The effects of natural substrates on the sporulation and viability of conidia and blastospores of *Metarhizium anisopliae*

Siamak Roshandel[1], Hassan Askary[2], Reza Talaei Hassanlouei[1], Hosein Allahyari[1]

1. Plant Protection Department. College of Agriculture and Natural Resources, Tehran University, Karadj, Iran

2. Biological Control Research Department, Iranian Research Institute of Plant Protection**,** Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

Corresponding author: Hassan Askary, email: askary@iripp.ir

Received: Mar., 26, 2015 4 (1) 94-104 Accepted: Nov., 01, 2016

Abstract

In this study the effects of natural grain-based media on the sporulation and germination of conidia and blastospores of *Metarhizium anisopliae* were investigated. Experiments were conducted with factorial arrangement in a completely randomized design with nine treatments and three replications. The obtained results showed that there was significant difference in the yield of conidia among different treatnents, two weeks after inoculation, ($F= 4.66$, $df= 7, 16$, $p<0.01$). Highest average yields were obtained as 3.1×10^8 and 3×10^8 conidia/g in rice flour and Sabouraud's Dextrose Agar with 1% yeast extract, respectively. The lowest level of conidia yield was 4×10^6 conidia/g which obtained in the wheat bran medium. There was significant difference between blastospores yields four days after inoculation of the media, ($F=2.57$, $df= 8, 18$, $p<0.05$). The highest and lowest average yield of blastospores were 6.1×10^8 and 4.5×10^8 blastospores/ml and were obtained in the wheat bran+rice bran+yeast extract and rice bran+yeast extract media, respectively. Analysis of variance of nutritional and chemical amendments which were added to blastospores media showed significant differences in the yield among different treatments ($F=5.02$, $df=13, 29$, $p<0.01$). The highest average yield of blastospores were 6.6×10^8, 6.1×10^8 and 5.7×10^8 blastospores/ml which were obtained in the rice bran+wheat bran+yeast extract+whey powder, rice bran+wheat bran+yeast extract, and wheat bran+rice bran+magnesium sulfate media, respectively. The lowest average yield of the blastospores was obtained in the media containing grape juice. Results also showed significant difference between the means of germination rate of conidia one, two and three month storage at 4°C and 25°C. The highest germination rate belonged to treatments stored in the refrigerator.

Keywords: culture substrate, mass production, media, *Metarhizium anisopliae*

Introduction

Because of detrimental effects of chemical pesticides on the environment and human health, use of integrated pest management (IPM) with innovative technologies is necessary. In this regard, significant progress has been achieved in the use of fungi as biological control agents. Today, more than 30 commercial fungal insecticides have been produced or introduced in the field (Wraight *et al.*, 2001). *Metarhizium anisopliae* (Ascomycota: Clavicipitaceae) is a well-known entomopathogenic fungus active against many insect pests (Zimmermann, 1993). It has more than 200 host species, which most of them are soil originated and were used successfully for control pests of greenhouse crops, pastures, forests and fruit trees (El Damir, 2006). Commercial mass production of entomopathogenic fungi on natural substrates is possible. In mass production systems, kind of culture medium, nutrients and environmental

growth conditions greatly affect yield, type, stability, viability, and virulence of fungal propagules (Wyss *et al.*, 2001; Ibrahim *et al.*, 2002; Shah *et al.*, 2005; Safavi *et al.*, 2007; Rangel *et al.*, 2008; Wu *et al.*, 2010; Bena-Molaei *et al.*, 2011). It is necessary to adopt strategies to optimize mass production of bio-control agents in order to be cost competitive with chemical pesticides and their application is feasible. Several studies were done on different nutrients for growth and sporulation of fungi such as *Beauveria bassiana*, *M. anisopliae* and *Isaria fumosorosea*. Mass production of *M. anisopliae* is mainly doing on solid fermentation which requires few weeks to maximize sporulation. Liquid fermentation is an alternative method which saves time for mass production of some fungi. Aerial conidia are more tolerant to drought than submerged conidia, more stable as a dry product (Jackson, 1997). So, researches were conducted to find substrates using natural products that are available and affordable. Most of entomopathogenic fungi which used as biological control agents are produced by two stage fermentation. At first stage, mycelia or blastospores of fungi produced as propagules for the second stage. Propagules produce in liquid medium at rotating flasks or fermenters then transferred to a solid medium to produce conidia. This two-stage fermentation process called biphasic fermentation is used for improving the industrial production as an alternative method that leads to shorter incubation period, produce more conidia and higher quality (Fargus *et al.,* 2001). In Brazil, *Isaria fumosorosea* and *Isaria farinosa* were produced by biphasic fermentation using agricultural waste products (Mascarin *et al.,* 2010). Conidial production of *B. bassiana*, *M. anisopliae* were evaluated in three level of humidity of substrates (substrate: water), on corn, wheat and millet. *M. anisopliae* produced more conidia than *B. bassiana*, by increasing water ratio (El Damir 2006). The effect of medium quality, light, temperature, and pH on the growth and sporulation of *Verticillium lecanii* DAOM198499 was evaluated. It was determined that the effect of medium quality was significant on blastospore production. Potato extract which was enriched with dextrose, peptone, and yeast extract was better than pure potato (Farsi *et al.,* 2005). In another study, rice and oats flour were used to produce *M. anisopliae* conidia. Existence of peptone, increased sporulation regardless of the medium used. However, the highest yield was obtained in oat flour. When oxygen increased, the number of *M. anisopliae* conidia was raised (Tlecuital-Beristain *et al.,* 2010). These researchers have recommended that recent conidial production methods for this fungus must be optimized on natural substrates (such as, rice, sorghum, barley residue, and even insect cadavers such as *Coptotermes formosanus*) to obtain maximal yield.

