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Mark A. Jackson^a; Angela R. Payne^a

^a National Center for Agricultural Utilization Research, Crop Bioprotection Research Unit, Agriculture Research Service, United States Department of Agriculture, Peoria, IL, USA

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Evaluation of the desiccation tolerance of blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) using a lab-scale, air-drying chamber with controlled relative humidity¹

MARK A. JACKSON & ANGELA R. PAYNE

National Center for Agricultural Utilization Research, Crop Bioprotection Research Unit,
Agriculture Research Service, United States Department of Agriculture, Peoria, IL, USA

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Abstract

The stabilization of living microbial agents for use as biological control agents is often accomplished through desiccation. Our air-drying studies with the entomopathogenic fungus *Paecilomyces fumosoroseus* have shown that the relative humidity (RH) of the drying air significantly affects the desiccation tolerance and the storage stability of blastospores. Drying air with a RH of more than 40% supported significantly higher rates of initial blastospore survival (68–82%) after drying compared to drying with lower relative humidity air. Drying air with a RH above 50% improved the shelf-life of the air-dried blastospore preparations. Adjustment of the pH or replacement of the spent medium with deionized water (d-H₂O) in the blastospore suspension had no significant impact on blastospore desiccation tolerance or storage stability. We have developed and describe a lab-scale, air-drying chamber that delivers air flow over the sample and that can be operated at controlled relative humidity.

Keywords: *Biocontrol, insects, fungi, blastospores, air-drying, relative humidity*

Introduction

The use of living microorganisms to control agronomic and urban pests such as weeds and insects has gained interest in recent years due to the increasing demand for organically produced foods, the development of pest resistance to registered chemical controls, and the desire to control pests in chemically sensitive environments with non-chemical agents (Moore & Prior 1993; Jackson 2000; Charudattan 2001; Whipps & Lumsden 2001). The commercialization of living microbial biocontrol products requires that they be economical, stable, and provide consistent pest control under field conditions (Zorner et al. 1993; Rath 2000; Auld et al. 2003; Fravel 2005;

Correspondence: Mark A. Jackson, PhD, Crop Bioprotection Research Unit, National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 N University St, Peoria, IL 61604, USA. Tel: +1 309 681 6283. Fax: +1 309 681 6693. E-mail: mark.jackson@ars.usda.gov

¹ Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Ghosheh 2005). All three commercial requirements have proven difficult particularly when failure for one of these requirements impairs the commercialization of a product. For fungal biocontrol agents which actively infect their host, a stable propagule must be economically produced and be viable and infective at the time of use (Wraight et al. 2001).

Worldwide, fungal biocontrol agents are often produced through the growth of the biocontrol fungus on solid media which results in the formation of infective conidia (Wraight et al. 2001). *Metarhizium flavoviride*, *Coniothyrium minutans*, and *Beauveria bassiana* are examples of fungal biopesticides that have been produced as conidial preparations using this technology (Cherry et al. 1999; Kiewnick 2000; Dalla-Santa et al. 2005; Ye et al. 2006). Conidia of these fungal biocontrol agents are typically air-dried on the solid-substrate growth medium and have been shown to have good shelf-life at refrigerated and room temperature (Moore et al. 1996; Cherry et al. 1999).

For large-scale biopesticide production, deep-tank fermentation is often a more cost effective method provided high concentrations of an infective propagule can be produced in submerged culture and these propagules can be stabilized (Bowers 1982; Stowell 1991). The use of osmoticants may sometimes be used to produce fungal propagules with enhanced desiccation tolerance (Harman et al. 1994; Pascual et al. 2003). Blastospores of bioinsecticide *Verticillium lecanii* and microcycle conidia of the bioherbicide *Colletotrichum gloeosporioides* var. *aeschyemene* have been commercially produced using deep-tank fermentation and dried as stable, infective propagules (Churchill 1982; Wraight et al. 2001). While many of the entomopathogenic fungi are dimorphic and produce high concentrations of 'yeast-like' blastospores in liquid culture that are infective to their respective insect hosts, these blastospores often survive drying poorly (Inch et al. 1986; Inch & Trinci 1987; Lane et al. 1991; Hegedus et al. 1992).

