STUDIES ON ENTOMOPATHOGENIC FUNGI: EVALUATIONS OF GERMINATION PROTOCOLS FOR ASSESSSING CONIDIAL QUALITY AND MODIFIED ATMOSPHERE PACKAGING FOR ENHANCING HIGH-TEMPERATURE SHELF LIFE

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STUDIES ON ENTOMOPATHOGENIC FUNGI: EVALUATIONS OF GERMINATION PROTOCOLS FOR ASSESSSING CONIDIAL QUALITY AND MODIFIED ATMOSPHERE PACKAGING FOR ENHANCING HIGH-TEMPERATURE SHELF LIFE

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Conidia of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* are the most common active ingredients in commercially available mycoinsecticides, and longer shelf-lives under non-refrigerated conditions could increase their limited market acceptance. Contrasting results from commonly used germination protocols constitute an important impediment to consistent, universally comparable efficacy and shelf-life studies. A series of laboratory experiments investigated the underlying causes of this problem. Lower viabilities due to imbibitional damage were observed with decreasing water activity (a_w) and decreasing temperature of the water/surfactant solutions used in preparing conidial suspensions. Dry conidia of *M. anisopliae* (strain CB-10) and *M. acridum* (strain IMI-330189), in contrast to those of *Beauveria bassiana (*strain GHA), were highly susceptible to imbibitional damage. Germination of the driest *M. anisopliae* conidia was drastically reduced to 66% after immersion at 25 ºC and to < 1% at 0.5 ºC. Significant loss of viability was prevented when conidia were immersed in warm water (ca. 34 ºC) or when slowly rehydrated by humidification prior to immersion. Inclusion/exclusion of debilitated conidia (conidia exhibiting delayed germination or hypersensitivity to imbibitional damage) in germination counts was identified as an additional factor contributing to variability in quality assessments. Germination protocols based on fast

rehydration and shorter incubation times are recommended as a means of discounting debilitated conidia in viability determinations. In shelf-life experiments, longevities of *B. bassiana* conidia were similar and significantly greater in storage containers flushed with CO_2, N_2, H_2, or He, than in containers flushed with high concentrations of O_2 . In atmospheres with 20% CO_2/80% N_2, viability measured after fast rehydration and incubation for 24 h remained high (87%) after a 16-month storage period at 25 ºC. Active packaging systems comprising aluminized bags and commercially available O_2- and moisture-absorbing materials, consistently preserved viability at levels of 80-89% for six months at 40 ºC and two months at 50 ºC. Results suggest that optimal a_w for long-term storage of conidia under anaerobic conditions are lower than previously reported from studies of storage in the presence of O_2. To our knowledge, these are the longest high-temperature survival times for *B. bassiana* conidia reported to date.

BIOGRAPHICAL SKETCH

Marcos Faria was born in Brasilia, Brazil. As a youth he spent much time on farms, sparking his interest in nature. He earned his B.S. in agronomy from the University of Brasilia, in 1988. After earning his degree he realized that his real interest was animals instead of soil or plants. Lacking both the capital to initiate a cattle or dairy business and the stimulus to pursue an animal science degree, he decided to work on smaller animals. His dislike for insects (contrary to his weird colleagues) motivated him to attain a scientist position with EMBRAPA (Brazilian Agricultural Research Corporation). His research focused on biological control of agricultural pests which led to an MSc in entomology from the University of Florida, in 1995. After several years of research he is fulfilling his father's dream of becoming a doctor (but a twisted one!), skilled in the art of ending his patients' lives thru fungal infections. He is very fortunate to be married to Cácia, a great woman and an ethical lawyer (which makes her even more special). They have a daughter named Ana Flávia who is the jewel of their lives. He has many outside interests and is a huge fan of NBA basketball and soccer world cups.

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CHAPTER 1

MYCOINSECTICIDES AND MYCOACARICIDES: A COMPREHENSIVE LIST WITH WORLDWIDE COVERAGE AND INTERNATIONAL CLASSIFICATION OF FORMULATION TYPES*

Abstract

A substantial number of mycoinsecticides and mycoacaricides have been developed worldwide since the 1960s. Here I present information generated from an updated, comprehensive list of these products. At least 12 species or subspecies (varieties) of fungi have been employed as active ingredients of mycoinsecticides and mycoacaricides for inundative and inoculative applications, although some are no longer in use. Products based on *Beauveria bassiana* (33.9%), *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%), and *B. brongniartii* (4.1%) are the most common among the 171 products presented in this Chapter. Approximately 75% of all listed products are currently registered, undergoing registration or commercially available (in some cases without registration), whereas 15% are no longer available. I was unable to determine the status of the remaining 10%. Insects in the orders Hemiptera, Coleoptera, Lepidoptera, Thysanoptera, and Orthoptera comprise most of the targets, distributed among at least 48 families. A total of 28 products are claimed to control acarines (mites and ticks) in at least 4 families, although only three products (all based

* Faria, M.R. de, Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biological Control 43, 237-256.

on *Hirsutella thompsonii*) were exclusively developed as acaricides. Eleven different technical grade active ingredients or formulation types have been identified, with technical concentrates (fungus-colonized substrates) (26.3%), wettable powders (20.5%) and oil dispersions (15.2%) being most common. Approximately 43% of all products were developed by South American companies and institutions. Currently, what may be the largest single microbial control program using fungi involves the use of *M. anisopliae* for control of spittlebugs (Cercopidae) in South American sugarcane and pastures.

Introduction

The first attempt to control a pest with a fungal agent was carried out in Russia in 1888, when the fungus now known as *Metarhizium anisopliae* (Metschn.) Sorokīn was mass produced on beer mash and sprayed in the field for control of the beet weevil *Cleonus punctiventris* (Germar) (Lord, 2005). Boverin, a *Beauveria bassiana*-based mycoinsecticide for control of the Colorado potato beetle and codling moth in the former USSR, was developed in 1965 (Kendrick, 2000). Mycar, a mycoacaricide based on *Hirsutella thompsonii* Fisher, was granted a full registration by the US Environmental Protection Agency in 1981 for control of the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead), in the United States (McCoy, 1986). Research and development efforts have increased markedly in recent years, and a considerable number of fungus-based biopesticides have been developed for control of insects and acarines (mites and ticks) in agricultural, urban, forest, livestock and aquatic environments. In the following discussion, these mycoinsecticides/mycoacaricides will be referred to as mycopesticides, a term that also embraces other fungal biological products not considered in this paper, such as mycoherbicides and mycofungicides.

There are many recent reviews discussing the efficacy, contemporary advances, future trends, and regulatory aspects of mycopesticides (Copping and Menn, 2000; Neale, 2000; Inglis et al., 2001; Wraight et al., 2001; Castrillo et al., 2005). Although numerous lists of developed mycopesticides are available (Tengerdy and Szakács, 1998; Butt et al., 1999; Butt and Copping, 2000; Hajek et al., 2001; Stewart, 2001; Wraight et al., 2001; Leite et al., 2003a; Copping, 2004; Hynes and Boyetchko, 2006), many of these are deficient, especially in terms of worldwide coverage.

This study aimed to assemble a comprehensive list of mycopesticides developed worldwide over the last four decades. In addition, I have attempted to review and standardize the nomenclature of formulation types so far employed for these biocontrol agents. Use of fungal agents through conservation and classical biological control strategies is discussed elsewhere (Pell et al., 2001; Hajek et al., 2003; Shah and Pell, 2003).

Products and Formulations

A complete list of mycopesticides for insect and acarine control from different regions of the globe was prepared from information obtained from a variety of sources. In addition to scientific publications, an array of technical publications, personal communications, company brochures, and websites, including those of manufacturers and governmental agencies, were used. Websites were accessed during the period between October, 2005 and September, 2006, and personal communications were obtained through April, 2007.

2.1. Fungal products

For the purpose of this paper, mycopesticides are defined as products based on living fungal propagules intended to control pests through inundative or inoculative

applications. Propagule types present in most products are classified as hyphae (mycelia), blastospores, or conidia. When the information could be found, conidia were further characterized as aerial or submerged conidia. When available information was insufficient to identify conidia versus blastospores, the propagules were listed simply as asexual spores. Target pests were identified to order and, when possible, to family. The diverse insects in the order Hemiptera were identified also to suborder. The classification of insects follows Triplehorn and Johnson (2005).

This work presents a comprehensive list of mycopesticides that are registered, undergoing registration or marketed, in some cases without being registered. A few preparations developed but never registered or commercialized, such as the ultra-low volume suspension of Mycotrol-OF, are also considered. Mycopesticides commercialized without registration and, in the other extreme, registered products that currently are not being commercialized, are included in the list of products as well. Names of manufacturers are listed according to best available information. However, due to difficulties in tracking the ever-changing relationships among companies, the manufacturer's names mentioned may in some cases refer to licensees or distributors that do not operate manufacturing facilities.

Products with no trade names (usually products undergoing a registration process or unformulated materials sold directly to end users) are included when it was possible to determine that they were commercialized for at least some period of time. For this reason, some names (or lack thereof) might be provisional. Products distributed without charge or sold at subsidized prices to end users by non-profit organizations, such as grower associations, as well as mycopesticides derived from on-farm production are not included. Technical products used solely for formulation of end-products also are not listed, e.g., Troy Boverin, Technical Laginex, PFR-97 MUP, and others. Finally, although molecular systematics studies have shown the proximity

of microsporidians to zygomycete fungi (Edlind et al. 1996; Keeling 2003), these microorganisms were not considered in this paper.

2.2. Fungal formulations

I have attempted to identify formulation types based on the CropLife International two-letter coding system for technical and formulated pesticides (CropLife International, 2002). The international coding system defines approximately 100 different formulation types for pesticides in general, and most of the formulation types described for the mycopesticides listed herein can be classified within this system. In a few cases, I found it difficult to match existing mycopesticide formulations with the formulation types defined by the international code. In these cases I have selected the defined type of formulation that best fits the mycopesticide and have added brief notations on specific incongruities. Some of the definitions I present also incorporate slightly modified definitions used by FAO/WHO (2002) to describe biopesticides based on entomopathogenic bacteria. Wording taken directly from the CropLife International (2002) or FAO/WHO (2002) definitions are presented in italics and quotation marks. Definitions are from the CropLife International coding system unless otherwise indicated.

2.2.1. Technical Grade Active Ingredients (Technical Products)

FAO/WHO (2002) applies the generic term technical grade active ingredient to both technical material and technical concentrate.

Technical material (TC): "*An active ingredient isolated (as far as is practicable) from the starting materials, solvents, etc., used to produce it*" (FAO/WHO, 2002). For entomopathogenic fungi, the starting materials are usually the liquid or solid culture substrates. Technical materials are usually the basis for all other formulation types, although in some circumstances they may be used as end-

products. According to the CropLife definition, technical materials may include *"associated impurities and small amounts of necessary additives"*. Purification "as far as practicable" is generally considered to result in impurity residues comprising <10% of the product weight (T. S. Woods, Chair, Specifications Expert Group, CropLife International, personal communication). In my understanding, conidia or other propagule types isolated from the culture together with associated impurities would fall into this category, also referred to as technical powder (Burges and Jones, 1998).

Technical concentrate (TK): A material consisting of the active ingredient together with related byproducts of the production process and free of any added modifying agents except for small amounts of stabilizers and free-flow agents, if necessary. This definition is a slight modification of the definition presented by FAO/WHO (2002) for bacterial technical concentrates. According to FAO/WHO, a TK should also be free of "visible extraneous matter", but the term extraneous matter is not defined, and in my judgment, the FAO/WHO definition fits fungal biopesticides that include components of the spent culture media, e.g., fungus-colonized cereal grains or whole-culture broths. In these cases, there have been no attempts to separate the active ingredient from the substrate. Included in this category, fungus-colonized solid substrates may contain variable proportions of sporulating mycelia and spores, depending on factors such as age of culture and batch. In some countries they are routinely used as end-products through direct incorporation into soil. Alternatively, the active ingredient may be extracted before application (e.g., by washing and sieving, often with the aid of surfactants). In this Chapter, for all technical concentrates based on solid substrates I consider the propagule type as being conidia + hyphae (C+H), although frequently the vast majority of propagules at the time of sale may be either spores or hyphae. For products produced in liquid media, mixtures of submerged conidia, blastospores, or hyphae may be present.

2.2.2. Formulation Types

Wettable powder (WP): "*A powder formulation to be applied as a suspension after dispersion in water.*" In my understanding, WP formulations must be ready-to-use. Thus, products such as hydrophobic technical materials that do not include additives that render them miscible in water (such as surfactants or clays) would not fall in this category.

Granule (GR): "*A free-flowing solid formulation of a defined granule size range ready for use.*" Although technical concentrates comprising microbe-colonized granular substrates may resemble and function as granular formulations, the term granule generally refers to more elaborated formulations with particles of controlled and uniform size and with the active ingredient strongly adhered to or incorporated into the granule. Thus, fungus-colonized cereal grains are not included under this definition.

Bait (ready for use) (RB): "*A formulation designed to attract and be eaten by the target pests.*" This definition is generally applicable to mycopesticides; however, because most fungal pathogens infect their hosts via direct penetration of the cuticle, ingestion may be of little consequence, and baits may therefore be based on attractants other than food.

Water dispersible granules (WG): "A formulation consisting of granules to be applied after disintegration and dispersion in water."

Contact powder (CP): "Insecticidal formulation in powder form for direct application." Free-flowing powders suitable for dusting are termed dustable powders (DP) under the CropLife International coding system. However, other than a few early preparations produced by nonprofit organizations, I did not identify any

mycoinsecticides formulated specifically for broadcast application as dusts. Thus, I have categorized all powders that do not fit any of the previously mentioned formulation types as contact powders.

Suspension concentrate (=flowable concentrate) (SC): "A stable suspension of active ingredient(s) in water, intended for dilution with water before use."

Oil miscible flowable concentrate (=oil miscible suspension) (OF): "A stable suspension of active ingredient(s) in a fluid intended for dilution in an organic liquid before use."

Ultra-low volume (ULV) suspension (SU): "A suspension ready for use through ULV equipment."

Oil dispersion (OD): "*A stable suspension of active ingredient(s) in a water-immiscible fluid, which may contain other dissolved active ingredient(s), intended for dilution in water before use.*" In practice, oil dispersions contain emulsifiers to render the mixture miscible in water for spraying (T. S. Woods, personal communication). The word "stable" in this and other of the abovementioned formulations indicates that the active ingredient does not settle out to a non-resuspendable cake during storage (T. S. Woods, personal communication). Here, I consider the definition to include suspensions that tend to settle, but which are designed to be readily resuspendable by the user via manual agitation. Oil dispersions of entomopathogenic fungi have been referred to most commonly in the literature as emulsifiable suspensions or emulsifiable oil suspensions and identified by the abbreviation ES. However, under the Croplife International code, the abbreviation ES refers to emulsions for seed treatments.

Table 1.1. Fungal species and varieties developed into mycoinsecticides/mycoacaricides and their commercial status.

Species / Varieties[1,2]	No. Products	Commercial status[1]		
		Active	Inactive	ND
Fungi: Anamorphic Hypocreales				
Aschersonia aleyrodis Webber	1 (0.6%)	0	1	0
Beauveria bassiana (Bals.) Vuill.	58 (33.9%)	45	9	4
Beauveria brongniartii (Sacc.) Petch	7 (4.1%)	5	0	2
Hirsutella thompsonii F.E.Fisher	3 (1.8%)	1	1	1
Isaria fumosorosea Wize	10 (5.8%)	7	1	2
Isaria sp.	1 (0.6%)	1	0	0
Lecanicillium longisporum (Petch) R.Zare & W.Gams	2 (1.2%)	2	0	0
Lecanicillium muscarium (Petch) R.Zare & W.Gams	3 (1.8%)	3	0	0
Lecanicillium sp.	11 (6.4%)	10	0	1
Metarhizium anisopliae (Metschn.) Sorokīn	58 (33.9%)	44	10	4
Metarhizium anisopliae var. *acridum* Driver & Milner	3 (1.8%)	3	0	0
Nomuraea rileyi (Farl.) Samson	1 (0.6%)	0	0	1
Fungi: Anamorphic species identified as "*Sporothrix insectorum*" de Hoog & H.C. Evans[4]	3 (1.8%)	2	1	0
Fungi: Zygomycota: Zygomycetes: Entomophthorales				
Conidiobolus thromboides Drechsler	2 (1.2%)	1	1	0
Chromista: Oomycota: Oomycetes: Pythiales				
Lagenidium giganteum Couch	1 (0.6%)	1[5]	0	0
Mixes (2 or more species)	7 (4.1%)	4	1	2
TOTAL	171 (100%)	129 (75.4%)	25 (14.6%)	17 (9.9%)

[1]Fungal classification based on Kirk et al. (2001).
[2]Scientific names according to database Index Fungorum (http://www.indexfungorum.org/).
[3]Availability of product is informed as follows: Active: product currently registered, undergoing registration or available (commercially or upon request) in the period between Oct/2005 and May/2006; Inactive: product no longer available in the market (or developed but never submitted to registration or marketed); ND: current status of product could not be determined.
[4]Further identification of commercial isolates required since isolates are not true *Sporothrix*.
[5]Registered but not currently commercialized.

Overview of Mycopesticides

Mycoinsecticides and mycoacaricides are listed alphabetically by genus and species in Table 1.1. To date, at least 12 species or subspecies (varieties) of fungi have been employed as the active ingredients in these products. This number will likely increase into the foreseeable future as molecular studies reveal cryptic species within large genera. Classifications at the generic level are also undergoing rapid change. In this work, I have adopted those recent taxonomic changes that I view as strongly supported in the published literature. Most notably, insect pathogenic fungi previously classified as *Verticillium* spp. have been placed in a new genus, *Lecanicillium* (Gams and Zare, 2001; Zare and Gams, 2001), and many insect pathogenic *Paecilomyces* species (including *P. fumosoroseus*) have been transferred to the genus *Isaria* (Hodge et al., 2005; Luangsa-Ard et al., 2005). Some species identified in the table are in urgent need of additional taxonomic work. Recent molecular studies, for example, indicate that isolates of fungi identified as *Sporothrix insectorum* de Hoog & H.C.Evans, currently used in Brazil for control of the lace bug *Leptopharsa heveae* Drake & Poor in rubber tree plantations, belong to more than one species and are not true *Sporothrix* species (K.T. Hodge, Cornell University, personal communication). In the realm of commerce, an unfortunate outcome of taxonomic revision is the emergence of disparities between the names of products and the fungi upon which they are based (as many product trade names are derived from the scientific names of their active ingredients). Product developers should consult fungal systematists before naming their products.

For most fungal species/subspecies listed, there is at least one product that is currently registered (or undergoing registration), or marketed; however, a few species are apparently no longer commercially available. These include *Aschersonia aleyrodis* Webber and *Nomuraea rileyi* (Farl.) Samson (the availability of the only listed *N.*

rileyi product could not be determined). *Lagenidium giganteum* Couch is registered but not currently available. With the exception of one species in the phylum Zygomycota and another in the kingdom Chromista (= Stramenipila or Straminopila), all agents comprising this list are anamorphic fungi. *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metschn.) Sorokīn, *Isaria fumosorosea* Wize, *B. brongniartii* (Sacc.) Petch, and *Lecanicillium* spp., are the main active ingredients of listed products. I believe that approximately two-thirds of all listed products are currently registered, undergoing registration or marketed.

During the last four decades, over 80 companies worldwide have developed 171 mycoinsecticides and mycoacaricides (Appendix 1). This contrasts sharply with the situation less than three decades ago, when only one commercial mycoinsecticide was available (Ignoffo and Anderson, 1979). Although most products are based on specific types of propagules (Section 2.1), the end product may contain small or even substantial amounts of other propagule types. Products based on aerial conidia may contain hyphae, and vice-versa, and mycoinsecticides produced through liquid fermentation may present a mix of submerged conidia, blastospores and hyphae (Leite et al., 2003a). The exact propagule composition of biopesticide products is rarely stated by manufacturers, and, in some cases, the specific propagule comprising the active ingredient is not indicated. For many of these products, the active ingredient is quantified in terms of colony forming units. Thus, in this listing I report only what I was able to identify from product claims and descriptions as the predominant propagule type. For six products, no identification of propagule type was possible. Based on the available information, I determined that a significant proportion of products (25.7% - most of these classified as technical concentrates) contain both asexual spores and hyphae. However, 67.5% of all products are described as being based exclusively on asexual spores, with aerial conidia being the most common

among all products (40.9%). Only 4.1% of listed products are claimed to contain only blastospores, whereas this kind of propagule is also present in two other products, one including submerged conidia and one including submerged conidia and hyphae. No products have been reported as containing only submerged conidia, and those based exclusively on hyphae account for only 2.4% of all products. The type of asexual spore could not be determined for 22.4% of products.

Formulation types could not be determined for 25.9% of all listed products, and despite conflicting data and lack of standardization among different sources, two technical products (TC and TK) and nine different formulation types were identified. The most common types were technical concentrates in the form of fungus-colonized substrates (26.3%), wettable powders (20.5%), and oil dispersions (15.2%). The remaining types include granules (2.9%), technical materials (2.9%), baits (1.8%), water dispersible granules (1.8%), oil miscible flowable concentrates (1.2%), ULV suspensions (0.6%), suspension concentrates (0.6%), and contact powders (0.6%).

