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Influence of culture conditions on production and freeze-drying tolerance of *Paecilomyces fumosoroseus* blastospores

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With the goal of developing a defined medium for the production of desiccation-tolerant blastospores of the bio-insecticidal fungus *Paecilomyces fumosoroseus*, we evaluated the impact of various media components such as amino acids, carbohydrates, trace metals and vitamins on hyphal growth and sporulation of *P. fumosoroseus* cultures and on the freeze-drying tolerance of blastospores produced under these conditions. A comparison of 13 amino acids as sole nitrogen sources showed that glutamate, aspartate, glycine and arginine supported biomass accumulations (12–16 mg ml^{-1}) and blastospore yields (6–11 $\times$ 10^8 blastospores ml^{-1}) comparable to our standard production medium which contains casamino acids as the nitrogen source. Using glutamate as the sole nitrogen source, tests with various carbohydrates showed that *P. fumosoroseus* grew best on glucose (18.8 mg biomass ml^{-1}) but produced similar blastospore concentrations (7.3–11.0 $\times$ 10^8) when grown with glucose, glycerol, fructose or sucrose. *P. fumosoroseus* cultures grown in media with sodium citrate or galactose as the sole carbohydrate produced lower blastospore concentrations but more-desiccation-tolerant spores. Zinc was the only trace metal tested that was required for optimal growth and sporulation. In a defined medium with glutamate as the nitrogen source, vitamins were unnecessary for *P. fumosoroseus* growth or sporulation. When blastospores were freeze-dried in the absence of a suspension medium, residual glucose (>2.5% w/v) was required for enhanced spore survival. Thus, a defined medium containing basal salts, glucose, glutamate and zinc can be used to produce optimal concentrations of desiccation-tolerant blastospores of *P. fumosoroseus*.

Keywords: *Paecilomyces fumosoroseus*; carbohydrates; amino acids; vitamins; trace minerals; growth; freeze-drying; water potential; trehalose

Introduction

Bemisia tabaci Gennadius (sweet potato whitefly, cotton whitefly, tobacco whitefly, including *B. argentifolii* silverleaf whitefly: *B. tabaci* biotype B [1]), is considered a major pest of economically important crops worldwide. Due to the increasing resistance of whiteflies to many synthetic insecticides, biological control is a promising control strategy. The deuteromycete *Paecilomyces fumosoroseus* is a pathogen of numerous insect species and is one of the most common fungi found attacking whitefly nymphs and adults [17]. *P. fumosoroseus* causes dramatic epizootics in *B. tabaci* in glasshouses, in open shade cloth-protected structures [24,25], and in the field [29].

For field application, an economical, large-scale mass production method for *P. fumosoroseus* must be developed. Submerged culture processes offer many advantages over conidial production in solid-state fermentation [19]. In liquid culture, *P. fumosoroseus* produces hyphae and blastospores [8]. Recent studies suggest that liquid culture-produced blastospores of *P. fumosoroseus* effectively control silverleaf whiteflies [15]. The commercial success of a liquid culture production method for blastospores of *P. fumosoroseus* requires short fermentation times and high yields of infective, stable blastospores. Blastospore stability as a dry preparation is generally required for use as a biocontrol agent.

Nutritional studies have been undertaken for improving the growth and sporulation of deuteromycetes like *Beauveria bassiana*, *Metarhizium anisopliae*, and *P. fumosoroseus* [5,6,13,14,26]. Studies with *B. bassiana* showed that nutrition regulated the production of conidia or blastospores in submerged culture [3,28]. Blastospores of *B. bassiana* produced in nitrogen-limited media contained more carbohydrate, glycogen and lipid and survived longer during storage in quarter strength Ringer's solution when compared to blastospores produced in carbon-limited media [18]. Our recent studies with submerged cultures of *P. fumosoroseus* have led to the development of a semi-defined liquid medium which yields high concentrations of desiccation-tolerant blastospores [15]. The nitrogen content of the medium affected blastospore yield and desiccation tolerance [15]. High concentrations of desiccation-tolerant blastospores were rapidly produced in media containing between 13 and 40 g casamino acids per liter.

