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INTRA-SPECIFIC VARIABILITY AMONG *METARHIZIUM ANISOPLIAE* STRAINS IN THEIR ABILITY TO PRODUCE BLASTOSPORES IN LIQUID CULTURE MEDIA

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Abstract

This study describes intra specific variability among *Metarhizium ansiopliae* strains in terms of their ability to produce blastospores in selected liquid culture media. Blastospore production of 10 strains was evaluated in 6 different media representing different carbon and nitrogen sources. Irrespective of strain, media containing corn steep and yeast extract provided highest blastospore yield. Among different strains F10, ERL607, ARSEF 3297 and ARSEF 4556 produced significantly ($p<0.001$) higher number of blastospores at 48 hours post inoculation (hpi). At 72 hpi, blastospore concentration was significantly higher for strains ARSEF 3297, CA1, F10, CA 22 and ARSEF 4556, while the strains, V275, UWS-2 and PW produced significantly lower number of blastospores. The single highest average production ($1.43x10^8 \pm 1x10^7$) was noticed in Adamek's modified medium(AMM) in case of CA1 at 72 hpi. Media influenced the morphology and budding pattern e.g. In Adamek's media (AM), blastospores were slender in shape and were observed frequently on apical as well as on lateral buds, whereas BH medium yielded blastospores which resembled aerial conidia. Blastospores produced on different media varied non-significantly among themselves as well as with aerial conidia in their virulence towards *Tenebrio molitor*. Considering the intra specific variability in blastospore production, this study highlights the importance of screening strains for their ability to produce blastospores prior to optimizing media and culture conditions for blastospore production.

Introduction

Insect pathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin, offers great potential as an alternative to chemical insecticides (Butt *et al*., 2001; Copping 2004). Several strains of this fungus have been shown to be highly efficacious against a variety of insect pests and being considered for commercial development (Ansari *et al*., 2008; Shah *et al*., 2007). At present solid state production e.g., mass production on rice grains is widely used for large scale production of *M. anisopliae*. Although high numbers of conidia are easily produced when *M. anisopliae* is grown on solid substrates, liquid fermentation is of commercial interest because of its short fermentation times (Ypsilos and Magan, 2005) and allows better control and manipulation of growth conditions (Humphreys *et al*., 1989). In liquid culture, like many deuteromycetes, *M. anisopliae* grows in yeast-like fashion producing high concentrations of vegetative propagules termed blastospores (Adamek 1963; Kleespies & Zimmermann 1992, 1998; Vega *et al*., 2003; Ypsilos & Magan, 2005). Blastospores of *M. ansiopliae* are typically larger than aerial conidia, which can be used as an alternative to solid state mass production.

Over the last 4-5 decades several researchers reported blastopsore production in *M. anisopliae*, however, most studies focused on optimizing cultural conditions for optimum blastospore production. These studies reported considerable variation in terms of *M. anisopliae* ability to yield high quantities of blastospores. For example, Kleespies & Zimmerman (1992) reported highest blastopsore yield of up to $1x10^8$ /ml. In another study, Kleespies & Zimmerman (1998) noticed that addition of 5% lecithin increased blastospore production to 1.9×10^8 /ml. In contrast, Vega *et al*., (2003) observed that irrespective of media composition *M. ansiopliae* failed to produce high quantities of blastospores as highest yield in their studies was 3.9×10^7/ml. Similarly Ypsilos & Magan, 2004; 2005 reported that depending upon media *M. anisopliae* produced blastopores in the range $1.5 x10^6$ to $5x10^7$ ml^{-1} of media. Recent studies by Jackson *et al*., (1997) also showed variability among three strains of *M. ansiopliae* in their ability to produce blastospores and/or microsclerotia. Since above studies were conducted using different strains and/or media, it is not clear whether blastospore production in *M. anisopliae* is dependent on strain or media composition. This study focuses on increasing our understanding of intra specific variability among certain *M. anisopliae* strains in term of blastospore production, so that media composition/conditions can be optimized for blastospore production on commercial scale.

