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The impact of nutrition on spore yields for various fungal entomopathogens in liquid culture

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Summary

Spore yields were measured for various fungal entomopathogens grown in six nutritionally different liquid media with low and high carbon concentrations (8 and 36 g l^{-1}, respectively) at carbon-to-nitrogen (C:N) ratios of 10:1, 30:1 and 50:1. Six fungi were tested: two *Beauveria bassiana* strains, three *Paecilomyces fumosoroseus* strains and one *Metarhizium anisopliae* strain. Spore yields were examined after 2, 4 or 7 days growth. In general, highest spore yields were obtained in media containing 36 g/l and a C:N ratio of 10:1. After 4 days growth, highest spore yields were measured in the three *Paecilomyces* isolates ($6.9–9.7 \times 10^8$ spores ml^{-1}). Spore production by the *B. bassiana* isolates was variable with one isolate producing high spore yields (12.2×10^8 spores ml^{-1}) after 7 days growth. The *M. anisopliae* isolate produced low spore concentrations under all conditions tested. Using a commercial production protocol, a comparison of spore yields for the coffee berry borer *P. fumosoroseus* and a commercial *B. bassiana* isolate showed that highest spore concentrations (7.2×10^8 spores ml^{-1}) were obtained with the *P. fumosoroseus* isolate 2-days post-inoculation. The ability of the *P. fumosoroseus* strain isolated from the coffee berry borer to rapidly produce high concentrations of spores prompted further testing to determine the desiccation tolerance of these spores. Desiccation studies showed that ca. 80% of the liquid culture produced *P. fumosoroseus* spores survived the air-drying process. The virulence of freshly produced, air-dried and freeze-dried coffee berry borer *P. fumosoroseus* blastospores preparations were tested against silverleaf whiteflies (*Bemisia argentifolii*). While all preparations infected and killed *B. argentifolii*, fresh and air-dried preparations were significantly more effective. These results suggest that screening potential fungal biopesticides for amenability to liquid culture spore production can aid in the identification of commercially viable isolates. In this study, *P. fumosoroseus* was shown to possess the production and stabilization attributes required for commercial development.

Introduction

Fungal entomopathogens such as *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, and *Paecilomyces fumosoroseus* (Wize) Brown & Smith, are known to be pathogenic to numerous insect species. The feasibility of using these fungi as biocontrol agents against important insect pests is dependent on numerous biological constraints, including the ability to produce high concentrations of stable propagules at a reasonable cost (Jaronski 1986; Latgé *et al.* 1986). On solid substrates, these fungi produce abundant aerial conidia which are amenable to storage as dry preparations; in submerged culture, these fungi often grow in yeast-like fashion producing high concentrations of vegetative propagules termed blastospores, which are typically larger than aerial conidia. Blastospores can be very infective when applied to the insect host, are generally not amenable to simple drying techniques, and tend to perish more rapidly during storage (Inch *et al.* 1986; Lane *et al.* 1991). Even with these shortcomings, the ability of many entomopathogenic fungi to produce high concentrations of blastospores in very short fermentation times has piqued commercial interest in this method of bioinsecticide production.

Jackson *et al.* (1997) reported the development of a liquid culture medium containing high concentrations of nitrogen, essential for the production of desiccation-tolerant blastospores of *P. fumosoroseus*. Compared to conidia, these blastospore preparations show great promise as a bioinsecticidal propagule due to their rapid rate of germination on agar and on the cuticle of insects, such as the silverleaf whitefly, *Bemisia argentifolii* Bellows & Perring (Vega *et al.* 1999). While nutritionally poor media are often required to stimulate liquid culture sporulation, various fungi produce high concentrations of blastospores in nutritionally-rich liquid media (Thomas *et al.* 1987; Jenkins & Prior 1993; Jackson *et al.* 1997). In addition, nutritional and environmental conditions in liquid media have been shown to impact spore yield, stability and biocontrol efficacy (Humphreys *et al.* 1989; Jackson & Bothast 1990; Lane *et al.* 1991; Jackson & Schisler 1992).