In order to understand how the components of our blastospore production medium impact on *P. fumosoroseus* growth, sporulation and blastospore desiccation tolerance, we sought to develop a totally defined blastospore production medium. Since the only undefined component of the medium is the nitrogen source, casamino acids and various synthetic amino acids were tested as replacement nitrogen sources. Glutamate provided a suitable nitrogen source

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for our defined medium. This glutamate-based defined medium was used to evaluate the impact of various carbohydrates, trace minerals and vitamins on blastospore yield and desiccation tolerance. In addition, the presence or absence of residual sugars in the suspension medium during freeze-drying was examined in terms of blastospore survival after drying.

Materials and methods

Paecilomyces fumosoroseus isolate

Paecilomyces fumosoroseus (Mycotech strain 612) was obtained from S Wraight, Mycotech Corporation, Butte, MT, USA. Stock cultures of *P. fumosoroseus* strain 612 were grown as a single-spore isolate on potato dextrose agar (Difco Laboratories, Detroit, MI, USA) at room temperature and stored as 1-mm potato dextrose agar plugs in 10% glycerol at –80°C. For liquid cultures, conidia were produced by growing glycerol stock cultures of *P. fumosoroseus* on potato dextrose agar at room temperature for 2 weeks.

Basal and defined media

The basal medium [15] contained per liter: glucose 80 g; casamino acids 13.2 g; KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 50 mg. Three individual trace mineral stock solutions were prepared to make final concentrations of (g L^{-1}): $ZnSO_4 \cdot 7H_2O$, 14 mg; $MnSO_4 \cdot H_2O$, 16 mg; $CoCl_2 \cdot 6H_2O$, 37 mg. A stock solution of vitamins was prepared to make final concentrations of (g L^{-1}): thiamine, riboflavin, pantothenate, niacin, pyridoxamine and thioctic acid, 500 μg of each; folic acid, biotin and vitamin B12, 50 μg of each. A stock solution of 20% glucose (w/v) was autoclaved separately. In the defined medium, casamino acids were replaced by glutamic acid, 9 g L^{-1}. This defined medium was used for all trace metal, vitamin and carbon source studies.

Nutritional studies

In nitrogen source studies, casamino acids were replaced by one of the following: l-arginine, l-aspartic acid, l-glutamic acid, glycine, l-histidine, l-isoleucine, l-leucine, lysine, methionine, phenylalanine, threonine, tyrosine or valine. These amino acids were incorporated individually to provide nitrogen at the casamino acids amino nitrogen concentration (0.85 g N L^{-1}). The carbohydrate used in these studies was glucose, 80 g L^{-1}.

In carbohydrate source studies, glucose (80 g L^{-1}) was replaced by one of the following: sodium acetate, sodium citrate, galactose, glucose, glycerol, fructose or sucrose at a concentration which provided the medium with 32 g carbon L^{-1}. For each carbohydrate, 20% (w/v) stock solutions were autoclaved separately. Initial water potential for media with various carbohydrates was measured using a thermocouple psychrometer (SC-10, Decagon Devices, Inc, Pullman, WA, USA). The nitrogen source for carbohydrate studies was glutamic acid, 9 g L^{-1} (0.85 g N L^{-1}).

The effect of vitamins on *P. fumosoroseus* growth and sporulation was tested by replacing the vitamin stock solution with deionized water. In trace metal studies, the effect of cobalt, manganese and zinc was tested individually by replacing each stock solution normally present in the medium with deionized water. For trace metal studies with iron, $FeSO_4 \cdot 7H_2O$ was not added to the medium.