Pest control by entomopathogenic fungi in large scale needs to mass amounts of fungal material (Jenkins *et al.,* 1998). So, the main goal of this research is investigation the effect of some natural substrates on quantity and quality of conidia and blastospores production of *M. anisopliae*.

Materials and Methods

Fungal isolates

M. anisopliae isolate, M14 obtained from Iranian Research Institute of Plant Protection and was cultured on Sabouraud's dextrose agar with 1% yeast extract (SDAY), incubated at 27±1°C, 60±5% RH and 16h light: 8h darkness photoperiod. Streptomycin sulfate 0.1% was added to all media to prevent bacterial contamination (Thomas *et al.,* 1987). Conidia were harvested from up to 14-day-old cultures and were suspended in 20 mL sterile distilled water containing 0.03% Tween 80 (Goettel and Inglis, 1997). Potato dextrose broth (PDB)

(Merck, Germany) was used for production of blastospores (Tlecuital-Beristain *et al.,* 2010). After sporulation of the fungus, conidia were harvested and transferred to Falcon® tube containing distilled water. The suspension was filtered through Whatman paper No. 1 to separate mycelia. Conidial concentrations were determined by improved Neubauer hemocytometer.

1. Evaluation of blastospores and conidia yield production in natural culture medium

1.1. Blastospore production rate in liquid substrates

Final treatments were obtained from rice bran, wheat bran and barley waste. Fifty grams of rice bran and wheat bran were added to one liter of sterile water. After 24 hours, extracts passed through Whatman paper No. 1 and were used as culture medium. Barley waste obtained from Behnoush Co. (Tehran, Iran). Fifty grams of the barley waste soaked in one liter of water and after 24 hours filtered through 100 mesh sieve and the extract was used as culture medium. Yeast extract in all treatments was 0.5 g/l (Jenkins & prior 1993). Treatments were: 1) wheat bran extract, 2) rice bran extract, 3) barley waste, 4) wheat bran extract+yeast extract, 5) rice bran+yeast extract, 6) barley waste+yeast extract, 7) wheat bran extract+rice bran extract, 8) wheat bran extract+rice bran extract+yeast extract and 9) potato+dextrose as a common medium. Erlenmeyer flasks containing the media were autoclaved for 30 min in 121C° and 1 atmosphere pressure. Flasks, then were inoculated with 2 ml of 2×10^7conidia/ml of *M. anisopliae* under aseptic conditions and were shaked four days at 180 rpm on a rotatory shaker (TM52A, Fan Azma Gostar Co., Iran) at room temperature (27±1°C) and 16:8 h (L:D) photoperiod. After fourth days the cultures were passed through filter paper (Whatman No. 1) to separate mycelia and the number of blastospores was determined by improved Neubauer hemocytometer (Bena-Molaei *et al.,* 2011). The experiment was conducted in a completely randomized design with three replicates.

