mg *(V. lecanii)*.

The quantity of spores harvested from the rice medium of different isolates through manual sieving varied from 8.0 to 22.7 per cent in *B. bassiana*, 2.4 to 30.6 per cent in *M. anisopliae* and 0.6 to 8.8 per cent in *V. lecanii*. Harvesting of aerial conidia of *B. bassiana* and *M. anisopliae* was easier as their spores were dusty and hydrophobic when compared to slimy and hydrophilic conidia of *V. lecanii*. Dense filamentous growth and slimy hydrophilic spores in V11, V1 2aand V1 3a isolates of *V. lecanii* resulted in poor spore harvest.

b. Viable spore count in spore dust

Bb 5a recorded the highest viable spore count of 4.7 x 10^{10} g^{-1} among the isolates of *B. bassiana*, Ma4 in *M. anisopliae* (2.5 x 10^{10} spores g^{-1}) and Vl5 in *V. lecanii* (1.7 x 10^9 spores g^{-1}). Miao *et al.* (1993) reported viable spore count of 10 x 10^{10} spores g^{-1} of *B. bassiana*, which is higher than the conidial production observed in Bb 5.

Taking into consideration of the quantity of spore dust production and viable spores in the spore dust, Bb5a produced maximum viable spore (13.16 x10^8cfu/g^{-1} rice). In the isolates of *M. anisopliae*, highest viable spore production was observed with Ma 2 isolate (5.91x10^8cfu/ g^{-1} rice). Although, Ma 4 isolate had highest spore production on rice (4.98 x 10^9 spores g^{-1}) and viable spore count (2.5 x 10^{10} spores g^{-1} spore dust), its overall production of viable spores (3.40 x10^8cfu/g^{-1} rice) was less than that of Ma 2 isolate, because of its lower spore harvest (13.60mg/ g^{-1} rice). In case of *V. lecanii*, highest viable spore production was observed with Vl 2a isolate (2.89 x10^6cfu/g^{-1} rice).

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