As indicated previously, assigning a formulation type to a product is not a straightforward step. Formulation definitions developed for chemical pesticides often are not precisely applicable to biopesticides. Revisions in the international code to accommodate current microbial formulation designations are needed. Also, there is a great deal of overlap among formulation definitions, and a single product may fall into more than one category. There is, therefore, a certain amount of subjectivity associated with selection of the proper code, and there are many circumstances in which designations adopted in this paper do not match those used in previous publications or by manufacturers.

Among listed products, at least 160 (93.6%) are claimed to have activity against insects, and 28 (16.4%) against acarines. Nematodes (2.9%), crustaceans (1.2%), and centipedes (1.2%) also are among the claimed target hosts, while targets

for 4.7% of products could not be determined. The sum of the above percentages is substantially greater than 100% due to the fact that most products are indicated as controlling pests in more than one order and usually multiple species within an order (data not shown). Targets are distributed among 10 insect orders: Hemiptera (59.6%), Coleoptera (40.9%), Lepidoptera (17.5%), Thysanoptera (14.6%), Orthoptera (9.4%), Diptera (7.0%), Hymenoptera (2.9%), Isoptera (2.3%), Siphonaptera (1.2%), and Blattodea (0.6%). The listed products are claimed to control targets in at least 48 insect families, with insects in the families Aleyrodidae, Curculionidae (including Scolytinae), Cercopidae, Scarabaeidae, Aphididae, and Thripidae being among the most common targets (Appendix 2). Among mycoacaricides, only three products, all based on *H. thompsonii*, were developed specifically for control of acarines. Target acarines are from at least four families, but predominantly focused on Tetranychidae.

Out of 129 products currently in the "active" category (registered, undergoing registration or available), over 90% were developed for inundation microbial control, while less than 10% were developed exclusively for inoculation control strategies (as defined by Eilenberg et al., 2001). The later strategy is based on mycoinsecticides with high mycelial concentration, for example formulated products based solely on hyphae, or fungus-colonized substrates for control of soil-inhabiting beetles.

Historically, countries in Asia, Latin America, and Eastern Europe have accounted for the greatest use of fungal pathogens. As reviewed by Feng et al. (1994), in the 1980s approximately 0.8-1.3 million hectares of forests in China were treated annually with *B. bassiana* for control of numerous pests; however, this use was largely government supported (noncommercial) and has declined markedly in recent years (Feng, 2003). Commercial production of mycopesticides is only beginning in China; launches by private companies of formulated products for management of grasshoppers and tea leafhoppers is anticipated (M.-G. Feng, Zhejiang University,

China, personal communication). There also have been high levels of noncommercial production of mycoinsecticides by grower cooperatives in Brazil during the 1970s and 1980s (Alves, 1998), by government laboratories in several Eastern European countries (especially Russia, Poland and Czechoslovakia) during the same time period (Lipa, 1985; Feng et al., 1994), and by government laboratories in Cuba since the 1990s (Rosset, 1997; Vega, 2005).

Products developed by South American companies and institutions represent 42.7% of all listed products, followed by North America (20.5%), Europe and Asia (12.3% each), Central America (7.1%), Africa (2.9%), and Oceania (2.3%). The data suggest that the largest current program using entomopathogenic fungi is based on *M. anisopliae* application for control of spittlebugs in sugarcane fields and pastures of South and Central America. Among the 58 listed products based on *M. anisopliae*, 37 (63.8%) are claimed to be active against cercopids, and over 90% of these are currently available in South and Central America. In Brazil, *M. anisopliae* is used to control a complex of spittlebugs, including *Mahanarva fimbriolata* (Stål) and *Mahanarva posticata* (Stål) in sugarcane fields, and *M. fimbriolata*, *Deois flavopicta* (Stål) and *Notozulia entreriana* (Berg) in pastures (Alves, 1998; Faria and Magalhães, 2001). In a single sugarcane operation, this fungus is annually applied on over 60,000 hectares for control of *M. fimbriolata* (J.E.M. de Almeida, Instituto Biológico, Brazil, personal communication). Other impressive numbers relate to the worldwide use of *M. anisopliae* for scarab control, and *B. bassiana* for control of weevils, whiteflies, scarabs, thrips, and aphids, each with at least 10 products currently in the "active" category.

In the literature there is a great deal of incomplete, inconsistent and even conflicting information regarding developed mycopesticides. Other difficulties in assembling an accurate list of products are related to incomplete information presented

on product labels or in product descriptions (when available), lack of up-to-date information (especially following corporate mergers), information in only one language, etc. Despite its imperfections, the list of products presented in this work represents a database that could be updated periodically, providing the scientific community with a valuable source of state-of-the-art information on fungus-based insecticides and acaricides.

APPENDIX 1

Fungi developed for control of insects and acarines. List organized by fungal species and within each species by region (Europe, Africa, Asia, Oceania, North America, Central America, and South America). Within each region, countries are listed in alphabetical order.

Country(ies) where undergoing registration, registered or marketed	Trade name[a]	Propagule(s) / Formulation[b]	Claimed Target(s) (Orders and Families)	Manufacturer	Source(s)[c]
Aschersonia aleyrodis					
Former USSR	Aseronia**	C / TC	Hemiptera (Aleyrodidae)	Gosagroprom, Former USSR	Ignoffo and Anderson (1979), M. Shternshis (pers. communication)
Beauveria bassiana					
Czech Republic	Boverol** (=Boverol-Spofa)	C / WP	Coleoptera (Chrysomelidae)	Fytovita, Czech Republic	Kreutz et al. (2004), Wraight et al. (2001), Z. Adamék (pers. communication)
Czech Republic	Boverosil**	C / WP (alone or combined with chemical pesticides)	Coleoptera (Curculionidae) + other stored-product pests (not specified)	NI	Feng et al. (1994), Hluchý and Samšiňáková (1989), Z. Adamék (pers. communication)
France	Ostrinil**	C+H / TK	Lepidoptera (Crambidae)	Natural Plant Protection (NPP), France	Wraight et al. (2001), Shah and Goettel (1999)
16Spain	Trichobass-L*	C / OD	Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae, Pieridae), Hemiptera (Aleyrodidae),	Trichodex S.A., Spain	Website[1]

Spain	Trichobass-P*	C / WP	Thysanoptera (Thripidae) + Acari (Tetranychidae) Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae) + Acari (Tetranychidae)	Trichodex S.A., Spain	Website[1]
South Africa	Bb Plus*	A / WP	Hemiptera (Aphididae) + Acari (Tetranychidae)	Biological Control Products SA (Pty) Ltd, South Africa	Website[2]
South Africa	Bb Weevil*	A / CP	Coleoptera (Curculionidae)	Biological Control Products SA (Pty) Ltd, South Africa	Website[2]
India	BioGuard Rich*	A / NI	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae, Aphididae), Lepidoptera (Crambidae), Thysanoptera (Thripidae)	Plantrich Chemicals & Biofertilizers Ltd, India	Website[3]
India	Bio-Power*	A / WP	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera: Auchenorrhyncha (Cicadellidae, Delphacidae), Lepidoptera (Plutellidae)	T.Stanes & Company Limited, India	Copping (2004), Website[4]

India	Racer*	A / WP	Lepidoptera (Noctuidae) + others (not specified)	Agri Life, India	Copping (2004), Website[5]
Japan	Biolisa-Madara*	C+H / TK (fiber band)	Coleptera (Cerambycidae)	Nitto Denko, Japan	M. Shimazu (pers. communication)
Russia	Boverin*	B / WP	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Biodron, Russia	Shternshis (2004), M. Shternshis (pers. communication)
Russia	Boverin*	B / NI (liquid suspension)	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Biodron, Russia	Shternshis (2004), M. Shternshis (pers. communication)
Former USSR	Boverin**	C or S+B / WP (alone or combined with chemical insecticides)	Coleoptera (Chrysomelidae), Lepidoptera (Tortricidae)	Glavmikrobioprom, Former USSR	Feng et al. (1994), Ferron (1981), Ignoffo and Anderson (1979)
Mexico	Bea-Sin*	C / WP	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	Wraight et al. (2001), A. Paez (pers. communication), Company brochure
Mexico	Bea-Sin*	C / OD	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Aleyrodidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	A. Paez (pers. communication), Company brochure
Mexico	Bio-Fung*	A / NI	Orthoptera	Centro de Sanidad Vegetal de Guanajuato (CESAVEG), Mexico	Guerra et al. (2001), P. Guerra (pers. Communication)

USA	Baits Motel Stay Awhile - Rest Forever* (previously: Healthy Indoors Brand, Ant and Cockroach Bait Station)	C / RB	Hymenoptera (Formicidae)	GlycoGenesys, Inc., USA (previously: SafeScience, Inc., USA)	Website[6], R. Pereira (pers. communication)
USA	Balence*	C / OD	Diptera (Muscidae)	Jabb of the Carolinas Inc., USA	Kaufman et al. (2005), Website[6], L. Castrillo (pers. communication)
USA, Mexico, Denmark, Italy, Spain, Sweden, Japan	BotaniGard ES*	C / OD	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Miridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Thysanoptera (Thripidae)	Laverlam International Corporation, USA (previously: Emerald BioAgriculture Corp., USA; Mycotech Corp., USA)	Kabaluk and Gazdik (2005), Wraight et al. (2001), Website[7]
USA, Mexico, Denmark, Italy, Spain, Sweden, Japan	BotaniGard 22 WP*	C / WP	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Thysanoptera (Thripidae)	Laverlam International Corporation, USA (previously: Emerald BioAgriculture Corp., USA; Mycotech Corp., USA)	Kabaluk and Gazdik (2005), Wraight et al. (2001), Website[7], Company brochure
USA	CornGard**	C / GR	Lepidoptera (Crambidae)	Mycotech Corp., USA	Hajek et al. (2001), Shah and Goettel (1999)

	USA, Mexico, Denmark, Italy, Sweden	Mycotrol ES*	C / OD	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Lepidoptera (Crambidae), Orthoptera (Acrididae, Tettigoniidae), Thysanoptera (Thripidae)	Laverlam International Corporation, USA (previously: Emerald BioAgriculture Corp., USA; Mycotech Corp., USA)	Kabaluk and Gazdik (2005), Wraight et al. (2001), Website[7]
	USA, Mexico, Denmark, Italy, Sweden	Mycotrol-O*	C / OD	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Lepidoptera (Crambidae, Noctuidae, Pieridae, Plutellidae), Orthoptera (Acrididae,	Laverlam International Corporation, USA (previously: Emerald BioAgriculture Corp., USA; Mycotech Corp., USA)	Website[7], Company brochure

			Tettigoniidae), Thysanoptera (Thripidae)		
USA	Mycotrol OF**	C / SU	Orthoptera (Acrididae, Tettigoniidae)	Mycotech Corp., USA	S. Jaronski (pers. communication)
USA	Mycotrol OF**	C / OD (also for ULV applications)	Orthoptera (Acrididae, Tettigoniidae)	Mycotech Corp., USA	S. Jaronski (pers. communication)
USA, Mexico, Denmark, Italy, Sweden	Mycotrol WP*	C / WP	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Fulgoridae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Thysanoptera (Thripidae), Lepidoptera (Crambidae), Orthoptera (Acrididae, Tettigoniidae)	Emerald BioAgriculture Corp., USA (previously: Mycotech Corp., USA)	Kabaluk and Gazdik (2005), Wraight et al. (2001), Website[6]
USA, Mexico, Greece, Italy, Spain, Switzerland	Naturalis L* (=Fermone Naturalis L-225)	C / OD	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Diptera (Ephydridae, Mycetophilidae, Sciaridae, Tipulidae), Hemiptera (Lygaeidae, Miridae,	Troy Biosciences Inc., USA	Kabaluk and Gazdik (2005), Website[6,8], K. Moran (pers. communication), Company brochure

			Cercopidae, Cicadellidae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae), Hymenoptera (Formicidae), Lepidoptera (Crambidae, Gelechiidae, Geometridae, Noctuidae, Tortricidae), Orthoptera (Acrididae, Gryllotalpidae), Thysanoptera (Thripidae) + Acari (Eriophyidae, Tetranychidae) + Crustacea + Diplopoda		
USA	Naturalis L – Home & Garden*	C / OD	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hymenoptera (Formicidae), Diptera (Tipulidae), Hemiptera (Lygaeidae, Cercopidae, Cicadellidae, Aleyrodidae, Aphididae,	Troy Biosciences Inc., USA	Website[8], K. Moran (pers. communication), Company brochure

				Pseudococcidae, Psyllidae), Lepidoptera (Crambidae, Geometridae, Noctuidae), Orthoptera (Acrididae, Gryllotalpidae), Thysanoptera (Thripidae) + Acari (Tarsonemidae, Tetranychidae) + Crustacea + Diplopoda		
	USA	Naturalis-O**	C / OD	Coleoptera (Chrysomelidae, Curculionidae), Hemiptera (Miridae, Cicadellidae, Aleyrodidae, Aphididae, Psyllidae), Lepidoptera, Thysanoptera (Thripidae)	Troy Biosciences Inc., USA	Company brochure
	USA	Organigard Emulsifiable Suspension Mycoinsecticide*	C / OD	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cicadellidae, Aleyrodidae, Aphididae, Pseudococcidae, Psyllidae),	Emerald BioAgriculture Corp., USA (previously: Mycotech Corp., USA)	Hajek et al. (2001), Website[6]

			Thysanoptera (Thripidae), Orthoptera (Acrididae, Tettigoniidae), Lepidoptera (Crambidae)		
Costa Rica, Panama	Beauvedieca*	C+H / TK	Coleoptera (Curculionidae)	Liga Agricola Industrial de La Caña de Azucar (LAICA), Costa Rica	Website[9]
Costa Rica, Panama	Beauvedieca*	C / NI	Coleoptera (Curculionidae)	Liga Agricola Industrial de La Caña de Azucar (LAICA), Costa Rica	Website[9]
Costa Rica	Nativo 2 SC*	A / NI	Coleoptera (Curculionidae)	Bayer Cropscience S.A., Colombia	W. Solano (pers. communication), Website[10]
Guatemala	Bichoxe***	A / NI	Hemiptera (Aleyrodidae)	Produtos Ecológicos, Guatemala	Alves et al. (2003)
Honduras, El Salvador, Guatemala, Jamaica, Nicaragua	Bazam*	C / WP	Coleoptera (Chrysomelidae, Curculionidae), Hemiptera (Aleyrodidae, Aphididae), Lepidoptera (Noctuidae, Plutellidae) + Acari (Tetranychidae)	Escuela Agrícola Panamericana, Honduras	Website[9], R. Trabanino (pers. communication)
Nicaragua	Mirabiol*	C+H / TK	Coleoptera (Curculionidae)	Union de Cooperativas Agropecuarias (UCA), Nicaragua	Website[9]
Argentina	Bb Vinchuca*	C / OD	Hemiptera (Reduviidae)	Laboratorios Biagro S.A., Argentina	R. Lecuona (pers. communication)

Argentina	Bb Moscas*	C / OD	Diptera (Muscidae)	Laboratorios Biagro S.A., Argentina	R. Lecuona (pers. communication)
Argentina	Bb Moscas*	C / RB	Diptera (Muscidae)	Laboratorios Biagro S.A., Argentina	R. Lecuona (pers. communication)
Brazil	Bovenat*	C+H / TK	Coleoptera (Curculionidae), Hemiptera (Aleyrodidae)	Natural Rural, Brazil	Website[11]
Brazil	Boveril WP ESALQ447*	C+H / TK	Coleoptera (Curculionidae)	Itaforte Industrial de BioProdutos Agro-Florestais Ltda., Brazil	R. Lopes (pers. communication)
Brazil	Boveril WP PL63*	C+H / TK	Coleoptera (Curculionidae) + Acari (Tetranychidae)	Itaforte Industrial de BioProdutos Agro-Florestais Ltda., Brazil	A. Ballarotti (pers. communication), R. Lopes (pers. communication)
Brazil	Boveriol*	C+H / TK	Isoptera (Rhinotermitidae, Termitidae)	Tecnicontrol Ind. e Com. de Produtos Biológicos Ltda., Brazil	Leite et al. (2003b), J. Almeida (pers. communication)
Brazil	No trade name*	C+H / TK	Coleoptera (Curculionidae)	Empresa Pernambucana de Pesquisa Agropecuária (IPA), Brazil	E. Marques (pers. communication)
Brazil	No trade name**	C+H / TK	Hymenoptera (Formicidae), Siphonaptera (Pulicidae)	Instituto de Biotecnologia Rangel Ltda. (Inbioter), Brazil	D. Rangel (pers. communication)
Brazil	No trade name*	C+H / TK	Coleoptera (Curculionidae)	Toyobo do Brasil Ltda., Brazil	J. Almeida (pers. communication)
Brazil	Bovemax*	C / OD	Coleoptera (Cerambycidae)	Turfal Ind. Com. Prod. Biol., Brazil	M.S. Leite (pers. communication)
Colombia	Ago Biocontrol Bassiana 50***	C / NI	Coleoptera, Diptera, Hemiptera, Lepidoptera	Ago Biocontrol, Colombia	Shah and Goettel (1999)
Colombia	Agronova*	A / NI	Coleoptera (Curculionidae,	Live Systems Technology S.A.,	Website[12]

			Scarabaeidae), Lepidoptera (Noctuidae, Nymphalidae, Sphingidae)	Colombia	
Colombia	Baubassil*	C / NI	Coleoptera, Hemiptera, Lepidoptera	Productos Biológicos Perkins Ltda, Colombia	Website[13]
Colombia, Dominican Republic	Bauveril*	A / WP	Coleoptera (Curculionidae, Scarabaeidae), Lepidoptera (Castniidae)	Laverlam S.A., Colombia	Alves et al. (2003), Website[14]
Colombia	BioExpert*	A / NI	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae)	Live Systems Technology S.A., Colombia	Website[12]
Colombia, Peru, Costa Rica, Dominican Republic, Honduras	Brocaril 50 WP*	A / WP	Coleoptera (Curculionidae)	Laverlam S.A., Colombia	Alves et al. (2003), Website[9,14, 15]
Colombia	Brocavec*	NI	NI	Empresa Colombiana de Productos Veterinarios Vecol S.A., Colombia	Website[16]
Colombia	Cebiopest*	NI	NI	Fundacion Centro de Biotecnologia Mariano Ospina Perez, Colombia	Website[16]
Colombia	Conidia***	C / WG	Coleoptera (Curculionidae)	Hoechst Schering AgrEvo, Colombia	Stetter and Lieb (2000), Wraight and Carruthers (1999)
Venezuela	Proecol***	C / NI	Lepidoptera (Noctuidae)	Probioagro S.A., Venezuela	Hajek et al. (2001), Wraight et al. (2001)
Beauveria brongniartii					
Austria, Italy	Melocont-Pilzgerste* (=Beauveria brong)	C+H / TK	Coleoptera (Scarabaeidae)	Kwizda Agro GmbH, Austria / Agrifutur	Henke et al. (2002),

				s.r.l., Italy	Wraight et al. (2001), H. Strasser (pers. communication)
Switzerland	Beauveria brongniartii Myzel*	C+H / TK	Coleoptera (Scarabaeidae)	LBBZ Arenenberg, Switzerland	S. Keller (pers. communication)
Switzerland	Beauveria Schweizer*	C+H / TK	Coleoptera (Scarabaeidae)	Eric Schweizer Samen AG, Switzerland	Shah and Pell (2003), Butt and Copping (2000)
Switzerland	Engerlingspilz*	C+H / TK	Coleoptera (Scarabaeidae)	Andermatt Biocontrol AG, Switzerland	Hajek et al. (2001), M. Andermatt (pers. communication)
Reunion Island	Betel*	C+H / TK	Coleoptera (Scarabaeidae)	Betel Reunion S.A., Reunion Island (subsidiary of Natural Plant Protection, France)	Hajek et al. (2001), Stetter and Lieb (2000), A. Bonhomme (pers. communication)
Japan	Biolisa-Kamikiri*	C+H / TK (fiber band)	Coleptera (Cerambycidae)	Nitto Denko, Japan	Hajek et al. (2001, 2006), Wraight et al. (2001), M. Shimazu (pers. communication)
Colombia	Ago Biocontrol Beauveria 50***	A / NI	Coleoptera, Hemiptera, Lepidoptera, Diptera	Ago Biocontrol, Colombia	Shah and Goettel (1999)
Conidiobolus thromboides					
South Africa	No trade name**	C / NI (liquid suspension)	Hemiptera (Aphididae), Thysanoptera (Thripidae)	Mycolab, South Africa	J. Hatting (pers. communication)
Colombia, Costa Rica, Honduras	Vektor 25 SL*	C / NI (liquid suspension)	Hemiptera (Aleyrodidae, Ortheziidae)	Laverlam S.A., Colombia	Website[9,14]
Hirsutella thompsonii					
India	MeteHit***	NI	Acari	Plantrich Chemicals & Biofertilizers Ltd, India	Santra (2004)