Various methods of drying microbial biocontrol agents have been evaluated including freeze drying, air drying, and spray drying (Quimby et al. 1999; Wraight et al. 2001; Larena et al. 2003a,b; Horaczek & Viernstein 2004). While freeze drying is the mildest form of drying, it is also the most expensive. For commercial use in agriculture, many biopesticidal products cannot afford the high cost of freeze-drying. Conversely, spray drying is the harshest method of microbial drying with cells being subjected to high pressures at the spray-nozzle orifice, temperature extremes, and to the physical trauma of rapidly losing 95% moisture content. While there are reports of spray drying being successfully used with liquid culture-produced spores of *M. anisopliae*, *M. flavoviride*, *B. bassiana*, and *P. fumosoroseus* (Stephan & Zimmerman 1998), studies on the use of spray drying for other liquid culture-produced propagules have been unsuccessful (Larena et al. 2003a,b; Horaczek & Viernstein 2004; Guijarro et al. 2006). Our studies on the spray drying of blastospores of *P. fumosoroseus* resulted in less than 10% blastospore survival at the laboratory and pilot-plant scale (data not shown).

The air-drying process has been successfully used to stabilize the fungal conidia and bacterial cells of numerous microbial biocontrol agents. Various methods of air-drying have been employed including fluidized-bed drying or air-drying by moving drier air over thin layers of conidia produced on solid substrates or liquid produced propagules. For solid substrate-produced conidia of *M. flavoviride* and *B. bassiana*, the air drying of conidia to low moisture content has been shown to improve storage stability (Moore et al. 1996; Hong et al. 2000; Ye et al. 2006). Fluidized-bed drying, a form of air

drying, has been used to produce stable fungal and bacterial biological control preparations (Jin et al. 1998; Larena et al. 2003a,b; Guijarro et al. 2006). Air drying has also been used to stabilize liquid culture produced microcycle conidia of the bioherbicidal fungus *C. gloeosporioides* (Churchill 1982). Our studies with blastospores of *P. fumosoroseus* have shown that freeze- and air-drying can be used to dry and stabilize these propagules with high rates of survival (Cliquet & Jackson 1997; Jackson et al. 1997, 2006).

Studies with the bioinsecticidal fungus *P. fumosoroseus* showed that desiccation tolerant, liquid culture-produced blastospores were produced only if specific nutritional conditions were used during their production (Jackson et al. 1997; Cliquet & Jackson 1999; Jackson 1999). Drying conditions, formulations, and blastospore preparation prior to drying have all been shown to impact blastospore survival after drying (Cliquet & Jackson 1997; Jackson 1999; Sandoval-Coronado et al. 2001; Jackson et al. 2006). In early studies, blastospore desiccation tolerance and storage stability were quite variable when blastospore preparations were dried in our biological containment hood (data not shown). We theorized that this variability in blastospore viability and storage stability was related to the ambient relative humidity of the air in our laboratory which showed dramatic seasonal variation with RH as low as 15% in the winter and as high as 60% in the summer.

In the studies presented, we evaluated the impact of the relative humidity of the drying air on the desiccation tolerance and, to limited degree, on the storage stability of blastospores of *P. fumosoroseus*. In addition, we considered the impact of the spent culture medium and the pH of the blastospore suspension on their desiccation tolerance and storage stability after air drying. A lab-scale, drying chamber was designed that delivered moving air of specified relative humidity for drying blastospore preparations of *P. fumosoroseus*. The design, operation, and achievable environmental conditions of this air-drying chamber are described.

