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Short Paper

Comparison of some natural broth media for production and virulence of *Beauveria bassiana* blastospores against the browntail moth, *Euproctis chrysorrhoea* (Lep.: Lymantriidae)

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Abstract: Effects of three nutritional levels of beet root molasses, cheese permeate, wheat bran extract, rice bran extract and Sabouraud's Dextrose Broth (SDB) were evaluated for blastospore production by two isolates of *Beauveria bassiana sensu lato.* at an interval of 24 h for seven days. Depending on the isolate, maximum blastospore production was obtained in 12% rice bran extract and 20% cheese permeates on the 7th day. Both isolates produced the fewest blastospores in 4% cheese permeate. Virulence of blastospores, produced in liquid media containing beet root molasses, permeate, wheat bran extract and SDB (as control), on third instar larvae of brown tail moth *Euproctis chrysorrhoea* indicated that there were no significant differences among these nutritional media for either one of the isolates. Considering blastospore quantity and quality in terms of virulence and local accessibility, cheese permeate was found to be the best medium for mass production of *B. bassiana* blastospores.

Keywords: *Beauveria bassiana*, *Euproctis chrysorrhoea*, blastospore, virulence, natural medium

Introduction

Entomopathogenic fungi play an important role in decreasing pest insect populations. Use of these fungi as microbial control agents for pest management in large scale depends on the capability of high spore production and at reasonable costs. The ability of pathogenic fungi to grow and produce spores on artificial media is one of the main advantages in the commercial development of these fungi (Cisneros and Vera, 2000; Dalla Santa *et al*., 2005). Approaches directed to medium optimization must consider not only spore yield but also spore qualities such as desiccation tolerance, stability as a dry preparation and biocontrol efficacy. In fact the important part of the success of microbial control programs often depends on an adequate mass-production method (Khachatourians, 1986; Kamp and Bidochka, 2002; Jenkins *et al*., 1998; Jenkins and Goettel, 1997).

Blastospores of mitosporic fungi produced in liquid media have potential to infect hosts (Kleespies and Zimmermann, 1992; Jenkins and Goettel, 1997). The type of growth medium and the nutritional and physical conditions of the mass production system greatly affect the number, type, stability, durability and virulence of fungal propagules (Ibrahim *et al*., 1993; Feng *et al*., 1994; Fargues *et al.*, 2002). There is not much information on the effects of culture media on virulence of entomopathogenic fungi (Ibrahim *et al*., 2002; Safavi *et al*., 2007).

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Bena-Molaei *et al.* (2011) studied the effect of culture substrates on virulence of *Beauveria bassiana* conidia against the browntail moth *Euproctis chrysorrhoea* (Lep.: Lymantriidae). Their results showed that there were significant differences among nutritional substrates for their effects on the virulence of produced conidia. Browntail moth, is one of the most important pests of oak and broad leaf trees (Nikdel *et al*., 2003). The objective of this research was the evaluation of some natural broth media for blastospore production of two isolates of *B. bassiana sensu lato.* We considered browntail moth larvae as a target host to test our hypothesis that growth media could affect the virulence of *B. bassiana* blastospores.

Materials and Methods

Media preparation for spore production assay

Cheap nutritional substrates were selected and evaluated for blastospore production. Substrates were beet root molasses, permeate, wheat bran extract and rice bran extract. In addition, Sabouraud's Dextrose Broth (SDB, Merck™) was considered as the control medium. Two isolates of *B. bassiana s.l.* (EUT105, of soil origin and EUT116 from a lepidopteran origin) were used. Both isolates are deposited at the fungal collection of the College of Agriculture and Natural Resources, University of Tehran.

Beet root molasses (obtained from the Naghadeh Sugar Factory, West Azerbaijan) and cheese permeate (obtained from Tabriz P-Azar Company) were dissolved in sterile distilled water and filtered through cheese cloth. Two hundred grams of each of wheat and rice bran were boiled separately in distilled water and filtered through cheese cloth. Sterile distilled water was then added to these 4 nutritional substrates to prepare final concentrations of 4, 12 and 20%. Experimental units were 250 ml Erlenmeyer flasks each containing 150 ml of one of the media. The flasks were autoclaved and then inoculated with one plug (0.5 cm) taken from actively growing fungal colonies on Sabouraud's Dextrose Agar + Yeast extract (SDAY) and then incubated at 24 ± 3 °C on a rotary shaker (GFL, Germany) at 120 rpm.

Blastospore production

Daily until 7 days post-inoculation, 1 ml of each suspension was added to 9 ml of sterile distilled water and blastospores were counted using a hemocytometer (Neubauer, Germany). This experiment was done in a factorial arrangement in a completely randomized design, each treatment had three replicates and the whole experiment was repeated once.

