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ORIGINAL PAPER

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Impact of carbon and nitrogen nutrition on the quality, yield and composition of blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*

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Abstract The impact of growing cultures of *Paecilomyces fumosoroseus* in liquid media containing four combinations of glucose and casamino acids (8 g l^{-1} or 80 g l^{-1} glucose, 1.32 g l^{-1} or 13.2 g l^{-1} casamino acids) was evaluated, based on blastospore production, germination rate, viability after freeze-drying and short-term storage stability. When blastospores were produced using a high casamino acid concentration, blastospore yields and germination rates were significantly higher ($13.2–18.5\times10^7$ blastospores ml^{-1}, 50–60% germination after 4 h), compared to cultures grown in media containing lower casamino acid concentrations ($0.4–2.3\times10^7$ blastospores ml^{-1}, 10–20% germination after 4 h). Chemical analyses of blastospore composition showed that accelerated blastospore germination may be related to increased proteinaceous reserves rather than to glycogen or lipid accumulation. Tolerance to freeze-drying by blastospores suspended in spent medium was enhanced by a high initial casamino acid concentration in the culture medium (75% survival) and by the residual glucose concentrations in the spent medium. Under the conditions of this study, the storage stability of blastospores of *P. fumosoroseus* was unaffected by the nutritional condition in which they were produced.

Keywords *Paecilomyces fumosoroseus* · Blastospores · Germination · Freeze-drying · Chemical composition

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Introduction

In general, the successful development of microbial biopesticides depends on the availability of a low-cost production process that yields high concentrations of viable, virulent propagules that can be stabilized to provide a product shelf-life of 12–18 months [9, 19]. The entomopathogenic fungus *Paecilomyces fumosoroseus* is being evaluated as a mycopesticide against numerous agricultural and urban insect pests, including sweetpotato whitefly, *Bemisia tabaci*, silverleaf whitefly, *B. argentifolii*, and Formosan subterranean termite, *Coptotermes formosanus* [1, 23, 28, 29].

In liquid culture, like many deuteromycetes, *P. fumosoroseus* grows hyphally for a short period of time and then yeast-like to produce blastospores [9, 13, 17]. Methods have been developed to produce high concentrations of desiccation-tolerant blastospores, using liquid culture fermentation [17, 18, 27]. These liquid culture-produced blastospores of *P. fumosoroseus* have been shown to rapidly infect and kill whiteflies and subterranean termites in laboratory bioassays and field trials [17, 21, 29]. The improved infectivity of blastospores compared to conidia of *P. fumosoroseus* appears to be associated with their rapid rate of germination [25]).

Lane et al. [20] evaluated the influence of carbon- and nitrogen-limited media on the production and quality of blastospores of the deuteromycete *Beauveria bassiana*. These authors noted that the germination rate and survival of *B. bassiana* blastospores during storage in liquid was associated with appropriate concentrations of nitrogen and carbon in the culture medium. The production of blastospores that germinate more rapidly has the potential to increase infectivity, as previously shown in studies on the pathogenicity of deuteromycetes like *Metarhizium anisopliae* and *P. fumosoroseus* on *Spodoptera frugiperda* and *Manduca* larvae, respectively [8, 10].

Our previous studies measured the influence of various carbon or nitrogen sources on the freeze-drying

tolerance of blastospores of *P. fumosoroseus* [3]. In the present study, we evaluated media containing two concentrations of casamino acids and two concentrations of glucose known to impact the yield and desiccation tolerance of blastospores of *P. fumosoroseus*. Under these differing nutritional conditions, the impact of carbon and nitrogen nutrition was evaluated by measuring blastospore yield, germination rate, desiccation tolerance and storage stability and comparing it to the utilization of substrates and the accumulation of protein, lipid and glycogen within developing blastospores.

Materials and methods

P. fumosoroseus isolate

P. fumosoroseus strain Mycotech 612 was obtained from S. Wraight (Mycotech Corp., Weslaco, Tex.). Stock cultures of *P. fumosoroseus* were maintained and spore inocula were produced as previously described [17].

Media and culture conditions

The basal salt medium supplemented with various glucose and casamino acid concentrations was previously described [16]. Carbon concentration and carbon:nitrogen ratio calculations were based on 40% carbon in glucose and 53% carbon, 8% nitrogen in casamino acids (Difco Laboratories, Detroit, Mich.). Initial water potential for media with various glucose and casamino acid concentrations was measured using a thermocouple psychrometer (SC-10; Decagon Devices, Pullman, Wash.). Culture media were inoculated with 1×10^4 conidia ml^{-1} obtained from sporulated, potato/dextrose agar plates, as previously described [17]. Cultures (100 ml) were grown in 250-ml shake-flasks at 300 rpm and 28°C. After 4 days of growth, cultures were harvested and blastospore concentrations were measured microscopically, using a hemacytometer.