Materials and methods

Culturing of Paecilomyces fumosoroseus

Paecilomyces fumosoroseus ARSEF 4491 (Wise) Brown & Smith was the isolate used in this study and was obtained during foreign exploration by staff (L. Lacey and A. Kirk) of the USDA/ARS, European Biological Control Laboratory, Montpellier, France (Lacey et al. 1993). The isolate is currently housed in the USDA's Agricultural Research Service Entomopathogenic Fungus (ARSEF) Culture Collection, Ithaca, NY. Stock cultures of *P. fumosoroseus* ARSEF 4491 were grown as single spore isolates on potato dextrose agar (PDA) for 3 weeks at room temperatures, cut into 1-mm² agar plugs and stock cultures of these agar plugs stored in 10% glycerol at -80°C. Conidial inocula were produced by inoculating PDA plates with a conidial suspension from frozen stock cultures and growing these cultures at room temperature (~25°C) for 2–3 weeks.

Blastospores of *P. fumosoroseus* were produced in 100 mL liquid cultures grown in 250 mL baffled, Erlenmeyer flasks. Liquid cultures were inoculated with conidia of *P. fumosoroseus* to provide a final concentration of 1×10^5 conidia/mL and were grown at 28°C and 300 rpm in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ). Blastospores of *P. fumosoroseus* were harvested for air-drying studies after 4 days growth. Blastospore concentrations were determined

microscopically using a hemacytometer. Blastospore concentrations in the 4-day-old cultures were $8\text{--}12 \times 10^8$ blastospores/mL.

The blastospore production medium that was used in these studies was previously shown to produce desiccation tolerant blastospores of *P. fumosoroseus* (Jackson et al. 1997; Jackson 1999). The basal component of the liquid culture medium contained per liter: KH_2PO_4 , 2.0 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 37 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 16 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500 μg each; folic acid, biotin, vitamin B12, 50 μg each. Carbon and nitrogen were provided in the medium by addition of glucose (Sigma Chemical, St. Louis, MO), 80 g/L, and Casamino acids (vitamin-assay, Difco, Detroit, MI), 13 g/L. All media had an initial pH of 5.5 and pH was uncontrolled during culture growth. Glucose stock solutions were autoclaved separately.

Blastospore harvesting and air-drying

Blastospores were harvested from the culture broth by first mixing liquid whole cultures of *P. fumosoroseus*, which consisted primarily of blastospores, with a filter aid, diatomaceous earth (HYFLO, Celite Corp., Lompoc, CA). In rinsed blastospore experiments, whole cultures of *P. fumosoroseus* were first centrifuged and the spent culture medium decanted. The blastospore pellet was then suspended in deionized water ($\text{d-H}_2\text{O}$) and centrifuged again. This process was repeated three times to remove spent media components prior to the addition of the diatomaceous earth. In all experiments, diatomaceous earth was added to whole cultures or rinsed blastospore suspensions at a rate of 5% (w/v). The approximate ratio (w/w) of *P. fumosoroseus* biomass to diatomaceous earth was 1:2. For pH studies, the blastospores suspension in spent media was adjusted to various pH values (pH 3, 4, 5, 6, 7, 8) with either 2 N HCl or 2 N NaOH and allowed to equilibrate for 1 h prior to dewatering. After 4 days growth, the typical pH value for *P. fumosoroseus* cultures in which pH was not controlled was ~ 5.25 .

For all treatments, the blastospore/diatomaceous earth preparations were vacuum-filtered on filter paper (Whatman #1, Maidstone, England) in a Buchner funnel to remove most of the liquid. The resulting filter cake ($\sim 70\%$ moisture) was crumbled and air-dried overnight in an air drying chamber that will be described later. All diatomaceous earth/blastospore preparations of *P. fumosoroseus* were air-dried for 12–20 h. Drying was halted when percent moisture remained constant. The moisture content of the dried blastospore preparations, expressed as (wet weight minus dry weight)/wet weight $\times 100$, was determined with a moisture analyzer (MARK I, Denver Instruments, Tempe, AZ). Dried blastospore preparations of *P. fumosoroseus* were stored in zip-lock bags at refrigerated temperatures (4°C) for 60 days.

