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Mass Production of Endomopathogenic Fungus *Metarhizium anisopliae* (Deuteromycota; Hyphomycetes) in the Laboratory

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Abstract: In the present study, mass production of *Metarhizium anisopliae* was carried out in the laboratory by using liquid and solid media. The spore count, radial growth, sporulation and biomass were maximum when *M. anisopliae* were cultured both czapeck dox agar and potato dextrose agar media. Hence, these two media can be used for successful mass production of fungus, *M. anisopliae*. Four types of solid media such as rice, maize, wheat and kodomillet were used for the mass production of *M. anisopliae* and highest sporulation was observed in *M. anisopliae* cultured in wheat ($8,300\times10^4$). Hence, wheat is recommended as best solid media for the mass production of *M. anisopliae*. The optimum temperature and ideal pH for the mass production of *M. anisopliae* was found to be 25-30°C and 7.0, respectively. Various culture containers such as Erlinmayer's flasks, petriplates and plastic bags were also used for the mass production of *M. anisopliae*. But very good result was obtained in High-Density Polyethylene (HDPE) bags. So this high-density polyethylene (HDPE) bags are ideal and also cost effective for the mass production of *M. anisopliae*.

Key words: *Metarhizium anisopliae,* czapeck dox agar media, potato dextrose agar media, radial growth, sporulation, petriplates, high-density polyethylene bags

INTRODUCTION

It is generally recognized that chemical pesticides contaminate ground water and enter food chains that have an impact on wide range of organisms. Furthermore, pesticide can pose hazardous to animal health and to the user spraying the chemical. Consumer perceptions worldwide are that chemical usage in agricultural production needs to be significantly reduced. Several fungal species including *Metarhizium anisopliae*, *Verticillium lecanii* and *Beauveria bassiana* are being used as biocontrol agents for a number of crop, livestock and human nuisance pests (Tanada and Kaya, 1993). Voluminous literature is available in abroad for the microorganisms to control insect pests. However, very limited studies are available in India in this context for fungus in general and M. *anisopliae* in particular. This is due to lack of proper technology. So economically viable mass culture technology is need of the hour. For mass culture technology, appropriate media, ideal temperature and proper containers are very much essential. Hence, the present study was designed to mass culture the endomogenous fungus, *M. anisopliae* for the production of virulent spores in various media *viz.*, liquid and solid media.

MATERIALS AND METHODS

The present study was carried out in N.S. College at Theni, Tamil Nadu, India during July 2005 to August 2006. The virulent spores of *M. anisopliae* were produced in the laboratory by two methods

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known as surface and submerged culture. In surface culture technique was the production of virulent spores in solid media (various cereal grains) and in submerged culture was the production of virulent spores in liquid media. After incubation, biomass and number of spores produced were calculated for both cultures.

The medial composition was prepared separately in each flask. These media were sterilized for 20 min at 121°C. After sterilization, it was poured in sterile petriplates and allowed to solidify in the pH of the medium, which was prepared near neutral (pH 7.0).

The original isolates of *M. anisopliae* used in the present study were obtained from infected larvae which collected from soil. Stock culture was maintained on culture tube slants of Potato Dextrose Agar (PDA). Inoculum was obtained from 2-week-old cultures. Fungal spores were taken from stock cultures and inoculated in PDA plates by stab culture technique. This inoculated PDA plates were incubated at 25°C for 2 weeks.

Agar blocks of actively growing culture on PDA plates were cut with a sterile cork borer (10 mm) and placed in the center of each plate. These inoculated plates were incubated at 25°C for 7 days. After 7 days, the radial growth of the fungus on the plates was determined by measuring the diameter of the fungus. To determine the extend of sporulation, a 6 mm block of fungal growth was taken after 7 days at 25°C from each plate and suspended in 0.1% Tween-80 solution. Spore count was done with a Neubeaur's haemocytometer.

The growth of *M. anisopliae* was also examined under static cultivation conditions. Fifty milliliters of broth medium in 250 mL flasks was inoculated with 10 mm culture blocks. All flasks were incubated at 25°C for 7 days. The broth was filtered through preweighed Whatman filter papers. The mycelial mat was washed thoroughly, oven dried and the dry mycelial weight was recorded.