For spent medium replacement studies, blastospores were pelleted by centrifugation (GSA rotor, RC5C Sorvall centrifuge; Kendro Laboratory Products, Asheville, N.C.) at 34 *g* for 5 min. The supernatant was discarded. The blastospore pellet was resuspended in an aqueous solution containing 2.5% glucose and centrifugation was repeated. The blastospore final pellet was resuspended in a 2.5% glucose solution.

Freeze-drying experiments

Freeze-drying was performed in a tray-dryer (Durastop-MP; FTS Systems, Stone Ridge, N.Y.), using an automatic-eutectic program that detects the eutectic point of the sample and sets the parameters for primary and secondary drying based on this information. The shelf temperature at the end of freeze-drying was set at 15°C. Blastospore suspensions derived from cultures grown in various nutritional environments were suspended in either spent medium or 2.5% glucose solution. The viability of these blastospore suspensions was assessed prior to freeze-drying. Blastospore suspensions (2.5 ml) were placed in individual 10-ml vials for freeze-drying. After drying, vials were sealed under vacuum with rubber stoppers. Blastospore viability was assessed at various time-points by opening three individual vials from each treatment and measuring blastospore germination.

Blastospore storage stability studies

In storage stability studies, we evaluated the viability of fresh, suspended blastospores and the viability of blastospores after freeze-drying. Blastospores produced in the various nutritional conditions were pelleted and resuspended in either spent medium or 2.5% glucose solution. For wet storage, blastospore suspensions (3 ml) were placed in sterile 15-ml capped centrifuge tubes and stored at 4°C. For freeze-dried blastospore preparations, vacuum-sealed vials containing dried blastospore preparations were stored at 25°C. Tubes and vials were sampled in triplicate for blastospore viability after various storage times. Because of the low blastospore yields in media containing 1.32 g l^{-1} casamino acids and 8 g l^{-1} glucose, storage experiments were not undertaken for this treatment.

Blastospore viability and germination rate studies

Except for the viability of fresh blastospore suspensions used as controls in freeze-drying experiments, blastospore viability for wet, freshly produced or stored blastospore suspensions was determined using the following method. A blastospore suspension (2 ml, approx. 10^8 blastospores ml^{-1}) was placed in a 250-ml baffled flask containing 50 ml of potato/dextrose broth (Difco). Cultures yielding less than 10^8 blastospores ml^{-1} were centrifuged and concentrated to obtain a spore concentration of 10^8 blastospores ml^{-1}. The flasks were placed on a rotary shaker and incubated at 300 rpm and 28°C. Samples were taken at 2, 4, 6 and 8 h post-inoculation and one drop of 2 N HCl was added to each 2-ml sample to halt the germination process. Blastospore germination was assessed microscopically by viewing 100 blastospores and counting the number of blastospores with germ tube formation. Germ tubes equal to or greater than the length of the spore were considered positive for germination.

For freeze-drying experiments, blastospore viability was determined for fresh suspensions and freeze-dried blastospore preparations. Freeze-dried blastospore pellets were vigorously vortexed with 3 ml of potato/dextrose broth. A 0.5-ml aliquot of the blastospore suspension was immediately transferred to a 15-ml sterile centrifuge tube containing 3 ml of potato/dex-

trose broth, capped and incubated at 300 rpm and 28°C for 6 h, at which time one drop of 2 N HCl was added to each tube to halt the germination process. Spore viability was assessed using the previously described germination assay.

For germination rate studies, blastospores were pelleted by centrifugation (GSA rotor) at 34 *g* for 3 min. The supernatant was discarded. The blastospore pellet was resuspended in potato/dextrose broth at a final concentration of approximately 10^6 blastospores ml^{-1}. Then 3 ml of the blastospore suspension was immediately transferred to a 15-ml sterile centrifuge tube, capped and incubated at 300 rpm and 28°C. A 0.1-ml suspension was sampled every hour and germination assessed microscopically as previously described.

