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Application of modified atmosphere packaging (gas flushing and active packaging) for extending the shelf life of *Beauveria bassiana* conidia at high temperatures

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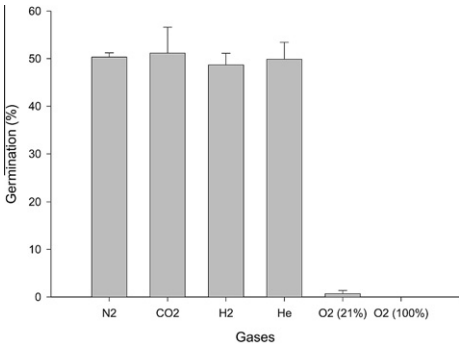
HIGHLIGHTS

- Shelf life of *Beauveria bassiana* conidia is extended in atmospheres with CO_2, N_2, H_2 or He.
- Viabilities $\geqslant$80% were recorded after 6 months at 40 °C in packages with O_2/moisture scavengers.
- Water activity under O_2-free storage should be lower than previously determined in open storage.
- An equilibration period at a moderate temperature is necessary when initial a_w of conidia is high.

GRAPHICAL ABSTRACT



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ABSTRACT

Shelf life determinations under non-refrigerated conditions, especially high temperature regimes characteristic of tropical/subtropical regions, deserve more attention. In this study, we investigated effects of modified atmosphere packaging (MAP) on longevity of conidia of *Beauveria bassiana* (Bb) strain GHA. Similar rates of conidial survival were observed after storage for 60 days at 50 °C in atmospheres of pure CO_2, N_2, H_2, or He (49–51% viability), but few conidia ($\leqslant$2%) survived storage in O_2-rich atmospheres. Viability of conidia stored in an atmosphere of 20% CO_2/80% N_2 decreased to <80% within 180 days at 40 °C and within 30 days at 50 °C but remained high (87%) after a 16-month storage period at 25 °C. O_2 concentrations in the storage containers ranged from 0.3% at the start to as high as 12.4% at the end of experiments (due to container leakage). When active packaging (hermetically sealed packages with O_2/moisture scavengers) was employed, shelf lives were substantially improved. Viabilities $\geqslant$80% were consistently recorded after 6 months at 40 °C or 2 months at 50 °C when a dual O_2/moisture absorber or a combination of sachets (dual O_2 absorber/CO_2 generator + desiccant) were used. Water activities (a_w) supporting greatest survival were $\leqslant$0.030, suggesting that optimal a_w for long-term storage under anaerobic conditions is lower than determined in previous studies of storage in the presence of O_2. Additionally, we have shown that actively packaged conidia with higher than desirable initial a_w should be allowed an equilibration period at a moderate temperature before exposure to high storage temperatures. Active packaging of dried conidia ($a_w \leqslant 0.032$) preserved 71% viability for 16 months at 40 °C and 63–65% for

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3 months at 50 °C. To our knowledge, these are the longest survival times yet reported for Bb conidia under high-temperature conditions.

1. Introduction

Improved shelf life of fungal propagules under non-refrigerated storage conditions is of paramount importance, since mycopesticides may be exposed to temperatures in the high 30 s to 50 °C for extended periods during transport, warehousing, or on-farm storage, particularly in the tropics or subtropics (Stathers et al., 1993; Alves et al., 1996; Hong et al., 1997; Burges, 1998; Jin et al., 1999; Roberts and Leger, 2004). Shelf life may vary according to species (Hong et al., 1997), isolate (Aregger, 1992; Hong et al., 2001), and propagule type (Elzein et al., 2004; Cliquet and Zeeshan, 2008). It is obvious that factors such as temperature (Daoust and Roberts, 1983; Sandhu et al., 1993; Moore et al., 1996; Hong et al., 1999), moisture (Sandhu et al., 1993; Connick et al., 1996; Hong et al., 2001), and packaging system (Clerk and Madelin, 1965; Jin et al., 1999; Abellana et al., 2000) play a vital role during storage.

Studies dealing with shelf life of entomopathogenic fungi have focused primarily on cold or moderate ambient temperatures (Walstad et al., 1970; Aregger, 1992; Silva, 2006). Also, most studies have involved "open" storage, in which air exchange takes place between the external and internal atmosphere of packages (non-hermetic containers). Variable results have been reported in open storage studies of *Beauveria bassiana* (Bb) conidia at high ambient temperatures ($\geqslant$30 °C). Marques and Alves (1996) reported that viability of conidia with 15.5% moisture content (MC) stored at 30 °C was greatly reduced within 30 days, while conidia formulated in sunflower oil retained 80% viability for 90 d but only ca. 30% viability for 120 d. Time for initial viability to drop to 80% was less than one week at 37 °C for a powder with unspecified moisture content (Jin et al., 1999). Significant reduction in viability of formulated aerial conidia with unspecified moisture content occurred 2–3 months after storage at 30 °C (Kassa, 2003).

When Bb conidia were stored in glass jars in which equilibrium relative humidities (ERHs) were controlled with saturated salt solutions to levels between 0% and 98%, highest viabilities were observed at lower temperatures and ERHs, and after one year at 40 and 30 °C viabilities were completely lost or sharply reduced, respectively, for all tested ERHs (Sandhu et al., 1993). Hermetic storage of formulated and unformulated Bb conidia with MC in the 11–19% range resulted in loss of $\geqslant$52% viability after 30 d at 35 °C (Silva, 2006). On the other hand, hermetic storage of Bb conidia with 4.6–5.2% MC led to estimated times for initial viability (96%) to drop to 80% of as long as 80 and 17 d at 40 and 50 °C, respectively (Hong et al., 2001).

To our knowledge, neither O_2-free hermetic packaging nor active packaging (AP), commercially used by the food industry since 1976 (Robertson, 2006), has been tested for storage of Bb. According to Robertson (2006), AP is defined as "packaging in which subsidiary constituents have been deliberately included in or on the packaging material or in the package headspace to enhance the performance of the package system". The objectives of this work were to elucidate the effects of various gaseous atmospheres on shelf life of Bb conidia and assess the potential of an anaerobic packaging strategy for extension of Bb conidia longevity under high- temperature regimes.

2. Material and methods

2.1. Conidial powders

Unformulated aerial conidia (technical powders) of Bb strain GHA were acquired from Laverlam International Corp. (Butte, MT, USA) and stored at −20 °C until use. Unless indicated otherwise, each of the replicate samples assigned to treatments in all tests comprised 0.6 g of conidial powder, an amount suitable for water activity measurements.

