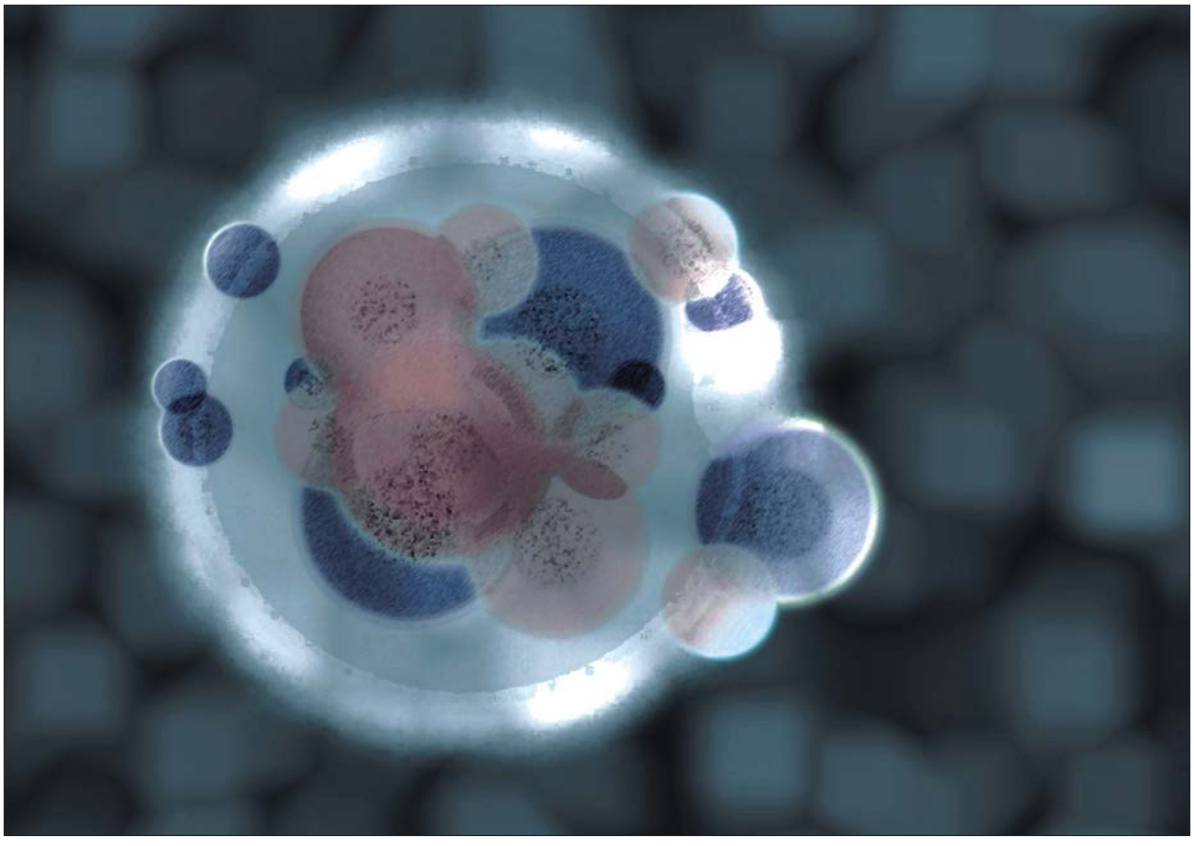

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 6 (2): 269-274, 2006
ISSN 1727-3048

Effect of Growing Media and Water Volume on Conidial Production of *Beauveria bassiana* and *Metarhizium anisopliae*

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Abstract: The objective of this study was to evaluate conidial production of two isolates of *Beauveria bassiana* (Balsamo) (5672 and CA 44) and one of *Metarhizium anisopliae* (Metschnikoff) (500B) using corn, wheat and millet and three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5). We found that *M. anisopliae* tended to produce more conidia overall than the *B. bassiana* isolates. For the *B. bassiana* isolates, conidial production on wheat was higher for the 1:1 and 1:1.5 ratio than the 1:0.5. No significant differences were found in conidial production for 5672 on corn and millet at the volume regimes tested; whereas, for CA-44 on corn and millet, conidial production tended to decrease with increasing water volume. In contrast, conidial production of *M. anisopliae* increased up to 8.6 and 2.9 times between the lowest and highest water volume ratio on corn and millet, respectively. Wheat tended to produce more conidia depending on the water volume used, although only slight increases were observed for 5672. We found that conidial production was positively influenced by increased water ratio, particularly for *M. anisopliae* (500B). Depending on the combination of isolate, growing media and water volume, differences up to 240 times were observed. Such difference can have dramatic effect on the economical production of entomopathogenic fungi for field application. Present results provide useful information for developing a simple mass production tactic for targeting Sunn Pest in their overwintering sites.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, mass production, substrate, water volume

INTRODUCTION

Entomopathogenic fungi as biological control agents show promise in reducing insect pest populations and damage in different agroecosystems (Inglis *et al*., 2001).