Inoculum preparation and culture conditions

Prior to inoculation, each medium was adjusted to pH 6 with either 2 N HCl or 2 N NaOH. Conidial suspensions used to inoculate media were obtained by rinsing the spores from a potato dextrose agar plate with sterile distilled water. The initial conidial concentration for all liquid cultures was 1×10^4 conidia ml^{-1}. Cultures (100 ml) were grown in 250-ml shake flasks at 300 rpm and 28°C. A pH of 6 was maintained by daily addition of 2 N NaOH or 2 N HCl.

Harvest and growth measurements

Cultures were harvested after 4 days' growth. Blastospore concentrations were measured microscopically using a hemacytometer after appropriate dilution. In all experiments except trace metal studies, blastospore concentrations were measured in whole cultures. Blastospore concentrations in trace metal studies were measured after mycelial biomass removal by filtration. For dry weight determinations, a 1.0-ml culture was vacuum-filtered on a predried, weighed 2.5-cm filter (GF/A; Whatmann Inc, Clifton, NJ, USA) and washed with 1 ml deionized water. Filters were dried at 100°C for 12 h and weighed. Each dry weight determination was repeated twice. Glucose concentrations in the spent medium were determined using a high performance liquid chromatographic (HPLC) system (Thermo Separation Products, Riviera Beach, FL, USA) fitted with an Aminex ion exclusion column (model HXP-87H, Bio-Rad Laboratories, Richmond, CA, USA).

Freeze-drying experiments

Blastospore concentrations were adjusted to 6.5–16×10^7 ml^{-1}. A 3-ml blastospore suspension was placed in each 10-ml vial. In some treatments, the spent medium was replaced by glucose at various concentrations to evaluate the effect of spent medium on blastospore desiccation tolerance. For spent medium replacement studies, blastospores were centrifuged at $100 \times g$ for 5 min and the supernatant was discarded. The pellet was then suspended in an aqueous glucose solution, centrifuged and the supernatant was discarded. The resulting blastospore pellet was resuspended in solutions containing various amounts of glucose. Freeze-drying of blastospores was performed in a tray-dryer (Durastop-MP, FTS Systems, Stone Ridge, NY, USA) using a eutectic-automatic program that detects the eutectic point of a sample vial and sets the primary and secondary drying parameters based on this information. The shelf temperature at the end of secondary drying was 15°C. After freeze-drying, vials were sealed under vacuum. Blastospore viability was assessed immediately after freeze-drying.

Blastospore viability

Five hundred microliters of fresh harvested blastospore suspension were poured into 15-ml sterile centrifuge tubes containing 3 ml potato dextrose broth (PDB). The capped tubes were placed horizontally on the shaker and incubated at 300 rpm and 28°C. After 6 h incubation, one drop of 2 N

HCl was added to each tube to halt the germination process. After freeze-drying, dried preparations of blastospores were vigorously vortexed with 3 ml of PDB. Suspensions were then processed as described for freshly harvested blastospores. The percentage of germinated blastospores was assessed microscopically by viewing 100 blastospores and counting the number of blastospores with germ tube formation. Survival after freeze-drying was determined as the ratio (% germination after freeze-drying/% germination of freshly harvested blastospores) × 100.

Statistical analysis

For each experiment, duplicate flasks were used. Three samples were taken from each flask for freeze-drying experiments. Each experiment was repeated at least twice. Analysis of variance was conducted on normalized data using Statistix 4.0 (St Paul, MN, USA). The mean of each treatment, in the different experiments, was compared using a Least Significant Difference (LSD) test. For data which were not suited for analysis of variance, standard errors were calculated.

Results

Growth requirements

Under the conditions of this study, aspartate, glutamate, glycine and arginine produced blastospore yields greater or equal to those obtained with casamino acids as the nitrogen source (Table 1). A comparison of sporulation when *P. fumosoroseus* was grown with individual amino acids as the nitrogen source showed that aspartic and glutamic acid produced the highest blastospore yields (~1×10^9 blastospores ml^{-1}), while isoleucine and threonine were poor nitrogen sources for blastospore production ($3–5 \times 10^7$ blastospores ml^{-1} (Table 1). In general, maximal dry weight accumulation was associated with maximal blastospore production, except for growth in media supplemented with tyrosine. In the tyrosine medium, poor sporulation (2.4×10^8 blastospores ml^{-1}) occurred in association with a moderately high dry weight accumulation (11.7 mg ml^{-1}). After 4 days' growth, high glucose consumption was, in general, associated with increased blastospore production and biomass accumulation (Table 1). The average glucose present in the spent medium after 4 days' growth was 66 ± 8.8 g L^{-1}, regardless of the amino acid added.