2.2. Germination counts

Two protocols were used for assessing pre- and post-treatment viability of conidial powders. In the first (slow-rehydration protocol), a small amount of conidial powder (0.15–0.2 mg) was picked up with a spatula, transferred to a glass Petri dish and incubated for 24 h in a 24-cm internal diameter glass "desiccator" with water-saturated atmosphere. The conidia were then transferred to screw-cap glass vials containing glass beads and 7 mL of 0.05% Lutensol® (Ethoxylated Tridecyl Alcohol, BASF Corporation, Florham Park, NJ, USA), possessing a hydrophilic–lipophilic balance number of 10. Suspensions were agitated for 10 min on a wrist action shaker (Burrel Scientific, Pittsburgh, PA, USA), and 10-µl aliquots were inoculated onto $1 \times 1 \times 0.3$ cm blocks (1 droplet/block) of a yeast extract agar-based solid medium (Meikle et al., 2003). The agar blocks (on glass slides) were placed in Petri dishes, and the dishes were sealed with Parafilm and incubated at 25 °C in darkness. Germination was assessed 24 h post-inoculation (p.i.). In the second germination protocol (fast-rehydration protocol), viability was determined as described above except that the dry conidia were directly suspended in the water/surfactant solution without prior exposure to a humid atmosphere. As it was previously shown that dry Bb conidia with high viability (high quality) are not susceptible to imbibitional damage at moderate temperatures (Faria et al., 2009), solutions used for preparing suspensions (water/surfactant) were equilibrated at room temperature. Conidia were considered to have germinated when a germ tube of any size was visible at 400X magnification with phase-contrast illumination. A minimum total of 200 conidia were examined in several microscope fields for each replicate suspension of each experimental treatment.

2.3. Gas flushing

2.3.1. Flushing with different gases

Conidial samples (0.6 g) were added to 3.4 cm diam. × 1.1 cm plastic sample cups (code 4-1110601; Novasina, Pfäffikon, Switzerland) and kept inside airtight 125-mL glass jars (Ball®, Jarden Corp., Muncie, IN, USA) sealed with metallic lids fitted with rubber septa. Each glass jar was flushed for 40 min at a 40 mL/min flow rate with pure carbon dioxide, nitrogen, helium or hydrogen, as well as 100% or 21% oxygen, balanced with N_2 (Airgas East, Inc., Salem, NH, USA). In jars not flushed with O_2, the concentration of this gas following this procedure was checked to make sure ambient air had been successfully removed from containers. Samples of gas (500 µL) were extracted from each jar with a gas-tight syringe (model 1750, Hamilton Company, Reno, NV, USA) and injected into a gas chromatograph (Varian Aerograph, Walnut Creek, CA, USA) equipped with a thermal conductivity detector. Peak heights were compared against a commercial standard containing 6.96% O_2 and 4.91% CO_2, balanced with N_2. Each gas exposure treatment was replicated three or four times. In order to minimize air leakage (O_2 ingress) into storage containers, the 125-mL glass jars were kept inside a larger airtight Ball® jar (0.95-L) containing the same mixture of gases. Using this setup, glass jars were incubated at 50 °C for 60 d. Following this storage, the O_2 concentration in each jar was re-assessed as an indication of leakage. Conidial water

activity (a_w) was measured at 25 °C with an a_w meter (LabMaster-a_w, Novasina, Pfäffikon, Switzerland), and germination counts were determined as previously described. The experiment was repeated on a different date, without the 21% O_2 treatment. Experimental temperatures for this and all subsequent experiments were ±1 °C, as determined from continuous monitoring with two digital data loggers per incubator (Hobo®, Onset Computer Corp., Bourne, MA, USA).

2.3.2. Flushing with 20% CO_2 and storage at different temperatures

Using the same set up described in the previous section, jars with conidial samples were flushed with 20% CO_2 (+80% N_2). After each storage period at 40 °C (45, 91, 180 and 240 d), four replicate jars were destructively sampled for measurement of the final O_2 concentration and a_w. The experiment was repeated, beginning on a different date. Following the same protocol, separate experiments were conducted investigating effects of storage at 25 °C (assessments at 46, 120, 180, 365, and 480 d) and 50 °C (assessments at 15, 30, 47, 61, 75, and 90 d). In all cases, viability was also assessed at 0 d (just prior to storage).

2.4. Active packaging of conidia

2.4.1. Comparison between aluminized films

In order to select an aluminized plastic film for the active-packaging experiments, conidial samples were held over $MgCl_2{\cdot}6H_2O$ for 2 d at 25 °C, resulting in a mean (±standard error) a_w of 0.321 ± 0.0006. Conidia were then transferred to pouches (10 × 12 cm) fabricated from two different aluminized films: one of unknown composition provided by a mycopesticide company (overall thickness 114 μm) and the other a three-layered laminate composed of 12 μm polyethylene terephthalate, 8 μm aluminum foil, and an 85 μm crystallized polypropylene heat-seal layer (manufacturer unknown). One dual-action O_2/H_2O-absorbing sachet (RP-3A, Mitsubishi Gas Chemical Co., Japan) was enclosed loose in each pouch before heat sealing and incubation at 40 °C for 5 months. Each treatment was replicated four times. After 5 months at 40 °C, final conidial a_ws and viabilities for samples stored with the respective films were virtually identical (90.9 ± 0.1 vs. 89.9 ± 2.0), and both materials were employed in the subsequent tests (one material used for all treatments within a test). Hereinafter, pouches composed of these laminates will be referred to as foil pouches. We opted not to measure final (post-storage-treatment) concentrations of O_2 in the aluminized pouches, which would have required installation of rubber septa, because we did not want to risk compromising container integrity. Heat-sealed aluminized pouches (high-barrier foil pouches) are specifically designed to function as hermetic storage containers.

2.4.2. Comparison between gas flushing and active packaging (AP) agents

Bb conidia were dried over NaOH in 125-ml glass jars for 1 d at 25 °C, resulting in 0.083 ± 0.001 a_w. A number of randomly selected samples were then transferred to glass jars and flushed with N_2. To minimize dusting during the flushing procedure, conidia in glass jars were kept inside small, uncovered plastic sample cups. O_2 leakage was reduced by using the previously-described double-container system. Each of the remaining 0.6-g samples was randomly assigned to one of three AP treatments comprising foil pouches (8 × 8.5 cm) with either one RP-3A O_2/H_2O-absorbing sachet, one O_2-absorbing film (code M-0034, lot 19208A, 89 × 64 × 0.3 mm; CSP Technologies, Auburn, AL, USA) plus one H_2O-absorbing film (CSP Technologies, code M-0026, lot 02208A, 64 × 38 × 0.6 mm), or one O_2/H_2O-absorbing film (CSP Technologies, code M-0033, lot 10808A, 76 × 76 × 0.6 mm). As a control, conidia were kept in polyethylene pouches (8 × 8.5 cm, wall thickness 30 μm; code P827-2.1.2; Empac Agroindustrial de Plásticos Ltda, Brasilia, Brazil) with one RP-3A O_2/H_2O-absorbing sachet.

Following preparation, all containers with conidia were pre-incubated at 25 °C for 5 d and then transferred to 50 °C. O_2 concentrations in glass jars flushed with N_2 were checked immediately prior to high-temperature incubation. For all treatments, three replicate containers were destructively sampled to assess conidial a_w and viability immediately before the transfer to 50 °C. Conidia were incubated at 50 °C for either 56 or 129 d. After storage, conidial a_w was measured and germination counts performed. For each treatment and assessment date, four independently prepared replicate pouches were destructively evaluated.