Materials and Methods

Sources and origin of fungal strains: Ten strains of *M. anisopliae* were passaged through *Galleria mellonella* larvae and isolated on Oatmeal dodine agar medium. Details of strain's origin and host are provided in Table 1. Single spore colonies were transferred to Sabouraud Dextrose Agar (SDA) and these 1st subcultures were used in subsequent studies. Conidia from each plate were harvested at 15 days post inoculation by scrapping with 0.03% tween. Conidial concentration was determined and adjusted to $1x10^7ml^{-1}$ using an improved Neubauer haemocytometer (Weber Scientific International Ltd. U.K.) prior to inoculating different liquid media.

Table 1. Different strains of *M. anisopliae* used in the experiments.

Isolate	Original host/Source	Country of origin
V 275	*Cydia pomonella*. Lepidoptera.	Austria
ARSEF 3297	*Boophilus* sp. (Acari: Ixoididae)	Mexico
ARSEF 4556	*Boophilus* sp. (Acari : Ixodidae)	USA
CA 1	Soil, *Galleria* baiting; avocado orchard	CA USA
F10	*Wiseana* spp. (Lepidoptera: Hepialidae),	New Zealand
PW	*Hylobius abietis* (Coleoptera; Curculionidae)	Republic of Ireland
ERL 700	*Heliothis zea* (Lepidoptera: Noctuidae)	FL USA
CA 22	Soil, *Galleria* baiting; avocado orchard	CA USA
ERL607	*Heliothis zea* (Lepidoptera: Noctuidae)	FL USA
UWS-2	*Hylobius abietis* (Coleoptera; Curculionidae)	Republic of Ireland

Determination of intra specific variability in blastospore production: Following screening of various culture media representing different carbon and nitrogen sources, osmolalites and pH, six media were selected to determine intra specific variation in blastospore production. Composition of selected media is described in Table 2. A total of 10 strains representing diverse environmental conditions and host origin (Table 1) were used in these studies. Aliquots of 25ml of the media were autoclaved in Erlenmeyer flasks (100 ml capacity) at 121°C for 20 min. Each flask was inoculated with the suspension of aerial conidia having the concentration of 1 x $10^7 ml^{-1}$. The inoculated flask cultures were incubated on G 24 incubator shaker at 120 rev min^{-1} at 26°C. It should be noted that while preparing AM and AMM, corn steep was added first without the addition of glucose and heated for 10 min. After that this was filtered through 2 layers of lens cleansing tissue (Whatman 105). Spore yields were examined after 24 hrs, 48 hrs and 72 hrs post inoculation. Osmolality (m mol Kg^{-1}) and pH of each media were also determined before inoculation and shown against each media in Table 4. Samples were taken every 24 hrs till 72 hrs post inoculation from each flask to count blastospores ml^{-1} using a Neubauer haemocytometer. Each treatment was replicated three times and whole experiment repeated 2 times.

Table 2. Composition of different media used in experiments.

Media	Corn steep (%)	Yeast extract (%)	Malt extract (%)	Glucose (%)	Tween-80 (%)	PEG-200 (%)	Peptone (%)	Glycerol (%)
A M	3	4	-	4	0.4	-	-	-
AMM	3	4	-	4	0.4	7	-	-
BH	-	1	3	-	-	-	-	-
BH M	-	1	2			7	-	-
SDB	-	-	-	2	0.4	-	0.5	-
SDBM	-	-	-	2			0.5	0.5

Studies on blastospore morphology using fluorescence microscope: Samples of ARSEF 4556 were examined to determine the influence of media on blastospore morphology i.e., cell wall, septa or bud scars using wide-field fluorescence microscope (Nikon Eclipse E 600). Blastopsores produced in 4 different media taken after 72 hrs were stained with Solo phenyl Flavine and Calcofluor (both prepared as 0.1% w/v stock solution in distilled water). Staining was carried out on fixed cells (2% formaldehyde, 5 min) and samples were examined at 40x, 60x and 100x. Images were recorded with Olympus digital camera mounted on the microscope using the Cool snap software (Fig. 1a-d).

Determining virulence of inoculum produced in different culture media

Preparation of the fungal inocula: Blastopsores from two strains yielding highest quantities of blastospores i.e., ARSEF 3297 and ARSEF 4556 cultured in 4 selected media were harvested by filtering through 2 layers of lens cleansing tissue (Whatman No.105) at 72 h post inoculation at 25°C. Blastospore were concentrated and washed twice with distilled water by centrifugation at 5000 rpm for 10 min (Couiter Bioresearch, Germany) and then resuspended in sterile distilled water slightly modifying the procedure of Fargues *et al*., 2002. Ten ml of each suspension was placed in sterilized universal bottle (30 ml) and vortexed for 40 seconds. Spore concentration was calculated using haemocytometer as mentioned before and suspensions were then adjusted to 10^7 spores ml^{-1}.