In this study, our objective was to determine if liquid culture fermentation could be used to produce high concentrations of stable, effective spores of various entomopathogenic fungi, including two that were isolated from the coffee berry borer and that might have potential for use as a biological control agent against this serious insect pest. To screen for amenability to liquid culture production, six fungal isolates were grown in liquid media that varied in carbon concentration and carbon to nitrogen ratio. Spore yields were measured for all isolates. For the high yielding *P. fumosoroseus* strain that was isolated from the coffee berry borer, additional studies were conducted to determine desiccation tolerance and biocontrol efficacy.

Materials and methods

Fungal cultures

Six different fungal entomopathogens were tested in this study: two strains of *Beauveria bassiana,* strain ATCC[1] 74250, GHA from Mycotech Corporation (now Emerald BioAgriculture, Lansing, MI) and strain ARSEF[2] 5460, isolated in Africa from coffee berry borers (*Hypothenemus hampei* (Ferrari); Coleoptera: Scolytidae); three strains of *Paecilomyces fumosoroseus,* strains ATCC 20874 and ARSEF 4502 isolated from *B. argentifolii* in Florida and Pakistan, respectively; and ARSEF strain 6730 isolated from a coffee berry borer collected in Africa; and *Metarhizium anisopliae* var. *anisopliae* ARSEF 4901 (the location where this strain was collected is not known). A strain of *P. fumosoroseus* isolated from *B. tabaci* in Texas (ARSEF 3581) was used as a standard for comparison in spore production and desiccation tolerance experiments. For stock cultures, all fungal strains were grown on potato dextrose agar (PDA) plates and 1 mm^2 agar pieces from these plates were stored at −80 °C in sterile cryovials containing 10% glycerol (in sterile distilled water). Conidial inocula for liquid culture studies were obtained from 2 to 3 week-old, sporulated dextrose agar plates that had been inoculated with stock cultures.

Media and culture conditions

In initial media screening experiments, all liquid cultures were inoculated with conidia obtained from sporulated PDA plates to provide an initial concentration of 5×10^6 conidia ml^{-1} culture broth. Six different nutritional environments were tested as described in Table 1. These media were composed of basal salts supplemented with trace metals, vitamins and various combinations of glucose and casamino acids (Becton Dickinson Microbiology Systems, Sparks, MD), as previously described (Jackson *et al.* 1997).

For blastospore production and desiccation tolerance studies, a high yielding strain of *P. fumosoroseus* (coffee berry borer strain; ARSEF 6730) was compared to our standard blastospore production strain *P. fumosoroseus* (ARSEF 3581); the two *P. fumosoroseus* strains were grown in the standard basal medium containing either 25 or 45 g casamino acids l^{-1} and 80 g glucose l^{-1}. The highest yielding strain of *B. bassiana* (ATCC 74250) was compared to a high yielding *P. fumosoroseus* strain (coffee berry borer strain; ARSEF 6730); both were grown in media containing 25 g casamino acids l^{-1}. In contrast to the initial experiments testing six different media (where conidia were used as the source of inoculum), these cultures were inoculated with blastospores obtained from 4 day-old cultures grown in the same medium and provided a final blastospore concentration of 5×10^6 blastospores ml^{-1}.

All liquid cultures were grown as 100 ml cultures in 250 ml baffled Erlenmeyer flasks at 28 °C and 300 rev min^{-1} in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ). Blastospore concentrations were measured microscopically using a hemacytometer at 2–4, or 7 days post-inoculation. For each experiment, two blastospore counts were made for each flask on each sampling date and three replicate

Table 1. Carbon concentration (g l^{-1}) and carbon to nitrogen ratio in liquid cultures used to assess yields of different fungal entomopathogens.