India	Mycohit*	NI/ WP (dusting during wet conditions is possible)	Acari (Eriophyidae)	Hindutan Antibiotics Ltd, India	Copping (2004), Kumar and Singh (2001)
USA	Mycar**	C / WP	Acari (Eriophyidae)	Abbott Laboratories, USA	McCoy (1978, 1996)
Isaria fumosorosea (formerly *Paecilomyces fumosoroseus*)					
Europe (many), Japan	PreFeRal* (=Preferred)	B / WG	Hemiptera (Aleyrodidae)	Biobest n.v., Belgium (under license from Certis, Inc., USA)	Saito (2005), Wraight et al. (2001), Website[17]
India	Priority*	A / WP	Acari (Eriophyidae,Tetranychidae)	T.Stanes & Company Limited, India	Copping (2004), Website[2]
Mexico	Pae-Sin*	C / WP	Hemiptera (Aleyrodidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	Wraight et al. (2001), A. Paez (pers. communication), Company brochure
Mexico	Pae-Sin*	C / OD	Hemiptera (Aleyrodidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	A. Paez (pers. communication), Company brochure
Mexico	P. fumosoroseus**	A / NI	Hemiptera (Aleyrodidae)	Centro de Sanidad Vegetal de Guanajuato (CESAVEG), Mexico	Guerra et al. (2001), P. Guerra (pers. communication)
USA, Mexico	PFR-97 20% WDG*	B / WG	Hemiptera (Aleyrodidae, Aphididae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Certis, Inc., USA (previous owner: Thermo Trilogy Corp., USA)	Wraight et al. (2001), Website[6]
Colombia	Ago Biocontrol Paecilomyces 50***	A / NI	Coleoptera + Nematoda	Ago Biocontrol, Colombia	Shah and Goettel (1999)
Colombia	Fumosil*	A / NI	Hemiptera (Aleyrodidae,	Productos Biológicos Perkins Ltda, Colombia	Website[13]

			Aphididae, Pseudococcidae), Thysanoptera (Thripidae)		
Colombia	Successor*	A / NI	Hemiptera (Aleyrodidae, Aphididae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Live Systems Technology S.A., Colombia	Website[12]
Venezuela	Bemisin***	C / NI	Hemiptera (Aleyrodidae)	Probioagro, Venezuela	Hajek et al. (2001), Wraight et al. (2001)
***Isaria* sp.** (formerly *Paecilomyces* sp.)					
India	PaciHit Rich*	NI (liquid suspension)	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Nematoda	Plantrich Chemicals & Biofertilizers Ltd, India	Website[1]
Lagenidium giganteum					
USA	Laginex AS*	H / SC	Diptera (Culicidae)	AgraQuest, Inc., USA	Scholte et al. (2004), Website[6], R. Riggs (pers. communication)
Lecanicillium longisporum (formerly *Verticillium lecanii*)					
Finland, Switzerland, UK, Japan	Vertalec*	B / WP	Hemiptera (Aphididae)	Koppert Biological Systems, Netherlands (previously: Tate and Lyle, UK)	Website[18], W. Ravensberg (pers. communication)
Brazil	Vertirril WP 1300*	C+H / TK	Hemiptera (Aleyrodidae, Ortheziidae)	Itaforte Industrial de BioProdutos Agro-Florestais Ltda., Brazil	A. Ballarotti (pers. communication), R. Lopes (pers. communication)
Lecanicillium muscarium (formerly *V. lecanii*)					
Netherlands, Denmark, Finland, France, Italy, Switzerland, UK, Turkey, Japan	Mycotal*	C / WP	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae)	Koppert Biological Systems, Netherlands (previously: Tate and Lyle, UK)	Website[18], W. Ravensberg (pers. communication)

Russia	Verticillin*	B / WP	Hemiptera (Aleyrodidae, Aphididae) + Acari (Tetranychidae)	Biodron, Russia	Shternshis (2004), M. Shternshis (pers. communication)
Russia	Verticillin*	B / NI (liquid suspension)	Hemiptera (Aleyrodidae, Aphididae) + Acari (Tetranychidae)	Biodron, Russia	Shternshis (2004), M. Shternshis (pers. communication)
***Lecanicillium* sp.** (formerly *V. lecanii*)					
Spain	Trichovert*	A / NI	Not informed	Trichodex S.A., Spain	Website[1]
Switzerland	MicroGermin Plus*	H / WP	Hemiptera (Aleyrodidae, Aphididae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Omya (Schweiz) AG, Switzerland	S. Keller (pers. communication)
India	Bio-Catch*	A / WP	Hemiptera (Aleyrodidae, Aphididae, Pseudococcidae)	T.Stanes & Company Limited, India	Copping (2004), Website[2]
India	Biovert Rich*	A / NI (powder)	"Insects" + Nematoda	Plantrich Chemicals & Biofertilizers Ltd, India	Website[1]
India	Mealikil*	A / WP	Hemiptera: Sternorrhyncha ("scales") + others (not specified)	Agri Life, India	Copping (2004), Website[3]
Mexico	Verti-Sin*	C / OD	Hemiptera (Aphididae), Thysanoptera (Thripidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	A. Paez (pers. communication), Company brochure
Honduras, El Salvador, Guatemala, Jamaica,	Verzam*	C/ NI	Hemiptera (Aleyrodidae,	Escuela Agrícola Panamericana,	Website[9], R. Trabanino (pers. communication)

Nicaragua			Aphididae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Honduras	
Brazil	No trade name*	C / OD	Hemiptera (Aphididae)	Turfal Ind. Com. Prod. Biol., Brazil	M.S. Leite (pers. communication)
Brazil	Vertinat*	C+H / TK	Hemiptera (Aleyrodidae, Ortheziidae)	Natural Rural, Brazil	Website[11]
Colombia	Ago Biocontrol Verticillium 50***	A / NI	Hemiptera, Diptera	Ago Biocontrol, Colombia	Shah and Goettel (1999)
Colombia, Peru, Costa Rica, Honduras	Vertisol 50 WP*	A / WP	Hemiptera (Aleyrodidae)	Laverlam S.A., Colombia	Website[9,14, 15]
Metarhizium anisopliae					
Austria, Italy	Granmet-P*	C+H / TK	Coleoptera (Scarabaeidae, Curculionidae, Nitidulidae)	Kwizda Agro GmbH, Austria / Agrifutur s.r.l., Italy	H. Strasser (pers. communication)
Germany, Switzerland	BIO 1020**	H / GR (more specifically, a Fine Granule-FG)	Coleoptera (Curculionidae)	Bayer AG, Germany	Hajek et al. (2001), Feng et al. (1994), D. Roberts (pers. communication)
Spain	Trichomet*	C / NI	Not informed	Trichodex S.A., Spain	Website[1]
Switzerland	BIO 1020*	H / GR	Coleoptera (Curculionidae)	Intrachem Bio SA, Italy	S. Keller (pers. communication)
Switzerland	Metarhizium Andermatt**	C+H / TK	Coleoptera (Scarabaeidae)	Andermatt Biocontrol AG, Switzerland	Hajek et al. (2001), M. Andermatt (pers. communication)
Switzerland	Metarhizium Schweizer*	C+H / TK	Coleoptera (Scarabaeidae)	Eric Schweizer Samen AG, Switzerland	Hajek et al. (2001), Wraight et al. (2001), S. Keller (pers. communication)

India	Bio-Magic*	A / WP (also applied undiluted into soil for control of grubs and weevils)	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Cercopidae) + others ("plant hoppers")	T.Stanes & Company Limited, India	Website[2]
India	Biomet Rich*	A / NI (liquid suspension)	Coleoptera, Isoptera	Plantrich Chemicals & Biofertilizers Ltd, India	Website[1]
India	Pacer*	A / WP	Isoptera	Agri Life, India	Copping (2004), Website[3]
Australia	BioCane Granules Biological Insecticide*	C+H / TK	Coleoptera (Scarabaeidae)	Becker Underwood Inc., USA - Australian division (previously: Bio-Care Technology Pty Ltd)	Samson et al. (2005), Milner et al. (2002), Website[19]
Australia	Chafer Guard Granules* (= Biogreen)	C+H / TK	Coleoptera (Scarabaeidae)	Becker Underwood Inc., USA - Australian division (under licence from Dept. Primary Industry and Adelaide Research and Innovation Pty Limited, Australia)	Website[19]
Mexico	Fitosan-M*	A / NI	Coleoptera (Scarabaeidae), Orthoptera	Centro de Sanidad Vegetal de Guanajuato (CESAVEG), Mexico	Guerra et al. (2001), P. Guerra (pers. communication)
Mexico	Meta-Sin*	C / WP	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Cercopidae), Orthoptera	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	Hajek et al. (2001), A. Paez (pers. communication), Company brochure
Mexico	Meta-Sin*	C / OD	Coleoptera (Curculionidae,	Agrobiologicos del Noroeste S.A. de C.V.	A. Paez (pers. communication),

			Scarabaeidae), Hemiptera (Cercopidae), Orthoptera	(Agrobionsa), Mexico	Company brochure
USA, Mexico	Bio-Blast Biological Termiticide*	C / TC	Isoptera (Kalotermitidae, Rhinotermitidae, Termopsidae)	EcoScience Corporation, USA	Wraight and Carruthers (1999), Rath (1995), Website[6], Company brochure
USA	Bio-Path Cockroach Control Chamber**	C / RB	Blattodea (Blattellidae, Blattidae)	EcoScience Corporation, USA	Evans (2003), Hajek et al. (2001), Gunner et al. (1995), J. Lord (pers. communication)
USA	Taenure Granular Bioinsecticide*	C / GR	Coleoptera (Curculionidae, Scarabaeidae), Diptera (Ephydridae, Mycetophilidae, Sciaridae, Tipulidae), Thysanoptera (Thripidae)	Novozymes Biologicals Inc., USA (previously: Earth BioSciences; (Taensa Co., USA)	Website[6], Company brochure
USA	Taerain**	C / OD	Hemiptera (Aleyrodidae), Thysanoptera (Thripidae) + Acari (Tetranychidae)	Earth BioSciences Inc., USA	Company brochure
USA	Tick-EX EC*	C / OD	Acari (Ixodidae) + Coleoptera (Scarabaeidae)	Novozymes Biologicals Inc., USA (previously: Earth BioSciences; Taensa Co., USA)	Website[6], Company brochure

USA	Tick-EX G*	C / GR	Acari + Coleoptera (Scarabaeidae)	Novozymes Biologicals Inc., USA (previously: Earth BioSciences; Taensa Co., USA)	Website[6]
Costa Rica, Panama	Metadieca*	C+H / TK	Hemiptera (Cercopidae)	Liga Agricola Industrial de La Caña de Azucar (LAICA), Costa Rica	Website[9]
Costa Rica, Panama	Metadieca*	C / TC	Hemiptera (Cercopidae)	Liga Agricola Industrial de La Caña de Azucar (LAICA), Costa Rica	Website[9]
Costa Rica	Technogreen Metarhizium 35 SL*	A / NI (liquid suspension)	Not informed	Biolaboratorios de Centroamerica S.A. (Biolab), Costa Rica	Website[10]
Guatemala	Salivase***	A / NI	Hemiptera (Cercopidae)	Produtos Ecológicos, Guatemala	Alves *et al.* (2003)
Honduras, El Salvador, Guatemala, Jamaica, Nicaragua	Metazam*	C / WP	Hemiptera (Cercopidae), Lepidoptera (Crambidae) + Acari (Ixodidae)	Escuela Agrícola Panamericana, Honduras	R. Trabanino (pers. communication)
Nicaragua	Metarhisa WP*	C / WP	Coleoptera (Curculionidae), Hemiptera (Cercopidae), Lepidoptera (Crambidae)	Nicaragua Sugar Estates Limited, Nicaragua	Website[9]
Brazil	BioCerto para Cigarrinhas*	C / OD	Hemiptera (Cercopidae)	Biocerto Ind. Com. Prod. Agrop. Ltda, Brazil	J. Falcão (pers. communication)
Brazil	BioCerto PM*	C / TC	Hemiptera (Cercopidae)	Biocerto Ind. Com. Prod. Agrop. Ltda, Brazil	J. Falcão (pers. communication)

Brazil	Biocontrol**	C / NI	Hemiptera (Cercopidae)	Agroceres, Brazil	Leite et al. (2003b), S. Alves (pers. communication)
Brazil	Biomax**	C / WP	Hemiptera (Cercopidae)	Labormax Produtos Químicos Ind. e Com. Ltda., Brazil	R. Pereira (pers. communication)
Brazil, Panama	Biotech*	C+H / TK	Hemiptera (Cercopidae)	Biotech, Brazil	Leite et al. (2003b), E. Marques (pers. communication)
Brazil	Conbio**	C+H / TK	Hemiptera (Cercopidae)	Equilíbrio Controle Biológico Ltda, Brazil	A. Batista Filho (pers. communication)
Brazil	Metabiol*	C+H / TK	Hemiptera (Cercopidae)	Tecnicontrol Ind. e Com. de Produtos Biológicos Ltda., Brazil	Leite et al. (2003b)
Brazil	Metanat*	C / OD	Hemiptera (Cercopidae, Aphididae)	Natural Rural, Brazil	Website[11]
Brazil	Metanat*	C+H / TK	Hemiptera (Cercopidae, Aphididae)	Natural Rural, Brazil	Website[11]
Brazil	Metaquino**	C+H / TK	Hemiptera (Cercopidae)	Com. Exec. Def. Fit. Lav. Can. PE (CODECAP), Brazil	Aquino *et al.* (1975)
Brazil	Metarril WP E9*	C+H / TK	Hemiptera (Cercopidae)	Itaforte Industrial de BioProdutos Agro-Florestais Ltda., Brazil	R. Lopes (pers. communication)
Brazil	Metarril SC 1037*	C / OD	Hemiptera (Cercopidae) + Acari (Ixodidae)	Itaforte Industrial de BioProdutos Agro-Florestais Ltda., Brazil	R. Lopes (pers. communication)
Brazil	Metarriz*	C+H / TK	Hemiptera (Cercopidae)	Biocontrol Sistemas de Controle Biológico, Brazil	J. Almeida (pers. communication)
Brazil	Methamax*	C / OD	Hemiptera (Cercopidae)	Turfal Ind. Com. Prod. Biol., Brazil	M.S. Leite (pers. communication)

Brazil	Methavida*	C+H / TK	Hemiptera (Cercopidae)	Methavida Controle Biológico Agrícola, Brazil	J. Almeida (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Bioagro Controle Biológico, Brazil	E. Marques (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Biocana Braz e Costa Ind. e Com. de Produtos Biológicos, Brazil	J. Almeida (pers. communication)
Brazil	No trade name**	C / NI	Hemiptera (Cercopidae)	BTA, Brazil	Leite *et al.* (2003b)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Empresa Mato-Grossense de Pesquisa, Assistência e Extensão Rural S/A (EMPAER), Brazil	M. Santaella (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Empresa Pernambucana de Pesquisa Agropecuária (IPA), Brazil	E. Marques (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO), Brazil	S. Alves (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Fitossan Assistência Fitossanitária e Controle Biológico Ltda., Brazil	J. Santos (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Fundação Agro-ambiental da Amazônia (FUNAM), Brazil	V. Rocha (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Instituto Biológico, Brazil	J. Almeida (pers. communication)

Brazil	No trade name**	C+H / TK	Hymenoptera (Formicidae), Siphonaptera (Pulicidae)	Instituto de Biotecnologia Rangel Ltda. (Inbioter), Brazil	D. Rangel (pers. communication)
Brazil	No trade name*	C+H / TK	Hemiptera (Cercopidae)	Toyobo do Brasil Ltda., Brazil	J. Almeida (pers. communication)
Colombia	Ago Biocontrol Metarhizium 50***	A / NI	Coleoptera, Hemiptera, Lepidoptera, Orthoptera	Ago Biocontrol, Colombia	Shah and Goettel (1999)
Colombia	DeepGreen*	A / NI	Coleoptera (Scarabaeidae), Hemiptera (Miridae, Cicadellidae)	Live Systems Technology S.A., Colombia	Website[12]
Colombia, Costa Rica, Honduras, Nicaragua	Destruxin* (=Dextruxin 50 WP)	A / WP	Coleoptera (Curculionidae, Scarabaeidae), Hemiptera (Miridae, Cercopidae, Delphacidae), Lepidoptera (Noctuidae)	Laverlam S.A., Colombia	Website[9,14]
Colombia	Metaril*	C / NI	Coleoptera (Curculiionidae, Scarabaeidae), Hemiptera (Miridae, Pentatomidae, Cercopidae, Delphacidae), Lepidoptera (Noctuidae)	Productos Biológicos Perkins Ltda, Colombia	Website[13]
Venezuela	Cobican***	C / NI	Coleoptera (Scarabaeidae), Hemiptera (Cercopidae, Aphididae)	Probioagro S.A., Venezuela	Alves et al. (2003), Wraight et al. (2001)

Venezuela	Metabiol***	A / NI	Hemiptera (Cercopidae)	Empresa Prave Agrobioténica S.A., Venezuela	Website[20]
Metarhizium anisopliae var. ***acridum***					
Mozambique, Namibia, Tanzania, South Africa, Sudan, Zambia	Green Muscle OF*	C / OF	Orthoptera (Acrididae, Pyrgomorphidae)	Biological Control Products SA (Pty) Ltd, South Africa (under license from CABI, UK)	Pettit and Jenkins (2005), Bateman (1997), Website[2]
Australia	Green Guard ULV*	C / OF	Orthoptera (Acrididae)	Becker Underwood Inc., USA - Australian division (under licence from CSIRO, Australia)	Milner and Hunter (2001), Website[19], P. Melville (pers. communication)
Australia	Green Guard SC*	C / TC (dry spores, surfactant solution and emulsifiable oil are sold together, but not mixed)	Orthoptera (Acrididae)	Becker Underwood Inc., USA - Australian division (under licence from CSIRO, Australia)	Website[19]
Nomuraea rileyi					
Colombia	Ago Biocontrol Nomuraea 50***	A / NI	Lepidoptera	Ago Biocontrol, Colombia	Shah and Goettel (1999)
"Sporothrix insectorum"					
Brazil	No trade name**	C+H / TK	Hemiptera (Tingidae)	Estação de Aviso Fitossanitário de São José do Rio Claro, Brazil	M.A. Leite (pers. communication)
Brazil	No trade name*	S+B+H / TK (liquid substrate)	Hemiptera (Tingidae)	Instituto Biológico, Brazil	Leite et al. (2003a), J. Almeida (pers. communication)

Brazil	Sporothrix ES*	C / OD	Hemiptera (Tingidae)	Biocerto Ind. Com. Prod. Agrop. Ltda, Brazil	J. Falcão (pers. communication)
Mix: ***B. bassiana***, ***M. anisopliae, I. fumosorosea***					
Mexico	Tri-Sin*	C / WP	Hemiptera (Psyllidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	A. Paez (pers. communication), Company brochure
Mexico	Tri-Sin*	C / OD	Hemiptera (Psyllidae)	Agrobiologicos del Noroeste S.A. de C.V. (Agrobionsa), Mexico	A. Paez (pers. communication), Company brochure
Mix: ***B. bassiana***, ***M. anisopliae*** + *Paecilomyces lilacinus*					
Colombia	Fungio WP*	NI	NI	Orius Biotecnología, Colombia	Website[21]
Colombia, Ecuador, Peru, Chile, Panama	Micosplag*	C / WP	Coleoptera (Chrysomelidae, Curculionidae, Scarabaeidae), Hemiptera (Lygaeidae, Miridae, Pentatomidae, Tingidae, Cercopidae, Delphacidae), Lepidoptera (Noctuidae, Nymphalidae, Crambidae) + Nematoda	Orius Biotecnología, Colombia	Website[15, 21], M. Higuera (pers. communication)
Mix: ***B. bassiana, M. anisopliae***, ***N. rileyi***, ***I. fumosorosea*** + *Bacillus thuringiensis*					
Colombia	Micobiol Completo***	A / NI	Coleoptera, Hemiptera, Lepidoptera, Diptera + Acari	NI	Alves et al. (2003)

Mix: ***B. bassiana***, ***H. thompsonii***, ***Lecanicillium* sp.**, ***M. anisopliae***, ***N. rileyi***, ***I. fumosorosea*** + *P. lilacinus*					
Colombia	Micobiol HE***	A / NI	Coleoptera, Diptera, Hemiptera, Lepidoptera + Acari + Nematoda	NI	Alves et al. (2003)
Mix (?): ***Lecanicillium*** spp. (formerly *V. lecanii*)					
Scandinavia	MicroGermin**	A / WP	Hemiptera (Aleyrodidae, Aphididae)	Christian Hansen BioSystems, Denmark	Butt *et al*. (1999), Helyer et al. (1992), W. Ravensberg (pers. communication)

[a]Availability of product is informed as follows: *active, ** Inactive, and *** current status could not be determined (see Table 1 for additional information).

[b]Categories adopted for propagule types of developed products: H: hyphae (mycelia), A: asexual spores (type not informed), (C) aerial conidia, (S) submerged conidia, (B) blastospores (= hyphal bodies). NI – not informed by consulted sources.

[c]Websites consulted:

[1]AMC Chemical and Trichodex (http://www.amcchemical.com/).

[2] Biological Control Products SA (Pty) Ltd (http://www.biocontrol.co.za).

[3]Plantrich Chemicals & Biofertilizers Ltd. (http://www.indiamart.com/biofertilizers/).

[4]T.Stanes & Company Limited (http://www.tstanes.com).

[5]Agri Life (http://www.somphyto.com).

[6]EPA – Environmental Protection Agency (http://www.epa.gov/oppbppd1/biopesticides/ingredients/).