Bioassays with browntail moth larvae

Egg masses and first instar larvae of the browntail moth, *Euproctis chrysorrhoea* were collected from Arasbaran forests, East Azarbaijan, Iran and maintained at 25 ± 2 °C, 50 ± 10% RH and 16:8 h (L: D) photoperiod. Fresh *Ulmus* (elm) leaves were used as food. Third instar larvae were used for bioassays. Blastospores were harvested from each medium on the 7^{th} day, filtered through two layers of cheese cloth and any possible contamination was checked. Three concentrations (10^5, 10^6 and 10^7 blastospore/ml) of each isolate produced on each medium were prepared. Third instar larvae were randomly selected from the stock colony and inoculated with 250 µL of prepared concentrations using a sprayer, depositing approximately 2.1×10^3 bl./cm^2 with 10^7 blastospore/ml. Inoculated larvae were transferred with a hair brush to octagonal dishes (4.5 × 11 × 11 cm) and control larvae were treated with sterile distilled water. Treated larvae were maintained at 25 ± 1 °C, 90 ± 10% RH and a photoperiod of 16h L: 8h D, fed daily and their mortalities were recorded every other day for 10 days. Dead larvae were placed in moist Petri dishes for observation of mycosis. The viability of blastospores was always more than 95% as determined according to Goettel and Inglis (1997). This experiment was done in a factorial arrangement in a completely randomized design and each treatment had four replicates, each replicate consisted of 11 larvae and 1536 larvae were used totally. The whole experiment was conducted twice.

Data Analysis

Observed total mortality percentages were corrected for control mortality that was always less than 5% using Schneider_Orelli formula

(Püntener, 1981) and then pooled data of two-time repeats were analyzed by SYSTAT12. The General Linear Model (GLM) procedure was used to perform an ANOVA with the Tukey HSD test for means comparison. In the case of determining difference among different concentrations, regression analysis was used.

Results

Analysis of variance with the GLM procedure showed that there were significant differences in production of blastospores among nutritional media for both isolates on the 4th day (EUT105: $F_{4,30} = 5.4$, $P < 0.005$ and EUT116: $F_{4,30} = 6.8$, $P < 0.005$) and on the 7th day (EUT105: $F_{4,30} = 3.1$, $P < 0.05$ and EUT116: $F_{4,30} = 9.3$, $P < 0.001$).

On the 4th day, the highest and lowest numbers of blastospore production for EUT105 (2.2×10^8 and 7×10^7 bl/ml) were recorded for 12% wheat bran extract and 4% molasses, respectively. In *B. bassiana* EUT116, 20% permeate (2.3×10^8 bl/ml) had the highest blastospore production with no significant difference with 12% rice bran extract and 12% wheat bran extract. The lowest blastospore production (3.4×10^7 bl/ml) occurred in 4% molasses (Table 1).

Mean comparisons on the 7th day showed that with isolate EUT105, 12% rice bran extract and 4% molasses had the highest (3.5×10^8 bl/ml) and lowest (1.3×10^8 blastospore/ml) blastospore yields, respectively. But there was no significant difference among the three concentrations of each medium with this isolate using Regression analysis. With EUT116, 20% permeate gave the highest (3.3×10^8 /ml) blastospore production (Table 2).

Table 1 Quantities of blastospores produced in five different media for two isolates of *Beauveria basiana* on 4th day post-inoculation.

Nutritional substrates	Yield (blastospores / ml)[2]					
	B. bassiana EUT105			*B. bassiana* EUT116		
	4%	12%	20%	4%	12%	20%
Permeate	9.6×10^7ab	1.4×10^8a	1.8×10^8a	1.5×10^8a	1.8×10^8a	2.3×10^8a
Beetroot molasses	7.0×10^7b	7.6×10^7a	1.3×10^8a	3.4×10^7c	7.4×10^7a	8.5×10^7ab
Rice bran	9.6×10^7ab	1.9×10^8a	1.0×10^8a	1.2×10^8ab	2.2×10^8a	9.0×10^7ab
Wheat bran	1.9×10^8a	2.2×10^8a	1.1×10^8a	1.2×10^8ab	1.9×10^8a	9.5×10^7ab
SDB[1]	9.0×10^7b	9.0×10^7a	9.0×10^7b	6.5×10^7bc	6.5×10^7a	6.5×10^7b

1. Sabouraud's Dextrose Broth, only one concentration was considered for this medium.
2. Means followed by the same letters in each column are not significantly different (Tukey's test at $P < 0.05$).