Tenebrio inoculation: Blastospores were assayed against 4th-5th instar of *Tenebrio molitar*. Larvae (batch of ten) were immersed in 10 ml aliquot ($1x10^7$/ml) suspension. Same concentration of dried aerial conidia (in 0.03% Tween) of both strains was used as positive control. Sterile distilled water served as control. Excess water was removed by filtering over a vacuum in a Buchner funnel. Batches of 10 larvae were incubated without food in 9 cm diameter Petri dishes lined with moist Whatman No. 1 filter paper and incubated at 25°C in the dark. Mortality was recorded daily for 8 days post inoculation. Each treatment was replicated three times and whole experiment conducted two times.

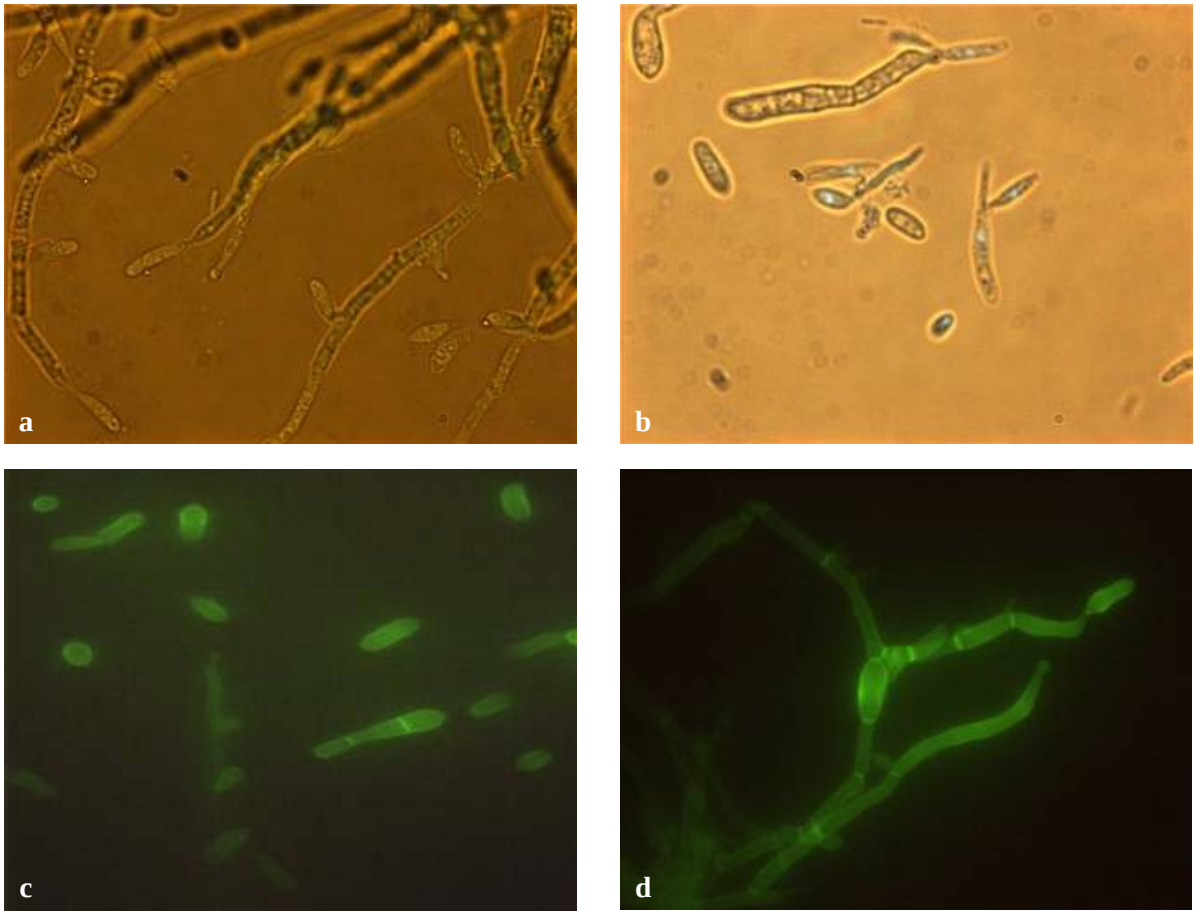

Fig. 1. Pattern of budding and spore shape of ARSEF 4556 in different media i.e., AM (a), note apical as well as lateral budding are prominent (a), single slender shape are dominant in AMM (b), Spores are much longer in BH medium and typically formed at apex (c), certain spore also show prominent observed septa thus making the spore tri-celled which could lead to further fragmentation and formation of 3 independent spores (c), blastospore in BHM are much slender than BH media (d). Samples a-b was treated with Calcofluor and c-d with Solophenyl flavine. All Photographs were captured with Olympus digital camera at 100x magnification using Cool snap software.

Statistical analysis: The whole study was repeated twice with each treatment replicated thrice unless stated otherwise. Data was subjected to one-way ANOVA followed by post hoc Tukey's HSD test for determining significant differences using the software SPSS 13.

Results

Intra specific variation in blastospore production: Culture media affected production of blastospores of all the strains tested. Only 4 of the evaluated media i.e., AM, AMM, BH, and BHM supported blastospore formation. Although SDB and SDBM media supported good mycelial growth, none of the strain produced blastospores in these media. Blastospore yields obtained for different strains indicate that strains responded differently to the media. After 48 hrs post inoculation three strains i.e., F10, ERL607 and ARSEF 4556 produced significantly ($p<0.05$) higher number of blastospores than other strains (Table 3). AM media yielded highest blastospore whereas BH media had least yield of blastopsore while AMM and BHM were intermediate. Two strains i.e., UWS-2 and PW produced no blastospores at 48 hrs post inoculation.