[7]Laverlam International Corporation (http://www.laverlamintl.com/).

[8]Troy Biosciences, Inc. (http://www.troybiosciences.com/).

[9]Bioplaguicidas.Org (http://www.bioplaguicidas.org/ (in Spanish).

[10]Servicio Fitosanitario del Estado – Costa Rica, Potecnet (http://www.protecnet.go.cr/InsumoSys/Principal.htm) (in Spanish).

[11]Natural Rural (http://www.naturalrural.com.br/) (in Portuguese).

[12]Live Systems Technology S.A. (http://lstsa.com/).

[13]Productos Biológicos Perkins Ltda (http://www.perkinsltda.com.co/) (in Spanish).

[14]Laverlam S.A. (www.laverlam.com.co/espanol/portada.htm) (in Spanish).

[15]Servicio Nacional de Sanidade Agraria (http://www.senasa.gob.pe) (in Spanish).

[16]Rede de Información y Comunicación Estratégica del Sector Agropecuario – AGRONET (http://www.agronet.gov.cl) (In Spanish).

[17]Biobest n.v. (http://www.biobest.be/).

[18]Koppert Biological Systems (www.koppert.nl/).

[19]Becker Underwood Pty Ltd (http://www.beckerunderwood.com/australia/).

[20]http://www.plagas-agricolas.info.ve/ (in Spanish).

[21]Orius Biotecnología (http://www.Oriusbiotecnologia.com/site/) (in Spanish).

APPENDIX 2

Number of mycopesticides and mycoacaricides per target orders and families.

	Fungal agents[1]																	
Orders and families	Aa	Bb	Bbr	Ct	Ht	If	Isp	Lg	Ll	Lm	Lsp	Ma	Mac	Nr	Si	Mix	**Total**	**%[2]**
Blattodea												1					**1**	**0.6**
Blattellidae												1					**1**	**0.6**
Blattidae												1					**1**	**0.6**
Coleoptera		37	7			1						22				3	**70**	**40.9**
Cerambycidae		2	1														**3**	**1.8**
Chrysomelidae		10														1	**11**	**6.4**
Curculionidae		31										10				1	**42**	**24.6**
Scarabaeidae		15	5									16				1	**37**	**21.6**
Nitidulidae												1					**1**	**0.6**
Family - Not informed			1			1						2				2	**6**	**3.5**
Diptera		6	1					1			1	1				2	**12**	**7.0**
Culicidae								1									**1**	**0.6**
Ephydridae		1										1					**2**	**1.2**
Muscidae		3															**3**	**1.8**
Mycetophilidae		1										1					**2**	**1.2**
Sciaridae		1										1					**2**	**1.2**
Tipulidae		2										1					**3**	**1.8**
Family - Not informed		1	1								1					2	**5**	**2.9**
Hemiptera	1	25	1	2		8	1		2	3	10	40			3	6	**102**	**59.6**
Heteroptera: Lygaeidae		2														1	**3**	**1.8**
Heteroptera: Miridae		8										3				1	**12**	**7.0**
Heteroptera: Pentatomidae												1				1	**2**	**1.2**
Heteroptera: Reduviidae		1															**1**	**0.6**
Heteroptera: Tingidae															3	1	**4**	**2.3**

Auchenorrhyncha: Cercopidae		2								37			1	**40**	**23.4**
Auchenorrhyncha:: Cicadellidae		9								1				**10**	**5.8**
Auchenorrhyncha: Delphacidae		1								2			1	**4**	**2.3**
Auchenorrhyncha: Fulgoridae		5												**5**	**2.9**
Sternorrhyncha: Aleyrodidae	1	20		1	8	1	1	3	5	1			1	**42**	**24.6**
Sternorrhyncha: Aphididae		12		1	3		1	2	5	3			1	**28**	**16.4**
Sternorrhyncha: Ortheziidae				1			1		1					**3**	**1.8**
Sternorrhyncha: Pseudococcidae		8			1				1					**10**	**5.8**
Sternorrhyncha: Psyllidae		9											2	**11**	**6.4**
Family - Not informed		2	1						2	1			2	**8**	**4.7**
Hymenoptera		4								1				**5**	**2.9**
Formicidae		4								1				**5**	**2.9**
Isoptera		1								3				**4**	**2.3**
Kalotermitidae										1				**1**	**0.6**
Rhinotermitidae		1								1				**2**	**1.2**
Termitidae		1												**1**	**0.6**
Termopsidae										1				**1**	**0.6**
Family - Not informed										2				**2**	**1.2**
Lepidoptera		20	1							5		1	3	**30**	**17.5**
Castniidae		2												**2**	**1.2**
Crambidae		9								2			1	**12**	**7.0**
Gelechiidae		1												**1**	**0.6**
Geometridae		2												**2**	**1.2**
Noctuidae		7								2			1	**10**	**5.8**
Nymphalidae		1											1	**2**	**1.2**
Pieridae		2												**2**	**1.2**
Plutellidae		3												**3**	**1.8**

Sphingidae	1														**1**	**0.6**
Tortricidae	2														**2**	**1.2**
Family - Not informed	4	1								1		1		2	**9**	**5.3**
Orthoptera	9									4	3				**16**	**9.4**
Acrididae	8										3				**11**	**6.4**
Gryllotalpidae	2														**2**	**1.2**
Pyrgomorphidae											1				**1**	**0.6**
Tettigoniidae	6														**6**	**3.5**
Family - Not informed	1									4					**5**	**2.9**
Siphonaptera	1									1					**2**	**1.2**
Pulicidae	1									1					**2**	**1.2**
Thysanoptera	14		1		3	1		1	3	2				1	**25**	**14.6**
Thripidae	14		1		3	1		1	3	2					**25**	**14.6**
Acari	11			3	3			2	2	5				2	**28**	**16.4**
Eriophyidae	1			2	1										**4**	**2.3**
Ixodidae										3					**3**	**1.8**
Tarsonemidae	1														**1**	**0.6**
Tetranychidae	9				3			2	2	1					**17**	**9.9**
Family - Not informed				1						1				2	**4**	**2.3**
Order(s) not informed	3								2	2				1	**8**	**4.7**

[1]Aa (*Aschersonia aleyrodis*); Bb (*Beauveria bassiana*); Bbr (*B. brongniartii*); Ct (*Conidiobolus thromboides*); Ht (*Hirsutella thompsonii*); If (I*saria fumosorosea*); Isp (*Isaria* sp.); Lg (*Lagenidium giganteum*); Ll (*Lecanicillium longisporum*); Lm (*L. muscarium*); Lsp *(Lecanicillium* sp.); Ma (*Metarhizium anisopliae*); Mac (*M. anisopliae* var. *acridum*); Nr (*Nomuraea rileyi*); Si ("*Sporothrix insectorum*"); Mix (mixture of two or more species).

[2]Percentages calculated by dividing the number of products per order/family by the total number of products (171).

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CHAPTER 2

IMBIBITIONAL DAMAGE IN CONIDIA OF THE ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA*, *METARHIZIUM ACRIDUM,* AND *METARHIZIUM ANISOPLIAE**

Abstract

When dried organisms are immersed in water, rapid imbibition may cause severe damage to plasma membranes; in unicellular organisms, such damage is usually lethal. This study investigated effects of water activity (dryness) of organisms and immersion temperature on imbibitional damage in three insect pathogenic fungi. Conidial powders of *Beauveria bassiana* (*Bb*), *Metarhizium anisopliae* (*Ma*) and *M. acridum* (*Mac*) were dried/hydrated to a broad range of water activities (0.023-0.961) prior to immersion in water at 0.5-33 ºC. Imbibitional damage in conidia of each fungus occurred rapidly, with no differences in viabilities observed following immersion for 2 vs. 60 min. Damage increased with decreasing water activity of the conidia and decreasing temperature of the immersion water. Dry (aw≤ 0.333) *Metarhizium* spp. conidia were highly susceptible to imbibitional damage, with viability declining to ≤ 5% after immersion at 0.5 ºC and ≤ 63% following immersion at 15 ºC. Germination

*Faria, M., Hajek, A.E., Wraight, S.P., 2009. Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Metarhizium anisopliae* var. *acridum*. Biological Control (in press).

of the driest *Ma* conidia was reduced to 66% after treatment at 25 ºC. In contrast, *Bb* was highly tolerant to damage, with significant reductions in viability (to levels as low as 43-65%) occurring only when dry conidia were immersed at 0.5 ºC. Damage was prevented when conidia were slowly rehydrated by humidification prior to immersion and immersion temperature was increased to 33-34 ºC; germination of all fungi was ≥ 94% under these optimal conditions. However, immersion of the driest *Bb*, *Ma*, and *Mac* powders in warm water (33 ºC) also resulted in high viabilities (95, 89, and 94%, respectively), and slow-rehydrated conidia also retained high viability (87, 92, and 83%, respectively) after immersion in ice-cold water (0.5 ºC). Formulation of conidia in pure (non-emulsifiable) paraffinic oil provided considerable protection from imbibitional damage. This study underscores a need for establishing standard protocols for preparing aqueous suspensions of sensitive fungi for both research and commercial applications.

Introduction

Many fungi display the ability to survive profound dehydration (Burges, 1998; Crowe et al., 1992). In desiccated (anhydrobiotic) states, spores are able to survive extreme environments characterized by dry heat, freezing/thawing, and acid conditions (Griffin, 1994). With respect to fungal entomopathogens employed as microbial biological control agents, enhanced thermotolerance imparted by desiccation (Hedgecock et al., 1995; Moore et al., 1996; Chen et al., 2008; Connick et al., 1998) is particularly significant as it can substantially increase the shelf-lives of fungus-based biopesticide products (Burges, 1998; Wraight et al., 2001).

When dried organisms are immersed in water, rapid imbibition may cause severe damage to plasma membranes (Crowe et al., 1992; Hoekstra et. al., 1999). In unicellular organisms, such injury usually leads to cell death (Nijsse et al., 2004).

Numerous studies with various organisms, including seeds, pollens, and yeasts, have demonstrated that imbibitional damage increases with decreasing moisture content (dryness) of the organism subjected to rapid rehydration and decreasing immersion temperature. Imbibitional damage can be prevented either by using warm water for rehydration or by slowly rehydrating the organism in a humid atmosphere (vapor phase rehydration) prior to immersion in water (see Crowe et al., 1992).

Imbibitional damage and methods for its prevention have been studied in yeasts (van Steveninki and Ledeboer, 1974; Crowe et al., 1992), but I am aware of only one reference to imbibitional damage among filamentous fungi, which cites *Metarhizium acridum* (Driver & Milner) Bischoff, Rehner, & Humber conidia dried to 4–5% moisture content (Moore et al., 1997) (*M. acridum* was formerly identified as *Metarhizium anisopliae* var. *acridum*; see Bischoff et al., 2009). Viability of conidia of this fungus that were slowly rehydrated by exposure to a water-saturated atmosphere for 5–40 minutes was > 70%, whereas viability of conidia that were rapidly rehydrated by immersion in water was < 25%.

Rehydration of conidia to levels of normal metabolism without membrane damage is of paramount importance for successful pest control using dry formulations of fungal pathogens. In-depth studies of imbibitional damage as a potentially important factor in the efficacy of mycopesticide products are lacking. The primary objective of this study was to quantify the effects of water activity and temperature as key factors determining severity of imbibitional damage in conidia of three commercially important species of entomopathogenic fungi. Broader objectives included increasing awareness and understanding of this phenomenon among mycopesticide researchers, producers, and users and assessing practical means of circumventing the problem. Toward the latter objective, an experiment was conducted to determine if oil formulation has potential to protect conidia from imbibitional damage.

Material and Methods

2.1 Source of fungi

Three fungi were tested: *Beauveria bassiana* (Bals.) Vuill. (*Bb*) strain GHA originally isolated from a chysomelid in the USA, *M. anisopliae* (Metschn.) Sorokīn (*Ma*) isolate CB-10 (ARSEF 7981) from a cercopid in Brazil, and *M. acridum* (*Mac*) isolate IMI-330189 (ARSEF 3341) from an acridid in Niger. Unformulated conidia of *Bb* were obtained from Laverlam International Corp. (Butte, MT, USA). *Metarhizium* isolates were lab-produced using a commonly employed biphasic process in which culture is initiated in liquid and completed on a semi-solid substrate. In the initial phase, flasks containing 100 mL liquid medium (20 g glucose, 20 g yeast extract L^{-1}) (Jenkins and Prior, 1993) were inoculated with conidia and incubated on a rotary shaker (150 rpm) for 6 days at 22–25 ºC. In the second phase, barley mixed with distilled water (dH_2O) was autoclaved for 20 min in polyethylene bags (100 g dry barley flakes + 60 ml dH_2O per bag). Each bag was then inoculated with 70 mL of liquid culture from the initial production phase. Bags were incubated at 25 ºC in darkness, and aerial conidia were harvested after two weeks by manually shaking the fungus-colonized substrate through a series of two sieves, 20 and 100 mesh. Conidia that passed through the 100-mesh sieve (150 μm pore size) were collected. The three stock technical powders were stored at –20 ºC.

2.2. Viability determinations

For viability determinations, 10 μL droplets of suspension comprising conidia suspended in water with a low concentration of surfactant (see below) or pure paraffinic oil were pipetted onto 1 x 1 x 0.3 cm blocks (1 droplet/block) of yeast extract agar-based solid medium (YEA) containing 0.5 g yeast extract, 100 mg gentamicin, 0.1 g Tween 80, and 16 g agar L^{-1} (Meikle et al., 2003), and incubated on

glass microscope slides in sealed Petri dishes in darkness at 25 ºC. After the desired incubation time, a coverslip was applied, and the sample was examined at 400X magnification with phase-contrast illumination. Conidia were considered germinated if a germ tube of any length was visible. A minimum total of 200 conidia were examined in several microscope fields for each replicate suspension of each experimental treatment.

In all experiments, incubators providing experimental temperatures were continuously monitored with digital data loggers (Hobo®, Onset Computer Corp., Bourne, MA, USA). Reported temperatures were ± 0.5 ºC for 25 ºC and ± 1 ºC for all other temperatures.

2.3. Initial tests to identify optimal procedures for assessing germination

Three different concentrations (0, 0.005, and 0.1 g L^{-1}) of benomyl (Bonide Chemical Co., Yorkville, NY, USA) were tested in YEA. Benomyl slows hyphal growth following germ tube emergence, allowing germination assessments over prolonged periods (Milner et al. 1991). Conidial samples of *Bb*, *Ma*, or *Mac* (0.6 g/sample) were collected from the stock powders and placed in 3.4 cm diam. x 1.1 cm plastic sample cups (Novasina, Pfäffikon, Switzerland). Water activity (a_w) was measured at 25 ºC with an electronic meter (LabMaster-a_w, Novasina, Pfäffikon, Switzerland); a_w of the samples ranged from 0.150 to 0.243. From each sample, three subsamples (0.15–0.2 mg picked up on the tip of a spatula) were taken. One of these subsamples was maintained in its original dry state, while the other two were slow hydrated via incubation at 25 ºC in a "desiccator" with a water-saturated atmosphere; one subsample was incubated for 40 min and the other for 24 h. Following these treatments, each of the three subsamples was transferred to a screw-cap glass vial (23 mL capacity) containing approximately 1 g of 2 mm glass beads and 7 mL of distilled

water (temperature not recorded) with 0.05% of the surfactant Lutensol® (Ethoxylated Tridecyl Alcohol, BASF Corporation, Florham Park, NJ, USA) possessing a hydrophilic-lipophilic balance number of 10 (Jin et al., 2008). Resulting suspensions (containing 1–3 x 10^6 conidia/ml) were agitated for 10 min with a wrist action shaker set at 6.7 oscillations/s (Burrel Scientific, Pittsburgh, PA, USA), and viability was determined using blocks of YEA with the above-indicated range of benomyl concentrations. Germination was recorded 18 h post-inoculation (p.i.) for *Bb* and 20 h p.i. for both *Ma* and *Mac*. This experiment was repeated with conidia dried to the 0.021–0.023 a_w range using the desiccant calcium sulphate (eight-mesh indicating drierite®, W.A. Hammond Drierite Co., Xenia, OH, USA), and germination was recorded 18, 21, and 25 h p.i. for *Bb*, *Ma* and *Mac*, respectively.

In a third experiment, the above-described procedure was repeated with the following alterations. The 0.6-g samples of each fungus were initially dried at 25 ºC for 5–9 days in hermetically sealed 125 mL glass jars (TMS Ball Corporation, Muncie, IN, USA) containing drierite. Distilled water plus 0.05% Lutensol (hereafter referred water+Lutensol) used in the fast-rehydration treatments was equilibrated at 25 ºC. Following the fast- vs. slow-rehydration treatments, conidia were inoculated onto blocks of YEA containing 0.005 g L^{-1} benomyl (hereafter referred to as YEA+benomyl), and germination counts were performed 24, 48, and 72 h p.i..

2.4. Effect of fast rehydration time on viability

Conidial samples of each fungal isolate were placed individually in 125-mL glass jars containing drierite and stored at 25 ºC for 10 days. After drying, subsamples of conidia were fast rehydrated into water+Lutensol previously equilibrated to 0.5 ºC in an ice bath (methods otherwise as described above). Suspensions were manually shaken for 5 seconds and then returned to the ice bath and incubated for an additional 2

or 60 min. After treatment, each suspension was vortexed for 10 sec at room temperature (23–24 ºC), and a sample was plated on YEA+benomyl. Conidia were incubated for 48 h at 25 ºC and germination was assessed.

2.5. Effects of conidial water activity and imbibition temperature on imbibitional damage

Conidial samples of the three fungal isolates were placed in hermetic 125-mL glass jars containing either dH_2O, drierite, or saturated salt solutions based on sodium hydroxide (NaOH), magnesium chloride hexahydrate ($MgCl_2.6H_20$), or sodium nitrite ($NaNO_2$) and stored at 25 ºC for three days in darkness. After storage, water activity was determined by rapidly transferring each sample into the water activity meter, previously calibrated with seven standards in the 0.064 to 0.973 a_w range. Average water activities (± standard deviation) of conidia held over drierite were 0.023±0.0023, 0.023±0.0025, and 0.026±0.0049 for *Bb*, *Mac* and *Ma*, respectively. Respective values generated by the other treatments were: 0.073±0.0020, 0.071±0.0058, and 0.069±0.0051 for NaOH; 0.335±0.0092, 0.321±0.0013, and 0.333±0.0116 for $MgCl_2.6H_20$; 0.627±0.0046, 0.634±0.0022, and 0.632±0.0037 for $NaNO_2$; and 0.953±0.0053, 0.961±0.0014, and 0.957±0.0035 for distilled water. After a_w of the conidial samples was determined, subsamples were collected and deposited in vials containing water+Lutensol that had been equilibrated in an ice bath (0.5 ºC) or in incubators set at 15, 25, or 33 ºC. Vials containing conidia were vigorously shaken for 5 seconds and returned to the target temperature for an additional 2 min. Germination was then assessed as described in section 2.4.

2.6. Assessing the protective potential of oil

An initial test examined the effects of aqueous versus oil immersion media on imbibitional damage. *Ma* conidia were dried over saturated NaOH solution at 25 ºC for 3 days in the hermetic jars, then immersed for 2 min in dH_20, dH_20+Lutensol, or pure paraffinic oil (without emulsifiers or other additives; lot number 1910308; provided by Laverlam International Corp.) at either 0.5 or 16 ºC. Dry conidia were also dusted onto YEA+benomyl in 60-mm Petri dishes equilibrated at either 1 or 16 ºC and held at these temperatures for 2 min. As controls, conidial samples were either fast rehydrated by immersion in warm water (without Lutensol) at 34 ºC for 2 min or slow rehydrated for 24 h at 25 ºC in a water-saturated atmosphere before being immersed in water equilibrated at 25 ºC. Following the imbibition treatments, samples of each water- or oil-based suspensions were inoculated onto blocks of YEA+benomyl equilibrated at room temperature and then incubated at 25 ºC for viability determinations at 24h p.i.; the 60-mm plates inoculated (dusted) with dry conidia were also incubated at 25 ºC for viability assessments. A complete list/description of the above-described treatments is presented in Table 2.5.

A second experiment was conducted to assess the potential for oil formulation to protect conidia from imbibitional damage. Dry *Ma* conidia were added to paraffinic oil equilibrated at room temperature, vortexed for 1 min, and then plated onto benomyl-amended YEA equilibrated at 1 ºC as explained above. In addition, dry conidia were dusted directly onto the surface of the medium in Petri dishes at the same temperature. As a control, conidia were fast rehydrated by immersion in warm water+Lutensol at 34 ºC for 2 min and then inoculated onto YEA+benomyl equilibrated at room temperature. Germination counts were recorded at 24 and 48 h p.i..