The viability of all air-dried *P. fumosoroseus* blastospore preparations was determined immediately after drying using a previously described 6-h blastospore germination assay (Jackson et al. 1997). Briefly, ~ 50 mg of each air dried blastospore preparation of *P. fumosoroseus* was suspended in 50 mL of potato dextrose broth in a 250-mL, baffled Erlenmeyer flask. The suspension was incubated at 300 rpm and 28°C in a rotary shaker incubator (New Brunswick NB 4000) for 6 h. Percent germination was determined microscopically by evaluating 100 discrete blastospores for germ tube formation. Germination was not assessed on clumps of blastospores where discrete

blastospores could not be viewed. Blastospores with germ tube formation equal to one-half the length of the blastospore were counted as germinated.

Studies were conducted to determine the impact of the relative humidity (RH) of the drying air on the desiccation tolerance of blastospores of *P. fumosoroseus*. Blastospore filter cakes were placed in shallow trays in the drying chamber and dried to less than 5% moisture with flowing air at various RH (≥ 50 , 40, and 30%). Dried blastospore preparations were stored in zip-lock bags at 4°C and their viability assessed after 1 and 2 months storage. Blastospore/diatomaceous earth preparations for all other air-drying studies concerning the impact of pH or spent media rinsing were dried with drying air of $\sim 55\%$ RH to less than 5% moisture. Treatments for all pH, media rinsing, and relative humidity experiments were run in triplicate and all experiments were repeated at least twice.

Air-drying chamber

In order to dry blastospore preparations with air that contained consistent RH levels, a controlled-humidity drying chamber was devised (Figure 1). The laboratory-scale drying chamber in which samples were dried under controlled humidity conditions with air moving over the sample is shown diagrammatically in Figure 1. In this drying chamber, the air source was compressed air which was very dry (RH 7–14%). In our system, 345 kPa air was provided by pressure regulation at an air flow of 10 L/min as