1.2. Conidia production rate in solid substrates

Treatments were: 1) rice grain, 2) wheat bran, 3) rice bran, 4) rice flour, 5) crushed rice (particle size of 2-3mm), 6) crushed corn (particle size of 3-5mm), 7) waste barley pulp (Behnoush Co.) and 8) SDA as control. About 500 g rice, crushed rice grain and crushed corn were washed separately, soaked in water for 2 hours, and then boiled. Grain transferred into cellophane plastic bags (50 × 30 cm) and then autoclaved. Also, 500 g of each powdery substrate was transferred to bags and then sprayed with distilled water until moisture reached to about 60%. All treatments were sterilized by autoclaving at 121 °C and 1 atmospheric pressure. All substrates inoculated in sterile conditions at 28°C, with 10 ml of 2×10^7 conidia/ml of *M. anisopliae*. Bags were closed by cotton and placed in room temperature (27±1°C) and 16:8 h (L:D) photoperiod. On day 14, the number of conidia was determined by improved Neubauer (Bena-Molaei *et al.,* 2011). The experiment was conducted in a completely randomized design with three replicates.

2. Evaluation of some chemicals and natural supplementary substrates on sporulation

Some chemicals and natural supplementary substrates were used in this experiment. Ammonium nitrate, magnesium sulfate (2 g/L) (Thomas *et al.,* 1987), whey powder (Kalleh Co., Iran) and grape juice concentrate (Varda Co., Iran) (12%) were added to primary media (selected from first experiment because of high production) included: (wheat bran+rice bran+yeast extract) and (wheat bran+yeast extract) which had a good result in virulence test (Roshandel *et al*, 2013). The experiment was done in factorial arrangement in a completely randomized design with three replications and the yield in terms of blastospore/ml

was calculated. Mean comparison was determined with F-LSD test by the SAS software version 9.2.

3. Evaluation of viability of conidia in natural substrates

One hundred grams of each treatments mentioned in last experiment (1-2) except SDA medium, transferred in plastic bags (3o×15 cm) and were stored in refrigerator and room temperature at 4°C and 25±2°C, respectively. After one, two and three months, $1ml^{-1}$ of diluted suspension of each treatments was distributed in petri dishes containing water agar 2%. After 24 h, the number of germinated conidia was counted by Olympus microscope (×1000 magnification) (Bena-Molaei *et al.*, 2011). This experiment was done in a completely randomized design with three replications. Mean comparison was determined with F-LSD test by the SAS software version 9.2.

Results

Analysis of variance showed significant difference between media in inoculation of conidia after 2 weeks. (F=4.66, df=7, 16, P<0.01). Highest yield obtained in rice flour and SDA treatments with 3.1×10^8 and 3×10^8 conidia/g, respectively. Following them, maximum conidial production belonged to crushed rice medium with 1.8×10^8 conidia/g. The means of conidial production on rice bran, corn, barley pulp (Behnoush®) and whole rice grain medium placed in same group. The lowest yield was 4×10^6 conidia/g in wheat bran medium (Table 1).

There was significant difference between blastospore yield production in different media, 4 days after inoculation (F=2.57, df= 8,18, P<0.05). The highest and lowest blastospore production belonged to wheat bran+rice bran+yeast extract and rice bran+yeast extract medium with 6.1×10^8 and 7.4×10^6 blastospores/ml, respectively (Table 2).

Table 1. Mean of sporulation by *Metarhizium anisopliae* on different solid culture media, 14 days after inoculation.

Culture media	Mean+SE (conidia/g)
Rice flour	$3.1\times10^8\pm 1.07\times10^4$ a
SDA (control)	$3\times10^8\pm5.9\times10^3$ a
Crushed rice	$1.8\times10^8\pm 4.8\times10^3$ ab
Rice bran	$1\times10^8\pm1.8\times10^3$ bc
Crushed corn	$8.8\times10^7\pm4.2\times10^3$ bc
Barley pulp (Behnoush®)	$7.4\times10^7\pm4.8\times10^3$ bc
Whole rice	$3.8\times10^7\pm4.4\times10^3$ bc
Wheat bran	$4\times10^6\pm1.6\times10^3$ c

Means with different letters are significantly different (P <0.05).

Table 2. Means of blastospore production by *Metarhizium anisopliae* on different liquid culture media, 4 days after inoculation

Culture media	Mean±SE (blastospore/ml)
Rice bran extract+Wheat bran+yeast extract	$6.1\times10^8\pm1.4\times10^4$ a
Rice bran extract+Wheat bran extract	$4.5\times10^8\pm2.02\times10^4$ a
PDB (control)	$3\times10^8\pm1.3\times10^4$ ab
Wheat bran extract+yeast extract	$2.4\times10^8\pm4.1\times10^3$ ab
Rice bran extract	$1\times10^8\pm1.02\times10^4$ bc
Wheat bran extract	$5.7\times10^7\pm7.3\times10^3$ bc
Barley pulp extract (Behnoush®) +yeast extract	$3.2\times10^7\pm1.8\times10^3$ bc
Barley pulp extract (Behnoush®)	$2.7\times10^7\pm4.8\times10^2$ bc
Rice bran extract+yeast extract	$7.4\times10^6\pm3.2\times10^2$ c

Means with different letters are significantly different (P <0.05).