2.7. Sorption isotherms

Sorption isotherms allow conversion of a_w measurements to moisture content (MC), and vice-versa. Saturated salt solutions were prepared according to the procedure described by Vertucci and Roos (1993). Small cups (3.4-cm diam., fabricated from aluminum foil) containing conidia were placed on plastic supports in hermetically sealed 0.95 L wide-mouth glass jars (TMS Ball Corporation, Muncie, IN, USA) containing drierite or different saturated salt solutions for a period of seven days at 25 ºC. Zinc chloride ($ZnCl_2$), sodium hydroxide (NaOH), lithium chloride (LiCl), magnesium chloride hexahydrate ($MgCl_2.6H_20$), potassium nitrite (KNO_2), sodium nitrite ($NaNO_2$), sodium chloride (NaCl), potassium chloride (KCl), and potassium sulfate (K_2SO_4) were used to create different equilibrium relative humidities. After storage, water activity was determined by rapidly transferring each sample cup into the calibrated water activity meter. Conidial samples were then weighed and placed in a drying oven at 105 ± 2 ºC for 24 h. After drying, each sample was cooled for 45 min in a sealed jar with drierite. The dry samples were then reweighed and wet-basis moisture content (w.b. MC) was calculated and expressed as the percent weight loss due to desiccation. In each test, one 1.0-g sample of conidia was exposed to each salt, and the entire procedure was repeated on three different dates.

2.8. Statistical analyses

For all experiments (other than sorption isotherms), four replicate samples of conidia were prepared (all mean viabilities and standard errors reported in the results are based on n = 4). In most cases, subsamples were assigned to the various treatments in a randomized complete block design. In the experiments that assessed the protective potential of oil, subsamples were assigned to the various treatments in a completely

randomized design. In the second test with oil-formulated conidia, the 24 and 48 h germination counts were recorded from independent samples (not repeated measures).

Percent germination data were normalized by arcsine transformation and analyzed by analysis of variance (ANOVA). Where indicated for analyses across fungi, viability data for each fungus were adjusted with respect to the maximum viability recorded for that fungus (each datum divided by the maximum). Means were compared by paired t-Test or Tukey-Kramer HSD (alpha = 0.05). ANOVAs and polynomial regression analyses were performed using the JMP statistical software package (SAS Institute Inc, Cary, NC, USA).

Results

3.1 Identification of optimal procedures for assessing germination

In the first test, a_w and immersion temperature were not standardized across fungal species, and therefore results from tests of each fungus were analyzed independently. For *Bb* and *Ma*, there were no significant benomyl x rehydration treatment interactions (*Bb*: $F_{2,15} = 0.54$, P = 0.59; *Ma*: $F_{2,15} = 0.40$, P = 0.68). Amendment of solid medium with benomyl had little or no effect on viability of these two fungi (Table 2.1). Slow rehydration had a significant positive impact on *Bb* germination, but the effect was small; germination was 91.8% following fast rehydration versus 96.2% after slow rehydration ($F_{1,15} = 59.1$, P< 0.0001). In contrast, slow rehydration increased viability of *Ma* conidia by an average of 34% (from 55.9 to 89.8%; $F_{1,15} = 947.8$, P< 0.0001).

Table 2.1. Effect of benomyl concentrations and slow rehydration times on percent germination of dry *Beauveria bassiana*, *Metarhizium acridum* and *M. anisopliae* conidia.

Benomyl ($g\ L^{-1}$)	Mean percent germination (± standard error) after slow rehydration for indicated times[1]											
	B. bassiana (*Bb*)				*M. acridum* (*Mac*)				*M. anisopliae* (*Ma*)			
	0	40 min	24h[2]	Mean[3]	0	40 min	24h	Mean	0	40 min	24h[2]	Mean
Test 1: water activity (a_w) not standardized prior to treatment[3]												
0	93.8±0.8	96.8±0.5	-	95.3±0.8 a	64.3±1.5	90.4±1.0	93.2±1.69	82.6±4.0 a	56.1±1.5	89.2±0.8	-	72.6±6.3 a
0.005	91.0±1.0	96.0±0.2	-	93.5±1.0 ab	29.3±3.0	51.4±4.0	88.3±2.3	56.3±7.5 b	54.6±1.7	89.9±0.3	-	72.3±6.7 a
0. 1	90.8±0.6	95.7±0.6	-	93.2±1.0 b	18.7±1.5	30.3±3.7	77.6±1.4	42.2±7.8 c	57.0±2.0	90.2±0.7	-	73.6±6.3 a
Mean	91.8±0.6 b	96.2±0.3 a			37.4±6.0 c	57.4±7.7 b	86.3±2.1 a		55.9±0.8 b	89.8±0.5 a		
Test 2: a_w standardized at 0.021 prior to treatment												
0	93.0±1.1	92.9±1.4	95.0±0.4	93.6±0.5 a	85.8±2.2	92.0±1.9	93.3±0.9	90.4±1.1 a	60.0±2.0	86.6±0.7	91.8±1.5	79.4±4.3 a
0.005	93.4±0.9	94.5±0.8	96.8±0.5	94.9±0.5 a	52.3±4.3	70.5±1.3	79.0±2.1	67.3±3.6 c	54.0±1.7	84.3±0.5	92.3±1.9	76.8±5.0 a
0.1	93.7±0.4	94.3±0.5	96.3±0.6	94.7±0.5 a	59.5±2.3	79.0±2.0	80.3±2. 6	72.9±3.2 b	60.5±1.2	86.3±2.7	90.8±1.8	79.2±4.1 a
Mean	93.4±0.4 b	93.9±0.4 b	96.0±0.4 a		65.9±4.6 b	80.5±2.8 a	84.2±2.2 a		58.2±1.2 c	85.7±0.8 b	91.6±0.9 a	

[1]Conidia were slow rehydrated by holding in a water-saturated atmosphere (humid chamber) for the indicated time and then suspended in water with 0.05% Lutensol and inoculated onto yeast extract agar for viability assessment; incubation times at 25 ºC were: 18 h for *Bb* in both tests, 20 h for the *Metarhizium* species in test 1, and 21 and 25 h for *Ma* and *Mac*, respectively, in test 2.
[2]Germination counts were not performed for *Bb* and *Ma* in test 1 due to excessive mycelial growth in the treatment without benomyl. [3]Initial water activity levels: 0.199 (*Bb*), 0.243 (*Mac*), and 0.150 (*Ma*).
[4]Within each test and fungal species, means followed by the same letter are not significantly different (Tukey HSD or t-test, $\alpha = 0.05$).

A benomyl x rehydration treatment interaction was detected in the case of *Mac* (rehydration x benomyl interaction: $F_{4,24} = 16.0$, $P < 0.0001$) ; however, the overall main effects of benomyl and rehydration were still tested, as trends were unambiguous (benomyl had a negative effect and slow rehydration had a positive effect on viability). Addition of benomyl to the germination medium reduced germination by an average of 26 or 40%, depending on concentration ($F_{2,24} = 181.3$, $P<0.0001$, Table 2.1). Slow rehydration increased germination by an average of 20 or 49%, depending on the duration of the slow rehydration period ($F_{2,24} = 253.1$, $P < 0.0001$). As the rehydration period was increased, the conidia became less susceptible to the benomyl effect.

In the viability checks of *Mac* in the first test, it was evident that many non-germinated conidia were swollen and apparently viable (data not shown). It was therefore decided to conduct a second test in which germination incubation for this fungus was increased from 20 to 25 h. In addition, conditions were standardized across fungal species by drying the conidia to 0.021-0.023 a_w and controlling imbibition temperature (set at 25 ºC). As a result, markedly higher viability percentages were recorded for *Mac*. Because tests 1 and 2 were conducted independently, however, differences were not statistically comparable.

An overall analysis of the adjusted data (see section 2.8) from test 2 revealed significant interactions among fungal isolate and both the rehydration and benomyl treatments (fungus x rehydration: $F_{4,78} = 28.6$, $P< 0.0001$; fungus x benomyl: $F_{4,78} = 32.4$, $P< 0.0001$). Slow rehydration had little effect on *Bb* compared to the *Metarhizium* isolates, and benomyl inhibited germination of *Mac*, but not *Bb* or *Ma* (Table 2.1). Due to these interactions, data were analyzed independently for each fungal isolate. In the cases of *Bb* and *Ma*, there were no benomyl x rehydration treatment interactions (*Bb*: $F_{4,24} = 0.38$, $P = 0.82$; *Ma*: $F_{4,24} = 1.03$, $P = 0.41$).

Apparently as a result of the increased incubation time, the benomyl x rehydration interaction for *Mac*, which was highly significant in the first test, was marginally insignificant in this case ($F_{4,24} = 2.71$, $P = 0.054$). Slow rehydration significantly increased viability of *Bb*, but only by a few percentage points, from 93.4 to 96.0% ($F_{2,24} = 13.7$, $P < 0.0001$). There was no significant effect of benomyl on *Bb* germination (Table 2.1). In contrast, slow rehydration for 40 min or 24 h increased viability of *Ma* by 28 or 33%, respectively ($F_{2,24} = 237$, $P < 0.0001$). As observed with *Bb*, benomyl had no significant effect on *Ma* germination. Slow rehydration for 24 h had an intermediate effect on *Mac* compared to *Bb* and *Ma*, increasing germination by 14–18%. Unlike *Bb* or *Ma*, *Mac* was significantly susceptible to benomyl. The two benomyl concentrations reduced viability from 90 to a mean of 70% ($F_{2,24} = 92.6$, $P < 0.0001$).

Considering the apparent fungistatic effect of benomyl, a third test was conducted to assess germination over longer incubation periods (24–72 h). An overall analysis of the adjusted data showed significant interactions among fungal species and both rehydration treatment and incubation time (fungus x rehydration: $F_{2,58} = 27.3$, $P = < 0.0001$; fungus x incubation time: $F_{2,58} = 4.72$, $P = 0.0023$) (Table 2.2). Most noticeably, conidia of *Bb* were less affected by increasing incubation or rehydration time than *Ma* or *Mac*. Data were consequently analyzed independently for each fungal isolate.

Significant incubation time x rehydration treatment interactions were observed for both *Bb* and *Mac* (*Bb*: $F_{2,15} = 5.10$, $P = 0.021$; *Mac*: $F_{2,15} = 8.82$, $P = 0.003$). This interaction was not significant for *Ma* ($F_{2,15} = 0.15$, $P = 0.86$), likely due to the greater standard errors associated with the data. The data in all cases indicated that viability of fast-rehydrated conidia increased more over 72 h than viability of slow-rehydrated conidia (Table 2.2).

The main effect of incubation time was highly significant for *Mac* ($F_{2,15}$ = 33.2, P = < 0.0001) and marginally insignificant for *Ma* ($F_{2,15}$ = 2.71, P = 0.10; one-tailed test). Increasing incubation time from 24 to 72 h increased germination by averages of 13, 7, and 1% for *Mac*, *Ma* and *Bb*, respectively (Table 2.2). At 72 h p.i., germination counts for all species became more difficult due to clumping caused by extensive hyphal elongation, and high levels of debris from hyphal degradation (vesicles etc.) in microscopic fields, although this was less pronounced for *Mac*.

The main effect of rehydration was significant for all fungal isolates, with slow rehydration increasing germination, particularly for *Metarhizium* species (Table 2.2). Slow rehydration increased germination by averages of 3, 15, and 26% for *Bb*, *Mac* and *Ma*, respectively (*Bb*: $F_{1,15}$ = 31.0, P = < 0.0001; *Mac*: $F_{1,15}$ = 124.5, P = < 0.0001; *Ma*: $F_{1,15}$ = 114.9, P = < 0.0001). Based on the results from the above-described experiments, the subsequent studies investigating effects of rehydration time, imbibition temperature, and water activity on imbibitional damage used the 0.005 g/L concentration of benomyl and an incubation time of 48 h for all of the fungi.

3.2 Effect of fast rehydration time on viability

Increasing the fast-rehydration immersion time from 2 to 60 min did not affect germination ($F_{1,18}$ = 0.0002, P = 0.99). Analysis of the adjusted data showed that the effects of fast rehydration varied greatly among fungal species ($F_{2,18}$ = 94.2, P < 0.0001), with no fungus x rehydration time interaction ($F_{2,18}$ = 0.03, P = 0.97) (Table 2.3). Following immersion of conidia in water+Lutensol at 0.5 ºC, *Bb* retained > 50% viability, whereas *Mac* retained only 12–13%, and viability of *Ma* was reduced to near zero.

Table 2.2. Effect of incubation times on percent germination of *Beauveria bassiana*, *Metarhizium acridum*, and *M. anisopliae* conidia following fast or slow rehydration.

Incubation time (h at 25 ºC)[1]	Percent germination (± standard error)								
	B. bassiana			*M. acridum*			*M. anisopliae*		
	Fast rehydrated[2]	Slow rehydrated[2]	Mean[3]	Fast rehydrated	Slow rehydrated	Mean[3]	Fast rehydrated	Slow rehydrated	Mean[3]
24	93.3±0.6	98.0±0.5	95.6±0.9 a	65.1±2.0	91.1±1.4	78.1±5.1 b	58.4±4.2	88.1±2.0	73.3±5.9 a
48	94.9±1.0	97.4±0.5	96.1±0.7 a	86.4±0.8	94.5±0.9	90.4±1.6 a	64.3±3.8	89.8±3.3	77.0±5.3 a
72	96.0±0.7	97.0±0.4	96.5±0.3 a	86.3±2.3	96.3±0.3	91.3±2.1 a	69.0±1.8	92.1±1.8	80.0±4.5 a
Mean[3]	94.7±0.5 b	97.5±0.3 a		79.3±3.2 b	94.0±0.8 a		63.9±2.0 b	90.0±1.5 a	

[1]Incubated on yeast extract agar with 0.005 g L^{-1} benomyl.
[2]Fast-rehydrated conidia were immersed in water with 0.05% Lutensol at 25 ºC without pre-rehydration, whereas slow-rehydrated conidia were held in a water-saturated atmosphere (humid chamber) at 25 ºC for 24 h before immersion.
[3]Within each fungal species, means followed by the same letter are not significantly different (Tukey HSD or t-test, $\alpha = 0.05$).

Table 2.3. Effect of imbibition time on percent germination of dry *Beauveria bassiana*, *Metarhizium acridum*, and *M. anisopliae* conidia following fast rehydration (immersion) at 0.5 ºC.

Initial hydration state of conidia[1]	Imbibition time at 0.5 ºC (minutes)	Percent germination ± standard error[2]		
		B. bassiana	*M. acridum*	*M. anisopliae*
Control (pre-rehydrated)	-	97.4 ± 0.5	94.5 ± 0.9	93.0 ± 0.7
Dry	2	55.4 ± 6.0 a	13.1 ± 5.2 a	0.8 ± 0.6 a
Dry	60	56.4 ± 4.8 a	12.1 ± 4.0 a	0.6 ± 0.5 a

[1]Control conidia were pre-rehydrated in a water-saturated atmosphere for 24 h at 25ºC prior to fast rehydration in water with 0.05% Lutensol at 25 ºC. Dry conidia were dried over drierite for 10 days at 25 ºC prior to fast rehydration in water+Lutensol equilibrated at 0.5 ºC.
[2]Viabilities were determined by plating on yeast extract agar with 0.005 gL^{-1} benomyl and incubating for 48 h at 25 ºC. Means within columns followed by the same letter are not significantly different (t-test, $\alpha = 0.05$).

3.2 Effects of conidial water activity and immersion temperature on viability

Overall analysis of the effect of conidial water activities and temperatures used for fast rehydration revealed highly significant interactions among fungal species and these two factors (fungus x water activity interaction: $F_{8,180} = 38.4$, P = < 0.0001; fungus x temperature interaction: $F_{6,180} = 2.37$, P = < 0.0001). Drying had a greater negative impact on viability of *Ma* than *Mac* or *Bb* (Table 2.4). Across temperatures, germination of *Bb* was substantially affected only at 0.5 ºC, whereas *Mac* and *Ma* viabilities were also greatly reduced at 15 and 15–25 ºC, respectively. Because of these interactions, each fungal species was analyzed independently.

Within each fungal species, a significant water temperature vs. water activity interaction was present (*Bb*: $F_{12,57} = 17.7$, P = < 0.0001; *Ma*: $F_{12,57} = 34.8$, P = < 0.0001; *Mac*: $F_{12,57} = 51.5$, P = < 0.0001); however, main effects were clear in that viability of all fungal species generally increased with increasing immersion temperature (*Bb*: $F_{3,57} = 274.0$, P = < 0.0001; *Mac*: $F_{3,57} = 964.1$, P = < 0.0001; *Ma*: $F_{3,57} = 472.6$, P = < 0.0001) and water activity (*Bb*: $F_{4,57} = 34.6$, P = < 0.0001; *Mac*: $F_{4,57} = 109.6$, P = < 0.0001; *Ma*: $F_{4,57} = 161.3$, P = < 0.0001) (Table 2.4). Dry conidia were highly susceptible to imbibitional damage when fast rehydrated under chilled conditions. Germination percentages for *Bb*, *Mac*, and *Ma* conidia dried to 0.023-0.026 a_w were 51.5, 5.3, and 0.9%, respectively, following fast rehydration at 0.5 ºC, whereas the respective germination percentages were 95.6, 93.9, and 94.0% for near-fully hydrated conidia imbibed at 33 ºC. With the exception of *Ma*, even the driest conidia were not susceptible to imbibitional damage when fast hydrated at 33 ºC, and in the case of *Ma*, dry conidia ($a_w \leq 0.333$) fast rehydrated at 33 ºC exhibited only an average 6.5% loss in viability when compared to near-fully hydrated conidia imbibed

Table 2.4. Effects of conidial water activity and imbibition temperature on percent germination of three entomopathogenic fungi.

Fungus	Water temperature (ºC)	Percent germination (mean ± standard error) at indicated water activity (a_w)[1]					
		0.023	0.073	0.335	0.627	0.954	Mean[2]
B. bassiana	0.5	51.5±3.3	42.5±1.7	64.9±6.1	87.6±2.1	86.6±1.3	66.6±4.4 b
	15	92.6±0.6	92.0±1.3	94.4±0.2	96.6±0.65	96.6±0.5	94.5±0.5 a
	25	92.3±0.3	95.3±1.2	94.1±1.2	96.1±0.8	96.1±0.8	94.8±0.5 a
	33	95.4±0.4	96.4±0.7	95.5±0.7	94.9±1.0	95.6±0.4	95.6±0.4 a
	Mean	82.9±4.7 c	81.5±5.9 c	87.2±3.6 b	93.8±1.1 a	93.8±1.1 a	
		0.023	0.071	0.321	0.634	0.961	Mean
M. acridum	0.5	5.3±0.9	3.9±1.2	4.3±2.1	28.3±1.3	83.0±2.4	24.9±7.0 d
	15	60.1±3.1	58.1±3.8	63.1±3.6	93.3±1.7	91.5±1.7	73.2±3.8 c
	25	89.5±1.2	91.4±2.1	91.8±0.7	92.3±0.4	91.9±1.3	91.4±0.5 b
	33	94.3±1.5	95.5±0.5	96.4±0.8	94.6±0.7	93.9±1.3	94.9±0.5 a
	Mean	62.3±9.2 c	62.2±9.5 c	63.9±9.5 c	77.1±7.3 b	90.1±1.3 a	
		0.026	0.069	0.333	0.632	0.957	Mean
M. anisopliae	0.5	0.9±0.4	1.0±0.8	0.3±0.3	7.9±1. 8	91.5±1.5	20.3±8.2 d
	15	28.3±2.8	27.4±1.1	40.6±2.5	78.1±2.7	93.1±1.2	52.1±6.7 c
	25	66.4±2.3	72.0±2.3	75.3±1.1	86.9±0.7	90.5±1.4	78.2±2.2 b
	33	88.8±1.7	86.3±2.0	87.3±1.7	89.9±1.6	94.0±0.8	89.2±0.9 a
	Mean	44.3±9.2 c	46.7±8.8 c	50.8±8.8 c	65.7±8.7 b	92.3±0.8 a	

[1]Conidia with different initial a_w were immersed in water with 0.05% Lutensol equilibrated at different temperatures and then incubated for 48 h on yeast extract agar with 0.005 g L^{-1} benomyl.
[2]For each fungal species, means followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

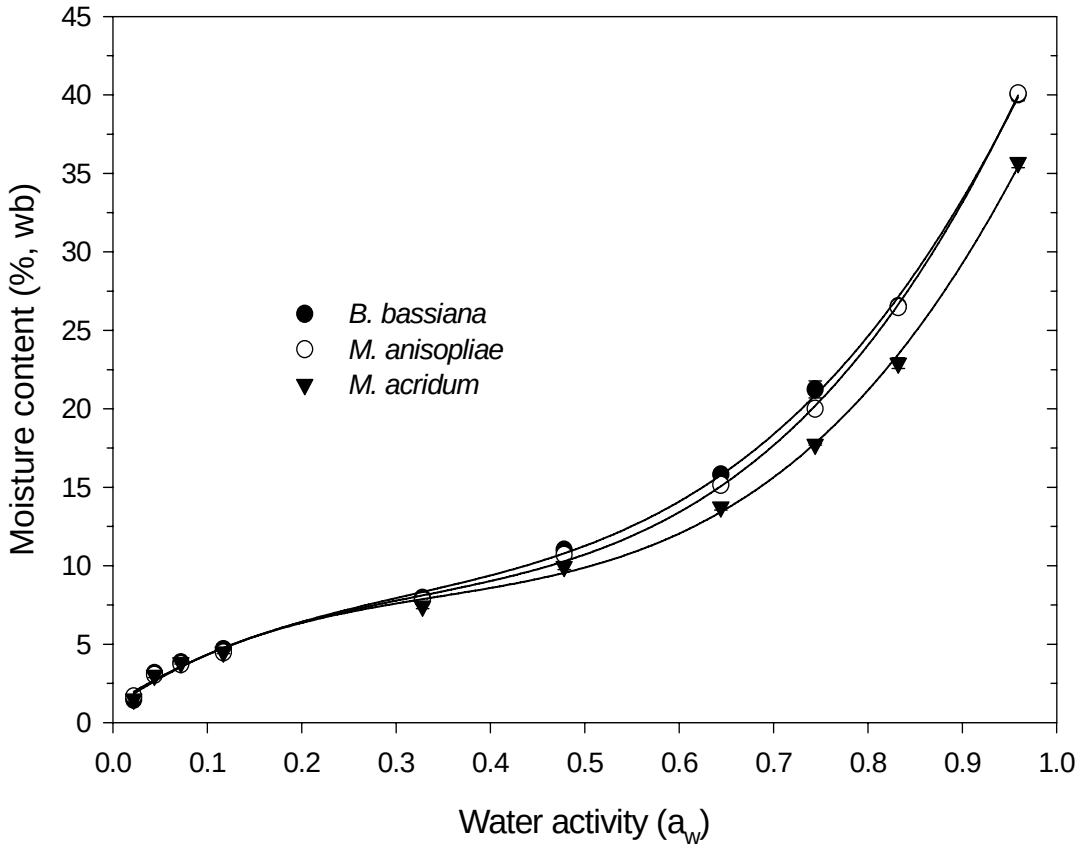

Figure 2.1. Relationships between conidial water activity and moisture content (%, wet-weight basis) determined at 25°C for *B. bassiana*, *M. anisopliae* and *M. acridum* for the range 0.021-0.960. Curves were fitted by cubic polynomial regressions and represent absorption and desorption isotherms above and below water activity in the range 0.222-0.303. *B. bassiana*: $y = 2.62 + 16.87*a_w + 27.85*(a_w-0.42)^2 + 85.73*(a_w-0.42)^3$ ($R^2=0.9991$), *M. anisopliae*: $y = 2.89 + 15.48*a_w + 28.23*(a_w-0.42)^2 + 90.68*(a_w-0.42)^3$ ($R^2=0.9996$), and *M. acridum*: $y = 4.00 + 11.29*a_w + 22.62*(a_w-0.42)^2 + 92.59*(a_w-0.42)^3$ ($R^2=0.9988$).

at the same temperature. Similarly, viabilities of near-fully hydrated conidia of *Bb*, *Mac*, and *Ma* ($a_w \geq 0.954$) were reduced only 9.0, 10.9, and 2.5%, respectively, when fast rehydrated at 0.5 ºC versus at 33 ºC. Based on sorption isotherms (Figure 2.1), at 25 ºC *Bb* conidia with 0.335, 0.627, and 0.954 a_w have w.b. MC equivalent to 8.4, 15.1 and 39.2%, respectively; *Mac* conidia with 0.321, 0.634, and 0.961 a_w have MCs equivalent to 7.8, 13.0, and 35.6%, respectively; *Ma* conidia with 0.333, 0.632, and 0.957 a_w have MCs equivalent to 8.2, 14.8, and 39.7%, respectively.