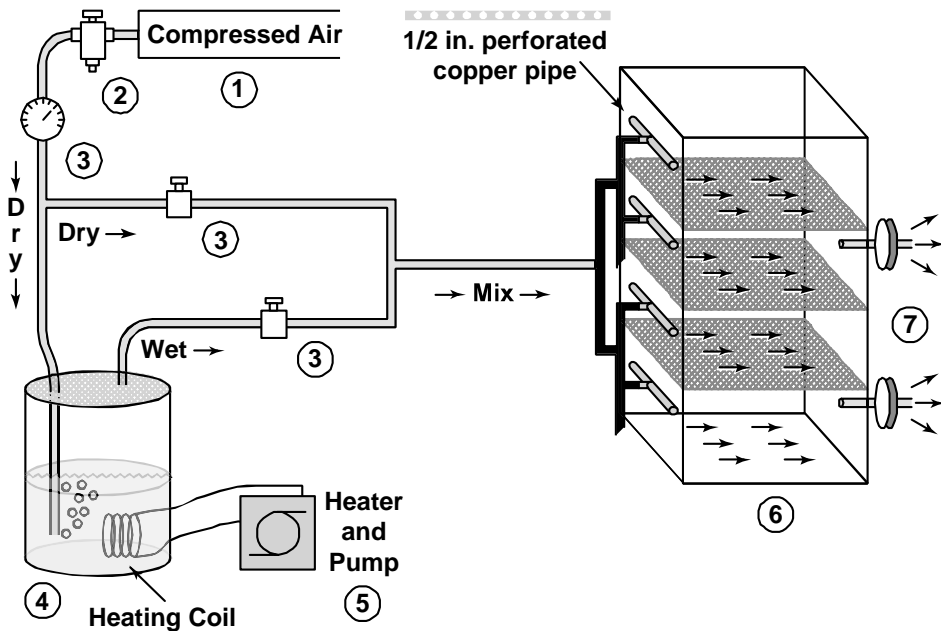


Figure 1. Diagram of controlled humidity, air drying chamber. Compressed air (1) was used as the air source with air pressure (2) regulated. Air flow was controlled with valves (3) that regulated the volume of air and the proportion of wet and dry air delivered to the drying chamber. Wet air was produced by bubbling air through a water bath (4) in which water temperature could be controlled with a heater (5) and heating coil. The drying chamber (6) contained shelves in which air manifolds produced a stream of air over samples. The air vents (7) for the drying chamber could be left open or filtered, depending on the samples being dried.

determined with an airflow meter (3, Cole Parmer Instrument Co., flow tube #N034-39C). The airflow was split and regulated to provide various volumes of dry air or wet air. Wet air was obtained by bubbling the dry compressed air through an 11-L filter/pressure vessel (Fisher Scientific, #09-753-48D) containing 4 L of water. To achieve consistent high RH values in the drying air, the water in the pressure vessel was heated with a heating coil. The water within the coil was heated by circulation through a column heater (Type F, Haake Co., Berlin, Germany). The wet air was used alone for drying or the wet air and dry air were metered to the drying chamber to achieve the appropriate RH value for the drying air. Uniform air flow over the blastospore/diatomaceous earth preparations was achieved for each shelf in the drying chamber by positioning a one-half inch perforated pipe at each shelf (Figure 1). At the bottom of each air-delivery manifold on the outside of the drying chamber, a drainage cap was installed so that condensate that formed during high-humidity drying could be removed. The vents on the far side of the cabinet were fitted with/without filters depending on the nature of the sample being dried.

Experiments were conducted to characterize the drying conditions obtainable with the air-drying chamber. The temperature of the water in the filter/pressure vessel was controlled at various temperatures by the column heater or set initially at a desired temperature and allowed to go to the lowest temperature possible. The water bath temperature, chamber temperature, chamber relative humidity, and % moisture of the filter cake were measured. Chamber temperature and chamber relative humidity were measured with a thermo-hygrometer (Fisher Scientific, model #11-661-13). Drying air with various RH was produced by metering wet and dry air into the chamber. All experiments were repeated at least twice.

Statistical analysis

Statistical analysis of data on blastospore survival after drying and blastospore storage were performed using a one-way analysis of variance and means were separated using Tukey–Kramer honestly significant difference (HSD). Data from different experiments was pooled. For data not appropriate for ANOVA, standard error for mean values is provided.

Results

In order to control air-drying, a chamber was needed that controlled humidity during drying with airflow through the chamber. The use of compressed air provided a very dry source of flowing air with a RH of 7–14. In our system, this dry, compressed air was bubbled through water to increase the RH of the drying air. The water container was fitted with a copper coil connected to a circulating water heater to increase or allow constant water temperature within the water bath and thus keep constant the RH of the drying air. When the water in the container was initially set at higher temperatures and uncontrolled, evaporative cooling by air flowing through the water consistently reduced water temperatures to 12–16°C within 4 h. While the initial relative humidity of the drying air was 96–71% for water heated to 35 or 20°C, respectively, the RH of the drying air was ~55% RH when the water bath temperature equilibrated at 12–16°C. The temperature in the drying chamber was always in the range of 20–22°C regardless of the RH of the drying air.

Table I. Influence of water temperature on the RH of drying air and its impact on the final moisture content of a filter cake (70% moisture) containing blastospores of *Paecilomyces fumosoroseus* and diatomaceous earth.