At 72 hrs post inoculation, AM and AMM yielded significantly ($p<0.05$) higher number of blastospores (Table 3). Blastospore yield was significantly ($p<0.05$) higher in the strains ARSEF 3297, CA1, ARSEF 4556, CA22 and F10, while the production in the strains V275, UWS-2 and PW was significantly ($p<0.001$) lower. Interestingly, irrespective of strain blastospore production decreased significantly at 72 hrs post inoculation in BH and BHM (Table 3). ARSEF 4556 produced highest number of balstopsores in all four media which supported balstospore induction while the production of other high yielding strains was media dependant (Table 3).

Morphology of spores and budding pattern in ARSEF4556: Culture media influenced blastospore morphology. Blastospores formed in AM media were apical as well as lateral whereas BH media had more number of apical blastospore (Fig. 1). Blastospores formed in AM media were also much longer than spores formed in BH medium. In AMM and BHM media single slender shaped media with typical budding were frequently observed (Fig. 1). BH media yielded shorter blastospores which resembled aerial conidia.

Table 3. Blastospore production ($X10^7ml^{-1}$) of different strains of *M. anisopliae* in selected media at 48 and 72 hours post inoculation. No blastospore production was observed after 24 hrs. The results are representatives of two similar experiments. Significance was determined using Duncan HSD test.

Fungal strain	Blastospore production ($x10^7ml^{-1}$) in selected culture media							
	AM		AMM		BH		BHM	
	48hpi	72hpi	48hpi	72hpi	48hpi	72hpi	48hpi	72hpi
3297	2.6^{b}	9^{ab}	2.8^{bc}	13.5^{ab}	1.3^{b}	0.2^{a}	2^{b}	-
275	0.07^{b}	0.3^{c}	0.05^{c}	0.2^{d}	-	-	0.03^{b}	-
Ca1	-	15^{a}	-	14.3^{a}	0.06^{b}	0.1^{bc}	1.0^{b}	0.2^{a}
E700	1.08^{b}	2.5^{bc}	2.7^{bc}	3.8^{cd}	-	-	0.1^{b}	0.06^{b}
4556	4.2^{ab}	13.1^{a}	0.03^{c}	8.4^{abcd}	5^{a}	0^{c}	8.5^{a}	0.03^{b}
CA22	1.2^{b}	14.1^{a}	-	3.5^{cd}	0.12^{b}	0.1^{bc}	0.3^{b}	0.06^{b}
607	6.4^{a}	5.2^{bc}	6.4^{ab}	6.1^{bcd}	0.05^{b}	-	0.3^{b}	0.4^{a}
F10	10^{a}	9.3^{ab}	7.7^{a}	9.3^{abc}	-	-	-	0.09^{b}
UWS2	-	2.7^{c}	-	0.3^{d}	-	-	-	-
PW	-	0.1^{c}	-	0.5^{d}	-	-	-	-