Analysis of variance of culture medium with nutrient supplements showed significant difference between treatments (F1=5.02, df= 12, 29, p <0.01). The highest average yield of blastospores belonged to wheat bran+rice bran+yeast extract+whey powder; wheat bran+rice bran+yeast exract and wheat bran+rice bran+magnesium sulfate media with 6.6×10^8; 6.1×10^8 and $7/5\times10^8$ blastospores/ml, respectively. There was no significant difference between means of blastospores yield in media wheat bran+rice bran+yeast extract+ammonium nitrate; wheat bran+rice bran+yeast+magnesium sulfate; wheat bran+rice bran+whey powder; wheat

bran; rice bran+ammonium nitrate; wheat bran+yeast extract+whey powder; wheat bran+yeast+magnesium sulfate and wheat bran+yeast+ammonium nitrate. The lowest average yield of blastospores belonged to wheat bran+rice bran+yeast extract+grape juice concentrate; wheat bran+yeast+grape juice concentrate and pure grape juice concentrate with 2.7×10^5, 8.6×10^5 and 7.4×10^5 blastospores/ml, respectively (Table 3).

Table 3. Means of blastospore production by *Metarhizium. anisopliae* on culture media with supplements, 4 days after inoculation.

Culture media	Mean±SE (blastospore/ml)
Wheat bran+Rice bran+yeast extract+whey powder	$6.6\times10^8\pm9.4\times10^3$ a
Wheat bran+Rice bran+yeast extract+ammonium nitrate	$4.3\times10^8\pm3.4\times10^3$ ab
Wheat bran+Rice bran+yeast extract+magnesium sulfate	$1.5\times10^8\pm4.3\times10^3$ ab
Wheat bran+Rice bran+yeast extract+grape juice concentrate	$7.2\times10^5\pm1.3\times10^2$ c
Rice bran+Wheat bran+whey powder	$2.9\times10^8\pm2.3\times10^3$ ab
Rice bran+Wheat bran+ammonium nitrate	$3.5\times10^8\pm1.1\times10^4$ ab
Rice bran+Wheat bran+magnesium sulfate	$5.7\times10^8\pm1.2\times10^4$ a
Rice bran+Wheat bran+grape juice concentrate	$6.1\times10^8\pm1.4\times10^2$ a
Whey powder	$3.8\times10^8\pm1.1\times10^4$ ab
Grape juice concentrate	$7.4\times10^5\pm1.2\times10^3$ c
Wheat bran+yeast extract+whey powder	$3.8\times10^8\pm2.9\times10^3$ ab
Wheat bran+yeast extract+ammonium nitrate	$4.8\times10^8\pm4.7\times10^4$ ab
Wheat bran+yeast extract+magnesium sulfate	$5.4\times10^8\pm1.1\times10^4$ ab
Wheat bran+yeast extract+grape juice concentrate	$8.6\times10^5\pm5.2\times10^2$ c

Means with different letters are significantly different (P <0.05).

Generally in all treatments, germination rate of conidia reduced by increasing the time of storage and temperature.

Analysis of variance of mean percentage germination of *Metarhizium anisopliae* conidia showed significant difference between treatments after one, two and three month storage at 4°C and 25°C. One month after conidia storage at room temperature, there was no significant differences between treatments (F=1.35, df= 7, 16, P<0.05). Significant difference was observed between treatments stored in refrigerator (F= 5.07, df= 7, 16, P<0.001) after one month, room temperature (F=5.43, df= 7, 16, P<0.001) and refrigerator (F=4.74, df= 7, 16, P<0.001), after two month. Also, significant differences were observed between treatments stored in room temperature (F=2.51, df= 7, 16, P>0.07) and refrigerator (F=9.39, df= 7, 16, P<0.001) during three months (Table 4). Highest and lowest germination rate of conidia belonged to SDA (control) and wheat bran medium as 97.03% and 75.87%, respectively, after one month storage in refrigerator. After two months storage of conidia in room temperature, maximum

and minimum germination rate belonged to SDA and crushed rice (62.09%, 60.09%) and rice flour medium (29.4%). After three months storage of conidia in refrigerator, SDA and crushed corn medium treatments had the most germination percentage and wheat bran had the lowest germination percentage of 24.1%. Germination rate of conidia reduced to 10.9% to 35.01% for treatments 3 months stored in room temperature.