3.4. Assessing the protective potential of oil as a carrier

As observed in the previous tests, viability of *Ma* conidia was high (in this case ≥ 82%) when conidia were slow rehydrated prior to immersion and when dry conidia were fast rehydrated in warm water (34 ºC). The technique used for assessing viability of conidia in aqueous suspension was equally effective for conidia formulated in pure paraffinic oil. Conidia in the oil droplets settled onto the agar surface and germinated normally. As the oil contained no emulsifier, there was no formation of oil micro-droplets, and conidia were readily identifiable.

A 2 x 4 factorial ANOVA of the data from the 0.5 and 16 ºC treatments across the three immersion media (water, water+Lutensol, and oil) revealed a highly significant immersion medium x temperature interaction ($F_{3,24} = 15.4$, $P < 0.0001$), and one-way ANOVA of the various treatments revealed highly significant differences ($F_{9,30} = 198.3$, $P < 0.0001$). Immersion of conidia in water or water+Lutensol at 0.5 or 16 ºC was extremely detrimental, reducing viability to low levels (0.1–24.8%), whereas immersion in oil at low temperatures had no negative effect (viability remained ≥ 83% and did not differ significantly from the controls) (Table 2.5). Viability after immersion in water+Lutensol at 16 ºC was lower than viability following immersion in water without Lutensol (12 vs. 25%), but the difference was

Table 2.5. Effects of immersion medium and immersion temperature on viability of *Metarhizium anisopliae* conidia.

Treatments / initial hydration state of conidia[1]	Immersion medium[2]	Immersion temperature (ºC)	% Viability (mean ± standard error)[3,4]
Controls			
Pre-rehydrated	Water	25	88.0 ± 1.9 a
Dry	Water	34	81.5 ± 1.2 a
Treatments			
Dry	Water	16	24.8 ± 3.8 b
Dry	Water	0.5	0.1 ± 0.1 c
Dry	Water + Lutensol	16	11.6 ± 2.6 b
Dry	Water + Lutensol	0.5	0.1 ± 0.1 c
Dry	Agar	16	20.6 ± 6.4 b
Dry	Agar	1	0.0 c
Dry	Paraffinic oil	16	84.1 ± 1.2 a
Dry	Paraffinic oil	0.5	83.4 ± 0.9 a

[1]Conidia were pre-rehydrated in a water-saturated atmosphere for 24 h or dried over a saturated NaOH solution for 3 days (in both cases at 25 ºC) prior to testing.
[2]Conidia were immersed in dH_2O, dH_2O with 0.05% Lutensol, or pure paraffinic oil or were dusted onto yeast extract agar with 0.005 g L^{-1} benomyl (YEA+benomyl) equilibrated at either 1 or 16 ºC.
[3]Following the immersion treatments, conidia were incubated on YEA+benomyl at 25 ºC for 24 h for viability determinations. Plates dusted with dry conidia were also incubated at 25 ºC for the same period. [4]Means followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$)

not statistically significant. Inoculation of dry conidia onto a cold agar substrate had the same negative effect on viability as immersion in cold water. Dry *Ma* conidia formulated in paraffinic oil retained 66% viability after inoculation onto agar equilibrated at 1 ºC, whereas only 0.1% of unformulated conidia survived this treatment ($F_{5,18}$ = 352.2, P = < 0.0001, Table 2.6).

Discussion

The principal finding of strong inverse relationships between imbibitional damage in conidia of filamentous fungi and both immersion temperature and water activity is in accord with the results of studies of imbibitional damage in seeds (Obendorf and Hobbs, 1970; Nijsse et al., 2004), pollen (Hoekstra, 1984; Hoekstra et al, 1988) and yeasts (van Steveninki and Ledeboer, 1974). Studies conducted over the past four decades have produced numerous hypotheses to explain imbibitional damage. One of the most prominent of these, referred to by Crowe et al. (1992) as the phase transition hypothesis, is based on observed changes in membrane permeability during temperature-dependent phase transitions. The phospholipid bilayer comprising biological membranes exists in either a gel phase or liquid crystalline phase depending on the phase transition temperature (generally designated as Tm, the melting temperature); the two phases coexist transiently at Tm. Dehydration greatly increases Tm, generally resulting in transition from the liquid crystalline phase to the gel phase. When a dry membrane in gel phase is immersed in water at a temperature < Tm, the membrane remains in gel phase until imbibition begins. Subsequent rehydration of the membrane reduces Tm, and reversion to the liquid crystalline phase is initiated. During this phase transition, leakage of cell contents occurs, and according to the

Table 2.6. Assessment of the protective effect of oil against imbibitional damage of *Metarhizium anisopliae* conidia.

Incubation time (h)	Percent germination (± standard error)[1]		
	Control[2]	Oil[3]	No carrier[4]
24	76.1 ± 2.8 b	63.3 ± 3.4 c	0.0 d
48	88.3 ± 1.3 a	65.8 ± 4.2 bc	0.1 ± 0.1 d

[1]Following the imbibition treatments, conidia were incubated 24 or 48 h on yeast extract agar with 0.005 gL^{-1} benomyl (YEA+benomyl) at 25 ºC for viability determinations. Means followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).
[2]Control (dry) conidia were immersed in warm (34 ºC) dH2O with 0.05% Lutensol prior to plating on YEA+benomyl.
[3]Conidia suspended in pure paraffinic oil were inoculated onto YEA+benomyl equilibrated at 1ºC.
[4]Dry conidia were dusted onto YEA+benomyl equilibrated at 1ºC.

phase transition hypothesis, leakage becomes severe and lethal when the transition takes place in the presence of liquid water (Crowe et al., 1989; Crowe et al., 1992). More recently, Hoekstra et al. (1999) hypothesized that membranes in gel phase have reduced elasticity, making them susceptible to mechanical damage from the pressure of penetrating liquid water. Regardless of the exact mechanism, the most prominent effect of imbibitional damage is a dramatic increase in cell permeability. The current hypotheses further suggest that imbibitional damage is prevented when membrane phospholipids melt to the liquid crystalline phase prior to or at the onset of imbibition. The transition can be induced either by slowly rehydrating membranes via exposure to water vapor (lowering Tm) or by heating membranes to a temperature > Tm (Crowe et al., 1992). In the latter case, heating of the membrane can occur very rapidly. Thus, when a dry membrane in gel phase is immersed in water at > Tm, transition to the liquid crystalline phase takes place before the liquid water can exert a lethal effect (Hoekstra et al., 1999).

Combinations of water activity and imbibition temperature that resulted in very high germination counts for conidia plunged into water are presented herein. Maximal germination counts for dry conidia of all fungal species were observed at 33 ºC, but I did not determine if higher viabilities (especially for dry Ma) could be achieved at higher imbibition temperatures. Incubation for either 2 or 60 minutes at 0.5 ºC resulted in equivalent levels of damage, indicating that this phenomenon takes place in a very short time. Over 90% of *Typha latifolia* pollen with low initial moisture content showed irreversible loss of viability when imbibed in ice-cold medium (Hoekstra, 1984). In the same study, evidence that imbibitional stress occurs in just a few seconds was reported for pollen dried to 7% MC.

When conidia were dried to a_w levels ≤ 0.335, substantial imbibitional damage (resulting in viabilities < 65%) was observed only at 0.5 ºC for *Bb*, whereas *Mac*

conidia were also susceptible to imbibitional damage at 15 ºC and *Ma* was also susceptible at both 15 and 25 ºC. These observations strongly suggest that *Bb* is less susceptible to imbibitional damage than the *Metarhizium* species. This trait may have contributed to the successful development of GHA and other *Bb* strains as biocontrol agents. The results also suggest that *Mac* may be less susceptible to damage than *Ma*. It is noteworthy in this regard that Magalhães and Boucias (2004) found no evidence of imbibitional damage when dried conidia of a Brazilian isolate of *Mac* were immersed in aqueous Tween solution without previous slow rehydration, but the temperature of the solution was not reported. It is important to underscore that the results presented herein are based on a single production batch of a single strain of each fungal species, and it is clear that rigorous conclusions regarding the relative susceptibilities of these fungi to imbibitional damage will require more extensive assays.

Interestingly, at the moderate imbibition temperature of 25 ºC, germination percentages for dry *Mac* were approximately 90%, which is considerably higher than the rate of 24.5% reported by Moore et al. (1997) for the same isolate. The conflicting results could be related to differences in the germination media, imbibition temperatures or, overall quality of conidia used in the two studies. Moore et al. (1997) did not report the temperature of the water+Tween solution used for fast rehydration; however, the conidia they subjected to this treatment had a low moisture content (4.5%, equivalent to an a_w of ca. 0.11), which, according to our results, would have made them highly susceptible to imbibitional damage at temperatures ≤ 15 ºC. In addition, conidial batches with low viability, possibly indicative of a stressed condition, might be more sensitive to imbibitional damage than batches exhibiting high viability. Viability of the conidial powder used by Moore et al. (1997) was 76-78% following slow rehydration for 40 min, whereas viability of the powder used in

the present study was > 90% (on benomyl-free medium). I also observed a 22% increase in germination of dry (non-slow-rehydrated) *Mac* conidia after incubation for 25 vs. 20 h at 25 ºC (86 vs. 64%, Table 2.1), and in view of this, it is possible that without slow rehydration, incubation of this fungus for 24 h at 25 ºC (the protocol reported by Moore and colleagues) is only marginally adequate for assessing germination, with potential for results to be significantly affected by small differences in incubation temperatures or other experimental parameters. Studies of the effects of initial a_w on the speed of germination of this and other biocontrol fungi (both in the presence and absence of benomyl) are warranted.

Conidia wettability can be correlated to presence and stability in the outer cell wall of a hydrophobic rodlet layer, present in *Bb*, *Ma*, and *Mac*, but absent or not well-organized in species that produce hydrophilic conidia (Boucias and Pendland, 1991). Possible roles of this hydrophobic layer include protection against dehydration, dispersal in air currents, and attachment to insect cuticle. Hydrophobicity seems to be at least partially mediated by components of rodlet layers called hydrophobins, a group of low-molecular weight proteins found in *Ma* (St. Leger et al., 1992). This extracellular polypeptide was also detected in *Bb* aerial conidia but not in blastospores, which lack a rodlet layer (Holder and Keyhani, 2005). Whether hydrophobicity is involved in differential water uptake by dry *Bb* and *Ma* conidia and consequently in variable levels of imbibitional damage to the plasma membrane is not clear. Both species seem to be equally hydrophobic, although size and arrangement of rodlets on the cell wall surface is quite distinct between these two species (Boucias et al., 1988).

Differences in desiccation tolerance between thick-walled conidia and thin-walled blastospores of *Mac* also have been reported and cell wall thickness suggested as a possible explanation (Leland et al., 2005). The *Mac* conidial wall comprises a double-layered cell wall with a total thickness of 0.17 μm (Leland et al., 2005).

Interestingly, the aerial conidia of *Ma*, which I found more susceptible to imbibitional damage than *Mac*, possess a triple-layered cell wall, yet total wall thickness is only 0.09-0.12 μm (Zacharuk, 1970). The importance of an outer barrier in avoiding imbibitional damage was demonstrated for lettuce seeds, which are tolerant to this kind of stress when intact, but highly sensitive if the endosperm layer is removed (Nijsse et al., 2004). These authors suggested that membrane injury could be prevented by a thick-walled endosperm acting as a barrier against rapid influx of liquid water combined with a network of intercellular spaces allowing diffusion of water vapor with consequently slower and more homogeneous rehydration. I hypothesize that the cell walls of conidia (especially the cell walls of *Bb*) act similarly to slow the uptake of water and allow slow and homogeneous membrane rehydration by water vapor, preventing or alleviating membrane injury. During ultrastructural studies with fungal conidia, Zacharuk (1970) observed that the cell wall was a barrier to rapid penetration of fixatives. Whether or not a thinner cell wall is involved in the increased susceptibility of *Ma* to imbibitional injury compared to *Mac* conidia remains to be proven, but cell walls likely play a major role in conferring varying degrees of tolerance to this kind of stress.

Tolerance to imbibitional damage was relatively high for dry *Bb* and *Mac* conidia at 25 ºC, but viability of both *Metarhizium* isolates dropped abruptly at 15 ºC. This is reason for concern for use of these fungi as pest control agents, as the temperature of water from underground sources may be this low, even in tropical areas. *Metarhizium* spp. comprise more than one-third of the mycoinsecticides and mycoacaricides developed worldwide and currently in the market, and the active ingredients in many of these products are dry conidia (in wettable powders or oil-based formulations) (see Chapter 1). There are numerous advantages to use of dry

conidia in biopesticide products, including facilitation of harvesting and formulation, reduced contamination, and reduction in shipping/storage volumes and weights. Even more importantly, maximum storage stability of conidia of commonly employed entomopathogenic fungi requires drying to a low moisture content (4–5% according to Hedgecock et al., 1995; Hong et al., 2001).

Avoidance of imbibitional stress via slow rehydration or suspension in warm water may be impractical for commercial applicators, depending on operational scale or other conditions. Nevertheless, development of either producer- or user-friendly protocols to cope with this problem should be considered a research priority. Our results suggest that oil formulation might be beneficial, but much additional research is needed. Applications of microbial biocontrol agents in pure oil, such as that used in the present study, do not involve mixing or other contact with water unless the target includes wet substrates. More significant would be demonstration of reduced imbibitional damage following aqueous suspension of conidia formulated in emulsifiable oils. When emulsifiable formulations are mixed in water, conidia are encapsulated in very small droplets of oil that might have only limited protective capacity. In addition to oils, however, there may be other formulation materials with potential, through encapsulation, to protect conidia or other fungal propagules from imbibitional damage.

Benomyl is a useful tool in assessing viability of entomopathogenic fungi. Slowing germ tube development enables researchers to incubate conidia for prolonged periods, providing greater flexibility in the set up and reading of viability assays (Milner et al., 1991; Goettel and Inglis, 1997). Nevertheless, it is important to recognize the capacity of this material to inhibit germination of at least some fungi. In the presence of benomyl, I observed substantially slower germination of *Mac*

compared to *Bb* or *Ma*. It should also be noted that conidia pretreated by exposure to a water-saturated environment (slow rehydrated) germinated more rapidly than dry conidia.

This study underscores a need for establishing standard protocols for preparing aqueous suspensions of sensitive fungi for research and commercial applications. Such information would be a particularly important addition to mycopesticide labels. The genera *Beauveria* and *Metarhizium* include diverse assemblages of species and strains with biopesticide development potential, and additional studies characterizing the range of susceptibilities of these fungi and others to imbibitional damage are urgently needed.

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CHAPTER 3

DEBILITATION IN CONIDIA OF THE ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* AND IMPLICATION WITH RESPECT TO VIABILITY DETERMINATIONS AND MYCOPESTICIDE QUALITY ASSESSMENTS

Abstract

Viabilities of entomopathogenic fungal conidia are most commonly determined by suspension of dry conidia from storage in a water/surfactant solution immediately prior to inoculation onto an agar-based germination substrate; conidia are then incubated at a moderate temperature (usually ca. 25ºC) for ≤ 24 h. In this study, germination rates determined from this fast rehydration (FR) protocol were compared to those obtained when dry conidia were subjected to slow rehydration (SR), by holding under high humidity conditions, prior to aqueous suspension. Differences in viability estimates obtained using the FR vs. SR protocols increased markedly after conidia were exposed to various stress factors in storage (high a_w, temp., and O_2), with the SR protocol producing higher estimates of viability in all cases. After *Beauveria bassiana* (Bb) conidia were stored under moist conditions for 21 days at 25ºC, the SR estimate of viability was >21% greater than the FR estimate. In jars flushed with different O_2 concentrations (balanced with N_2) and stored at 50 ºC for 34 days, proportional differences between protocols varied, depending on water activity, from 18–44% in jars flushed with 100% N_2 (0% O_2) to as high as 63–93% when treated with 21–22% O_2. Atmospheres with different CO_2 concentrations had no impact on the differences between estimates. For conidia stored over a broad range of moderate to high temperatures in the absence of O_2, SR-FR differences were ≤ 9% at 25–40 ºC but 30%

at 50 ºC. Germination of stressed Bb and *Metarhizium anisopliae* (Ma) conidia increased substantially when incubation time on the germination substrate was increased from 24 to 72 h, whereas germination of non-stressed conidia showed little change. Testing ultimately revealed that stress-induced changes in conidial quality were the underlying causes of the observed differences in viability estimates from the various protocols. Conidia debilitated by stress were characterized by hypersensitivity to lethal imbibitional damage (damage that is mitigated by slow rehydration) or by slow germination. These findings indicate that for Bb and Ma, viability protocols incorporating short incubation times (≤ 24 h) and fast rehydration, by excluding debilitated conidia from counts, may provide more reliable assessments of overall mycopesticide quality.

Introduction

A considerable number of mycoinsecticides have reached the marketplace, and millions of hectares are treated annually with entomopathogenic fungi worldwide (Chapter 1). Despite recent advances, use of mycopesticides is proportionally limited when compared to their chemical counterparts, even in countries in which many mycopesticides are produced. The growing adoption of these microbial biocontrol agents depends on factors such as 1) development of better products, 2) development and implementation of truly integrated pest management strategies in which biological options are emphasized (Lomer et al., 1999; Thomas, 1999; Lacey et al., 2001), 3) the capacity of biopesticide manufacturers/retailers to maintain marketing and product-support teams, 4) cultural changes (acceptance by farmers of slow-acting, narrow-host-range products), and 5) sound, knowledge-based recommendations for product use. With respect to development of better products, this may encompass increased concentrations of active ingredients, more predictable shelf-lives under non-

refrigerated conditions (Hong et al., 1997; Wraight et al., 2001), improved shipping and handling characteristics, greater UV-tolerance (Inglis et al., 2001), and ultimately, greater efficacy and reliability under field conditions (Lacey et al., 2001).