2.4.3. Comparison among AP sachets

Bb conidial samples were stored in 125-mL glass jars over the desiccant calcium sulphate (eight-mesh indicating drierite, W.A. Hammond Drierite Co., Xenia, OH, USA) for 2 d at 25 °C. Conidial a_w just before packaging was 0.019 ± 0.0005. Alternatively, conidia were stored over a saturated NaCl solution for 2 d at 25 °C, which resulted in 0.738 ± 0.0007 a_w prior to packaging. Samples were then transferred to foil pouches (10 × 12 cm) containing one of the following AP sachets: RP-3A O_2/H_2O absorber, Ageless® ZPT 1000 O_2 absorber (Mitsubishi Gas Chemical Co., Japan), OxyFree™ 504A O_2/CO_2 absorber (Tianhua Tech, China), OxyFree™ 504E O_2 absorber/CO_2 generator (Tianhua Tech, China), or drierite H_2O absorber (56.7 g). As a control, foil pouches without any AP sachet were employed. Pouches were incubated at 50 °C without a pre-incubation equilibration period, and conidial a_w was quantified and germination counts were performed after 45 d. Each treatment (sachet type vs. initial a_w) was replicated four times.

2.4.4. Combination of AP sachets for shelf-life extension

Conidial samples were dried over drierite for 2 d at 25 °C (resulting in 0.020 ± 0.0008 a_w) and then transferred to 16 × 20 cm foil pouches with different sachets: one RP-5A O_2/H_2O absorber (same as RP-3A but indicated for larger packages), one 504E O_2 absorber/CO_2 generator, or one 504E plus one sachet of drierite (56.7 g). Each treatment was replicated three times, and conidial a_w and germination assessments were performed 148 and 180 d post-storage at 40 °C.

2.4.5. Effect of a pre-incubation regime on shelf-life

Due to lower than expected viabilities after a 6-month period at 40 °C in the previous experiment, we decided to test the importance of allowing conidial powders with high initial a_w to equilibrate to lower O_2/moisture levels within the package before exposure to high temperature regimes. Bb samples were kept over drierite or NaCl for 2 d at 25 °C, resulting in 0.020 ± 0.0008 and 0.740 ± 0.0018 a_ws, respectively. Then, conidia were transferred to foil pouches each with one RP-3A O_2/H_2O absorber sachet and pre-incubated for an additional 5 days at 25 °C (equilibration period) before storage at target temperatures (25, 40, and 50 °C). Alternatively, samples were kept over drierite or NaCl for 7 d at 25 °C, transferred to foil pouches with RP-3A sachets and immediately stored at target temperatures without a 5-d pre-incubation period at 25 °C. Each treatment was replicated four times and assessment of conidial a_w and germinations were carried out after 60 d at 50 °C, and 180 d at either 25 or 40 °C.

2.4.6. Effects of a_w on long-term conidial survival at 40 and 50 °C under anaerobic conditions

Samples of conidia (0.6 g) were initially dried over drierite in 125-mL glass jars for 3 days at 25 °C and then transferred to foil pouches. Conidial a_w was then adjusted to different levels via the following treatments: (1) addition of sachet 504E (O_2 scavenger/CO_2 and H_2O generator), (2) addition of sachet RP-3 K (O_2 scaven-

ger/H_2O generator), (3) addition of sachet RP-3A (O_2/H_2O scavenger), and (4) hydration of conidia for 6.5 h over 30 mL distilled water (in glass jar) prior to addition of sachet RP-3A. For each treatment, three independently prepared replicate pouches were destructively evaluated after 7 days at 25 °C (equilibration period) in order to determine viabilities of conidia prior to high-temperature storage, and four pouches were destructively evaluated in assessments following long-term storage for 3 months at 50 °C or 16 months at 40 °C. The experiment was repeated on a different date, the only difference being that drierite (10 g) was also enclosed in each pouch with a 504E sachet (treatment 1).

2.5. Statistical analyses

In experiments conducted to determine the effect of different gases on storage of Bb conidia, jars flushed with gases other than O_2 in which considerable leakage took place (final O_2 concentration >3.5%) were discarded. With the exception of the tests with helium (see Section 3), no more than one or two of the eight replicates of each treatment were discarded. In gas flushing experiments with 20% CO_2/80% N_2 at different temperatures, all glass jars were considered in statistical analyses independently of O_2 ingress, as final conidial viabilities across replicates within treatments were similar, regardless of final O_2 concentration. Data in the form of percentages were arcsine square root transformed and examined using a one-way analysis of variance. Means were compared by the Tukey–Kramer HSD test or *t*-test and considered to be statistically different at the 5% significance level. Data analyses were performed using the JMP statistical package (SAS Institute Inc, Cary, NC, USA).

3. Results

Actual temperatures in closed, equilibrated incubators set at 25, 40, and 50 °C were ±0.5, ±1, and ±1 °C, respectively (in the 40 and 50 °C treatments, temperatures momentarily dropped 2–3 °C and 4–6 °C, respectively, when incubator were opened). Germination percentages determined after fast versus slow rehydration are reported in most cases, but we regard estimates from the fast-rehydration protocol as being better indicators of conidial quality (Faria et al., 2010). Thus, unless otherwise stated, the use of the terms "viability" and "germination" refer to germination percentages measured following fast rehydration. In all experiments of the present study, the estimated initial viabilities determined by the fast- and slow-rehydration protocols were $\geqslant$96.1% and $\geqslant$96.8%, respectively.

3.1. Flushing with different gases

Final a_w for conidia in the first series of assays (with lower a_w) did not vary with gas treatment ($P = 0.41$, $F_{[5,13]} = 1.1$); overall mean a_w was 0.099 ± 0.0248. Significant germination differences were recorded after 60 d at 50 °C ($P < 0.0001$, $F_{[5,13]} = 122.0$), and while exposure to N_2, CO_2, H_2, and He produced equivalent viabilities in the 49–51% range, very low germination rates and no survivors were recorded for 21% and 100% O_2, respectively (Fig. 1A). Final O_2 concentrations did not differ among jars flushed with gases other than O_2 ($P = 0.29$, $F_{[3,9]} = 1.5$), ranging from 0.3% to 1.1%.

In the second series of assays (with higher a_w), excessive O_2 leakage occurred inexplicably in all containers flushed with helium (final O_2 >3.5%), and the data were discarded. As in the first assay, the treatments produced no significant differences in final conidial a_w ($P = 0.29$, $F_{[3,11]} = 1.4$); a_w averaged 0.119 ± 0.0021 across treatments. Flushing with 100% O_2 resulted again in no survivors (21% O_2 was not tested). Storage with all other gases resulted in low, equivalent viability (range of 10–13%) (Fig. 1B).