Table 4. Mean of conidial germination rate for *Metarhizium anisopliae* one, two and three month after storage at 4°C and 25°C.

Substrate	1 month		2 months		3 months	
	Refrigerator	Room	Refrigerator	Room	Refrigerator	Room
Whole rice	93.2±3.52 a	70.65±2.47 ns	80.65±4.4 ab	59.79±2.23 a	67.5±7.5 ab	33.08±8.3 ns
Rice bran	80.47±2.9 bc	72.6±2.12 ns	80.14±2.39 ab	60.01±2.21 a	61.6±2,26 ab	28,88±3.6 ns
Barley pulp	80.7±3.2 bc	65.3±2.45 ns	67.7±1.99 bc	50.22±3.27 ab	50.56±2.57 bc	15.57±2.14 ns
Rice flour	80.8±2.2 bc	69.2±2.86 ns	70.2±2.55 bc	29.4±1.41 c	20.79±2.4 d	11.89±1.71 ns
Crushed rice	87.3±2.33 ab	72.9±1.47 ns	76.34± 3.7 bc	60.09±2.09 a	31.21±2.74 cd	10.9±098 ns
Crushed corn	96.06±2.03 a	66.4±3.04 ns	93.31±1.7 a	51.36±2.8 ab	84.85±1.39 a	15.21±1.1 ns
Wheat bran	75.87±1.86 c	67.7±2.36 ns	64.27±2.31 c	42.86±0.79 bc	24.11±1.08 d	20.47±1.34 ns
SDA as control	97.03±1.88 a	73.2±1.46 ns	93.87±2.77 a	62.09±2.23 a	87.81±1.4 a	35.01±1.77 ns

Means with different letters are significantly different (P <0.05).

Discussion

Mass production of entomopathogenic fungi has been received many attentions. Nutritional elements play important role in conidia and blastospore production rate in most fungi. In this subject, cereals and industrial organic by-products are the most important source of hydrocarbons and other nutrients for entomopathogenic fungi. There have been numerous studies on the effect of nutrition on growth and production of the fungus *Beauveria bassiana* and *M. anisopliae* (Jenkins & Prior, 1993; Jenkins *et al.*, 1998; Jackson *et al.*, 2003; Shah *et al.*, 2005; Bena-Molaei *et al.*, 2011; Ortiz-Urquiza *et al.*, 2013).

In present study, the most conidia yield of *M. anisopliae* isolate M14 was 3.1×10^8 conidia/g on rice flour medium and highest rate blastospore yield was 6.6×10^8 blastospores/ml^{-1} in wheat bran+rice bran+yeast extract+whey powder medium which is in agreement to other studies. Feng *et al.* showed that *B. bassiana* produced 10^{10} conidia/g on wheat bran medium (Feng *et al.,* 1994). In another study, isolates of *B. bassiana* produced $3.1\text{-}7.3\times10^7$ conidia/g of medium (Arcas *et al.,* 1999). Researchers suggested that not only kind of medium is important factor in conidia production of entomopathogenic fungus but also, the moisture content of substrates play important role in different isolates of *B. bassiana* on wheat, maize and millet. El Damir also showed that kind of substrate and media is effective on conidia production of *M. anisopliae* (El Damir, 2006). Cereal grains are generally rich from carbohydrates and other substrates for conidia production (Bena-Molaei *et al.*, 2011). One of the other important factors on conidia production is ratio of surface area to volume of the medium (Jenkins & Prior, 1993; Jenkins *et al.*, 1998). Media with a high surface to

volume ratio are suitable for production. Our results indicated that rice flour produced more conidia (3.1×10^8 conidia/g) than the other media. These results are supported by other researcher which reported that culture media based on rice increases conidia production of *M. anisopliae* (Dorta *et al.,* 1990; Jackson, 1997; Jenkins *et al.,* 2007). In LUBILOSA program, rice grain was the base of media for conidia production of *M. anisopliae* (Jenkins *et al.,* 2007). Suitable texture of rice flour may lead to better maintenance of required water for conidia production by the fungus in the space between the particles of substrate and better exchange of oxygen and CO_2 in the medium. However, it has been mentioned that wheat bran medium is suitable for conidia production of *B. bassiana* (Bena-Molaei *et al.*, 2011) because of rich crude protein, minerals and amino acids (such as methionine and cysteine), but in our experiment caused low production of conidia by *M. anisopliae*. Researchers suggested that the balance between nutrient and C:N ratio was important in the proliferation of entomopathogenic fungi (Jenkins & prior 1993; Jackson *et al.,* 2006; Tlecuital-Beristain *et al.,* 2010; Issaly *et al.,* 2005). For example, adding inorganic nitrogen source to culture media of *Metarhizium flavoviride* caused production of abundant mycelium without blastospores (Issaly *et al.,* 2005). Also, in a combination of 22:4, C:N ratio with 3% sucrose and 1% Casamino acid, the fungus *B. bassiana* produced 5.65×10^7 spores/ml., five days after inoculation (Pham *et al.,* 2009).