In defined media with glutamate as the nitrogen source, *P. fumosoroseus* was able to grow and sporulate in the absence of added cobalt, iron or manganese (Figure 1). In media deficient in zinc, growth and sporulation were significantly reduced. Supplementing defined media with vitamins produced no significant difference in biomass accumulation or blastospore yields in *P. fumosoroseus* cultures after 4 days' growth (data not shown). In a glutamate-supplemented defined basal medium, the use of the carbon sources glucose, glycerol, fructose or sucrose all supported statistically similar high spore yields (Table 2). Biomass accumulation was highest in media containing glucose.

Table 1 Growth, sporulation and glucose utilization by 4-day-old cultures of *Paecilomyces fumosoroseus* grown on different amino-acids with glucose (80 g L^{-1}) as the carbon source

Nitrogen source[a]	Blastospores $\times 10^8$ ml^{-1}	Biomass (mg ml^{-1})	Glucose uptake (g L^{-1})
ASP	11.0 (1.5)[b]	16.0 (1.4)	20.2
GLU	10.0 (1.1)	14.9 (0.8)	23.0
GLY	6.6 (1.2)	16.1 (0.6)	31.0
Casamino acids	6.1 (2.1)	14.7 (0.3)	24.8
ARG	6.0 (1.3)	12.2 (1.4)	12.2
LYS	4.3 (0.4)	5.9 (0.6)	18.0
LEU	4.1 (0.2)	7.3 (0.6)	11.2
TYR	2.4 (0.4)	11.7 (0.4)	5.8
MET	1.5 (0.1)	3.7 (0.3)	10.5
PHE	1.4 (0.5)	5.6 (0.8)	13.3
VAL	1.0 (0.2)	2.5 (0.3)	6.0
HIS	1.0 (0.3)	5.4 (1.3)	17.2
ILE	0.5 (0.1)	2.6 (0.4)	7.0
THR	0.3 (0.1)	3.0 (0.8)	5.5

[a]Each amino-acid was added at a concentration of 0.85 g N L^{-1} which corresponds to the percentage of amino-nitrogen provided by casamino acids.
[b]Values in brackets are the standard error for the associated spore or biomass concentration.

Tolerance to freeze-drying

The survival of *P. fumosoroseus* blastospores grown in glutamate as the sole nitrogen source was 67% (±13%) when blastospores were suspended in spent medium and freeze dried (data not shown). The viability of freeze-dried blastospores was 12% when the blastospores were rinsed and resuspended in deionized water prior to freeze-drying (Figure 2). Survival increased when blastospores were rinsed and resuspended in a glucose solution. A significant increase in survival (53%) after freeze-drying was observed when blastospores were rinsed and resuspended in a solution containing 2.5% glucose. Even higher blastospore survival (80%) was obtained when blastospores were rinsed and resuspended in 10–25% glucose (Figure 2). Non-rinsed blastospore survival (67%) was comparable to blastospore survival (70%) after suspension in 6% glucose prior to freeze-drying.