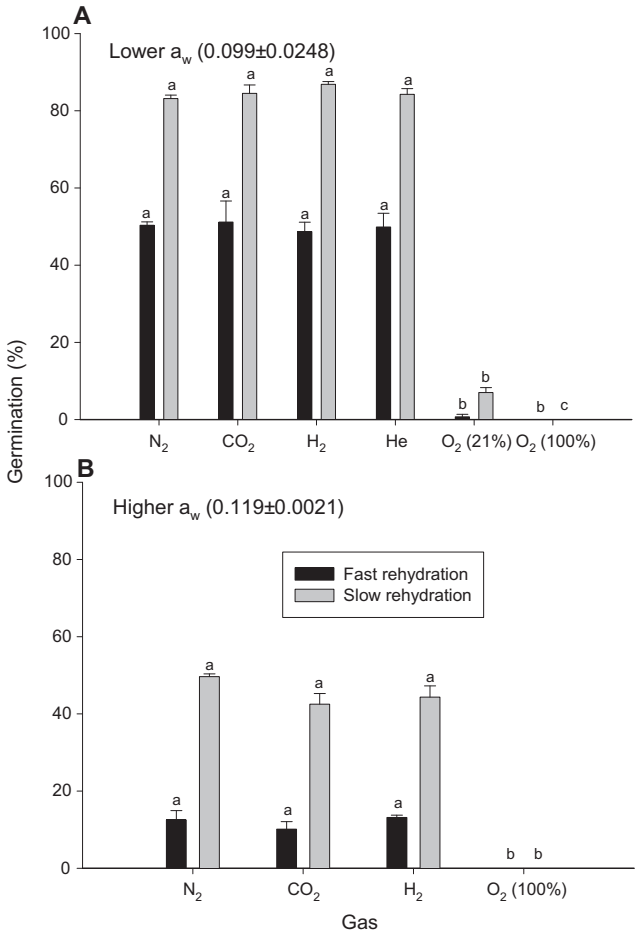

Fig. 1. Effect of different gases on percent viabilities (mean ± SE) after fast vs. slow-rehydration protocols for *Beauveria bassiana* conidia after storage at 50 °C for 60 days. In both experiments, viabilities for the 100% O_2 treatment were 0%. Within each germination protocol, bars (±SE) with the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

These germination rates were markedly lower than the range of 49–51% observed in the assays with lower a_w. Final O_2 concentrations (1.6–1.9%) did not differ among jars flushed with gases other than O_2 ($P = 0.73$, $F_{[2,8]} = 0.3$).

3.2. Flushing with 20% CO_2 and storage at different temperatures

In the 25 °C storage experiment, a significant drop in viability was observed ($P = 0.0002$, $F_{[5,18]} = 8.8$), but the decrease was small and gradual, from 96% to 90% within 365 d and to 87% within 480 d (Fig. 2A). Conidial a_w increased from 0.104 at day 46 to 0.204 at the end of the experiment ($P < 0.0001$, $F_{[4,15]} = 36.3$), and O_2 concentrations increased from an average of 0.5–12.4% ($P < 0.0001$, $F_{[4,15]} = 38.0$).

In the experiment at 40 °C (Fig. 2B), a 2-factor ANOVA (main effect of assay and storage time) followed by the Tukey HSD test revealed a statistically significant loss of viability during the first 3 months of storage, but the decrease was only 6 percentage points (from 93% to 87%). This was followed by a rapid decline to just 4% viability within 240 d (overall ANOVA $P < 0.0001$, $F_{[4,34]} = 361.7$). During the interval between 45 and 240 days, mean O_2 concentration increased from 1.2% to 6.6% ($P = 0.0002$, $F_{[3,28]} = 9.4$) and a_w increased from 0.104 to 0.145 ($P < 0.0001$, $F_{[3,27]} = 35.4$).

At 50 °C, initial viability dropped quickly, from 96% to 81% within the first 15 d to 10% after just 90 d of storage ($P < 0.0001$, $F_{[6,21} = 129.1$) (Fig. 2C). O_2 concentration increased from an average

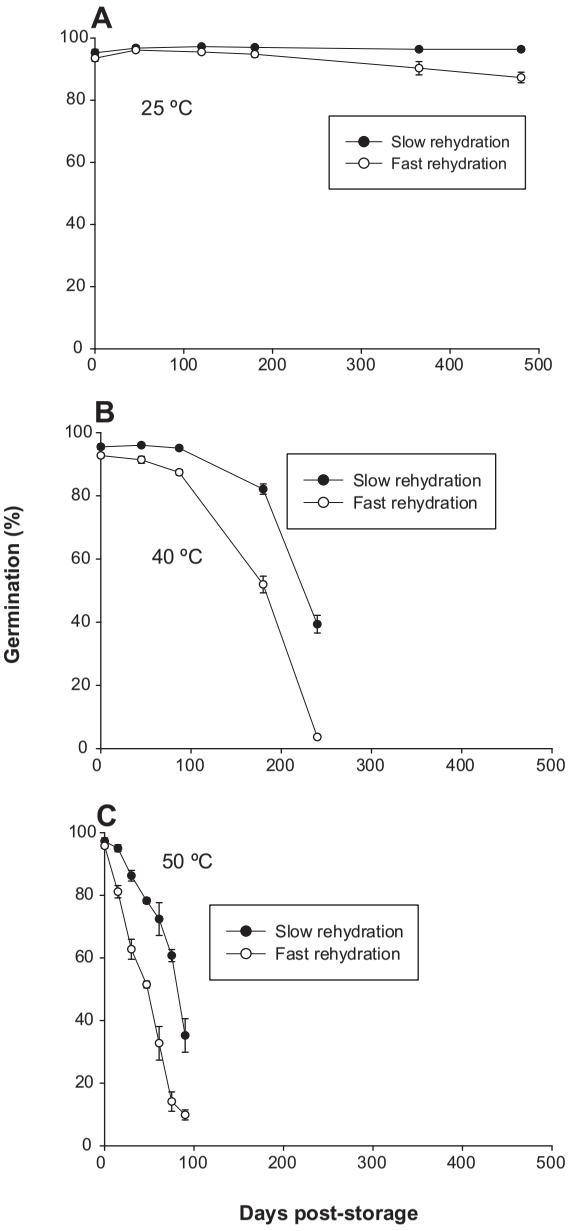

Fig. 2. Viabilities after fast- vs. slow-rehydration protocols of *Beauveria bassiana* conidia flushed with 20% CO_2 (+80% N_2) after storage at either 25 (A), 40 (B), or 50 °C (C).

of 0.8% at 15 d to 3.2% at 90 d ($P = 0.0074$, $F_{[5,18]} = 4.5$), whereas conidial a_w did not change significantly during this period (from 0.104 at 15 d to 0.098 at 90 d; $P = 0.3448$, $F_{[5,18]} = 1.2$).

3.3. Comparison between gas flushing and AP agents

Use of foil pouches, each with one O_2/H_2O-absorbing RP-3A sachet, significantly lowered conidial a_w to 0.059 following a 5-d equilibration period at 25 °C ($P = 0.016$, $F_{[4,10]} = 5.2$) (Table 1). Considerable differences in viabilities were observed among treatments 56 d ($P < 0.0001$, $F_{[4,10]} = 5.2$) and 129 d after storage at 50 °C ($P < 0.0001$, $F_{[4,15]} = 427.9$). The combination of one O_2- and one H_2O-absorbing film provided satisfactory viabilities up to 56 d post-storage, but results after 129 d were not as good as the use of RP-3A. O_2 concentration in glass jars flushed with N_2 was 0.2% immediately prior to incubation at 50 °C. Gas flushing

Table 1
Water activities (a_w) and viabilities of *Beauveria bassiana* conidia stored at 50 °C in gas-flushed jars or foil pouches with absorbing sachets/films.