In some research, it have been reported that adding supplements like whey powder increased conidia production of *B. bassiana* (Jenkins *et al.,* 2007, Kassa *et al.,* 2007, Bena-Molaei *et al.,* 2011). Our results also showed that conidia production of *M. anisopliae* increased in rice bran+wheat bran+yeast extract+whey powder medium. However, there was no significant difference between this treatment and rice bran+wheat bran+yeast extract medium. In presence of grape juice in culture media, *M. anisopliae* produced less conidia in all treatments. We suggest that it may be due to unbalanced levels of carbohydrates that have not been appropriate for tested fungus. Low production in some culture media can be caused by weakness of the nutrients (Bena-Molaei *et al.,* 2011). The positive role of cations such as Zn^{++} was referred to produce mycelium and propagules of the fungus *Paecilomyces fumosoroseus* (Assaf *et al.*, 2009). In present study, adding chemical supplement such as magnesium sulfate to wheat bran+rice bran medium caused augmentation in conidia production (Table 3).

Storage of fungal conidia in optimal condition is one of important factors in mass production of entomopathogenic fungi. Our results showed that low temperature is a critical factor to save more viability of *M. anisopliae* conidia. Germination rate of *M. anisopliae* conidia was more higher when conidia stored in low temperature (about 5 to 8 ºC) compared to higher temperature (about 24 to 28 ºC) after one, two and three months storage.

The results of this study demonstrated some aspects of nutritional requirements and critical condition for conidial storage of *M. anisoplia* (isolate of M14), as basic information needs for mass process.

Acknowledgements

This publication is a part of the first author's Ph.D. thesis at the University of Tehran, which was supported under grant number 73132800.6.08. Also, we wish to thank the staff of the Biological Control Research Department of Iranian Research Institute of Plant Protection especially Arezoo Yousefi and Maryam Kalantari for their helps in conducting this research.

References

Arcas, J.A., Diaz, B.M., & Lecuona, R.E. 1999. Bioinsecticidal activity of conidia and dry mycelium preparations of two isolate of *Beauveria bassiana* against the sugarcane borer, *Diatraea saccharalis*. Journal of Biotechnology, 67: 151-158.

Assaf, A., Escobar, F. & de la Torre, M. 2009. Culture medium improvement for *Isaria fumosorosea* submerged conidia production. Biochemical Engineering Journal, 47: 87-92.

Bena-Molaei, P., Talaei-Hassanloui, R., Askary, H., and Kharazi-Pakdel, A. 2011. Study on potential of some solid natural substances in production of *Beauveria bassiana* (Ascomycota, Cordycipitaceae) conidia. Journal of Entomological Society of Iran, 30(2): 1-15. (In Farsi).

Dorta, B., Bosch, A., Arcas, J.A., & Ertola, R.J. 1990. High level of sporulation of *Metarhizium anisopliae* in a medium containing by-products. Applied Microbiology Biotechnology, 33: 712-715.

EL Damir, M. 2006. Effect of growing media and water volume on conidial production of *Beauveria bassiana* and *Metarhizium anisopliae*. Journal of Biological Sciences, 6(2): 269-274.

Fargus, J., Smits, N., Vidal, C., Vega, F., & Mercadier, G. 2001. Effect of liquid culture media on morphology, growth, propagule production, and pathogenic activity of the hyphomycete, *Metarhizium flavoviridae*. Mycopathologica, 154: 127-138.

Goettel, M.S. & Inglis, G.D. 1997. Fungi: Hyphomycetes. pp. 213-249. In: Lacey L.A. (ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, USA.

Farsi, M.J., Askary, H., Talebi Jahromi, KH., & Kharazi Pakdel, A. 2005. Effect of important ecological factors on blastospore production of *Verticillium lecanii* DAOM 198499 in liquid medium. Iranian, Journal of Agriculture Science, 36(1):109-119. (In Persian).

Feng, M.G., Liu, B.L., & Tzeng, Y.M. 2000. *Verticillium lecanii* spore production in solid-state and liquid-state fermentations. Bioprocess and Biosystems Engineering, 23: 25-29.