To study the effect of different temperatures on growth and sporulation, 10 mm culture blocks were inoculated on PDA medium and potato dextrose broth in flasks. The flasks were incubated for 10 days in BOD incubators at 10, 20, 25, 30, 35 and 40°C. Radial growth; spore load and dry mycelial weight were determined.

For mass scale production, various cereal grains such as rice, maize, wheat and kodomillet were tested for their efficiency on supporting the growth of *M. anisopliae*. The cereal grains were macerated separately in a household grinder and broken grains of similar particle size were collected. Twenty-five grams of such grains were distributed in 250 mL Erlinmayer's flasks and washed thoroughly with water. Ten milliliters of water was then added to each flask. Subsequently, the grains containing flasks were cooked and sterilized. Then the flasks were inoculated with 5 mL of *M. anisopliae* spore suspension at 1×10 spores mL^{-1} suspension. Finally the flasks were incubated at 25°C for 2 weeks and the numbers of spores per gram of crushed grains were calculated for each flask.

Fifteen grams of broken grains were taken and washed thoroughly with water and washed grains were distributed in petriplates. Fifteen milliliters of water was then added to each plate. The grains were cooked and sterilized in the plates. Subsequently, the plates were inoculated with 5 mL of *M. anisopliae* spore suspension at 1×10 spores mL^{-1} suspension. Finally, the plates were incubated at 25°C for 2 weeks and the numbers of spores per gram of crushed grains were calculated for each plate.

High-density polyethylene (HDPE) bags obtained from local market were used for the mass production of *M. anisopliae*. Fifty grams of rice grains were mixed with 50 mL of water in bags measuring 23×17 cm. The bags were sealed on a burner flame and autoclaved at 151 bs for 1 h. After autoclaving, the bags were cooled and the cooked grains were separated and equally distributed by thorough shaking. Under aseptic conditions in a laminar flow chamber, the corners of the bags were clipped and the substrates then inoculated with 5 mL of *M. anisopliae* spore suspension at 1×10 spores mL^{-1} suspension. The inoculated bags were then resealed on the burner flame and thoroughly shaken to distribute the inoculum. They were incubated at 25°C for 2 weeks and the spore

load was recorded. For preparation of the spore powder, the rice grains covered with the spore mass were collected from the bags and oven dried at 40°C for 24 h. The dried grains were then macerated to make a powder formulation. The powder was dissolved in 0.1% Tween 80 solutions to calculate the spore count.

RESULTS AND DISCUSSION

The optimal nutrient medium is necessary for the adequate growth of microorganisms in general and fungi in particular (Altomare *et al*., 1999). In the present study, two types of media were selected one was liquid media and another was solid media The highest spore count, radial growth, sporulation and biomass production was recorded for both Czapeck dox agar and Potato dextrose agar medium of the present study (Table 1). Czapeck dox agar medium is the best medium because in addition to agar and sucrose it also had minerals, which are badly needed for the growth of fungi. This may be the reason for good performance of this medium rather than other media. The fungus, *T. harzianum* was also reached the maximum level of biomass in the sucrose (as in Czapeck dox agar) yeast medium (Altomare *et al*., 1999). Leland (2001) produced submerged conidia of fungi, *M. anisopliae* in Jenkin's medium. Both Jenkins and Czapeck dox agar medium had sucrose is one of its ingredients. So Czapeck dox agar medium of the present study is considered as ideal medium for the mass culture of *M. anisopliae*.

Many workers support potato dextrose medium is the best medium for the culture of fungi. The Fungus, *T. harzianum* was cultured well in Difco potato dextrose agar (Knudsen *et al*., 1991). Leland (2001) was used potato dextrose agar for the stock culture of *M. anisopliae*. The Potato agar medium is confirmed as a best agar medium for the growth and spore production of *P. tabaccinum* (Zhang *et al*., 2001). The fungus, *M. anisopliae* cultured aerobically on potato dextrose agar (Wes Watson and Schal, 2002). Many earlier researchers successfully used Sabouraud's dextrose agar medium for the mass culture of fungi. However, surprisingly in the present study, the spore count, sporulation and biomass production was less in the Sabouraud's Dextrose Agar (SDA) medium. Hallsworth and Magan (1996) offered Sabouraud Dextrose Agar (SDA) medium for the successful culture of fungus, *B. bassiana*, *M. anisopliae* and *P. farinosu*. Endomogenous fungi, *B. bassiana* and *M. anisopliae* were cultured on Sabouraud's Dextrose Agar (SDA) plates and conidia were successfully harvested (Luz *et al*., 1994; Kaaya *et al*., 1996). Leland (2001) selected Sabouraud's Dextrose Agar (SDA) for the stock culture of *M. anisopliae*.