Water container temperature (C)	Wet air (% airflow)	Dry air (% airflow)	Chamber (%RH±S.E.)	Filter cake (%M±S.E.)
25	100	0	98.0±0.0	47.0±0.7
20	100	0	85.5±1.5	19.0±2.4
18	100	0	79.0±0.6	3.4±0.5
No temperature control (12–16°C)	100	0	55.1±1.0	3.9±0.2
	50	50	42.5±0.5	2.1±0.1
	0	100	6.7±0.3	1.3±0.1

Water temperature controlled with heating coil; airflow; 10 L min⁻¹.

In studies where the water in the container was controlled at a constant temperature, drying air with a much higher RH could be maintained (Table I). Bubbling air through water that was kept above 18°C resulted in high RH drying air which produced blastospore/diatomaceous earth preparations with moisture contents higher than the desired 5% moisture (Table I). When the water temperature was controlled at 18°C, the resulting drying air had a RH of 79% and produced blastospore/diatomaceous earth preparations with moisture content of 3.9% (Table I). When the water temperature of the water bath was uncontrolled, the use of 100% wet air or 100% compressed dry air or blends of wet and dry air resulted in drying air with RH values between 57 and 7%.

In studies that evaluated the impact of pH on the desiccation tolerance of blastospores of *P. fumosoroseus*, no significant difference was seen in initial survival after drying (72–83%) or in survival after storage for 2 months at 4°C regardless of whether blastospore/diatomaceous earth preparations were pH adjusted to 3, 4, 5, 6, 7, or 8 (Table II). Similarly, no significant difference was seen in blastospore desiccation tolerance or storage stability regardless of whether the blastospores were rinsed with d-H₂O prior to drying or dried in the presence of spent culture media, 80

Table II. Influence of pH and d-H₂O rinsing on the desiccation tolerance and storage stability of blastospores of *P. fumosoroseus*.

Blastospore preparation (n)	% Moisture (after drying)	% Viability (6-h germination)*		
		After drying	1 Month	2 Months
Unrinsed (11)	4.5	82	58	72a,b
Rinsed (6)	3.5	80	54	79a
pH 3 (6)	3.8	77	70	66a,b,c
pH 4 (5)	4.9	72	57	51c
pH 5 (6)	4.3	83	64	64a,b,c
pH 6 (6)	4.1	79	60	57b,c
pH 7 (6)	4.3	78	66	62a,b,c
pH 8 (6)	3.8	74	68	64a,b,c

Blastospore/diatomaceous earth preparations dried at room temperature in a controlled humidity drying chamber (>50% RH), airflow, 10 L min⁻¹. Dried blastospore preparations stored in zip-lock bags at 4°C. *Mean values in columns followed by different letters are significantly different by analysis of variance using Tukey–Kramer HSD.

and 82% survival after drying, respectively (Table II). Only blastospores dried in spent media adjusted to pH 4 had significantly lower viability after 2 months storage at 4°C when compared to the blastospores dried in spent media that had not been pH adjusted (Table II).

When studies were conducted to evaluate the impact of the RH of the drying air on the desiccation tolerance of blastospores of *P. fumosoroseus*, drying air with a RH value of 40 or above supported high blastospore survival rates (64–82%) that were not significantly different (Table III). Drying blastospores of *P. fumosoroseus* with air that had a RH value of 40% or less reduced the survival of blastospores during storage at 4°C (Table III). Even though initial blastospore survival and moisture content were not affected by drying at a RH of 40% or above, those blastospore preparations dried at a RH above 50% showed significantly higher viability after 2 months storage at 4°C when compared to blastospores air-dried with 40% RH air or 30% RH air (Table III).

Discussion

Previous studies in our laboratory where blastospores/diatomaceous earth preparations of *P. fumosoroseus* were air-dried in biological containment hoods showed variation in initial blastospore survival rate and storage stability. In evaluating our laboratory environment, the RH of our laboratory air showed significant variation. We observed seasonal variations in RH that ranged between 15% in the winter and 60% in the summer. This led to our development of an air-drying chamber where we could control humidity while moving air over our blastospore preparations to remove excess water. Although controlled humidity environments can be developed with salt solutions of various concentrations, the drying process is slower and may result in unwanted microbial contamination particularly when many samples or samples of large mass are to be dried (Higginbottom 1953). A reasonably rapid method of drying living cells for use as biocontrol agents was needed to reduce unwanted microbial contamination and to lower drying costs.