Ibrahim, L., Butt, T., & Jenkinson, P. 2002. Effect of artificial culture media on germination, growth, virulence and surface properties of the enthomopathogenic Hyphomycete *Metarhizium anisopliae*. Mycological Research, 106: 705-715.

Issaly, N., Chauveau, H., Aglevor, F., Fargues, J., & Durand, A. 2005. Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. Process Biochemistry, 40: 1425-1431.

Jackson, M.A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology & Biotechnology, 19: 180-187.

Jackson, M.A., Erhan, S., & Poprawski, T.J. 2006. Influence of formulation additives on the desiccation tolerance and storage stability of blastospores of the entomopathogenic fungus *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes). Biocontrol Science and Technology, 16(1): 61-75.

Jackson, M.A., Cliquet, S. & ItenL, B. 2003. Media and fermentation processes for the rapid production of high concentrations of stable blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*, Biocontrol Science and Technology, 13: 23-33.

Jenkins, N.E., & Prior, C. 1993. Growth and formation of true conidia by *Metarhiziumflavoviride* in a simple liquid medium. Mycological Research, 97(12): 1489-1494.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J., & Lomer, C.J. 1998. Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News and Information, 19: 21-31.

Jenkins, N.E., Ali, B.S. & Moore, D. 2007. Mass production of entomopathogenic fungi for biological control of insect pest. pp. 287-293. In: Parker, B.L., Skinner, M., EL Bouhssini, M. & S. G. Kumair (eds), Sunn Pest Management, A Decade of Progress 1994-2004. ICARDA, Aleppo, Syria, Arab Society for Plant Protection.

Kassa, A., Brownbridge, M., Parker, B., Skinner, M, Gouli, V., Gouli, T., Guo, S.M., & Lee, F. 2007. Evaluation of whey powder as a basal media ingredient for mass production of *Beauveria bassiana* and *Metarhizium anisopliae*. Proceedings of the 40th Annual meeting of the society for invertebrate pathology and 1st international forum on the entomopathogenic nematodes and symbiotic bacteria, 12-16 Aug., Quebec City, Universite Laval, Canada, 97.

Mascarin, G.M., Alves, S.B., & Lopes, R. 2010. Culture media selection for mass production of *Isaria fumosorosea* and *Isaria farinose*. Brazilian Archives of Biology and Technology, 53(4): 753-761.

Ortiz-Urquiza, A., Keyhani, N.O., & Quesada-Moraga E. 2013. Culture conditions affect virulence and production of insect toxic proteins in the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Science and Technology, 23(10): 1199-1212.

Pham,T.H., Kim, J.J., Kim, G.S., & Kim, K. 2009. Production of blastospores of entomopathogenic fungus *Beauveria bassiana* in a submerged batch culture. Mycobiology, 37(3): 218-224.

Safavi, S.A., Shah, F.A., Pakdel, A.K., Rasoulian, G.R., Bandani, A.R., & Butt, T.M. 2007. Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*, FEMS Microbiology Letters, 270: 116-123.

Rangel, D.E.N., Alston, D.G., & Roberts, D.W. 2008. Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. Mycological Research, 112: 1355-1361.

Roshandel, S., Talaei Hassanlouei, R., Askary, H., Allahyari, H. & Marzban, R. 2013. Effect of culture substrates on virulence of *Metarhizium anisopliae* conidia and blastospores against Sunn pest, *Eurygaster integriceps*. Iranian Journal of Plant Protection Science, 44(2): 225-234.

Shah, F., Wang, C.S., & Butt, T.M. 2005. Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae FEMS.* Microbiology Letters, 251(2): 259-266.

Tlecuital-Beristain, S., Gonzalez, G.V., Diaz-Godinez, G., & Lovea, O. 2010. Medium selection and effect of higher Oxygen concentration pulses on *Metarhizium anisopliae* Var *Lepidiotum* conidial production and quality. Mycopathologia, 169: 367-394.

Thomas, K.C., Khachatourians, G., & Ingledew, W.M. 1987. Production and properties of *Beauveria bassiana* conidia cultivated in submerged culture. Canadian Journal of Microbiology, 33: 12-20.

Wraight, S.P., Jackson, M.A. & Kock, S.L. 2001. Production, stabilization and formulation of fungal biocontrol agents. pp. 253-287. In: Butt, T.M., Jackson, C.W. & Magan, N. (eds), Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI publishing, Wallingford,

Wu, J.H., Ali, S., Huang, Z., Ren, S. X., & Cai, S.J. 2010. Media composition influences growth, enzyme activity and virulence of the entomopathogen *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae). Pakistan Journal of Zoology, 42: 451-459.