Development of mycoinsecticides and assessment of their quality rely to a great extent on viability determinations, and numerous techniques for measuring viability are available (Goettel and Inglis, 1997). Germination protocols with pre-incubation regimes including slow rehydration of dry conidia in high humidity environments have been shown to prevent imbibitional damage and thereby boost germination percentages when compared to fast rehydration of dry conidia (Moore et al., 1997; Magalhães and Boucias, 2004; Chapter 2). In some cases, fast-rehydration and longer incubation times achievable through use of media amended with a hyphal-growth inhibitor (benomyl) have been recommended (Milner et al., 1991; Meikle et al., 2003). In fact, Burges (1998) warned about the risk of performing viability counts after incubation for only 24 h due to exclusion of slow-germinating spores in the viability rate, a recommendation based on reports of substantial proportions of slow-germinating conidia in stored formulations. After seven years of storage at 6 ± 2 ºC, for example, viability of fast-hydrated *Beauveria bassiana* conidia was 0% after incubation for 24 h but 100% after 72 h (Alves et al., 1996). For dried *M. acridum* (formerly identified as *M. anisopliae* var. *acridum*) stored in oil for 37 months at 17 ºC, germination counts were 47 and 79% after incubation for 24 and 48 h, respectively (Moore et al., 1995).

After initiating studies on shelf-life of *Beauveria* and *Metarhizium* conidia I observed that germination counts following slow rehydration were usually higher than counts following fast-rehydration protocols, especially after storage under harsh conditions. Faced with uncertainties as to which protocol to rely on, I decided to launch a more detailed investigation. This study employed several stress factors,

including temperature, water activity, and oxygen concentration to alter the physiological condition of *B. bassiana* and *M. anisopliae* conidial powders. Viability was then quantified using two recommended techniques incorporating rapid versus slow rehydration. The principal objective of this study was to enhance our understanding of differences between germination protocols and increase awareness of important considerations when choosing among techniques designed to assess conidial quality. An additional objective included characterization of storage conditions promoting conidial longevity.

Material and Methods

2.1 Source of fungi

Fresh batches of a technical *B. bassiana* (Bb) product based on aerial conidia of isolate GHA were provided by Laverlam International Corp. (Butte, MT, USA). Aerial conidia of the isolate CB-10 (= ARSEF 7981 or CG 858) of *M. anisopliae* (Ma; classified according to Bischoff et al., 2009) were lab-produced on cooked rice (Chapter 2) and stored for 14 months at -20 ºC until the start of experiments.

2.2 Germination counts

For viability determination through the slow rehydration (SR) protocol, a small portion of conidial powder (0.15–0.2 mg) was picked up on the tip of a spatula, transferred to a glass Petri dish, and incubated (slow hydrated) at 25 ºC for 24 h in a 24-cm internal diameter glass "desiccator" with water-saturated atmosphere. The hydrated conidia were then added to a screw-cap glass vial 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 (Jin et al., 2008). The temperature of water+surfactant was not controlled, but it was in

the range 20-25 ºC throughout. The suspension was agitated for 10 min on a wrist-action shaker (Burrel Scientific, Pittsburgh, PA, USA), and 10-µl aliquots were plated on 1 x 1 x 0.3 cm blocks (1 droplet/block) of yeast extract agar-based solid medium (YEA) (Meikle et al., 2003) amended with 0.005 g L^{-1} of benomyl (Bonide Chemical Co., Yorkville, NY, USA). Viability of conidia was also assessed through a conventional protocol by suspending usually dry conidia in water+surfactant and plating directly onto YEA immediately after removal from storage containers, omitting the slow hydration step. This treatment will be referred to as fast rehydration (FR) protocol. Following inoculation in both cases, the glass slides containing the agar blocks were incubated in parafilmed Petri dishes at 25 ºC in darkness, and germination counts were performed 24 h post-inoculation (p.i.). Conidia were considered to have germinated when a germ tube was microscopically 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. Temperatures in incubators were monitored with digital data loggers (Hobo®, Onset Computer Corp., Bourne, MA, USA). Reported temperatures were ± 0.5 ºC for 25 ºC and ± 1 ºC for all other temperatures.

2.3 Effects of water activity on conidial viability

Constant equilibrium relative humidities (ERHs) were attained in airtight glass jars (0.95 L) with rubber-lined metallic lids (Ball®, Jarden Corp., Muncie, IN, USA) modified to include a 13-mm rubber septum. In each of 4 replicate jars, 1.0 g of Bb conidial powder contained in aluminum foil dishes was stored for 21 days at 25 ºC over the desiccant calcium sulphate (eight-mesh indicating Drierite, W.A. Hammond Drierite Co., Xenia, OH, USA), distilled water, or saturated salt solutions (NaOH, $NaNO_2$, NaCl, and K_2SO_4). Respiratory activity of conidia was indirectly measured via

determinations of O_2 and CO_2 concentrations at end of the experiment. Five-hundred µl of "air" was retrieved 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. Readings were compared against a commercial standard containing 6.96% O_2 and 4.91% CO_2 balanced with N_2 (Airgas East Headquarter, Salem, NH, USA). Following the gas chromatograph measurements, each jar was opened and the sample was quickly transferred to a water activity (a_w) meter (LabMaster-a_w, Novasina, Pfäffikon, Switzerland) set to 25 ºC. Germination after slow versus fast rehydration was then determined as described in the previous section.

2.4 Effects of carbon dioxide and oxygen on conidia viability

A_w of Bb conidial samples (0.6 g/sample) held in 3.4 cm diam. x 1.1 cm plastic cups (Novasina, Pfäffikon, Switzerland, code 4-1110601) was standardized by storage at either 10 or 25 ºC in glass jars containing NaOH for a 48 h period. A_w was measured and the samples were immediately transferred to 125-mL glass jars (Ball®, Jarden Corp., Muncie, IN, USA) and sealed with metallic lids possessing rubber septa. Conidial a_w following 48-h incubation over NaOH and just before transfer to glass jars was 0.078 (10 ºC) and 0.090 (25 ºC) for the CO_2 experiments, and in the 0.084-0.090 range (25 ºC) for the O_2 experiments. Lab RH during transfer of samples was in the mid 20's and low 40's during the first and second set of experiments with each gas, respectively. Samples were flushed for 40 minutes at 40 mL/min flow rate with variable CO_2 (0 to 100%) or O_2 concentrations (0 to 22.4%), balanced with N_2, and in some instances were flushed with compressed air. Concentration of O_2 in every jar following flushing was checked to make sure ambient air had been successfully removed from storage containers. Each treatment was replicated four-fold, and both

experiments were repeated on a different date. A preliminary short-term experiment at high storage temperatures revealed that the described containers were not completely sealed, as indicated by slow increases in O_2 concentration. Further investigation showed that O_2 ingress was largely prevented by sealing each 125-ml jar inside a 0.95-L jar containing the same mixture of gases. Using this double container set up, glass jars were incubated at 50 ºC for 34 days, and O_2 concentration (i.e., as leakage indicative), a_w, and germination counts were determined as previously described.

2.5 Effects of temperature on conidial respiration and viability

In a time-course experiment, samples of dry Bb conidia (0.6 g/sample) possessing initial a_w of 0.090±0.001 were placed in 125-mL glass jars and flushed with 100% N_2 as described above. Using the double-container set up, storage was carried out at 25, 40, and 50 ºC. O_2 concentration in jars, a_w, and germination counts were determined at all temperatures after storage for 15, 29 and 61 days. Four jars were used for each temperature/time. O_2 concentration, a_w, and germination counts were determined as mentioned earlier. As in all previous experiments, samples were assessed destructively.

2.6 Relationship between conidial debilitation and imbibitional damage

This experiment was performed with debilitated versus non-debilitated conidial powders of Bb and Ma. Debilitated (stressed) conidia were produced by incubation at 50 ºC in jars with saturated $NaNO_2$ solution (64.4% ERH); debilitation incubation times were 13 h for Ma and 48 h for Bb. Non-debilitated conidia remained in storage at -20 ºC. Four replicate samples of each species/treatment combination (0.6g /sample) were then held over Drierite for three days at 25 ºC to standardize a_w (procedure previously shown to dehydrate conidial powders to a a_w of ca. 0.021). Conidia were then slow-rehydrated

for 24 h prior to immersion in water+surfactant at 34 ºC, or fast-hydrated (immersed in water+surfactant without previous slow hydration) at either 25 or 0.5 ºC. Fast rehydration at ca. 25 ºC is known to cause significant imbibitional damage in dry Ma conidia (Moore et al., 1997; Chapter 2). Non-stressed GHA conidia, on the other hand, are highly sensitive to imbibitional damage only at very low temperatures (Chapter 2). Thus, for Bb the same procedure was followed except that the water+surfactant solution was set to 0.5 ºC (using an ice bath) instead of 25 ºC. Viabilities were determined following all treatments, with germination counts performed at 24, 48, and 72 h p.i following incubation at 25 ºC. Based on germination rates previously mentioned, some derived parameters were calculated. The full potential (overall) viability was considered as the SR germination when imbibition was performed at 34 ºC and after incubation at 25 ºC for 72 h, and this represents the best estimate of total living conidia in the powder. The difference between the overall viability and FR germination at 24 h p.i. and 34 ºC refers to the percentage of debilitated conidia in a conidial powder. This includes both conidia expressing delayed germination (difference for FR germination at 34ºC between 72 and 24 h p.i**)** and conidia unable to germinate at 34 ºC unless if slow hydrated (difference between overall viability and FR germination at 34 ºC/72 h p.i).

2.7 Statistical analyses

Jars flushed with CO_2 and/or N_2 in which considerable leakage took place (final O_2 concentration > 2%), and jars flushed with O_2 in which the final concentration was approx. 2% higher or lower than the target concentration, were excluded from statistical analyses. Percent germination data were arcsine square root transformed to make sure assumptions of normality and homogeneity of variance were met, and examined using two- or three-way analysis of variance (ANOVA). Means were compared by the Tukey-Kramer HSD test and considered to be statistically

different at the 5% significance level. All data analyses were performed using the JMP statistical package (SAS Institute Inc, Cary, NC, USA). The experiment investigating the relationship between conidial stress and imbibitional damage incorporated a nested or repeated measures design (estimates of germination over time were derived from subsamples of treated samples), and data were therefore analyzed using multivariate ANOVA (MANOVA), an analytical approach that does not depend on the sphericity assumption (Zar, 1999). Significance of interaction terms was tested using several alternative multivariate tests, including Wilk's Lambda and Pillai's Trace; results reported herein are those generated by the Pillai's Trace option. Means separation following MANOVA was conducted as recommended by Stevens (2002). The JMP multivariate analysis platform provides estimates of the degree of deviation from the sphericity assumption, reporting both the greenhouse-Geisser (G-G) and Huynh-Feldt (H-F) estimations of the epsilon parameter. Tukey's HSD was considered valid for means testing in all cases as the mean of the G-G and H-F epsilon values exceeded 0.7 (see Stevens, 2002). The Tukey-Kramer option was accessed via the JMP standard least squares, random effects platform (standard univariate ANOVA, split plot design).

Results

3.1 Effect of conidial water activity

After storage in the glass jars with Drierite or the various saturated salt solutions for 21 days at 25 ºC, water activities of conidia ranged from 0.021 to 0.972, equivalent to 2.1-97.2% in terms of equilibrium relative humidity (a_w*100) (Table 3.1). Final O_2 concentrations were negatively correlated ($r = -0.752$, $P < 0.0001$) and CO_2 concentrations positively correlated ($r = 0.986$, $P < 0.0001$) with a_w. In jars with

Table 3.1. Viability of *Beauveria bassiana* conidia estimated by two different germination protocols following storage at different water activities for 21 days at 25 ºC.

Treatment	Final conidial water activity (a_w)	Final O_2 (%) in jar	Final CO_2 (%) in jar	Germination (%)[1]				Proportional difference between viability estimates[2]	
				Slow rehydration (SR protocol)		Fast rehydration (FR protocol)			
Control	-	-	-	97.9±1.01	A	97.5±0.54	A	0.4±0.67	A
Drierite	0.021± 0.0003	19.1± 0.42	Not detected[3]	97.8±0.32	A	94.9±1.42	AB	2.9±1.57	B
NaOH	0.070± 0.0003	19.1± 0.46	Not detected	97.1±0.38	A	94.6±0.43	AB	2.6±0.55	BA
$NaNO_2$	0.636± 0.0030	18.7± 0.36	0.4± 0.04	97.6±0.47	A	92.1±0.66	B	5.6±0.89	B
NaCl	0.743± 0.0024	17.3± 0.04	2.5± 0.12	95.3±1.33	A	75.0±2.73	C	21.3±2.76	C
K_2SO_4	0.957± 0.0015	1.0± 0.10	14.1± 0.27	0.8±0.83	B	0.2±0.17	D	-	-
H_2O	0.972± 0.0012	1.1± 0.10	14.2± 0.21	0.0		0.0		-	-

[1]Germination percentages (means ± standard errors) followed by the same letter are not significantly different (Tukey-Kramer HSD, $\alpha = 0.05$).
[2]Proportional differences (means ± standard errors) followed by the same letter are not significantly different (Tukey-Kramer HSD, $\alpha = 0.05$).
[3]CO_2 not detectable for concentrations below approximately 0.2%.

moist conidia ($a_w \geq 0.957$), CO_2 reached over 14%, and O_2 dropped to ca. 1%. In jars with dry conidia ($a_w \leq 0.070$), low concentrations of CO_2 were not detectable, because very small CO_2 peaks were overshadowed by larger adjacent nitrogen peaks, which in the standard gas had an 18-fold greater concentration compared to CO_2. Two-way ANOVA indicated highly significant main effects of a_w and germination protocol ($F_{1,28} = 878.5$, $P < 0.0001$; $F_{1,28} = 56.5$, $P < 0.0001$, respectively) and a strong a_w x germination protocol interaction ($F_{4,28} = 9.7$, $P < 0.0001$). As a_w of the conidial samples increased from 0.021 to 0.743, the proportional difference between the estimates of viability from the SR versus FR protocols increased from 3 to 21% (Table 3.1). When a_w increased from 0.743 to 0.957, viability dropped to < 1%, regardless of the germination protocol.

3.2 Effect of carbon dioxide and oxygen

3.2.1. Carbon dioxide

Following CO_2 (balanced in N_2) storage at 50 ºC and 0.098 a_w for 34 days, ANOVA (excluding the air storage treatment) revealed no interaction between germination protocols and CO_2 concentrations ($F_{3,24} = 1.1$, $P = 0.36$) (Figure 3.1A). Mean germination determined from the FR protocol (average over the different CO_2 treatments) was 12 percentage points lower than the estimate from the SH protocol (80 vs. 92%) (protocol main effect $F_{1,24} = 128.8$, $P < 0.0001$). ANOVA indicated a marginally significant effect of CO_2 concentration on germination (CO_2 main effect $F_{3,24} = 3.1$, $P = 0.044$); however, mean germination across CO_2 concentrations varied only from 90–95% following SR and 76–82% following FR and did not show a significant trend ($r = 0.177$, $n = 32$, $P = 0.33$). Compared to storage in a CO_2 atmosphere, storage in air resulted in markedly lower germination rates (< 40%

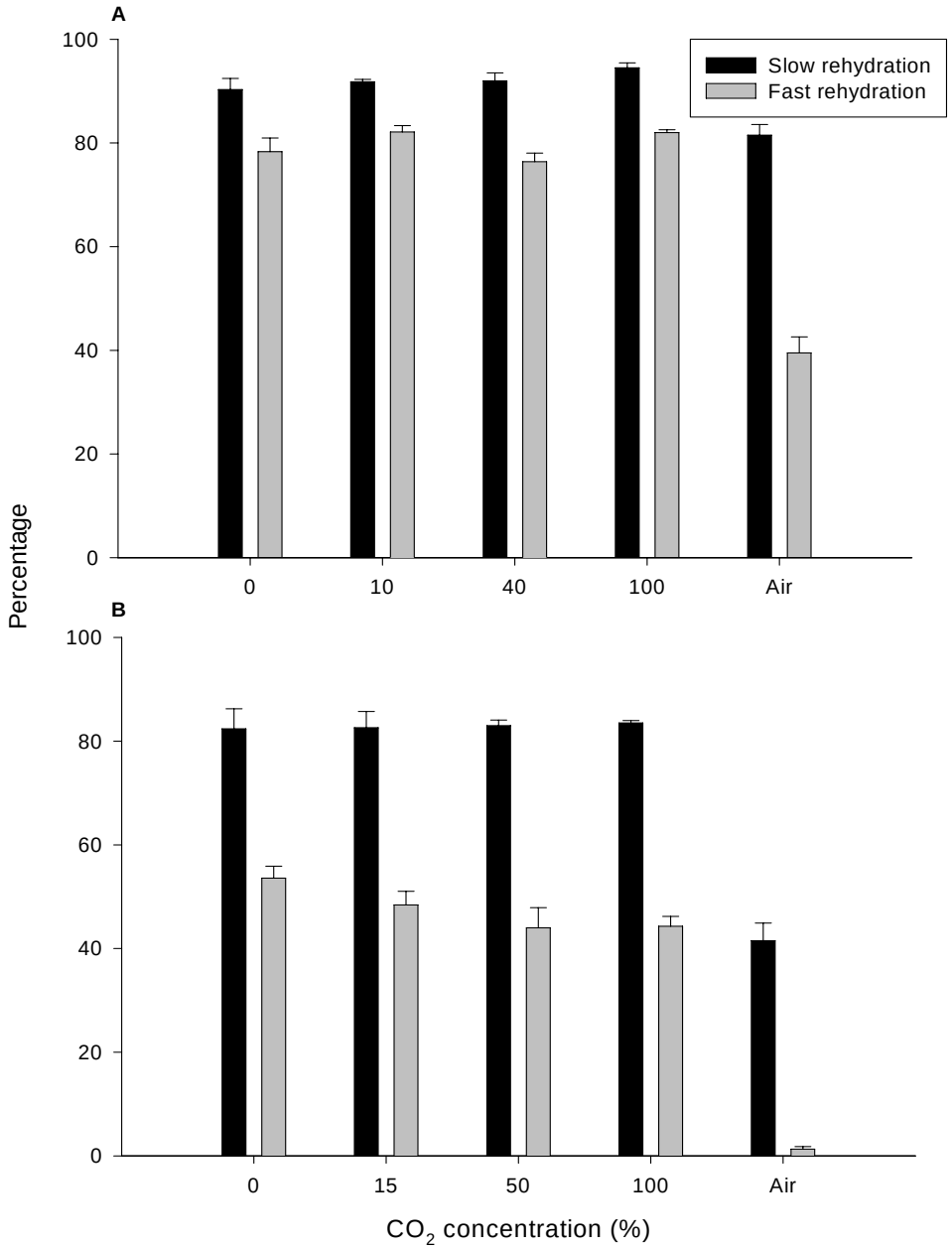

Figure 3.1. Effects of CO_2 concentration on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the fast- or slow-rehydration germination protocol after storage at 50 ºC for 34 days. Top graph (A): conidia with 0.098 ± 0.005 a_w in all treatments; bottom graph (B): conidia in all CO_2 treatments with 0.143 ± 0.002 a_w.

following FR and < 82% following SR) and an increase in the difference between the mean FR vs. SR estimates (from 12 to 42 percentage points). Similar results were recorded following CO_2 storage at the higher a_w of 0.143, i.e., there was no germination protocol x CO_2 concentration interaction ($F_{3,21} = 1.0$, $P = 0.40$) and no effect of CO_2 concentration ($F_{3,21} = 0.82$, $P = 0.49$) (Figure 3.1B). Also, as observed at the lower a_w, the effect of germination protocol was highly significant ($F_{1,21} = 249.5$, $P < 0.0001$); however, storage under wetter conditions resulted in lower viability throughout and a substantially greater difference between the two germination protocols (mean difference of 35 percentage points at 0.143 a_w vs. 12 percentage points at 0.098 a_w). After storage in air, germination following the FR protocol was 1.3% compared to 41.5% following the SR protocol, a proportional difference much greater than that observed at the lower a_w (97 vs. 13%).

In the first CO_2 experiment, average a_w for conidia flushed with compressed air was 0.098, a value similar to that recorded in the CO_2-flushed samples and equivalent to 4.3% moisture content according to isotherms established for this isolate (Chapter 2). In the second experiment, however, the final a_w in the air treatment was higher than that measured in the CO_2 treatments (0.163 a_w or 5.7% moisture content, vs. 0.143 a_w or 5.3% moisture content in the CO_2-flushed samples). This higher moisture level likely contributed to the extreme difference in germination following the two germination protocols.