Compositional analyses

Glycogen was extracted according to the method described by Inch et al. [13]. The glucose released from glycogen was used as a measure of the intracellular glycogen. For intracellular glucose determination, the whole *P. fumosoroseus* culture was rinsed in phosphate buffer (pH 6.5) containing polyethylene glycol 200 (14%) and sodium azide (0.1%). This suspension developed a water potential of −2,500 kPar to prevent any cell leakage. Blastospores were centrifuged at 34 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in phosphate buffer with 0.1% sodium azide. Mechanical disruption of blastospores was performed using a mini bead-beater analytical cell disrupter (Biospec Products, Bartlesville, Okla.) with 0.5 mm diam. glass beads. Glucose was measured colorimetrically using oxidase–peroxidase coupled to *o*-dianisidin (Sigma, St Louis, Mo.). Dry weights, lipids, protein and carbohydrate concentrations in whole cultures were measured as previously described [14].

Glucose concentrations in the supernatant were determined using high performance liquid chromatography as previously described [15].

Statistical analysis

Duplicate flasks were used for each treatment in all experiments and all experiments were repeated twice. Duplicate samples from each blastospore preparation were used to measure blastospore viability. Descriptive statistics and analysis of variance (ANOVA) were performed with StatGraphics ver. 4.0 (Manugistics, Rockville, Md.). Mean values were subjected to ANOVA and were separated using the least significant difference (LSD) test (*P*-value = 0.05).

For data not suitable for ANOVA, standard error values were estimated as a measure of variance.

Results

Yields and germination of freshly harvested blastospores

Blastospores were produced in media supplemented with two casamino acid concentrations (1.32 g l^{-1}, 13.2 g l^{-1}) and two glucose concentrations (8 g l^{-1}, 80 g l^{-1}), resulting in four different growth media (Table 1). Maximal blastospore yields after 4 days of growth were produced in media with 13.2 g l^{-1} casamino acids, regardless of the initial glucose concentration (8 g l^{-1}, 80 g l^{-1}). Glucose was exhausted after 2 days of growth in medium supplemented with 8 g l^{-1} glucose and 13.2 g l^{-1} casamino acids.

The germination rate of blastopores freshly harvested from media containing a high initial casamino acid concentration (13.2 g l^{-1}) was 50% higher (95% germination) than the germination rate of blastospores produced in a low casamino acid concentration (1.32 g l^{-1}; 23% and 46% germination), regardless of the initial glucose concentration in the medium (Table 1).

The germination kinetics of blastospores produced in the four culture conditions showed that blastospores produced in media with 13.2 g l^{-1} casamino acids germinated more rapidly than those produced in media

Table 1 Influence of casamino acid and glucose concentrations on the production, germination and freeze-drying tolerance of blastospores of *P. fumosoroseus*. *Spent medium*: blastospores were suspended in spent medium prior to freeze-drying. *Glucose*: blastospores were suspended in 2.5% glucose solution prior to freeze-drying. *ND* Not determined. Data followed by the same letter are not significantly different (LSD test, $P = 0.05$)

Initial culture conditions			Growth parameters					Blastospore viability (% germination)		
			Glucose (g l^{-1}) in spent medium				Blastospore yield $\times 10^7$ ml^{-1} after 4 days of growth	After 4 days of growth	After freeze-drying	
Casamino acids (g l^{-1})	Glucose (g l^{-1})	Water potential (kPa)	Day						Spent medium	Glucose
			1	2	3	4				
1.32	8	ND	8.5	6.5	4.5	3	5.36c	23.5c	12.0b	11.7 ± 4.1
1.32	80	−1,480	81	74	74	72	8.47c	46.2b	7.7b	17.5 ± 8.7
13.2	8	−900	8	0	0	0	59.22b	94.0a	16.2b	86.0 ± 1.8
13.2	80	−2,400	85	80	69	49	84.75a	96.0a	74.7a	73.5 ± 5.8