A successful microbial insecticide should be able to produce high quantities of inocula (Goettel and Roberts, 1992; Mamania, 1991; McCoy, 1990; Soper and Ward, 1981). These inocula should be constant in their virulence characteristics and compatible with formulation and application technology (Jenkins *et al*., 1998). Goettel and Roberts (1992) indicated three methods of mass production for entomopathogenic fungi: 1) *in vivo*, 2) submerged culture and 3) surface culture.

In vivo production is generally not economical and should be used only for obligatory fungi (Goettel and Roberts, 1992). Blastospores produced from submerged cultures are less virulent and live for a shorter time than conidia from surface culture (Roberts and Sweeney, 1982). Conidia are preferred for field application over blastospores and mycelia because of their stability under dry conditions (Soper and Ward, 1981). Submerged culture is often used as a first phase of a mass production protocol (Goettel and Roberts, 1992). Different growing media are developed for mass production of specific entomopathogenic fungi. For example, *Metarhizium anisopliae* (Metschnikoff) can be grown on rice (Daoust and Roberts, 1983, Kaay and Hassan, 2000), crushed grain or maize (Shashi *et al*., 1999). *Beauveria bassiana* (Balsamo) Vuillemin can be grown on bran (Goettel, 1984), rice powder, compost, groundcorn (Hussy and Tinsley, 1981), whole cowpea grain (Shashi *et al*., 1999), or preserved cadavers of the hosts (Luz and Fargues, 1998).

The type of growing medium affects conidial production of entomopathogenic fungi. Nelson *et al*., (1996) investigated the effects of three growing media (rice, wheat and barley) on spore production of *M. anisopliae*, *B. bassiana* and *B. brongniartii* (Saccardo). They concluded that these fungi produced more spores on rice over other growing substrates when incubated for 3 weeks at 23°C and under natural daylight. However, in another study, higher spore production of *M. anisopliae* was obtained when it was grown on rice bran and rice husk substrate mixtures than when it was grown on rice grains (Dorta *et al*., 1990). A total amount of 39.33×10^7 conidia mL^{-1} water of *B. bassiana* was produced using a medium of rice hulls, saw dust and rice at a ratio of 75:25:100 (Puzari *et al*., 1997), compared to production of 1×10^9 conidia mL^{-1} on molasses yeast broth (Shashi *et al*., 1999). Conidial production of

B. bassiana was estimated to be 14.9×10^7 conidia/insect and 10.6×10^9 conidia g^{-1} when it was grown on insect cadavers of *Blissus antillus* Leonard and on rice media, respectively (Samuels and Coracini, 2004).

Spore production of entomopathogenic fungi is affected by moisture content of the growing substrate (Hajek *et al*., 1990; Ignoffo, 1992; Roberts and Campbell, 1977). The optimal moisture content of the solid medium is the most important factor for the growth and nutrient consumption of the fungi (Young *et al*., 2002). Final quantities of inocula produced by entomopathogenic fungi in mass production protocols depend on the moisture content of the growing substrate (Jenkins *et al*., 1998). It has been reported that spore germination, germ tube extension and infection for most entomopathogenic fungi require at least 95% RH (Hallsworth and Magan, 1999). Conidial germination of *B. bassiana* and *M. anisopliae* only occurred when the ambient %RH >92 (Walstad *et al*., 1970).

The objective of this study was to evaluate conidial production of two isolates of *B. bassiana* (5672 and CA 44) and one *M. anisopliae* (500B) using corn, wheat and millet at three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5).

MATERIALS AND METHODS

Fungal isolates and vegetative cultures: Two isolates of *B. bassiana* (5672 and CA 44) and one *M. anisopliae* (500B) were used in this study. These isolates are maintained at the University of Vermont in the Entomology Research Laboratory (Burlington, VT) long term storage facility. A test tube of each isolate was used to start vegetative cultures. Approximately 10 mL of a 0.01% water solution of Tween 80 was added to each test tube and vortexed thoroughly for 5-10 min and 0.1 mL of each suspension was transferred into to a 9 cm diam Petri dish containing quarter–strength sabauroud dextrose agar (SDAY/4) [neopeptone 2.5 g, dextrose 10 g, yeast extract 2.5 g, agar 15 g, citric acid 0.4 mL (50 g in 100 mL dH_2O) and water 1] and incubated at 25±2°C in the dark.