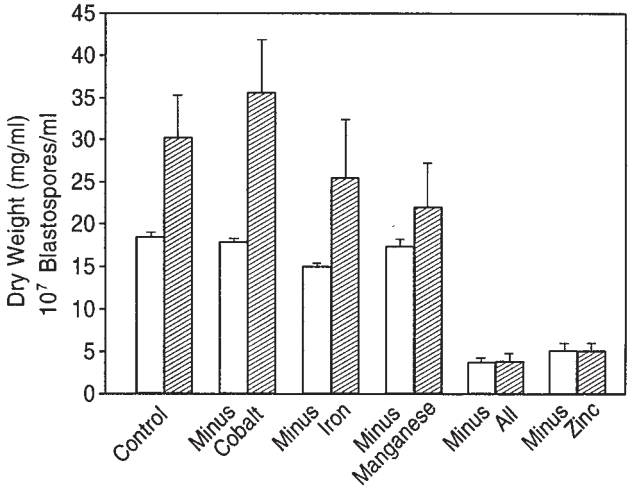

Figure 1 Influence of trace metals on biomass accumulation (□) and sporulation (▨) by liquid cultures of *Paecilomyces fumosoroseus* grown in a glutamate-glucose basal medium, 4-day-old cultures. Bars represent standard errors.

Table 2 The impact of carbohydrate source on growth and sporulation of *Paecilomyces fumosoroseus* in a defined medium with glutamic acid (9 g L^{-1}) as the nitrogen source

Carbohydrate[a]	Initial concentration (g L^{-1})	Carbohydrate concentration at 4 days (g L^{-1})	Blastospores[b] $\times 10^8$ ml^{-1}	Biomass[b] (mg ml^{-1})	Initial water potential (bars)
Glucose	80	48.6	11.0 a[c]	18.8 a	–9.7
Glycerol	81	63.2	9.2 a	10.0 c	–23.2
Fructose	80	51.5	7.7 a	14.6 a	–11.1
Sucrose	76	61.4[d]	7.3 a	16.9 a,b	–5.4
Galactose	80	65.0	4.2 b	9.1 c	–10.8
Sodium citrate	130	83.3	0.4 c	ND	–36.1
Acetic acid	181	ND	ND	poor growth	ND

[a]Each carbon source was added at a concentration providing 32 g carbon L^{-1}.
[b]Measurements taken from 4-day-old cultures.
[c]Mean values followed by the same letter are not significantly different using the Newman–Keuls test ($P = 0.05$).
[d]Sucrose hydrolysed to glucose: 9.9 g L^{-1}; fructose: 51.5 g L^{-1}. ND = not done.

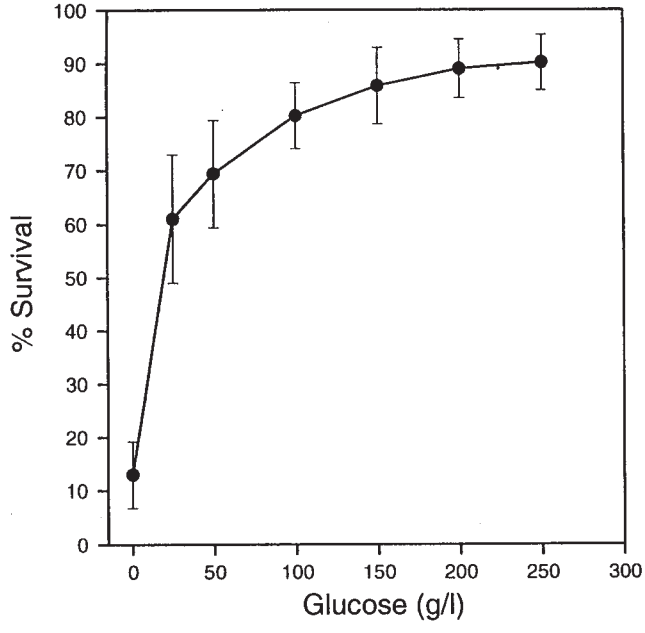

Figure 2 The survival of freeze-dried blastospores of *Paecilomyces fumosoroseus* after rinsing and resuspension in solutions containing various concentrations of glucose. Bars represent standard errors.