We have developed a simple controlled-humidity, air-drying system that utilized common laboratory resources and equipment (Figure 1). By using compressed air, a constant flow of low RH drying air could be obtained for various drying purposes. Even without a heating coil, bubbling air through a water bath produced drying air with a relative humidity of ~50–60%. A heating coil in the water bath extends the range of RH for drying air to near 100%. From a practical point of view, drying air

Table III. Desiccation tolerance and storage stability of blastospores of *Paecilomyces fumosoroseus* after air-drying in a chamber with moving air of controlled relative humidity.

Inlet RH of drying air (<i>n</i>)	% Moisture* (after drying)	% Viability (6-h germination)*		
		After drying	1 Month	2 Months
>50% (11)	4.5a	82a	58a	72a
40% (6)	2.7b	64a	21a,b	13b
30% (6)	2.4b	35b	8b	1b

Dried blastospore preparations were stored in zip-lock bags at 4°C. *Mean values in columns followed by different letters are significantly different by analysis of variance using Tukey–Kramer HSD.

with a very high RH is incapable of producing a dry end product (Table I). The use of controlled RH drying air allowed for reproducibility in obtaining blastospore preparations with predictable moisture content that were slowly dried. This controlled RH drying chamber may be useful for drying other microbial biocontrol preparations or other laboratory materials where RH regulated drying is required.

When the desiccation tolerance of blastospore/diatomaceous earth preparations was evaluated after drying with controlled RH air, drying air with a relative humidity above 40% resulted in similar levels of desiccation tolerance for blastospores of *P. fumosoroseus* (64–82% survival). Furthermore, drying air with a RH greater than 50% was needed to produce a storage stable blastospore preparation (Table II). Drying air with RH values greater than 50% were obtained without water bath heating. It should be noted that the drying environment will increase in RH due to water evaporation from the sample. As samples become drier, the RH of the drying air returns to the set RH value which will affect the final moisture content of the dried sample. These results suggest that the slower drying or a higher level of cellular moisture during air-drying may be necessary for cells to acclimate to a desiccated state or accumulate compounds necessary for sustained survival during storage in a desiccated state. It has been noted that mycelial preparations of the aphid-pathogenic fungus *Erynia neoaphidis* and conidia of *Metarhizium flavoviride* had better shelf-life when dried more slowly at higher relative humidity (Hong et al. 2000; Shah et al. 2000). Additional studies are needed to determine the physiological changes associated with cellular desiccation during air-drying and in particular how these changes differ between cells that are rapidly dried and those that are slowly air-dried.

The fact that *P. fumosoroseus* blastospore/diatomaceous earth preparations were ~2/3 diatomaceous earth served two purposes during drying. The non-compressible nature of diatomaceous earth aided blastospore separation from the culture broth and kept blastospores separated during the drying process, reducing cell fusion during drying and enhancing blastospore dispersion when dried preparations were resuspended in water. In addition, the low water-carrying capacity of diatomaceous earth allowed blastospore/diatomaceous earth preparations to attain lower moisture contents during drying with higher RH air.

In conclusion, air-drying can be a cost effective way of stabilizing blastospores of the entomopathogenic fungus *P. fumosoroseus*. These results have demonstrated the importance of controlled-humidity during air-drying for studies focused on evaluating the desiccation tolerance and storage stability of fungal biocontrol agents. Additional studies are needed and are underway to evaluate the impact of the RH of drying air and storage environment on the long-term storage stability for blastospores of *P. fumosoroseus*.

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