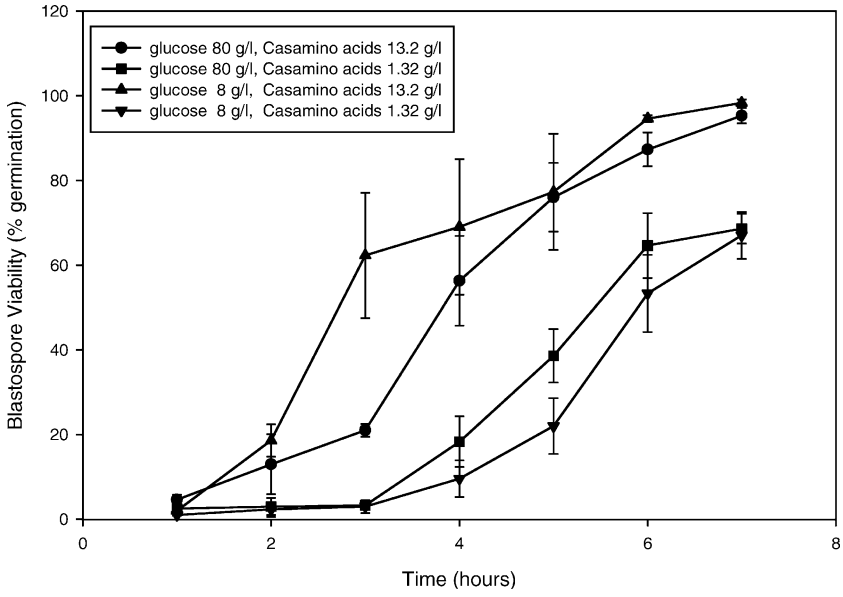

Fig. 1 Influence of the casamino acids and glucose concentrations in the culture medium on the germination rate of blastospores of *P. fumosoroseus*

with 1.32 g l^{-1} casamino acids (Fig. 1). Fifty percent of blastospores grown in media containing 13.2 g l^{-1} casamino acids germinated within the first 2–4 h, whereas 5–6 h of incubation was necessary for 50% germination of blastospores grown in 1.32 g l^{-1} casamino acids. The germination rate of blastospores produced in media with a low casamino acids content and incubated for 6 h is different in Fig. 1 (approx. 55% germination) and in Table 1 (23–46% germination). These differences in the germination rate are probably related to the modification of protocol used to evaluate the percent germination. In the kinetics experiment, viability of blastospores was assessed using flasks, whereas centrifuge tubes were used to determine the viability of blastospores in freeze-drying experiments. The oxygen transfer is likely to be higher in flasks than in tubes, thus leading to an increase in the germination rate of blastospores in flasks.

Blastospore viability after freeze-drying

Survival of non-rinsed and rinsed blastospore was optimal (74.7% survival) after freeze-drying when *P. fumosoroseus* cultures were grown in media with high nitrogen and carbon concentrations (13.2 g l^{-1} casamino acids, 80 g l^{-1} glucose, Table 1). Blastospores produced in media containing a high initial casamino acid