Container/treatment[2]	Day 0[1]			Day 56			Day 129		
		Percent germination after			Percent germination after			Percent germination after	
	Initial a_w	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration
Glass jar flushed with N_2	0.116 ± 0.0059 ab	95.0 ± 0.7 a	97.0 ± 0.3 a	0.127 ± 0.0049 a	9.0 ± 1.8 d	59.7 ± 2.4 b	0.075 ± 0.0065 a	0.1 ± 0.1 cd	1.3 ± 0.3 d
Foil pouch + RP-3A O_2/H_2O-absorbing sachet	0.059 ± 0.0009 b	96.5 ± 0.5 a	98.0 ± 0.5 a	0.022 ± 0.0000 c	89.4 ± 0.7 a	94.4 ± 0.7 a	0.019 ± 0.0003 b	54.9 ± 1.1 a	84.8 ± 1.7 a
Foil pouch + M-0033 O_2/H_2O-absorbing film	0.068 ± 0.0018 b	96.0 ± 0.5 a	98.3 ± 0.1 a	0.023 ± 0.0003 c	33.8 ± 2.2 b	70.4 ± 2.0 b	0.020 ± 0.0003 b	1.0 ± 0.5 c	12.9 ± 1.8 c
Foil pouch + M-0034 O_2-absorbing film + M-0026 H_2O-absorbing film	0.071 ± 0.0058 ab	97.3 ± 0.3 a	98.5 ± 0.3 a	0.023 ± 0.0008 c	86.0 ± 2.2 a	90.1 ± 1.0 a	0.020 ± 0.0003 b	24.8 ± 0.8 b	72.5 ± 1.6 b
Polyethylene pouch + RP-3A O_2/H_2O-absorbing sachet	0.150 ± 0.0318 a	96.2 ± 0.5 a	97.3 ± 0.1 a	0.046 ± 0.0020 b	16.0 ± 0.9 c	33.8 ± 3.9 c	0.031 ± 0.0010 b	0.0 d	0.0 e

[1] Data recorded immediately prior to high-temperature storage, following an initial 5-day pre-incubation (equilibration) period at 25 °C.
[2] Within columns, means (±standard errors) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

Table 2
Water activities (a_w) and viabilities of *Beauveria bassiana* conidia stored for 45 days at 50 °C in foil pouches with different absorbing and/or generating sachets.

AP sachet	Low initial a_w (0.019)			High initial a_w (0.738)		
		Percent germination after			Percent germination after	
	Final a_w[1]	Fast rehydration	Slow rehydration	Final a_w[1]	Fast rehydration	Slow rehydration
Ageless O_2 absorber; H_2O generator	0.807 ± 0.0012 a	0.0 c	0.0 c	0.819 ± 0.0015 a	0.0 c	0.0 c
Drierite H_2O absorber	0.022 ± 0.0003 de	5.2 ± 0.7 b	9.0 ± 1.0 b	0.023 ± 0.0007 e	7.3 ± 1.3 b	10.8 ± 0.9 b
RP-3A O_2/H_2O absorber	0.019 ± 0.0003 e	79.0 ± 1.3 a	95.5 ± 0.3 a	0.020 ± 0.0003 e	72.8 ± 3.2 a	96.3 ± 0.6 a
504A O_2/CO_2 absorber; H_2O generator	0.704 ± 0.0003 c	0.0 c	0.0 c	0.729 ± 0.0009 c	0.0 c	0.0 c
504E O_2 absorber; CO_2/H_2O generator	0.761 ± 0.0035 b	0.0 c	0.0 c	0.798 ± 0.0024 b	0.0 c	0.0 c
No sachet (control)	0.027 ± 0.0003 d	3.8 ± 0.4 b	8.3 ± 0.9 b	0.709 ± 0.0012 d	0.0 c	0.0 c

[1] Within each column, means (±standard errors) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

Table 3
Effects of an O_2 absorber/CO_2 generator, with or without a desiccant sachet, on water activities (a_w) and viabilities of *Beauveria bassiana* conidia stored in foil pouches at 40 °C for either 5 or 6 months.

Sachet type	Stored 148 days			Stored 178 days		
		Percent germination after			Percent germination after	
	a_w[1,2]	Fast rehydration	Slow rehydration	a_w[1,2]	Fast rehydration	Slow rehydration
504E O_2 absorber; CO_2/H_2O generator	0.793 ± 0.0038 a	0.0 b	0.0% b	0.809 ± 0.0168 a	0.0 b	0.0 b
504E + Drierite H_2O absorber	0.030 ± 0.0003 b	81.0 ± 4.5 a	95.8 ± 0.4 a	0.030 ± 0.0003 b	79.3 ± 1.9 a	95.0 ± 0.3 a
RP-5A O_2/H2O absorber)	0.026 ± 0.0000 b	83.5 ± 2.2 a	96.5 ± 1.0 a	0.028 ± 0.0003 b	81.8 ± 0.4 a	93.7 ± 1.2 a

[1] Initial a_w was 0.020 ± 0.0008, and conidia were not pre-incubated (equilibrated) at moderate temperature before exposure to 40 °C.
[2] Within each column, means (±standard errors) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

or use of a polyethylene pouch (+RP-3A) resulted in poor viabilities compared to active packaging with a dual O_2/H_2O absorber.

3.4. Comparison among AP sachets

Use of the various AP sachets resulted in highly significant differences in conidial viability both for conidia with low ($P < 0.0001$, $F_{[5,12]} = 1631.4$) and high initial a_w ($P < 0.0001$, $F_{[5,12]} = 522.4$) (Table 2). As expected, considering the absorption qualities of the different sachets, the final a_w of conidia in treatments with either low ($P < 0.0001$, $F_{[5,12]} = 69{,}902$) or high initial a_w ($P < 0.0001$, $F_{[5,12]} = 84{,}526$) were also markedly different. Use of sachets that released moisture during storage (Ageless, 504A, and 504E), or that absorbed moisture but not O_2 (drierite), resulted in lower viability compared to use of a dual-action O_2/H_2O absorber (RP-3A).

3.5. Combination of AP sachets for shelf-life extension

The AP sachet that absorbs O_2 but releases moisture (504E) was efficient when tested in conjunction with a desiccant (drierite), but the use of sachet 504E alone resulted in total loss of viability (Table 3). The combo approach was as good as the use of a dual O_2/H_2O absorber (RP-5A), both at 148 d ($P < 0.0001$, $F_{[2,6]} = 309.0$) and 178 d post-storage at 40 °C ($P < 0.0001$, $F_{[2,6]} = 2035$).

3.6. Effect of a pre-incubation regime on shelf-life

Final conidial a_w did not vary among treatments within each of the storage temperature regimes (Table 4). Viabilities after 180 d at 25 °C were high (91–94%) for all treatments, except high initial a_w/no pre-incubation, which reduced viability to 88%; however, there were few significant differences ($P = 0.0205$, $F_{[3,8]} = 5.8$ and see Table 4). After 180 d at 40 °C, viability was 87–89% for all treatments and significantly lower (75%) in the high initial a_w/no pre-incubation treatment ($P = 0.0068$, $F_{[3,8]} = 8.7$). Finally, after 60 d at 50 °C the same trend was again observed, with viabilities from all treatments in the range 83–86% except for the high initial a_w/no pre-incubation treatment, which significantly reduced viability to 60% ($P < 0.0001$, $F_{[3,8]} = 37.8$).