Preparation of the liquid fungal inocula: A suspension of each isolate was prepared after 2 weeks and diluted to 1×10^7 conidia mL^{-1}. One mL was then added to a 500 mL Erlenmeyer flask containing liquid SDAY/4. The mixture was shaken on a rotary shaker at 150 rpm for 5 days at 25±2°C. The liquid culture of each isolate was filtered through three layers of cheesecloth into a sterile flask to collect blastospores. Blastospores were counted using a Levy hemocytometer (Hausser Scientific, Horsham, PA USA) 0.100 mm deep and the final suspension of each isolate was adjusted to 5×10^7 for inoculating the solid substrate.

Preparation and inoculation of the solid substrates: Three solid substrates of whole millet grain, whole wheat grain and cracked corn grain were used. A volume of 300 mL of each grain was placed in an autoclave bag (30.5×60.9 cm). Three volumes of tap water (grain: water): 1:0.5, 1:1 and 1:1.5 were used with each substrate and replicated four times. The pH of the water was adjusted to approximately 5 by adding 0.4 mL of 2.6 M citric acid per liter In general, fungi grow well in their submerged culture at pH 5-6 (Park *et al*., 2001). Bags were cooked at 100±5°C for 1 h in a water bath, autoclaved for 1 h at 121°C and 15 psi and cooled overnight. A volume of 150 mL of the stock suspension of 5×10^7 conidia mL^{-1} of each isolate was prepared as described in section 2.2 and each bag was inoculated with 3 mL of the suspension. Viability of the inoculum was determined by plating the diluted suspension on SDAY/4. A clean paper cup (9 cm diam× 6 cm height), open at both ends, was placed on the open side of the bag to hold the end of the bag open. The opening was covered with three layers of paper towel (22×22 cm) (POR Proctor and Gamble, USA, Bounty) and cotton cloth and then a sheet of aluminum foil was added to maintain high humidity inside the bags. A rubber band was used to fasten the paper towel and cloth to the cup. Established bags were incubated in the dark at 25°C for 2 weeks. Bags were left for 1 week at room temperature and then transferred to the growing room at 25°C where they were opened and held in the dark. Bags were mixed thoroughly to avoid creating hard clusters of the mixture of fungi/media and to facilitate homogenous fungal growth.

After 1 week in the growing room in dark conditions, conidial production was determined by combining 1200 mL of Tween 80 (0.01%) into each bag and shaking thoroughly to obtain a homogenous suspension. From each bag, a 10-mL sample of the suspension was taken and added with 9 mL of Tween 80 (0.01%). After appropriate dilutions were made, conidial concentration was counted using a Levy hemocytometer (Hausser Scientific, Horsham, PA USA) 0.100 mm deep.

Data analysis: The results were converted to conidia g^{-1} of initial growing media. Counts of conidia g^{-1} were log (10) transformed. Data were analyzed in SAS (SAS Institute, 2002) using analysis of variance (ANOVA) based on the factorial design with three factors (isolates, water volume and growing media). An $\alpha = 0.05$ was used in the statistical analysis.

RESULTS

Isolates, water volume and growing media significantly interacted ($p\leq0.05$) in their influence on conidial production (Table 1). Because of observed higher

Table 1: ANOVA table for log (10) transformed conidia g^{-1} of two of *B. bassiana* (5672 and CA-44) and one *M. anisopliae* (500B) using corn, wheat and millet and three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5)

Source	df	Type III SS	Mean square	F-value	p>F
Isolate	2	270.13	135.06	511.81	<0.0001
Growing media	2	5.04	2.52	9.56	0.0002
Water volume	2	12.33	6.16	23.36	<0.0001
Isolate*growing media	4	10.22	2.56	9.68	<0.0001
Isolate*water volume	4	27.09	6.77	5.67	<0.0001
Growing*water volume	4	26.50	6.62	25.10	<0.0001
Isolate*growing media*water	8	6.22	0.78	2.94	0.0070
Volume error	67	17.6810519	0.2638963		
Corrected total	93	427.5671301			

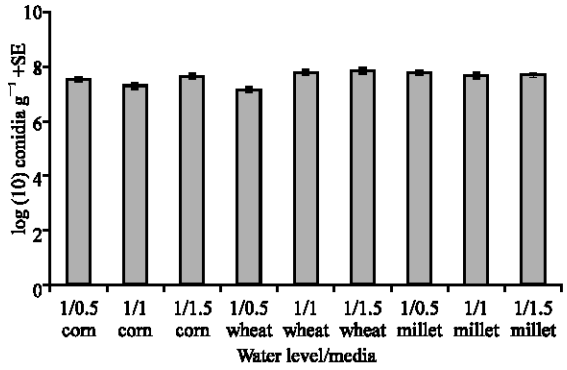

Fig. 1: Number of conidia g^{-1} (log 10 transformed) of isolate 5672 *Beauveria bassiana* produced on corn, wheat and millet and three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5)