[C g l^{-1}] – C:N	Glucose (g l^{-1})	Casamino acids (g l^{-1})
[8] – 10:1	10.0	10.0
[8] – 30:1	16.6	3.4
[8] – 50:1	18.0	2.0
[36] – 10:1	45.0	45.0
[36] – 30:1	75.0	15.0
[36] – 50:1	81.0	9.0

[1] ATCC denotes the American Type Culture Collection (Manassas, Virginia, USA).

[2] ARSEF denotes the US Department of Agriculture, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (Ithaca, New York, USA; Humber 1992).

flasks were used for each different medium. All experiments were repeated at least twice.

Drying studies

Blastospore desiccation tolerance was evaluated by air-drying blastospore preparations to less than 4% moisture. Briefly, whole cultures containing blastospores were mixed with diatomaceous earth (HYFLO, Celite Corp., Lompoc, CA) at a rate of 1 g diatomaceous earth/2×10^{10} blastospores. Spent media was removed by vacuum filtering the suspension on filter paper (Whatman No. 1). The blastospore-diatomaceous earth filter cake (~65% moisture) was crumbled into a shallow aluminum pan and air dried with moist air (RH > 65%) overnight at 23 °C. Moisture levels in dried blastospore preparations were measured using a moisture analyser (Mark I, Denver Instruments, Tempe, AZ). For freeze-drying studies, whole cultures of *P. fumosoroseus* containing blastospores were mixed 1:1 with a 20% lactose, 2% bovine serum albumin solution, as previously described (Jackson *et al.* 1997). Spore viability after drying was assessed by placing 100 mg of the dried blastospore preparation in 50 ml of potato dextrose broth in a 250 ml baffled flask. After 6 h incubation at 28 °C and 300 rev min^{-1} in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ), percentage viability was determined by examining 100 spores microscopically for the emergence of a germ tube.

Bioassays

Bioassays against the coffee berry borer were not possible due to logistic problems in rearing the insect; therefore, to assess the pathogenicity of *P. fumosoroseus* ARSEF 6730, laboratory bioassays against the silverleaf whitefly (*B. argentifolii*) were conducted using freshly produced, freeze-dried and air-dried *P. fumosoroseus* blastospores. Silverleaf whitefly bioassays were conducted with third instar nymphs of *Bemisia argentifolii*, as previously described (Poprawski & Jackson 1999). Briefly, bioassays were conducted using five replicated melon leaves per blastospore treatment with a negative control (0.01% Tween 80). Each melon leaf, infested with 25 early third instar *B. argentifolii* nymphs, was sprayed with a blastospore suspension delivering 1000 blastospores mm^2 (equivalent to a field application of 1×10^{13} spores ha^{-1}). Whitefly bioassays were repeated three times using blastospore preparations from three different production and drying runs. Data was analysed as a one-way ANOVA using JMP (SAS Institute, Inc. 2000); means were separated using Tukey–Kramer's adjustement for multiple comparisons.

Statistical analysis

A cube-root transformation was used on blastospore counts in order to meet the homogeneity of variances assumption. A three-way fixed effect ANOVA was conducted to identify statistically significant effects containing taxon, treatment, and day for the cube-root count data using SAS Proc Mixed (SAS Institute, Inc. 1997). Mean comparisons were conducted using the Tukey–Kramer adjustment (Kramer 1956) for multiple comparisons; letters indicating statistical significance or similarity were obtained using the SAS macro pdmix6124 (Saxton 1997).

Results

Cultures of both *B. bassiana* and *P. fumosoroseus* produced higher yields than *M. anisopliae* under all the treatments tested (Table 2). Blastospore yields obtained for the *B. bassiana* strains indicate that strains respond differently to the media. For example, spore production in *B. bassiana* ATCC 74250 was higher than in *B. bassiana* ARSEF 5460 in all treatments tested. In contrast, such a difference was not as marked among the three *P. fumosoroseus* strains. A low carbon concentration (8 g l^{-1}) combined with a high C:N ratio (either 30:1 or 50:1) resulted in the lowest spore yields for *P. fumosoroseus* as opposed to a high carbon concentration (36 g l^{-1}) and a low C:N ratio (10:1) which resulted in the highest yields 7 days post-inoculation.