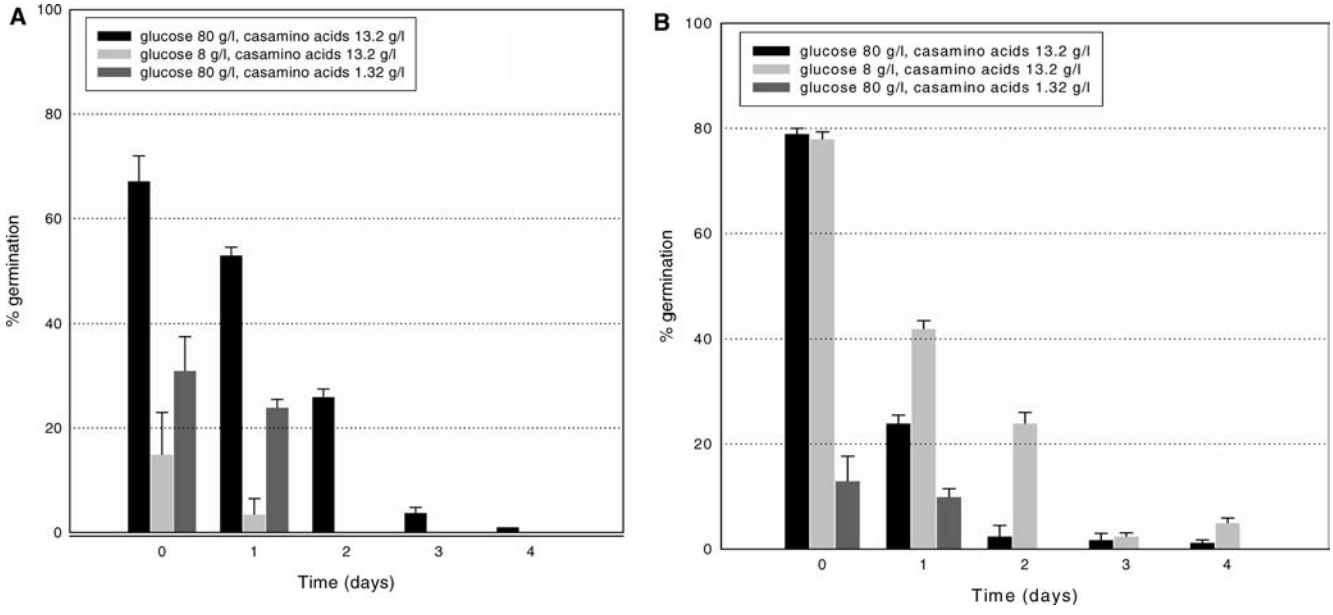

Fig. 2 Influence of medium composition on the survival of freeze-dried blastospores of *P. fumosoroseus* stored at 25°C. **a** Blastospores suspended in spent medium prior to freeze-drying. **b** Blastospores suspended in 2.5% glucose prior to freeze-drying

Table 2 Compositional analysis of *P. fumosoroseus* biomass after 4 days of growth

Initial culture conditions		Endogenous reserves (mg/mg DW)×100			
Casamino acids (g l^{-1})	Glucose (g l^{-1})	Dry weight (mg ml^{-1})	Lipid	Glycogen	Protein
1.32	8	2.5 ± 0.3	15.6 ± 3.8	3.8 ± 0.9	8.9 ± 0.6
1.32	80	2.9 ± 0.2	21.8 ± 2.5	1.4 ± 0.6	10.0 ± 0.6
13.2	8	7.2 ± 0.2	2.6 ± 0.5	0.8 ± 0.04	28.4 ± 2.3
13.2	80	14.8 ± 0.3	8.2 ± 0.2	2.8 ± 0.6	18.8 ± 1.5

concentration (13.2 g l^{-1}) but different glucose levels showed significant differences in freeze-drying tolerance (74.7%, 16.2% survival) when freeze-dried in spent medium. The freeze-drying tolerance was similar when these blastospores were suspended in 2.5% glucose solution prior to freeze-drying (Table 1). With a high initial glucose concentration (80 g l^{-1}) in the medium, blastospores produced in media containing a high initial casamino acid concentration had significantly higher survival rates (74.7%) compared to blastospores produced in media with lower casamino acid concentrations (12.0%, 7.7%). This difference in blastospore survival was associated with the initial level of casamino acids in the culture medium and remained significant when blastospores were suspended in a 2.5% glucose solution prior to freeze-drying (Table 1).

Blastospore viability during short-term storage

Freshly harvested blastospores were maintained in spent medium or in a 2.5% glucose solution and stored at 4°C. The germination rate of blastospores at 4 weeks storage was less than 30%, regardless of whether blastospores were produced in high or low casamino acid concentrations (13.2 g l^{-1} or 1.32 g l^{-1}), or whether blastospores were produced in high or low glucose concentrations (80 g l^{-1} or 8 g l^{-1}). The mortality rate was similar for blastospores suspended in spent medium or in 2.5% glucose solution (data not shown).

Freeze-dried samples were kept under vacuum and stored at 25°C. In general, storage was detrimental for freeze-dried blastospores in all treatments (Fig. 2). No freeze-dried blastospores germinated after 4 days of storage at 25°C, regardless of whether blastospores were suspended in spent medium (Fig. 2a) or in 2.5% glucose solution prior to freeze-drying (Fig. 2b). The mortality rate of blastospores produced in 80 g l^{-1} glucose and suspended in the spent medium was comparable, regardless of the initial casamino acid concentration in the medium (1.32 g l^{-1} or 13.2 g l^{-1}).

Endogenous reserves

The accumulation of endogenous reserves in *P. fumosoroseus* blastospores was affected by the casamino acid concentrations in the culture medium (Table 2). The intracellular lipid content increased when *P. fumosoroseus* blastospores were produced in media with a low casamino acid concentration (15.6–21 mg lipids mg^{-1} dry weight; DW) compared to the lipid content in blastospores produced in media with a high casamino acids content (2.6–8.2 mg lipids mg^{-1} DW). Intracellular proteins accumulated when *P. fumosoroseus* blastospores were produced in media containing a high concentration of casamino acids (28.4–18.8 mg proteins mg^{-1} DW).

Discussion

These studies demonstrated that carbon and nitrogen nutrition significantly impact the yield and quality of blastospores produced by cultures of *P. fumosoroseus*. Higher blastospore yields and biomass accumulations were produced in cultures grown in media supplemented with higher levels of glucose and nitrogen (Tables 1, 2). In media supplemented with lower concentrations of casamino acids, nitrogen depletion was likely responsible for the decrease in blastospore yields observed. Previous studies with *P. fumosoroseus* and other dimorphic, entomopathogenic fungi that grow yeast-like to produce blastospores showed that media supplemented with the highest nitrogen concentration supported the highest blastospore production rates [17, 26].