Table 2 Quantities of blastospores produced in five different media for two isolates of *Beauveria basiana* on 7th day post-inoculation.

Nutritional substrates	Yield (blastospores / ml)[2]					
	B. bassiana EUT105			*B. bassiana* EUT116		
	4%	12%	20%	1.9×10^8a	12%	20%
Permeate	1.3×10^8a	2.6×10^8a	3.2×10^8a	6.8×10^7b	3.2×10^8a	3.3×10^8a
Beetroot molasses	1.3×10^8a	1.5×10^8a	1.6×10^8a	1.8×10^8a	1.3×10^8b	1.8×10^8ab
Rice bran	2.8×10^8a	3.5×10^8a	3.0×10^8a	2.2×10^8a	2.9×10^8ab	1.4×10^8b
Wheat bran	2.7×10^8a	3.0×10^8a	1.7×10^8a	1.7×10^8a	3.3×10^8a	2.1×10^8ab
SDB[1]	2.4×10^8a	2.4×10^8a	2.4×10^8a	1.9×10^8a	1.7×10^8ab	1.7×10^8ab

1. Sabouraud's Dextrose Broth, only one concentration was considered for this medium.
2. Means followed by the same letters in each column are not significantly different (Tukey's test at $P < 0.05$).

Bioassay with third instars larvae of browntail moth

The nutritional substrates had no significant effects on blastospore virulence on browntail moth larvae for either isolate (EUT105: $F_{4,45}$ = 1.8, $P > 0.05$ and EUT116: $F_{4,45}$ = 2.3, $P > 0.05$). Applying 10^7 blastospore/ml with deposition rate of approximately 2.1×10^3 bl./cm^2, of EUT105 produced mortalities ranging from 73 to 82% and it was between 69 to 85% for EUT116 (Table 3).

Table 3 Percent mortality of the browntail moth, *Euproctis chrysorrhoea* third-instar larvae caused by two isolates of *Beauveria bassiana* blastospores obtained from different culture substrates.

Nutritional substrates	% Mortality ± SE[1]	
Permeate	*B. bassiana* EUT105	*B. bassiana* EUT116
Beetroot molasses	72.9 ± 13.3	81.9 ± 13.9
Wheat bran	77.2 ± 10.2	79.7 ± 9.5
Rice bran	81.8 ± 9.2	86.2 ± 11.0
SDB	75.5 ± 9.7	69.3 ± 10.7
Permeate	81.1 ± 10.4	84.2 ± 10.0

1. mortality at 10^7 blastospores / ml.

Discussion

For growth, *B. bassiana* needs carbon sources such as dextrose which can be replaced by polysaccharides such as starch or lipids. But the nutritional requirements of each species or isolate of entomopathogenic fungus must be considered separately (Smith and Grula, 1981; Taborsky, 1992). In our study, type of nutritional media significantly affected the production of *B. bassiana* blastospores. Increasing the concentration of permeates and molasses led to higher blastospore production, while wheat bran extract and rice bran extract didn't increase the quantity of blastospores produced. We also demonstrated that liquid culture composition influences spore production. Complementary nourishment of media with agricultural by-products enhanced chlamydospore production in liquid culture of *Fusarium oxysporum* (Elzein and Kroschel, 2004). Puzari *et al*. (1997) reported that 3.9×10^8 conidia/ml of *B. bassiana* were produced using a medium of rice hulls and saw dust, whereas 1×10^9 conidia/ml were produced on molasses yeast broth (Shashi *et al*., 1999). There is far less information for blastospore production using natural substrates.

Verhaar and Hijwegen (1993) reported production of 3×10^9 spore/ml of *Verticillium lecanii* in oat flour media. Farsi *et al*. (2003) obtained 1.02×10^8 spores/ml of *V. lecanii* in 5% molasses media. In our study, 1×10^8-2×10^8 and 1.5×10^8-3.5×10^8 blastospore/ml in our used media were produced on the 4th and 7th day, respectively. Thus, blastospore production was in optimal range. Rice bran extract, wheat bran extracts and permeates showed higher blastospore production than molasses and control. These media present good carbon and nitrogen sources and some essential macronutrients and microelements. Thus it seems that these media support better fungal growth providing nutritional requirements and good blastospore production. Media including rice bran and wheat bran are good sources for starch and permeate and molasses are very rich in sugar (Kamyab, 2009). However the amount of protein, phosphorous, magnesium, methionine and cysteine in molasses is less than in the other studied media and probably could be the reason for lower blastospore production. As in the case of *P. fumosoroseus* (Now *Isaria fumosorosea*), higher concentrations of nitrogen can increase blastospore production (Jackson, 1997; Cliquet and Jackson, 2005). Although molasses has more calcium and potassium than the other substrates used (Kamyab, 2009), there must be an equilibrium among nutritional requirements other than carbon and nitrogen; their ratios could be effective factors in production and quality of fungal spores (Jackson and Bothast, 1990; Schisler *et al*., 1991).