The spore count (24), radial growth (2.5 cm), sporulation (600×10^4) and biomass (0.465) production was determined for *M. anisopliae* in the Malt extract agar medium of the present study. Where as the spore count (100), radial growth (2.9 cm), sporulation ($2,500\times10^4$) and biomass (0.67 g)

Table 1: Results of spore count, radial growth, sporulation and biomass of *M. anisopliae* cultured in different media

Media	Spore count	Radial growth (cm)	Average	No. of cells in 1 mm^2	No. of cells mL^{-1} Average x^4	Sporulation	Biomass (g)
Sabouraud's Dextrose Agar (SDA)	12	2.7	12/16 = 0.75	0.75	300×10^4	300×10^4	0.37
Sabouraud's Maltose Agar (SMA)	29	2.3	29/16 = 1.812	1.812	725×10^4	725×10^4	0.45
Sabouraud's Maltose Agar +1% Yeast Extract (SMAE)	100	2.9	100/16 = 6.25	6.25	$2,500\times10^4$	$2,500\times10^4$	0.67
Malt Extract Agar (MEA)	24	2.5	24/16 = 1.5	1.50	600×10^4	600×10^4	0.465
Potato Dextrose Agar (PDA)	112	2.7	112/16 = 7	7.00	$2,800\times10^4$	$2,800\times10^4$	0.692
Czapeck Dox Agar (CDA)	132	3.3	132/16 = 8.25	8.25	$3,300\times10^4$	$3,300\times10^4$	0.81

production of *M. anisopliae* in Sabouraud's maltose agar + 1% yeast extract was reasonably good in the present study (Table 1). The fungus, *B. bassiana* also grow very well in Sabouraud's agar medium + yeast extract (Knudsen *et al*., 1991). Sabouraud's dextrose broth with 1% yeast (SDBY) was used for the production of *B. bassiana* (Bextine and Thorvilson, 2002). In all the media used in the present study had carbohydrate source one of its ingredients. The addition of glucose, maltose or complex sugar-rich media increased the biocontrol effect (Druvefors *et al*., 2005). Sugars can improve the viability of freeze-dried cultures that are important components of the formulation of biocontrol agent (Abadias *et al*., 2001; Costa *et al*., 2000) (Table 2).

Temperature is one of the ecological factor which effectively control the growth of microorganisms especially fungi. Many researchers studied on the influence of temperature on germination, development and sporulation of entomopathogenic fungi (Maniania, 1993). In the present study, the fungus; *M. anisopliae* was exposed with different temperatures ranging from 10 to 40°C. Radial growth, sporulation and biomass were high at 25 and 30°C. So 25-30°C is supposed to be the optimum temperature for spore production of *M. anisopliae*. Growth was completely inhibited at 40°C (Table 2). Many scientists around the world accepted 25-30°C as optimum temperature for the culture of *M. anisopliae* and also other species of fungi. Spores of fungi germinated at temperatures between 15 and 35°C and peak germination occurred at 25-30°C for *B. bassiana* (Tang *et al*., 1992) and also for *M. anisoplia* (Bartlett and Jaronski, 1988). Ambient temperature for the conidia production in *B. bassiana* and *M. anisopliae* was 20-25°C (Maniania, 1993). The fungus, *B. bassiana* grew optimally at about 25°C where as *M. anisopliae* grew at 30-35°C and *P. farinosus* grew at about 20°C. The growth rates for each species were optimal at 25°C . The optimal temperature for spore production in *P. tabaccinum* was 20-30°C (Zhang *et al*., 2001).