3.7. Effect of a_w on long-term viability of conidia stored anaerobically at 40 and 50 °C

Anaerobic packaging of conidia with different a_w was achieved, and initial viabilities (after fast rehydration) across treatments were not different following a 7-day equilibration period at 25 °C (Table 5), either in experiment 1 ($P = 0.560$, $F_{[3,8]} = 0.7$) or experiment 2 ($P = 0.248$, $F_{[3,8]} = 1.7$). Initial a_w significantly affected conidial survival at both 40 and 50 °C.

In experiment 1, percent germination after 3 months at 50 °C ranged from 0 or near zero (conidial $a_w \geqslant 0.214$) to 66% ($a_w = 0.026$) ($P < 0.0001$, $F_{[3,12]} = 625.3$). The most severely affected conidia (those exhibiting ⩽0.1% viability) were a darker color than less-affected conidia. Conidia dried to a very low a_w of 0.026 retained 71% viability after storage for 16 months at 40 °C. A doubling of initial a_w to 0.05 resulted in a significantly lower viability (53%). It was observed also that spore powders with an initial a_w of 0.050 exhibited slight caking (clumping) when sampled with the spatula, whereas samples labeled $a_w \leqslant 0.032$ retained their original loose consistencies.

In experiment 2, viabilities after 3 months at 50 °C ranged from 0 ($a_w = 0.214$) to 65% ($a_w = 0.025$) ($P < 0.0001$, $F_{[3,12]} = 514.1$). After 16 months at 40 °C, viability of conidia initially dried to very low a_ws of either = 0.025 or 0.032 was 71% in both cases ($P = 0.5810$, $F_{[1,5]} = 0.3$).

4. Discussion

Shelf lives observed in the present study are longer than previously reported for Bb. Modified atmospheres following flushing with gases other than O_2 (CO_2, N_2, H_2, and He) resulted in comparable viabilities following a 2-month storage at 50 °C. When an atmosphere with 20% CO_2 (+80% N_2) was tested in jars, times for conidial viability to drop to 80% (fast-rehydration protocol) were >91 and >15 d at 40 and 50 °C, respectively. These times are similar

Table 4
Effects of initial a_w and pre-incubation at a moderate temperature (25 °C) on germination of *Beauveria bassiana* conidia stored in foil pouches with an O_2/H_2O-absorbing sachet.

Condition	Day 180 at 25 °C			Day 180 at 40 °C			Day 60 at 50 °C		
		Percent germination after			Percent germination after			Percent germination after	
	Final a_w[1,2]	Fast rehydration	Slow rehydration	Final a_w[1,2]	Fast rehydration	Slow rehydration	Final a_w[1,2]	Fast rehydration	Slow rehydration
Low initial a_w/pre-incubation	0.029 ± 0.0000 a	93.2 ± 0.4 ab	95.2 ± 1.7 a	0.028 ± 0.0003 a	87.8 ± 0.9 a	96.3 ± 0.8 a	0.022 ± 0.0000 a	84.8 ± 3.5 a	97.7 ± 0.8 ab
Low initial a_w/no pre-incubation	0.029 ± 0.0003 a	94.0 ± 1.1 a	94.8 ± 0.5 a	0.028 ± 0.0003 a	88.8 ± 0.8 a	95.0 ± 0.5 a	0.022 ± 0.0000 a	86.3 ± 3.8 a	96.2 ± 1.9 ab
High initial a_w/pre-incubation	0.029 ± 0.0000 a	91.0 ± 1.3 ab	93.7 ± 0.9 a	0.028 ± 0.0000 a	88.0 ± 2.6 a	95.0 ± 0.9 a	0.021 ± 0.0000 a	82.5 ± 1.0 a	98.2 ± 1.0 a
High initial a_w/no pre-incubation	0.029 ± 0.0003 a	88.3 ± 0.4 b	91.0 ± 2.3 a	0.028 ± 0.0003 a	75.3 ± 2.2 b	93.8 ± 0.7 a	0.021 ± 0.0000 a	60.0 ± 3.0 b	94.7 ± 1.0 b

[1] Within each column, means (±standard errors) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).
[2] Low and high initial a_ws were 0.020 ± 0.0008 and 0.740 ± 0.0018, respectively.

Table 5
Long-term effects of water activity (a_w) on viability of *Beauveria bassiana* strain GHA conidial powders stored at 40 and 50 °C under anaerobic conditions.

	Day 0[2]		Stored 3 months at 50 °C		Stored 16 months at 40 °C	
Treatment[1]	Initial a_w	Percent germination[3]	Final a_w	Percent germination	Final a_w	Percent germination
Experiment 1[4]						
Desiccation + RP-3A (O_2/H_2O absorber)	0.026 ± 0.000	92.5 ± 1.76 a	0.027 ± 0.000	65.5 ± 1.67 a	0.020 ± 0.000	71.0 ± 1.51 a
Hydration + RP-3A	0.050 ± 0.001	89.3 ± 1.59 a	0.026 ± 0.000	45.8 ± 2.98 b	0.020 ± 0.000	52.7 ± 5.02 b
Desiccation + RP-3K (O_2 absorber; H_2O generator)	0.214 ± 0.003	91.7 ± 0.88 a	0.306 ± 0.004	0.1 ± 0.13 c		nd[5]
Desiccation + 504E (O_2 absorber; CO_2/H_2O generator)	0.710 ± 0.002	91.0 ± 1.76 a	0.702 ± 0.002	0.0 c		nd
Experiment 2[4]						
Desiccation + RP-3A	0.025 ± 0.000	95.5 ± 1.09 a	0.028 ± 0.000	64.5 ± 0.98 a	0.020 ± 0.000	71.4 ± 0.92 a
Desiccation + 504E + drierite	0.032 ± 0.001	95.2 ± 0.14 a	0.030 ± 0.000	62.9 ± 3.04 a	0.024 ± 0.000	70.6 ± 1.16 a
Hydration + RP-3A	0.079 ± 0.002	97.5 ± 0.66 a	0.028 ± 0.000	60.5 ± 2.25 a		nd
Desiccation + RP-3K	0.214 ± 0.003	95.5 ± 0.90 a	0.306 ± 0.002	0.0 b		nd

[1] Initial a_w of conidia was adjusted either by desiccation over drierite for 3 d or hydration over water for 6.5 h prior to packaging in foil pouches with indicated sachet.
[2] Data recorded immediately prior to high-temperature storage, following an initial 7-day equilibration period at 25 °C.
[3] Germination recorded 24 h after fast rehydration.
[4] Within each experiment and column, means (±standard errors) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).
[5] Not determined.

to estimates calculable from data published by Hong et al. (2001), in which Bb conidia dried to ca. 5% moisture content and stored in air in hermetically sealed containers retained 80% viability for 80 and 17 d at 40 and 50 °C, respectively. These were previously the longest shelf lives recorded for this fungal species at these high temperatures. However, when optimal active packaging (with sachets that absorb both O_2 and moisture in hermetic packages) was employed for isolate GHA, viabilities consistently in the 80–90% range were recorded following 6 months at 40 °C or 2 months at 50 °C. Even after 16 months at 40 °C, actively packaged conidia displaying $a_w \leqslant 0.032$ (in equilibrium with 3.2% ERH at 25 °C) exhibited germination >70% (a sorption isotherm relating a_w to moisture content of Bb strain GHA conidia is presented in Faria et al. (2009)).