No significant difference in blastospore survival after freeze-drying was noted when *P. fumosoroseus* blastospores were produced in media with glutamate, arginine, aspartic acid, glycine, histidine, leucine, lysine, tyrosine or casamino acids as the sole nitrogen source, regardless of whether the blastospores were suspended in spent media or suspended in a 6% glucose solution prior to freeze-drying (data not shown). Elimination of the vitamin mixture or the trace metals cobalt, manganese or iron from our standard medium had no significant impact on the desiccation tolerance of the blastospores produced judged by survival after freeze-drying (data not shown). Blastospores produced in glutamate-supplemented media with galactose or sodium citrate as the sole carbon source were more desiccation-tolerant compared to blastospores produced in media with glucose, fructose, sucrose or glycerol, regardless of whether they were suspended in spent media or 2.5% glucose prior to freeze drying (Figure 3).

Discussion

The source of nitrogen or carbon can affect the growth and sporulation of *Paecilomyces fumosoroseus* in submerged culture. In general, most of the carbohydrates and amino acids tested supported both hyphal growth and sporulation. Under the conditions of this study, cultures grown for 4 days in media with aspartate or glutamate produced blastospore concentrations higher than those obtained using casamino acids as the nitrogen source (Table 1). The ability of tyrosine to inhibit sporulation while supporting biomass accumulation suggests that commercial production media should contain reduced levels of this amino acid.

Phenylalanine inhibited growth but stimulated submerged culture sporulation of the fungus *Beauveria bassiana* [6]. Amino acids other than phenylalanine or tyrosine inhibited sporulation of the fungus *Colletotrichum trunca-*

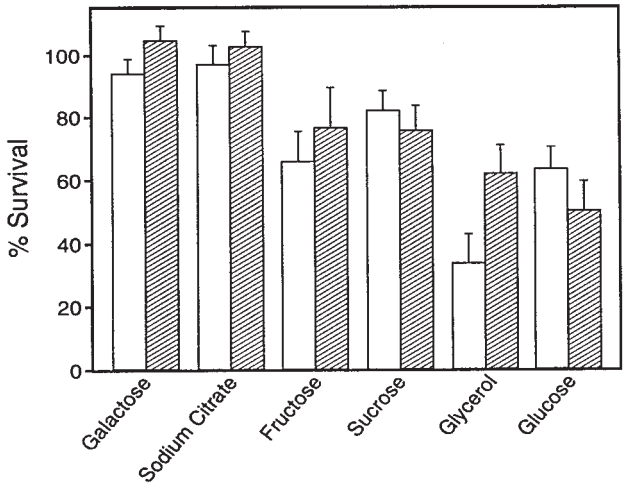

Figure 3 The survival of blastospores of *Paecilomyces fumosoroseus* after freeze-drying when produced in a glutamate-supplemented basal medium with various carbon sources. Prior to freeze-drying, blastospores were suspended in spent culture medium (□) or in a 2.5% glucose solution (▨). Bars represent standard errors.

tum in submerged culture [16]. Our data on *P. fumosoroseus* coupled with these data suggest that the effect of amino acids on submerged culture fungal sporulation is dependent on the fungus being studied.

When *P. fumosoroseus* was grown in defined media containing various individual carbohydrates, numerous carbohydrates supported blastospore yields similar to levels obtained with cultures grown in media containing casamino acids and glucose. These results corroborate previous studies with other entomopathogenic fungi in demonstrating the capacity of these organisms to utilize numerous carbon sources [5,14,21].

The composition of the suspension medium had a significant impact on the survival of blastospores after freeze-drying. When blastospores of *P. fumosoroseus* were rinsed and resuspended in deionized water, survival after freeze-drying was 12%. This survival rate is comparable to values noted for water-rinsed *Escherichia coli* and *Bacillus thuringiensis* cells after drying [20]. Water removal from membrane lipid bilayers during drying leads to destructive events such as protein fusion, liquid crystalline to gel phase transition and increased membrane permeability [7]. The molecular size of sugars affects membrane preservation during drying with disaccharides like trehalose, lactose and sucrose protecting membranes better than their subunits, glucose and galactose [7]. In addition, suspending fungi in various sugars resulted in differences in desiccation tolerance [2,27].