pH of the medium is another important factor in spore production. Optimum pH for *B. bassiana* growth is 5.7-5.9 and for spore formation it is 7-8 (Taborsky, 1992). There is a close relationship between oxygen and moisture

content of media and decrease of available oxygen which can retard fungal growth. According to our results, increasing nutrient concentration from 12 to 20% in wheat bran extract and rice bran extract didn't result in higher blastospore production. We speculate that decreasing oxygen content could be a reason for this.

Considering factors such as availability, price and feasibility of production, extraction and filtration of wheat and rice bran, these media are more time-consuming and not cost-effective. The blastospores of *B. bassiana* isolates produced on the tested culture media demonstrated high virulence against the browntail moth larvae although there was no significant difference due to nutritional contents of the media. In contrast, Ibrahim *et al.* (2002) reported that culture media influenced fungal virulence. They showed that conidia of *M. anisopliae* grown on Sabouraud dextrose agar modified (SDAM) with KCl and minimal medium (MM) were more aggressive to *Myzus persicae* and *Meligethes aeneus* than conidia derived from Sabouraud dextrose agar (SDA) or yeast extract agar. This could be related to fungus reservoir during the growth on different nutrients. It is known that *B. bassiana* needs nutrition for penetrating insect integument and growth (St. Leger *et al*., 1989). Thus low nutrition reservoir of blastospores or conidia can be a probable factor for low virulence.

This is the first study of *B. bassiana s.l.* blastospores against the browntail moth providing evidence for its control potential against this insect. On the basis of analysis for yield data, harvesting blastospores was dependent on the medium and isolate. For *B. bassiana* EUT105, using 12% wheat and rice bran extract after 48 hours, using 20% molasses after 72 hours and using 20% permeate after 96 hours is suggested. Whereas in the case of *B. bassiana* EUT116, the third day is the best time for blastospore harvesting from all media. In conclusion, cheese permeate medium is introduced as an appropriate medium for mass production of *B. bassiana* blastospores due to production of heavy blastospores of good virulence to browntail moth larvae and more importantly, lower cost of spore production compared with SDB medium. As this medium is a waste product in local cheese production, it could be provided at high volume with very low cost. Introducing this production medium could be considered from the two aspects in future; researchers can attempt to optimize it and fungal-product factories can use it locally for the production of semi-massive-scale biopesticide on the basis of blastospores.

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مقایسه چند محیط غذایی طبیعی برای تولید بلاستوسپور قارچ *Beauveria bassiana* و زهرآگینی علیه پروانه دم‌قهوه‌ای بلوط، *Euproctis chrysorrhoea* (Lep.: Lymantriidae)

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چکیده: اثر سه سطح از محیط‌های غذایی شامل ملاس چغندرقند، آب پنیر، عصاره سبوس گندم، عصاره سبوس برنج و محیط مایع سابورود دکستروز برای تولید بلاستوسپورهای دو جدایه از قارچ *Beauveria bassiana sensu lato* در فواصل زمانی ۲۴ ساعته به‌مدت ۷ روز مورد بررسی قرار گرفت. بسته به جدایه، بیشینه تولید بلاستوسپور در روز هفتم در عصاره سبوس برنج ۱۲ درصد و آب پنیر ۲۰ درصد بود. برای هر دو جدایه، کم‌ترین مقدار بلاستوسپور تولیدی از آب پنیر ۴ درصد به‌دست آمد. زهرآگینی بلاستوسپورهای تولید شده در محیط‌های مایع حاوی ملاس چغندرقند، آب پنیر، عصاره سبوس گندم و سابورود دکستروز (به‌عنوان شاهد) روی لارو سن سوم پروانه دم‌قهوه‌ای، *Euproctis chrysorrhoea* نشان داد که در مورد هر دو جدایه بین این محیط‌های غذایی تفاوت معنی‌داری وجود ندارد. با توجه به کمیت و کیفیت بلاستوسپورها ازنظر زهرآگینی و دسترسی محلی، آب پنیر به‌عنوان محیط مناسب برای تولید انبوه بلاستوسپورهای قارچ *B. bassiana* معرفی می‌شود.

واژگان کلیدی: *Beauveria bassiana*، پروانه دم‌قهوه‌ای بلوط، بلاستوسپور، زهرآگینی، محیط غذایی طبیعی