- = Less than $1x10^4$/ml blastospore observed

Means followed by same letter within a column varied non-significantly ($p<0.05$)

Table 4. Virulence LT_{50} (days post-inoculation) of blastospores and aerial conidia of two strains of *M. anisopliae* grown on various media. The results are representative of two similar experiments.

Culture media	pH	Osmolality m mol. Kg^{-1}	Virulence LT_{50} (days post-inoculation)	
			ARSEF 3297	ARSEF 4556
AM	4.9	650	5.77^{b}	3.96^{a}
AMM	5	1230	3.79^{a}	3.59^{a}
BH	6	180	4.17^{a}	4.16^{a}
BHM	6.1	480	3.93^{a}	3.84^{a}
Aerial conidia (in 0.03% tween)	4.9	820	4.40^{a}	4.31^{a}

All the means within a column followed by the same letter are not significantly different ($p<0.001$) using Tukey test

Pathogenicity of blastospores and aerial conidia: Virulence of blastospore varied non-significantly among the blastospores produced in different media. Similarly virulence of blastospores varied non-significantly with that of aerial conidia (Table 4). However, modified media were found more effective in terms of taking less time as compared to the existing media and that of aerial conidia. Osmolality and pH had no direct effects on the virulence.

Discussion

Blastospore production in *M. anisopliae* appeared to be strain dependent with certain strains exhibiting significantly higher ability to form blastospores than others. Nutritional conditions also strongly influenced blastospore production but in a strain dependant manner. The results of our study corroborates earlier reports on the effect of nutrition on propagules production in *Metarrhizium* spp., and other entomopathogenic hyphomycetes (Kleespies & Zimmermann, 1992 ; Jackson *et al*., 1997; Vidal *et al*., 1998; Inch *et al*., 1986; Fargues *et al*., 2005). These authors noted variable blastopsore production in different media by different strains, however, each author had used different media and/or strain, therefore, exact role of either media or strain on blastospore production couldn't be established. In our study, we observed that even slight variation in nutrition can significantly influence blastospore for certain strains. For example, addition of 7% PEG significantly reduced blastospore production in ARSEF 4556 and CA22 as compared to AM media. Similarly F10 yielded significantly higher blastospore at 48hrs when grown in AM media than in AMM media, although these differences were not seen at 72 hrs. With regard to nutritional composition of media, our results are in line with earlier which suggested that corn steep (CS) solid and yeast extract are best nitrogen sources for blastospore induction in *M. anisopliae*. Ypsilos & Magan, (2005) also reported, corn steep solid and cottonseed flour (7%) to be the best nitrogen sources for optimum blastospore production of *M. anisopliae* but neither of these two nitrogen sources gave as high blastospore production as when 3% CS was used in combination with 4% yeast extarct (Adamek's medium modified to 0.98 a_w using PEG 200; Ypsilos & Magan, 2004). Interestingly, in BH and BHM media, there was maximum concentration of

blastospores after 48 hrs and then a significant decrease in the yield after 72 hrs was noticed. From this it appears that some elements present in yeast extract and malt extract promote high number of blastospores in comparatively shorter duration and this media has the potential to be exploited more through manipulation of the nutritional environment. Nutrition also appeared to influence blastospore morphology and induction; microscopic observation showed that AM media supported formation of both apical as well as lateral spore buds, which in turn resulted in higher blastospore yields. Similarly blastospores formed in AM media were relatively larger than those formed in BH medium.

Overall, this study showed intra specific variability among *M. anisopliae* strains in terms of their ability to produce blastospores. Since certain strain irrespective of media composition failed to produce blastospores, it is important to select strains which are good in blastospore production, therefore screening for strains for blastospore production should be considered prior to developing any media for blastospore production. We are currently evaluating optimized media for increasing blastopsore yield and quality of high blastospore yielding strains.

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