Shelf life of the mycoherbicide *Sclerotinia minor* along with its substrate was also substantially increased when ambient air was replaced by CO_2 and/or N_2 (Teshler et al., 2007). Likewise, shelf lives of air-dried mycelial alginate pellets of the nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydospora* were improved under vacuum, CO_2, or N_2-rich environments when compared to air atmosphere (Duan et al., 2008). According to these authors, replacement of O_2 by other gases slows metabolism and limits oxidative reactions, allowing for shelf life extension of fungi. We recently demonstrated that atmosphere in which air was replaced by CO_2/N_2 increased longevity of Bb conidia (Faria et al., 2010). All other attempts to extend the shelf life of this fungus have been carried out in air, even though the beneficial effects of O_2 exclusion (or increased CO_2 concentration) during short-term storage of a fungus identified as *Metarhizium anisopliae* was demonstrated decades ago by Clerk and Madelin (1965). Indeed, these authors suggested the adoption of modified atmosphere packaging as a strategy for prolonging conidial longevity, so the limited progress made since then is surprising. On the other hand, much of our knowledge of optimal storage conditions for microbial biocontrol agents is developed and held in secret or patented by commercial enterprises. Most notably, a patent by Jin et al. (1999) claimed that *Metarhizium* conidia dried to very low a_ws with drierite and stored in O_2-free atmospheres created by use of the Ageless sachets inside moisture- and gas-impermeable foil pouches showed 74% viability after 2 months at 37 °C, whereas 0% viability was reported in foil pouches either without an O_2 absorber or with ERH as high as 40 or 100%. Preservation of dry mycelia of *Batkoa* sp. and *Furia* sp. for 3 months at 23 °C by use of Ageless and silica gel also proved successful (Leite et al., 2002), but we are not aware of additional studies on modified atmosphere packaging of entomopathogenic fungi.

The present study focused almost exclusively on conidial survival under high-temperature storage conditions; however, the excellent shelf life exhibited by Bb conidia stored at the moderate temperature of 25 °C in glass jars flushed with CO_2/N_2 (Fig. 2A) is also noteworthy. We unfortunately did not include a normal atmosphere (air) control in the experiment, but 87% viability after 16 months is nevertheless one of the longest recorded shelf lives of this fungus in the absence of refrigeration. In 25 °C storage studies reported by Jaronski (1997), a wettable powder formulation of Bb strain GHA retained 89% viability after nearly 9 months, and a technical powder remained 70% viable after 1 year.

Gas flushing was shown to be less efficient than high barrier foils (+AP agents) for shelf life extension of Bb conidia in the present study due to higher than desirable a_w following the flushing procedure, persistent problems with air leakage, and likely also due to the incapacity of gas flushing protocols to remove all O_2. Teshler et al. (2007) reported a residual O_2 concentration of 0.26% following gas flushing of foil bags. On the other hand, in our study a_w remained at constant low levels following hermetic packaging with foil and use of an efficient O_2/moisture absorber. In anhydrobiotic organisms, isolated enzymatic reactions leading to production of free radicals and free-radical mediated non-enzymatic reactions may take place. For example, phospholipid degradation reactions may occur, with detrimental accumulation of their byproducts (fatty acids) in membranes (McKersie et al., 1988). However, aging under O_2-free and extremely dry atmospheric conditions is considerably slower than aging under non-hermetic conditions.

Shelf life predictability for mycopesticides is difficult due to variability with respect to active ingredients (composition and quality of propagules may vary from batch to batch) and uncontrolled relative humidity and temperature regimes during transportation and storage. Improved predictability can be achieved by use of controlled conidial a_w and gas composition, which can be accomplished by appropriate AP packaging systems as previously discussed. In non-hermetic packaging, availability of air to conidia is far greater (Hong et al., 2005), and since O_2 penetration through currently used storage containers is not standardized, considerable variation in terms of O_2 concentration and conidial a_w and consequent shelf life is not surprising. As shown in this work, adoption of plastic polymers with high permeability to O_2 and moisture are totally undesirable for packaging of mycopesticides, even if combined with an efficient AP sachet.

Most of the AP sachets used in the food industry that we tested were not effective for extending Bb conidia longevity, either because conidial a_w increased to undesirable levels or O_2 was not removed. A dual O_2/moisture absorbing sachet was more efficient than sachets with only one attribute. Although CO_2 is known to possess fungistatic activity against some growing fungi (Tabak and Cooke, 1968; Abellana et al., 2000), no deleterious effect has been observed on stored entomopathogenic conidia, which creates the possibility for use of active packaging through dual O_2 absorbing/CO_2-emitting sachets. This was the reason for inclusion of gas flushing experiments with ca. 20% CO_2 in this and previous work (Faria et al., 2010). Unfortunately, moisture released when the O_2-absorbing/CO_2-emitting sachet was used alone resulted in increased conidial a_w and, therefore, reduced longevity. However, association of this kind of sachet with a desiccant sachet proved successful, and the development of efficient three-way O_2 absorbing/CO_2 generating/moisture absorbing sachets would be welcomed, especially if considerably less expensive than the commercial O_2/moisture absorbing sachets currently available.

Active packaging is not new in insect pathology, and combination of O_2 absorbing sachets and desiccants (Jin et al., 1999; Leite et al., 2002), and adoption of dual O_2/moisture absorbing sachets by some companies have been observed by the authors. Although active packaging is an attractive way of replacing gas flushing to control atmosphere composition in commercial packages, we have noticed mistaken suppositions concerning the overall O_2- and moisture-absorbing capabilities of AP sachets. The Ageless® system uses an O_2-scavenging technology based on iron powder oxidation, and 1 g of this inorganic substance reacts with 300 cc of O_2, reducing its concentration to less than 0.03% (Flodberg, 1997; Vermeiren et al., 1999). Unlike Ageless, that requires moisture to activate its O_2-absorbing mechanism, the Revolutionary Preservation (RP) system™ also absorbs moisture and corrosive gases, and is comprised of 5–15% unsaturated organic compounds; 10–45% calcium oxide, 10–50% mordenite, 5–15% activated carbon, and 10–30% polyethylene (Day, 2005). RP-3A and RP-5A sachets used in this study are capable of absorbing both the moisture and oxygen potentially present in 300 and 500 mL of air, respectively. Due to the limited moisture absorbing capacity of AP sachets, avoidance of large amounts of high moisture material (such as colonized substrates) into packages and pre-drying of mycoinsecticides prior to packaging are strongly recommended in order to extend shelf-life.

Field dosages of fungal conidia employed for control of pests such as highly susceptible locusts can be as low as 25 g per hectare

(Kooyman, 2007). In experiments with a "commercial" volume of pure conidia (30 g), lowering the initial a_w from 0.321 (equivalent to 8.2% MC) to 0.074 (equivalent to 3.6% MC) was achieved when one RP-5A sachet was enclosed per pouch (data not shown). For large volumes of conidia in commercial packages, strategies such as the use of more potent O_2/moisture absorbers (higher RP numbers) or combination of O_2 absorbers with potent desiccants could work satisfactorily, although selection of the best packaging strategy depends on various parameters such as composition and size of the package, volume and initial a_w of the mycoinsecticide, target a_w, O_2- and moisture-absorbing capacities of the sachets, and the rates of O_2 and moisture absorption by AP sachets. The latter is required for definition of the optimal equilibration period at mild temperatures before storage under high temperature regimes.