The optimal pH is necessary for the normal growth and spore production of fungi. In general, growth of entomopathogenic fungi is optimal over a broad range of pH. Some other fungal species grow over a narrow pH range and optimum growth appears to correspond to a specific pH value (Prosser and Tough, 1991). In the present study the neutral pH (7.0) was maintained throughout the study period. Medium pH did not affect mycelial growth and spore germination of *P. tabaccinum* but the optimal pH for spore production was 7.0 (Zhang *et al*., 2001). The growth of fungus was declined when it was exposed in low pH (Brown, 1988).

The mechanism involved in control and regulation of mycocellial growth are better studied on solid medium than submerged cultures, as fungi are adapted to growth on solid substrates (Prosser and Tough, 1991). The use of various cereal grains and other vegetable products, such as potato, sweet potato, tapioca and carrot is costly and may not be economically feasible for mass scale production. However, the cereal grains (rice, wheat, maize and kodomillet) used in the present study is less costly. The results in the present study obtained with different cereal grains revealed that wheat grains supported maximum sporulation, followed by maize and rice. Kodomillet gave a very poor hyphal yield (Table 3). Conidia of *B. bassiana* and *M. anisopliae* were produced successfully on a substrate of maize and rice (Kaaya and Mwangi, 1998). The inoculated rice grains of the present study showed

Table 2: Radial growth, sporulation and biomass production of *M. anisopliae* exposed to different temperatures on PDA agar media

Temperature	Radial growth (cm)	Spore count	Average	No. of cells in 1 mm^2	No. of cells mL^{-1} Average x^4	Sporulation	Biomass (g)
10	0.0	0	0	0.0	0	0	0.03
20	2.7	48	48/16=3	3.0	$1,200\times10^4$	$1,200\times10^4$	0.57
25	3.2	112	112/16=7	7.0	$2,800\times10^4$	$2,800\times10^4$	0.725
30	3.4	128	128/16=8	8.0	$3,200\times10^4$	$3,200\times10^4$	0.76
35	2.8	68	68/16=4.2	4.2	$1,700\times10^4$	$1,700\times10^4$	0.68
40	0.0	0	0	0.0	0	0	0.00

Table 3: Results of spore count of *M. anisopliae* cultured in different cereal grains

Substrate	Spore count	Average in 1 mm^2	No. of spores	No. of cells mL^{-1} Average x^4
Rice	288	288/16=18	18	$7,200\times10^4$
Wheat	332	332/16=20.7	20.7	$8,300\times10^4$
Maize	304	304/16=19	19	$7,600\times10^4$
Kodomillet	112	112/16=7	7	$2,800\times10^4$

sporulating mass after 2 weeks. It was found that amount of water added to the grains at the time of white mycelial growth by the third day of inoculation and were completely covered with a green autoclaving played a critical role. Excess water resulted in grain clumping and hence less consumption of the substrate, as evidenced by higher amounts of unconsumed substrate. It is also resulted in low spore count in the final product. Too little water during sterilization resulted in charring and browning of the grains and in poor fungal growth. Shaking of the flasks after inoculation or mixing the grains with a sterile rod helped to equalize the distribution of the inoculum on all the grains increased the spore count. The fungi also mass cultured in different containers. The endomopathogenic fungi, *B. bassiana* and *M. anisopliae* conidia were produced in bottles (500 mL) containing rice as culture medium (Filho *et al*., 2002). In the present study also various containers like Erlinmayer's flasks, petriplates and high-density polyethylene (HDPE) bags were used for the mass production of *M. anisopliae*. The high-density polyethylene (HDPE) bags obtained from the local market were used for mass production of the fungus on rice. Plastic bags of 12×17 cm were found ideal for growth and sporulation. They were easy for inoculation and handling during the growth period. Large bags gave rise to the problem of grain clumping during autoclaving. All the bags were frequently shaken during the vegetative growth phase for the first 6 days to ensure complete coverage of all the grains and break-up of small clumps.

In submerged cultures, the spores were produced 24 h after inoculation and optimum spore production was achieved on 3rd and 4th day. After 4th day although the spore density increases, the quality of the suspension decreases because the initial spores rapidly lose its viability, new spores are formed more slowly and thus the number of virulent spores in the culture decreases. In aerial cultures, the sporulation starts only on the 6th day. Although the spore production duration was more the virulent spores were comparatively high.

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