The high freeze-drying tolerance of blastospores rinsed and resuspended in glucose solutions compared to water-rinsed blastospores suggests a protective effect of glucose during freeze-drying. This assertion is supported by the finding that blastospores suspended in spent culture medium, which contained 66 ± 8.8 g L^{-1} glucose, had a high survival rate (67–83%) after freeze-drying. Suspending blastospores in glucose solutions (5% or more) prior to freeze-drying enhances desiccation tolerance.

No difference was found in the freeze-drying tolerance of non-rinsed or glucose-rinsed blastospores grown in media with various amino acids, deficient in trace minerals, or deficient in vitamins. Thus, vitamin and trace metal supplements are unnecessary for producing desiccation-tolerant blastospores of *P. fumosoroseus*. Whether these nutrients are important for product shelf-life or biocontrol efficacy will require additional studies.

Although produced in lower concentrations, blastospores produced in medium containing sodium citrate or galactose survived drying better than blastospores produced in fructose, sucrose, glycerol or glucose (Figure 3). Variation in desiccation tolerance does not appear to be related to dissimilar blastospore concentrations during freeze-drying since previous experiments showed that blastospore concentrations (1 or 1/10, diluted with the spent medium) had no significant effect on tolerance to drying (data not shown). Likewise, the suspension medium during drying was not a factor since blastospore survival in spent culture medium or a 2.5% glucose solution was similar. This finding that improved survival was not solely dependent on the residual sugars in the medium but also on the nutritional environment during growth suggests a physiological modification in the blastospore due to the carbohydrate added to the medium. Similarly our studies with *P. fumosoroseus* showed that the nitrogen content of the growth medium could affect blastospore desiccation tolerance when drying was carried out in the presence of residual sugars [15]. Hallsworth and Magan [9,10,11] showed that pH, temperature and water activity influenced the accumulation of polyols in the conidia of various entomopathogenic fungi grown on solid media, including *P. fumosoroseus*.

How growth in sodium citrate- or galactose-supplemented medium imparts desiccation tolerance to blastospores of *P. fumosoroseus* produced in liquid culture, is unclear. It is possible, that in the case of sodium citrate, the lowered water potential may have improved the desiccation tolerance of the blastospores. Some yeasts and filamentous fungi respond to low water potential by accumulating compounds, like polyols and potassium, a mechanism known as osmoregulation [4,22,23]. The accumulation of trehalose by *Trichoderma harzianum* spores is increased when the fungus is grown at a low water potential and has been associated with spore survival after drying [12]. The water potential developed by the sodium citrate medium is −36.1 bars and is likely to remain low during growth, since sodium citrate is only slightly utilized during growth. On the other hand, the high rate of survival of blastospores produced in a galactose-supplemented medium, initial water potential of −10.8 bars, suggests that factors other than osmotic stress may be involved in enhancing the desiccation tolerance of blastospores of *P. fumosoroseus*.

In conclusion, *P. fumosoroseus* is an omnivorous fungus capable of growth and sporulation on numerous carbon and nitrogen sources. Maximal sporulation occurred when cultures were grown on aspartate or glutamate as sole nitrogen source. This finding led to the development of a glucose-glutamate supplemented defined basal medium which required only zinc supplementation for optimal sporulation in submerged culture. The high yields of desiccation-tolerant spores and the low cost of this glucose-glutamate based medium are attributes which recommend its use for the commercial production of blastospores of *P. fumosoroseus*. In contrast, the use of sodium citrate or galactose as a carbohydrate source enhanced the desiccation tolerance of the *P. fumosoroseus* blastospores but supported significantly lower spore yields. The selection of an appropriate commercial production medium for blastospores of *P. fumosoroseus* will require additional studies directed at determining the shelf-life and subsequent biocontrol efficacy of dried blastospore preparations produced under these differing nutritional conditions.

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