No difference in blastospore yield or desiccation tolerance was seen for cultures of *P. fumosoroseus* ARSEF 6730 grown in media containing either 25 or 45 g casamino acids l^{-1} when compared to our standard blastospore production strain, *P. fumosoroseus* ARSEF 3581 (Table 3). *P. fumosoroseus* ARSEF 6730 blastospore yields were significantly higher after 2 days growth when a blastospore rather than conidial inoculum was used (Tables 2 and 4). For cultures of *B. bassiana* ATCC 74250, the use of blastospore or conidia inoculum had no impact on blastospore yields after 2 days growth.

Whitefly bioassays showed that all blastospore preparations of *P. fumosoroseus* ARSEF 6730 were infective (Table 5). Air-dried and fresh blastospore preparations caused significantly higher mortality for *B. argentifolii* when compared to freeze-dried blastospore preparations. Control mortality for all tests averaged less than 1%.

Discussion

The selection of fungal entomopathogens for further development as commercial insect biocontrol agents is possibly the most critical step in this process. Once the pathogen is selected for biopesticide development, a tremendous amount of manpower and capital is mustered for bioassays, field trials, production and stabilization studies, development of formulation and application technology, not to mention product registration. All is for naught if the fungal biopesticide

Table 2. Blastospore yields for different fungal entomopathogens grown in liquid media with various carbon concentrations ([C g l^{-1}]) and carbon-to-nitrogen (C:N) ratios.

Strain and Medium [C g l^{-1}] – C:N	Blastospore yield (spores $ml^{-1} \times 10^7$)		
	Day 2	Day 4	Day7
B. bassiana (ARSEF 5460[a])			
[8] – 10:1	4.2 b,c[b]	4.6 c	24.2 a,b
[8] – 30:1	3.3 c	4.3 c	17.5 a,b
[8] – 50:1	3.8 c	5.3 b,c	10.5 c
[36] – 10:1	5.1 a,b	11.4 a	27.3 a
[36] – 30:1	5.1 a,b	4.9 b,c	11.9 b,c
[36] – 50:1	5.5 a	6.5 b	21.3 a
B. bassiana (ATCC 74250)			
[8] – 10:1	9.8 c	36.3 a,b	57.7 c
[8] – 30:1	10.6 b,c	36.2 a,b	40.2 c,d
[8] – 50:1	11.4 a,b,c	25.6 b	26.0 d
[36] – 10:1	11.6 a,b	23.4 b	122.7 a,b
[36] – 30:1	12.3 a,b	32.8 b	123.9 a
[36] – 50:1	13.5 a	58.0 a	97.0 a,b
M. anisopliae (ARSEF 4901)			
[8] – 10:1	1.5 a	1.4 a	3.3 b
[8] – 30:1	0.4 a	1.6 a	1.9 b
[8] – 50:1	0.4 a	2.0 a	1.8 b
[36] – 10:1	0.8 a	3.1 a	6.6 a
[36] – 30:1	1.2 a	1.5 a	3.9 b
[36] – 50:1	0.8 a	3.7 a	3.9 b
P. fumosoroseus (ARSEF 6730)			
[8] – 10:1	22.9 b	16.2 b	17.2 b
[8] – 30:1	3.9 c	13.3 b	9.9 e
[8] – 50:1	3.7 c	5.4 b	5.1 f
[36] – 10:1	41.2 a	69.0 a	223.6 a
[36] – 30:1	35.6 a,b	57.0 a	98.1 b
[36] – 50:1	21.9 b	14.0 b	57.1 c
P. fumosoroseus (ATCC 20874)			
[8] – 10:1	36.5 b	50.9 b	50.0 b
[8] – 30:1	11.5 c	24.3 c	27.3 c,d
[8] – 50:1	11.4 c	19.6 c	23.8 d
[36] – 10:1	60.1 a	97.1 a	268.7 a
[36] – 30:1	42.9 a,b	79.3 a,b	43.4 b,c
[36] – 50:1	39.3 a,b	68.8 a,b	52.0 b
P. fumosoroseus (ARSEF 4502)			
[8] – 10:1	25.9 a	39.8 b	24.7 d
[8] – 30:1	19.6 a	16.0 c	17.1 d
[8] – 50:1	11.2 b	12.7 c	10.1 e
[36] – 10:1	28.1 a	83.1 a	133.2 a
[36] – 30:1	26.6 a	75.4 a	85.9 b
[36] – 50:1	22.4 a	40.5 b	52.0 c