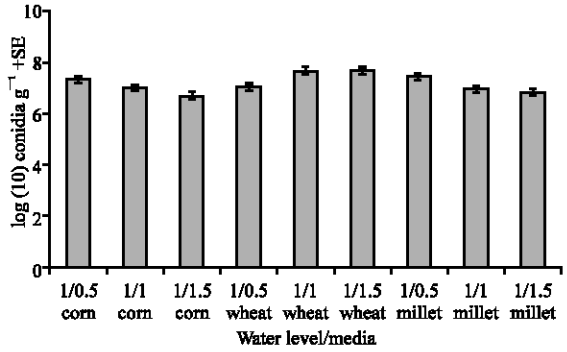

Fig. 2: Number of conidia g^{-1} (log 10 transformed) of isolate CA-44 *Beauveria bassiana* produced on corn, wheat and millet and three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5)

order interactions multiple comparisons of means of main factors were not performed. However, exploring the trends that lead to this interaction reveals the nature of the influence these factors had on conidial production.

Metarhizium anisopliae tended to produce more conidia overall than the *B. bassiana* isolates. For the *B. bassiana* isolates, conidial production on wheat was higher for the 1:1 and 1:1.5 ratio than the 1:0.5 (Fig. 1 and 2). No apparent differences were found in conidial

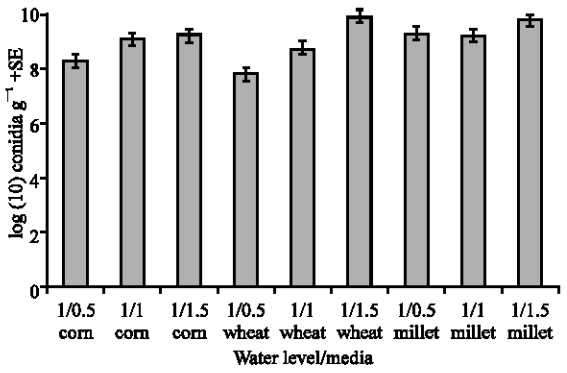

Fig. 3: Number of conidia g^{-1} (log 10 transformed) of isolate 500B *Metarhizium anisopliae* produced on corn, wheat and millet and three water volumes (substrate: water) (1:0.5), (1:1) and (1:1.5)

production for 5672 on corn and millet at the volume regimes tested (Fig. 1); whereas, for CA-44 on corn and millet, conidial production tended to decrease with increasing water volume (Fig. 2). In contrast, conidial production between the lowest and highest water volume ratio of *M. anisopliae* increased up to 8.6 and 2.9 times on corn and millet, respectively (Fig. 3). Depending on growing media, conidial production of 1:1 ratio was similar to either the lower or higher water treatment, except for on wheat where it was intermediate.

DISCUSSION

High spore production capability is a requirement for the successful development of microbial agents for pest management (Goettel and Roberts, 1992). The appropriate moisture level and growing substrate are essential for optimal mass production of entomopathogenic fungi (Jenkins *et al*., 1998; McCoy, 1990; Young *et al*., 2002). In this study, we used corn, wheat and millet as potential growing substrates for mass production of entomopathogenic fungi in a Sunn Pest management program. These substrates are available at a relatively low price where Sunn Pest is a problem in the Near and Middle East.

In general, a large number of conidia were produced on all three growing substrates. Even so, depending on the combination of isolate, growing media and water volume used, differences up to 240 times were observed. Wheat tended to produce more conidia depending on the water volume used, although only slight increases of conidial production on wheat over other growing media were observed for 5672. Such differences can have dramatic effect on the economical production of entomopathogenic fungi for field application.

The significance of growing substrate types in mass productions of *M. anisopliae* and *B. bassiana* was investigated in several studies. Dorta *et al.* (1990) tested conidial production of *M. anisopliae* on rice bran, rice husk mixtures and rice. They found that *M. anisopliae* produced 5-15 times more conidia on rice bran and rice husk mixtures than on rice (Dorta *et al.*, 1990). Puzari *et al.* (1997) reported that a total amount of 39.33×10^7 conidia mL^{-1} water of *B. bassiana* were produced using a medium of rice hulls, saw dust and rice at a ratio of 75:25:100 (Puzari *et al.*, 1997), where as in a separate study production of 1×10^9 conidia mL^{-1} were produced on molasses yeast broth (Shashi *et al.*, 1999).