Wyss, G.S., Charudattan, R., & DeValerio, J.T. 2001. Evaluation of agar and grain media for mass Production of conidia of *Dactylaria higginsii*. Plant Disease, 85: 1165-1170.

Zimmermann, G. 1993. The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. Pesticide Science, 37(4): 375-379.

بررسی تاثیر محیط‌های غذایی طبیعی روی میزان تولید و زنده‌مانی اسپور و بلاستوسپورهای قارچ *Metarhizium anisopliae*

سیامک روشندل[۱]، حسن عسکری[۲]، رضا طلایی حسنلویی[۳]، حسین اللهیاری[۳]

۱- گروه گیاه‌پزشکی، پردیس کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران

۲- بخش تحقیقات کنترل بیولوژیک، موسسه تحقیقات گیاه‌پزشکی کشور، سازمان تحقیقات، آموزش و ترویج کشاورزی، تهران، ایران

۳- گروه گیاه‌پزشکی، پردیس کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران

مسئول مکاتبات: حسن عسکری، پست الکترونیک: askary@iripp.ir

تاریخ دریافت: ۹۴/۰۶/۰۱ ۴ (۱) ۹۴-۱۰۴ تاریخ پذیرش: ۹۵/۰۸/۱۱

چکیده

در این تحقیق میزان تولید و جوانه زنی اسپور و بلاستوسپورهای قارچ *Metarhizium anisopliae* که روی بذرهای مختلف تولید شده بودند، مورد ارزیابی قرار گرفت. آزمایش‌ها به‌صورت فاکتوریل و در قالب طرح کاملاً تصادفی که دارای ۹ تیمار در سه تکرار بودند، انجام شد. نتایج اختلاف معنی‌داری را بین تعداد کنیدی تولید شده، دو هفته پس از کشت نشان داد ($F= 4.66, df= 7, 16, p<0.01$). بیشترین میانگین تولید محصول به‌میزان $۳/۱\times۱۰^{۸}$ و ۳×۱۰^{۸} کنیدی/گرم متعلق به آرد برنج و محیط کشت SDA (شاهد) بود. کمترین میانگین تولید محصول به‌میزان ۴×۱۰^{۶} کنیدی/گرم متعلق به سبوس گندم بود. همچنین اختلاف معنی‌داری بین میزان تولید بلاستوسپور بعد از ۴ روز کشت در محیط‌های مایع مشاهده شد ($F= 2.57, df=8, 18, p<0.05$). بیشترین و کمترین میزان تولید بلاستوسپور به‌میزان $۶/۱\times۱۰^{۸}$ و $۴/۵\times۱۰^{۸}$ در هر میلی‌لیتر به‌ترتیب متعلق به عصاره‌ی سبوس گندم+سبوس برنج+مخمر و سبوس برنج+مخمر بودند. تجزیه‌ی واریانس برای تیمارهای مواد افزودنی شیمیایی و عناصر غذایی که در محیط‌های کشت مایع اضافه شده بودند نشان داد که اختلاف معنی‌داری بین تیمارها وجود دارد ($F=5.02, df= 13, 29, p<0.01$) بیشترین میزان تولید بلاستوسپور در هر میلی‌لیتر $۶/۶\times۱۰^{۸}$، $۶/۱\times۱۰^{۸}$ و $۵/۷\times۱۰^{۸}$ به‌ترتیب متعلق به تیمار عصاره‌ی سبوس برنج+سبوس گندم+مخمر+آب پنیر؛ تیمار عصاره‌ی سبوس برنج+عصاره‌ی سبوس گندم+مخمر و تیمار عصاره‌ی سبوس گندم+عصاره‌ی سبوس برنج، سولفات منیزیوم و کمترین میزان محصول تولید شده متعلق به تیمار آب انگور بود. نتایج نشان داد که میانگین درصد جوانه‌زنی کنیدی‌های *M. anisopliae* بعد از یک، دو و سه ماه که در شرایط ۴ و ۲۵ درجه‌ی سلسیوس نگهداری شده بودند، تفاوت معنی‌داری با هم داشتند. درصد جوانه‌زنی در تمام تیمارهایی که در یخچال نگهداری شده بودند بیشتر از تیمارهایی بودند که در دمای معمولی اتاق حفظ شده بودند.

واژه‌های کلیدی: جوانه‌زنی، تولید انبوه، قارچ، عناصر غذایی، محیط کشت، *Metarhizium anisopliae*