Cultures were grown in baffled Ehrlenmeyer flasks in a rotary-shaker incubator at 300 rev min^{-1} and 28 °C.

[a] ARSEF denotes the USDA, ARS, Collection of Entomopathogenic Fungal Cultures (Ithaca, New York, USA; Humber 1992); ATCC denotes the American Type Culture Collection (Manassas, Virginia, USA).

[b] Numbers within a column for each specific strain, followed by different letters are significantly different using Tukey–Kramer's adjustment for multiple comparisons. Comparisons were made using cubic root transformed data.

Table 3. Spore yields and desiccation tolerance for *Paecilomyces fumosoroseus* strains ARSEF 6730 and 3581 grown in liquid media containing 25 or 45 g casamino acids l^{-1}.

Strain	Casamino acids (g l^{-1})	Day 2 yield (spores $ml^{-1} \times 10^7$)	Germination after drying (%)	Moisture after drying (%)
P. fumosoroseus	25	65	77	3.9
(ARSEF 6730)	45	59	81	3.8
P. fumosoroseus	25	72	71	3.2
(ARSEF 3581)	45	67	74	3.5
		nsd[a]	nsd	nsd

Desiccation tolerance is measured as survival after air-drying.

[a] nsd = Mean value shown in columns are not significantly different using Tukey–Kramer's adjustment for multiple comparisons.

Table 4. Blastospore yields for *Paecilomyces fumosoroseus* and *Beauveria bassiana* cultures grown in standard production media containing 25 g casamino acids l^{-1}.

Strain	Blastospore yield (spores $ml^{-1} \times 10^7$)			
	Day 2	Day 3	Day 4	Day 7
P. fumosoroseus (ARSEF 6730)	72 a[a]	118 a	107 a	112 a
B. bassiana (ATCC 74250)	15 b	45 b	60 b	104 a

[a] Numbers within a column followed by different letters are significantly different using Tukey–Kramer's adjustment for multiple comparisons.

Table 5. Mortality of third instar silverleaf whitefly (*Bemisia argentifolii*) nymphs sprayed with air-dried, freeze-dried, and fresh blastospores of *P. fumosoroseus* ARSEF 6730.

Spore preparation	Nymph mortality (%)
Fresh	80 a[a]
Air-dried	88 a
Freeze-dried	65 b
No spores	0.5 c

[a] Numbers within a column followed by different letters are significantly different using Tukey–Kramer's adjustment for multiple comparisons.

cannot be economically produced and stabilized with a good shelf-life. While many fungal pathogens are capable of infecting and killing agronomically important insect pests like the coffee berry borer, few can be produced and stabilized at a cost that provides benefit in an agricultural setting (Wraight *et al*. 2001) To this end, we have attempted to use differing nutritional environments to screen fungal entomopathogens for amenability to liquid culture production with the goal of identifying fungal isolates worthy of additional evaluation for use as a biopesticide against the coffee berry borer.