Minimum desirable shelf lives for biological insecticides are 3–6 months under 'ambient storage' for products supplied by contract for application at a specific time, and 18 months otherwise (Couch and Ignoffo, 1981). Shelf life (which we define as the time during which $\geqslant$80% viability is retained under a certain temperature) of ca. one year has been recorded for the relatively thermotolerant *Metarhizium acridum* with 6.2% MC (but not 7.0% MC) at 27–32 °C stored under vacuum (Hong et al., 1999), and paraffinic oil formulations of Bb at 25 °C (Wraight et al., 2001). Longevities observed in our study at 25 and 40 °C would comply with Couch & Ignoffo's recommendations. Gas flushing and storage of Bb conidia with MC = 4.4% led to high germination rates (87%) after 16 months at 25 °C, and it is very likely that 80% viability would have been retained for at least 18 months. We also achieved a shelf life of at least 6 months at 40 °C when conidia were actively packaged with MC = 2.1–2.4%, which is sufficient time for deliveries under contract in regions with effective mean temperatures of approximately 40 °C. Even longer shelf lives could be expected in more commonly encountered tropical climates where conditions are less extreme. Temperatures as high as or higher than 50 °C can be reached in some regions (Hong et al., 1997) or during transportation (Ostrem and Godshall, 1979), and we achieved a 2-month shelf life at this extreme temperature. Our principal objective in testing at such a high constant temperature, however, was to accelerate the initial experiments (reduce response times to expedite identification of important variables for subsequent research). This strategy was successful in testing the effects of different gases, comparing different packaging strategies (gas flushing vs. active packaging), and selecting AP sachets with greatest potential to extend the shelf life of Bb conidia, as subsequently demonstrated in experiments carried out at 40 °C.

Definitions of shelf life such as viability after storage (Duan et al., 2008) are incomplete, and inclusion of a time-related component and storage conditions are necessary. So, shelf life should be defined as the period of time that a mycopesticide can be stored under specified conditions without considerable loss of attributes that would compromise its effectiveness. This is adapted from definitions used by food technologists (Man and Jones, 2000; Robertson, 2006). Viability is the most common attribute used by insect pathologists to assess conidia quality, and according to Jenkins and Grzywacz (2000) it should be greater than 85%, although in this work we targeted 80%. Therefore, time for initial viability to drop to 80% at a given temperature (as well as RH for non-hermetic packages), could be assigned as the shelf life for standardized mycoinsecticides under those conditions. As a guideline to regulators and manufacturers, an open dating system that clearly displays the packaging date and the estimated shelf lives under specific conditions, would be highly desirable. The specific conditions should refer to the ones that users must follow and/or the ones that are likely to be adopted by them before product application. For instance, display on commercial packages of time for initial viability to drop to 80% at both 4 °C (refrigeration) and, for example, 35 °C (for products commercialized in regions or seasons with effective mean temperatures around this value) would be very useful.

In addition to the storage factors previously mentioned (see Introduction), pre-storage factors such as the initial quality of fungal propagules, which in turn is influenced by culturing conditions (Agosin et al., 1997; Frey and Magan, 2001; Tarocco et al., 2005), drying and harvesting processes (Sandoval-Coronado et al., 2001; Bateman, 2004; Jackson and Payne, 2007), and formulation (Sandoval-Coronado et al., 2001; Batta, 2003; Friesen et al., 2006) have profound impacts on longevity. We have shown that in-package drying of conidia under moderate conditions before exposure to high temperature regimes is required to allow undesirably high initial a_ws to reach desirable levels and, therefore, avoid premature death or conidial debilitation.

Post-storage factors, such as germination protocol, although not directly related to shelf life, can also greatly affect viability estimates. Results from this and previous studies have indicated, for example, that estimates of viability obtained for conidial preparations removed from dry storage vary markedly depending on the speed of rehydration and length of the incubation period (Moore et al., 1997; Faria et al., 2009, 2010). Shelf lives presented in this study were estimated based on a fast-rehydration protocol (without previous exposure of conidia to a slow-rehydration regime inside a wet chamber for 24 h). Estimates of time for initial viability to drop to 80% would be much greater (in some cases doubled) if estimates from the slow-rehydration protocol were accepted as the standard. However, the biological/microbial control significance of this improvement is questionable, and we have shown that viability determinations following the more gentle process of slow rehydration are inflated by germination of debilitated conidia, including those that have lost the capacity for rapid germination or are hypersensitive to imbibitional damage (Faria et al., 2010). In the aforementioned study (Faria et al., 2010), we ultimately recommended incubation for generally $\leqslant$24 h following fast rehydration in a protocol aimed at providing the most conservative/rigorous assessment of mycoinsecticide quality. With respect to Bb strain GHA, we suggested an incubation time of 16–18 h. The lack of accord with the current study, in which we have used a 24 h incubation time, is explained by the fact that the experiments reported herein were initiated prior to the studies that led us to propose the above-cited recommendations. It is likely that if we had performed our assessments after 18 rather than 24 h, we would have recorded somewhat lower estimates of germination, translating to shorter shelf lives. These observations underscore a great need for empirical studies of diverse pest-pathogen systems to determine which assessment methods provide the most meaningful estimates of viability (i.e., which methods produce viability estimates that are the best indicators of pathogen virulence and biopesticide quality).

Water activity, a term initially proposed by microbiologists (Troller, 1980), is defined as the ratio of the water vapor pressure of a material to the vapor pressure of pure water at the same temperature (Robertson, 2006). It is a straightforward measure of water available for chemical and biological reactions and, therefore, a meaningful parameter in studies with dehydrated microorganisms. A_ws of pre-dehydrated conidia kept in hermetic foil pouches with drierite or O_2/moisture absorbers were consistently in the 0.019–0.030 range (1.9–3.0% ERH). This small variation was observed between readings performed in wintertime (colder and drier air in the laboratory) and seasons with higher T/RH. The importance of drying aerial Bb conidia for extended shelf life has been demonstrated previously (Clerk and Madelin, 1965; Feng et al., 1994; Shimizu and Mitani, 2000). In hermetic storage studies in which air was not removed from packages, Hong et al. (2001) reported that longevity of two Bb isolates was not significantly increased when storage moisture content was reduced below

4.6–5.2%, corresponding to moisture contents in equilibrium with about 11–14% ERH at 20 °C (see Faria et al., 2009 for sorption isotherms that allow conversion of a_w measurements to MC). In our study, best conidial a_ws were consistently associated with drierite, which dried the conidia to considerably <5% MC (see also Faria et al., 2010). It is possible that under anaerobic conditions (<0.03% O_2) optimal water activities for storage are lower than under aerobic atmospheres.

A great number of mycoinsecticides are commercialized worldwide (Faria and Wraight, 2007) and improvements in their longevity under non-refrigerated conditions are urgently needed for greater market acceptance. Mycoinsecticides capable of displaying predictable and satisfactory germination counts following long-distance transportation and realistic uncontrolled storage should be a goal for product developers. We have shown that MAP, especially active packaging, could be broadly adopted in order to extend shelf life of Bb and possibly other fungal agents.

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