Growing substrate appears to be less important than water content in conidial production, however, there appears to be an interaction between both. There have been other studies that examine the interaction of water content and growing substrate on conidial production of entomopathogenic fungi. The lack of published research is probably because data are being produced as part of commercial enterprises. We found that conidial production was positively influenced by increased water ratio, particularly for *M. anisopliae* (500B) (Fig. 1-3). No significant differences were found in conidial production for 5672 on corn and millet at the volume regimes tested. Conidial production of *M. anisopliae* was higher for the 1:1.5 than the 1:0.5 and 1:1 ratio on the three test substrates.

Total spore production of entomopathogenic fungi depend on the water volume used in mass production designs (Jenkins *et al.*, 1998; Young *et al.*, 2002). Different growing substrates require different water volumes (Jenkins *et al.*, 1998). Jenkins *et al.*, (1998) indicated that moisture content levels between 1:0.35- 1:0.60 (wet substrate: water) are commonly used in mass production of entomopathogenic fungi. Magalhães and Frazao (1996) found that high spore production of *M. flavoviride* Gams and Rozsypal (CG 423) was obtained when water content in the rice, rice husk + bran rice and parboiled rice ranged from 1:0.30-1:1.20 (volume: water). We conclude that the optimal conditions for mass production differs depending on isolate, water volume and substrate used and specific attention must be given to these variables when establishing a mass production protocol.

We found that *M. anisopliae* produced higher conidial quantities than *B. bassiana* isolates on growing substrates and water volumes tested. Magalhães *et al.* (2000) found the same results when examining the spore production of *M. anisopliae* and *B. bassiana* in cadavers of grasshopper *Rhammatocerus schistocercoides* (Rehn) at 100% RH. Sun *et al.* (2003) reported that *B. bassiana* isolates produced more overall conidia than *M. anisopliae* isolates when grown on termite (*Coptotermes formosanus* Shiraki). This dissimilarity of our finding may be related to the isolate, growing substrate, %RH or temperature factors tested. For instance, according to our previous research on spore production, we found that at different temperature and %RH levels, isolates from *B. bassiana* and *M. anisopliae* responded differently (unpublished data). At ≤95% RH, no sporulation for *M. anisopliae* isolates was observed and little or no sporulation occurred at low temperature (15°C), *B. bassiana* isolates tended to produce conidia at low temperature (15°C) with several isolates producing spores at 95% RH. In that study, at high RH 100% and temperatures 20 and 30°C, *M. anisopliae* isolates generally tended to produce more conidia than *B. bassiana* isolates.

Our data helps in predicting quantities of entomopathogenic fungi necessary for field application. For instance, when wheat is used as growing substrate at the preferred water volume to substrate ratios 1:1 and 1:1.5, total quantities of wheat required are 158.5 and 128.9, 208.6 and 205.8 and 17.7 and 1.16 kg ha^{-1} of 5672, CA-44 and 500B, respectively using a recommended application rate of 1×10^{13} conidia ha^{-1} (Magalhães and Boucias, 2004). These feasible differences in conidial production, depending on isolate and water volume used, provide practical information to be used in Sunn Pest management.

Large scale applications of entompathogenic fungi for pest management in their natural habitats require development of appropriate mass production techniques that provide large quantities of fungal spores using simple, economic and efficient methods (Jenkins *et al.*, 1998). Additionally, integrating our understanding of the biology and ecology of Sunn Pest will help in its management. Sunn Pest has one generation per year and it spends around 9 mo in overwintering sites in litter around tree trunks and under bushes (Banks *et al.*, 1961; Miller 1991; Parker et., 2002; Radjabi and Termeh, 1992; Sheikh and Al Rahbi, 1996). Sampling to identify areas for application of entomopathogenic fungi is an important step to save resources. Directing the applications of entomopathogenic fungi to litter under bushes and trees where the overwintering adults are mainly located

(Parker *et al*., 2002) and ignoring other areas in overwintering sites may increase the economic feasibility of using fungi, not only by reducing the amount of fungi required but also reduction in time and labor for making applications.

Present results provide useful information for developing a simple mass production tactics for targeting Sunn Pest in their overwriting sites. This approach for mass production can be used by scientists, technicians and trained farmers in IPM programs for Sunn Pest.

ACKNOWLEDGMENTS

I thank Dr. Scott D. Costa for reviewing the work and Dr. Vladimir Gouli and Svitlana Gouli for providing me with necessary skills to complete this study.

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