Additionally, *P. fumosoroseus* blastospores produced in media supplemented with a higher casamino acid concentration contained higher concentrations of protein, lower concentrations of lipid and germinated more rapidly than blastospores produced in media with a lower casamino acid concentration (Fig. 1, Table 2). Accelerated blastospore germination rates may be related to increased proteinaceous reserves rather than to glycogen or lipid accumulation. Increased protein content in conidia of the bioherbicidal fungus *Colletotrichum truncatum* was also shown to increase the rate of spore germination and the frequency of appressoria formation on *Sesbania exaltata* seedlings [14, 22]. In contrast to these findings, Lane et al. [20] found that *Beauveria bassiana* blastospores produced in nitrogen-limited media also accumulated higher concentations of lipids and glycogen but had an enhanced, rather than lessened, germination rate on leafhoppers (*Nephottetix virescens*) wings. These findings reveal that the fungal environment and/or fungal organism can interact with the culture conditions and strongly influence the germination rate of blastospores and conidia. Additional

experiments are needed to determine whether these increased proteinaceous reserves are actually involved in enhancing the germination rate of blastospores of *P. fumosoroseus* and, if they are, how they carry out this function.

In media with low carbon and high nitrogen concentrations, the drastic decrease (78%) in blastospore viability after freeze-drying was likely related to the total exhaustion of glucose in the spent medium (Table 1). Blastospore survival was restored when these blastospores were suspended in 2.5% glucose solution prior to freeze-drying, as previously described [3]. Other studies have also shown that the presence of glucose in the supernatant stabilized fungal cells during desiccation [2, 24]. The beneficial effect of sugars on membrane and protein stability is widely reported [4–7]. Similarly, our previous studies with *P. fumosoroseus* blastospores showed that the freeze-drying of blastospores produced under appropriate nutritional conditions in a carbohydrate-rich suspension (10% lactose, 1% bovine serum albumin) supported high blastospore survival rates [17].

When produced in carbon-rich media that provided adequate residual glucose concentrations in the spent medium, blastospores produced in media with a higher concentration of casamino acids survived freeze-drying better than blastospores produced in media with a lower concentration of casamino acids (Table 1). This difference in blastospore tolerance to freeze-drying appears to be related to the initial casamino acids content in the growth medium and the increased accumulation of protein (Tables 1, 2). Whether accumulation of these endogenous, proteinaceous reserves is correlated to enhanced desiccation tolerance requires further investigation.

Studies on desiccation tolerance show that the accumulation of intracellular compounds such as trehalose and glycerol, and K^+, as a consequence of lowering the water potential of the growth medium, can improve cell survival [11, 12]. In this study, the variation in water potential related to the difference in casamino acid concentration (1.3 g l^{-1} or 13.2 g l^{-1}) is 150 kPa. This difference in osmotic pressure is probably too small to induce the accumulation of intracellular osmolytes.

Under the conditions of this study, modifications of casamino acid and glucose concentrations in the culture medium had no impact on the shelf-life of blastospores suspensions stored at 4°C, nor on freeze-dried blastospores stored at 25°C. These results disagree with other studies with blastospores of *B. bassiana* that showed that nitrogen-limited blastospores survived longer than carbon-limited blastospores and accumulated more endogenous reserves [20]. These reserves were depleted during storage, allowing the fungus to survive longer. In our experiments, carbon- and nitrogen-limited blastospores accumulated lipids with no significant effect on shelf life. Additionally, our previous studies with *P. fumosoroseus* blastospores freeze-dried in the presence of disaccharides such as lactose and sucrose showed very good storage stability at 4°C ([17]; unpublished data). While carbohydrates like glucose appear to enhance the desiccation tolerance of blastospores during freeze-drying [3], storage stability is unaffected.

In conclusion, a higher nitrogen concentration in the liquid production medium significantly improved the yield, germination rate and desiccation tolerance of blastospores of *P. fumosoroseus*. A more rapid germination rate for blastospores of *P. fumosoroseus* should improve its potential for infecting the target insect host by reducing the time requirements for free moisture and lessening the effects of insect grooming and molting. Although high levels of glucose do not necessarily increase blastospore yields, residual glucose in the spent medium does appear to stabilize blastospores of *P. fumosoroseus* during the freeze-drying process.

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