The nutritional environments tested in these studies represented rich and weak media (high or low carbon concentrations) at differing carbon-to-nitrogen (C:N) ratios (10:1, 30:1, 50:1). Previous studies with the plant pathogenic fungus *Colletotrichum truncatum* showed that liquid culture sporulation was enhanced by growth in weak media with a moderate C:N ratio (30:1) and that biocontrol efficacy against the weedy plant *Sesba-*

nia exaltata was enhanced when conidia were produced in weak media with a lower C:N ratio (Jackson & Bothast 1990; Schisler *et al*. 1991). Furthermore, rich media inhibited submerged sporulation while triggering the formation of microsclerotia of *C. truncatum* (Jackson & Schisler 1995). Conversely, cultures of the entomopathogenic fungus *P. fumosoroseus* tend to produce 'yeast-like' blastospores in liquid media with highest blastospore yields being produced in rich media (Jackson *et al*. 1997). These results suggest that some fungi, like *C. truncatum*, that must grow vegetatively and then differentiate to produce conidia, may require the depletion of essential nutrients to trigger sporulation thus benefitting from the lower nutrient levels in weak media while dimorphic fungi, like *P. fumosoroseus*, that produce blastospores vegetatively obtain optimal spore concentrations with optimal biomass accumulation.

The screening studies showed interesting trends that appear to be genus-specific. All of the *P. fumosoroseus* isolates tested, produced highest spore concentrations when grown in the richest nutritional environment (highest carbon concentration, lowest C:N ratio) suggesting that blastospores are the predominant form of growth (Table 2). The *M. anisopliae* isolate tested sporulated poorly in all media but did produce significantly higher spore concentrations by day 7 in the richest medium (Table 2). The slow spore production rate and low yields do not make this a strong candidate for further evaluation. The two *B. bassiana* isolates evaluated in this study showed markedly different sporulation patterns. In general, *B. bassiana* ATCC 74250 produced higher spore concentrations at all sampling times (Table 2). Interestingly, the day 4 measurement showed that both the weak media with higher nitrogen content (lowest C:N ratios) and the rich medium with the lowest nitrogen content all produced the highest spore concentrations. By day 7, all the cultures grown in rich media, regardless of C:N ratio, had produced significantly higher spore concentrations compared to the cultures grown in weak media. Since *B. bassiana* has been reported to produce blastospores and submerged conidia in liquid culture (Thomas *et al*. 1987; Rombach 1989; Hegedus 1990), it is possible that the 7 day cultures are producing submerged conidia in response to nutrient depletion. Further studies are required to confirm this hypothesis. The relatively slow rate of spore production and low yields do not support further evaluation of *B. bassiana* ARSEF 5460 isolated from the coffee berry borer (Table 2).

From the screening experiment, *P. fumosoroseus* ARSEF 6730 and *B. bassiana* ATCC 74250 were candidates for further evaluation. A comparison of spore production rates and yields for these two fungi using optimal production conditions for *P. fumosoroseus* blastospores (Jackson and Cliquet, unpublished) showed that the *B. bassiana* has the potential to attain comparable high spore yields but requires twice the fermentation time (Table 4). Unfortunately, less than 30% of the *B. bassiana* spores survive air-drying (data not shown). Further studies directed at identifying nutritional conditions that may enhance spore production rates and spore desiccation tolerance are warranted.

When blastospores of *P. fumosoroseus* ARSEF 6730 were produced using optimal production conditions and compared to our standard production strain, *P. fumosoroseus* ARSEF 3581, no significant difference was seen in yields or in desiccation tolerance (Table 3). This finding is consistent with our previous production studies with other strains of *P. fumosoroseus* (Jackson *et al*. 1999). As no coffee berry borer colonies were available for efficacy testing, the bioefficacy of blastospores of *P. fumosoroseus* ARSEF 6730 were successfully used to infect and kill the silverleaf whitefly. Testing of this *P. fumosoroseus* isolate against the coffee berry borer will be conducted when feasible. These results have demonstrated that this isolate of *P. fumosoroseus* possesses production and stabilization characteristics required for commercial production and warrants further evaluation as a biopesticide for control of the coffee berry borer.

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