3.2.2. Oxygen

In experiments in which the effect of O_2 (balanced in N_2) was assessed, residual levels of O_2 in jars flushed with 100% N_2 was in the 0.2-0.5% range, similar to residual levels in the CO_2 experiments. In the first experiment, with conidia at 0.100

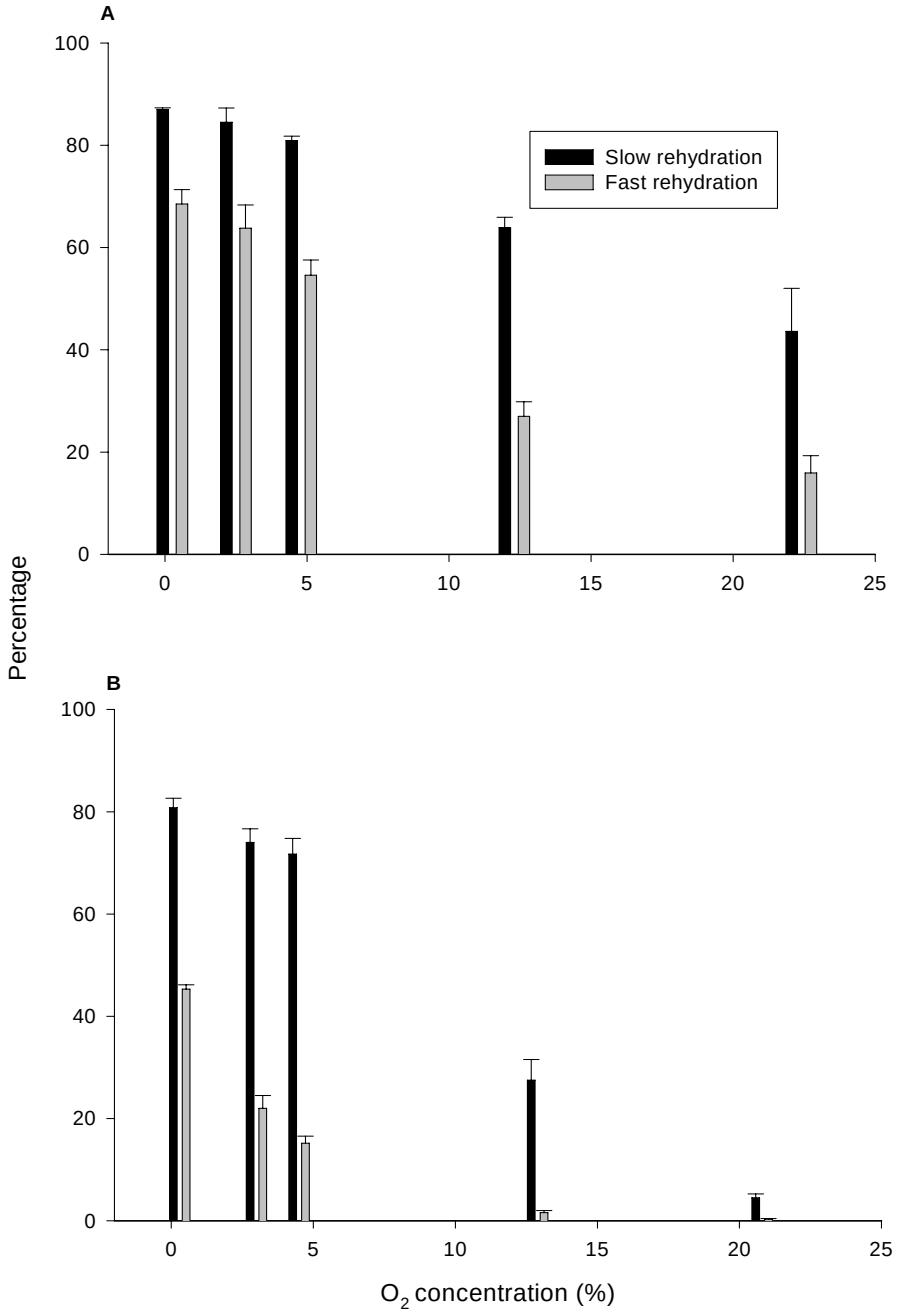

Figure 3.2. Effects of O_2 concentration on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the fast- or slow-rehydration germination protocol after storage at 50 ºC for 34 days. Top graph (A): conidia with 0.100±0.006 a_w; bottom graph (B): conidia with 0.147±0.005 a_w.

a_w, ANOVA revealed no significant germination protocol x O_2 concentration interaction ($F_{4,26} = 1.1$, $P = 0.39$) (Figure 3.2A). Both main effects were highly significant; mean germination decreased from 78 to 30% following storage in atmospheres with 0.3 vs. 22.4% O_2, respectively ($F_{4,26} = 58.3$, $P < 0.0001$), and germination following the FR protocol averaged 27 percentage points lower than the SR protocol ($F_{1,26} = 118.6$, $P < 0.0001$).

In the second experiment with conidia at a higher a_w (0.147), a strong interaction was noted between germination protocol and O_2 concentration ($F_{4,28} = 20.2$, $P < 0.0001$) (Figure 3.2B). The proportional difference between the germination estimates increased with increasing O_2 concentration (FR estimates were 21–25% lower than the SR estimates at 0.3–2.5% O_2 vs. 58–63% lower at 12–22% O_2). Both main effects were also highly significant (germination protocol $F_{1,28} = 657.5$, $P < 0.0001$; O_2 $F_{4,28} = 329.9$, $P < 0.0001$), and the negative effects of O_2 on conidial germination were more pronounced than observed under the drier conditions (a_w = 0.100) of the first test.

3.3 Effect of temperature

Plots describing the time-course of SR and FR germination counts in an N_2-rich atmosphere are shown in Figure 3.3. A three-factor ANOVA (time, temperature, and germination protocol) indicated significance of all main effects and 2-way interactions (all P values ≤ 0.0001) as well as the 3-way interaction ($F_{6,76} = 3.0$. $P = 0.01$). Increases in both temperature and time were associated with increasing proportional differences between the estimates of germination generated by the

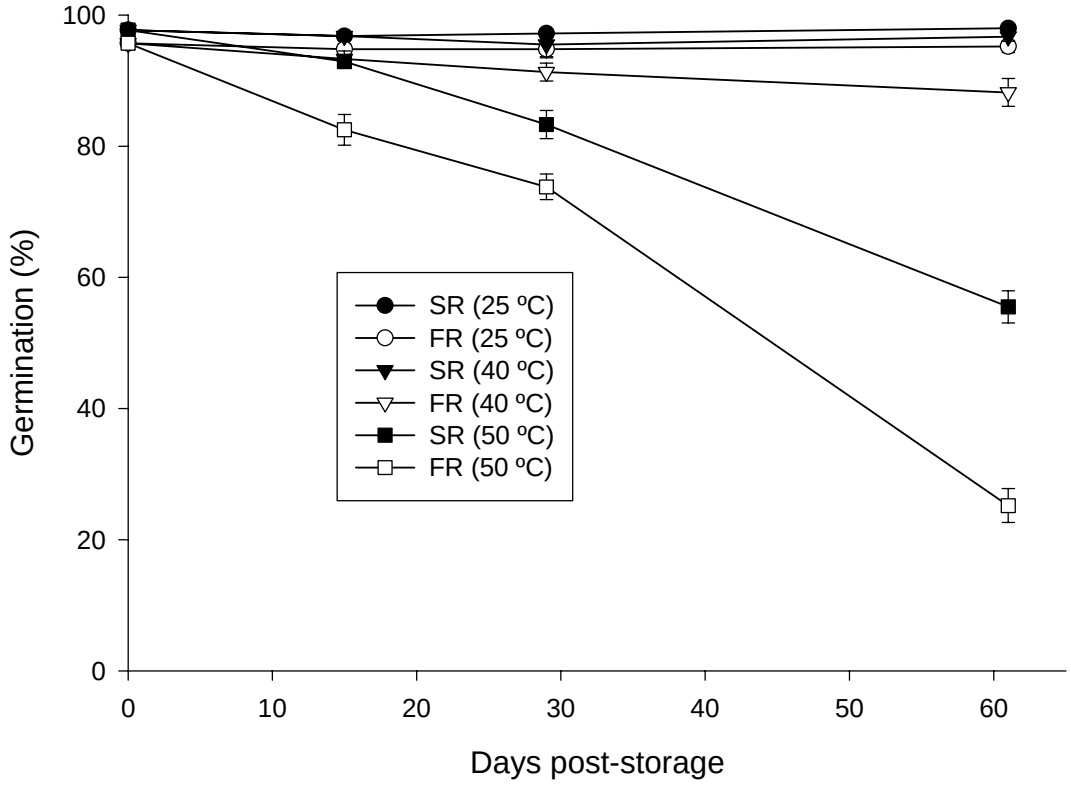

Figure 3.3. Effects of temperature on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the slow-rehydration (SR) or the fast-rehydration germination protocol (FR). Samples gas-flushed with 100% N_2.

alternative protocols (temperature x germination protocol interaction $F_{2,76} = 12.3$, $P < 0.0001$). After storage for two months, mean FR germination counts were 3% lower than the SR counts at 25ºC and 55% lower at 50ºC.

3.4 Relationship between conidial stress and imbibitional damage

3.4.1. Beauveria bassiana

Germinability of conidia was dramatically affected by the short-term exposure to the high-stress conditions of 50ºC/64.4% ERH (Table 3.2). Germination rates of debilitated conidia were not only substantially lower than germination rates of non-debilitated conidia across all comparable pre-germination treatments (stress main effect: $F_{1,18} = 1046$, $P < 0.0001$), but the repeated measures ANOVA also indicated highly significant 2-way interactions of stress x germination protocol ($F_{2,18} = 28.1$, $P < 0.0001$) and stress x incubation time ($F_{2,17} = 22.3$, $P < 0.0001$). FR had a much greater negative effect on stressed conidia than on non-stressed conidia, and stressed conidia germinated more slowly than non-stressed conidia. The 3-way interaction was not significant ($F_{4,36} = 1.65$, $P = 0.18$). In the remaining analyses, data from the stressed versus non-stressed conidial populations were analyzed separately.

Analysis of results with the non-stressed conidia indicated no rehydration protocol x time interaction ($F_{4,18} = 0.43$, $P = 0.79$). The main effect of incubation time was also insignificant; germination did not vary with time, regardless of the rehydration protocol (time main effect: $F_{2,8} = 0.29$, $P = 0.75$). On the other hand, germination protocol had a significant effect on viability assessments ($F_{2,9} = 182.4$, $P < .0001$). Germination following FR at 34ºC was consistently lower than germination

Table 3.2. Effects of conidial quality and rehydration protocol on germination of *Beauveria bassiana* strain GHA conidia, subjected (48h at 64.4% RH and 50 ºC) or not to stressful conditions during storage.

Incubation time (h)	Germination (%)[1]			Debilitated conidia (based on germination recorded after SR and FR at 34 ºC)		
	Slow rehydration (SR protocol followed by immersion at 34ºC)	Fast rehydration (FR protocol)		Conidia exhibiting delayed germination (%)[2]	Conidia susceptible to imbibitional damage (%)[3]	Total debilitated conidia (%)
		34 ºC	0.5 ºC			
	Non-stressed conidia					
24	97.3±0.5 a	95.5±1.0 a	50.1±4.0 b	-	-	-
48	97.8±0.4 a	95.1±0.6 a	52.9±4.7 b	-	-	-
72	97.5±0.5 a	95.5±1.2 a	51.6±4.2 b	0.0±0.9	2.0±1.4	2.0±1.2
	Stressed conidia					
24	55.6±3.4 bc	13.6±2.2 e	0.5±0.4 f	-	-	-
48	69.8±3.6 ab	29.9±3.8 d	3.5±1.2 f	-	-	-
72	77.8±2.2 a	43.5±3.9 cd	3.8±0.6 f	29.9±5.6	34.3±6.0	64.2±2.6

[1]Germination percentages (means ± standard errors) followed by the same letter are not significantly different (Tukey-Kramer HSD, $\alpha = 0.05$).
[2]Difference between FR/34 ºC germinations at 72 vs. 24 h.
[3]Conidia unable to germinate at 34 ºC unless slowly rehydrated (difference between SR/72h, the overall viability, and FR/34ºC/72h germination).
[4]Sum of items 2 and 3 above.

following SR, but the mean difference was small (97.5 vs. 95.5%) and not statistically significant according to the Tukey test. In contrast, FR at 0.5ºC produced a significant 46% reduction in viability compared with SR.

In the case of the stressed conidia, germination increased with incubation time across all rehydration treatments (Table 3.2). The increases were more or less consistent across germination protocols, and this result was reflected in a marginally insignificant germination protocol x time interaction ($F_{4,18} = 2.5$, $P = 0.081$). The main effects of both incubation time and rehydration protocol were highly significant ($F_{2,8} = 39.1$, $P < 0.0001$; $F_{2,9} = 328.3$, $P < 0.0001$, respectively). In contrast to the results with non-stressed conidia, there was a significant negative effect of FR at 34ºC; after 72 h, germination of conidia subjected to FR was 44% compared to 78% for conidia subjected to SR (Table 3.2). Few conidia survived FR at 0.5ºC. Non-stressed conidia exhibiting delayed germination were not observed (95.5 vs. 95.5% germinations after 24 vs. 72 h), whereas 30% of the stressed conidia required > 24 h to germinate following SR (13.6 vs. 43.5%).

3.4.2. Metarhizium anisopliae

Overall results with Ma (Table 3.3) were remarkably similar to those with *B. bassiana*, except that in the case of the non-stressed conidia, the main effects of both incubation time and germination protocol were significant ($F_{2,8} = 22.3$, $P = 0.0005$; $F_{2,9} = 60.6$, $P < 0.0001$, respectively), and the germination protocol x time interaction was significant, though only marginally ($F_{4,18} = 3.1$, $P = 0.041$). ANOVA of the data from the stressed conidia was complicated by the minimal germination observed within 24 h after FR with consequently zero or near-zero variance. Excluding the 24-h data, the main effects were highly significant ($P \leq 0.0002$), and there was no evidence of a 2-way interaction ($F_{2,8} = 0.5$, $P = 0.60$). Large proportions of the conidia exposed to the

Table 3.3. Effects of conidial quality and rehydration protocol on germination of *M. anisopliae* (isolate CB-10), subjected (13h at 64.4% RH and 50 ºC) or not to stressful conditions during storage.

Incubation time (h)	Germination (%)[1]			Debilitated conidia (based on germination recorded after SR and FR at 34 ºC)		
	Slow rehydration (SR protocol followed by immersion at 34ºC)	Fast rehydration (FR protocol)		Conidia exhibiting delayed germination (%)[2]	Conidia susceptible to imbibitional damage (%)[3]	Total debilitated conidia (%)[4]
		34 ºC	25 ºC			
	Non-stressed conidia					
24	91.3±1.0 abc	81.3±1.9 cd	49.9±2.0 e	-	-	-
48	93.6±0.8 ab	86.6±2.9 bc	70.9±2.1 d	-	-	-
72	96.5±0.5 a	86.3±2.3 bc	72.0±5.3 d	5.0±1.3	10.2±2.3	15.3±2.0
	Stressed conidia					
24	28.3±10.4	0.0±0.0	0.1±0.1	-	-	-
48	70.3±4.9 a	27.4±3.6 c	11.5±1.9 e	-	-	-
72	85.2±2.8 b	41.5±2.6 d	20.1±1.5 ce	41.5±2.6	43.5±5.2	85.2±2.8

[1]Germination percentages (means ± standard errors) followed by the same letter are not significantly different (Tukey-Kramer HSD, $\alpha = 0.05$).
[2]Difference between FR/34 ºC germinations at 72 vs. 24 h.
[3]Conidia unable to germinate at 34 ºC unless slowly rehydrated (difference between SR/72h, the overall viability, and FR/34ºC/72h germination).
[4]Sum of items 2 and 3 above.

stressful storage conditions exhibited slow germination and high sensitivity to FR. Following SR, 57% of conidia required > 24 h to germinate, and only 42 and 20% of conidia remained viable after FR at 34 and 25ºC, respectively.

Discussion

In the present study, viability of a highly stressed Ma conidial powder determined following fast rehydration was 0% after incubation for 24 h and > 40% after 72 h; germination reached 85% following slow rehydration and incubation for 72 h. These observations highlight the great extent to which germination protocols may impact results of experiments involving entomopathogenic fungi. The ungerminated conidia of both Ma and Bb observed after incubation for 72 h appeared dead (not swollen) and, therefore, it is very likely that the full potential (overall) germination rates reported here are close to actual values. I have shown that exposure of Bb and Ma conidia to unfavorable (stressful) storage conditions of high a_w, temperature, and/or O_2 (factors that boost metabolic activity) results in increased sensitivity to imbibitional damage and reduced capacity for rapid germination (within 24h) following fast rehydration. Slow, vapor phase rehydration of conidia and incubation for a prolonged period enabled many stressed conidia to successfully germinate. In all experiments, increasing the level of stress (i.e., increasing a_w, temperature, or O_2 concentration) resulted in increasing proportions of affected conidia. I refer to these conidia as stressed or "debilitated" conidia, which fall into two categories: those expressing delayed germination and those with greater susceptibility to imbibitional damage than non-stressed conidia and, therefore, unable to germinate unless slowly rehydrated. There likely exists a third category of debilitated conidia: those conidia alive at the time of rehydration but unable to initiate germ tube formation. Such conidia could clearly be

characterized as moribund (approaching death) and might be detectable through use of fluorescent staining procedures not attempted in the present study (Schading et al., 1995).

The likely mechanism underlying imbibitional damage during rehydration of dry cells was unveiled two decades ago (Crowe et al., 1989), and there is abundant evidence supporting the existence of a gel-to-liquid crystalline phase transition when the phospholipids in dry cell membranes are rehydrated. Leakage of cell contents takes place when dry membranes are plunged into water at a temperature below the phase transition temperature (generally referred to as the melting temperature, T_m). Imbibitional damage can be avoided by slowly rehydrating dry cells in a water-vapor saturated atmosphere, or by immersion in water at a temperature > T_m (Crowe et al., 1992; Chapter 2). For the dry, non-debilitated Bb powder, it is clear that T_m is below 34 ºC, since both the FR and SR germination protocols performed at this imbibition temperature generated equally high estimates of viability. A T_m of < 34 ºC is in accord with values known for other microorganisms; it is well known, for example, that fast rehydration of dry *Saccharomyces cerevisiae* cells should be performed using water warmed to 38-42 ºC (Echigo et al., 1966; van Steveninki and Ledeboer, 1974).

Regarding the high-viability (non-debilitated) Ma powder, 24% of conidia were susceptible to imbibitional damage at 25 ºC, whereas 65% were susceptible following exposure to stress. For Bb conidia fast rehydrated at 0.5 ºC, conidial mortality due to imbibitional damage was 46% in a non-stressed powder versus 74% in a stressed powder (at this low temperature, but not at 25 ºC, non-debilitated Bb conidia are quite susceptible to imbibitional damage (Chapter 2). It has been shown that stress induces production of fatty acids by de-esterification of membrane phospholipids and that this process may drive up T_m (McKersie et al., 1989; van Bilsen and Hoekstra, 1993; van Bilsen et al., 1994).

Imbibitional damage is an irreversible physical process (Hoekstra and van der Wal, 1988), which instantaneously kills sensitive unicellular organisms, as previously shown in studies of pollen (Hoekstra, 1984) and conidia of entomopathogenic fungi (Chapter 2). This fact explains the few significant differences in viability observed across incubation times following fast rehydration of non-stressed conidia at the low, imbibitional damage-inducing temperatures. In low-quality (stressed) powders, due to presence of conidia expressing delayed germination, germination rates tended to increase with increasing incubation time and the trends were similar, regardless of the rehydration protocol. Thus, the populations of conidia that survived imbibitional damage exhibited patterns of delayed germination very similar to those of conidia that were protected from imbibitional damage by the slow rehydration protocol (Tables 2 and 3). This interesting result (insignificant or only marginally significant interaction between incubation time and rehydration protocol) supports a hypothesis that the underlying causes of delayed germination and increased sensitivity to imbibitional damage are very different and exhibit independent action.

Delayed germination has been associated with diverse factors, such as incompatibility with formulants and other chemicals (Alves et al., 1998; Mohan et al., 2007; Chapter 2), UV radiation (Zimmermann, 1982; Moore et al., 1993; Braga et al., 2001; Ghajar et al., 2006), temperatures either below or above the optimal range following inoculation on nutrient media (Luz and Fargues, 1997; Devi et al., 2005), exposure to substrates with low water activities (Luz and Fargues, 1997; Milner et al., 1997; Andersen et al., 2006), short-term exposure of aqueous conidial suspensions to heat before inoculation on suitable media (Zimmermann, 1982; Rangel et al., 2005; Fernandes et al., 2008), and long-term storage at above-freezing temperatures (McClatchie et al., 1994; Moore et al., 1995; Alves et al., 1996; Magalhães and Boucias, 2004). With respect to long-term storage, delayed germination for Bb isolate

447 was first noticed after a 30-month storage of conidial powders at 6 ± 2 ºC (Alves et al., 1996). After seven years at this temperature, germination of unformulated conidia was reported to be 0% after incubation for 24 h but 100% after 72 h. In contrast, delayed germination was not observed for conidia stored frozen for the same period of time. Delayed germination was also observed for conidia of isolate IMI 330189 of *M. acridum* dried with silica gel and kept in oil, for which delayed germination was noticed after 18 months of storage at either 8 or 17 ºC (Moore et al., 1995). After 37 months, ca. 30% of conidia stored at 17 ºC did not germinate within 24 h but did so within 48 h, whereas at 8 ºC viability was higher, and ca. 21% of living conidia did not germinate within 24 h. Dry conidia of *M. acridum* isolate CG 423 showed 21% viability at 24 h but 88% at 48 h after 6 months at ca. 25 ºC (Magalhães and Boucias, 2004).

Delayed germination is a physiological phenomenon that becomes more pronounced as conidia age naturally or which can be expressed within a very short period of exposure to stressful conditions during storage. The effect of storage on retarded germination was briefly discussed by Burges (1998), who attributed delayed germination of dry conidia to the "extra time taken for recovery from physiological dehabilitation in storage". A deeper understanding of the physiological processes associated with delayed germination is clearly needed. Despite similar appearances of conidia comprising a population, the population may indeed be highly heterogeneous (Gottlieb, 1978). Intra-populational heterogeneity in conidia, including differences in age and maturity (Moore et al., 1996) and cold tolerance (Daoust and Roberts, 1983) have been shown or suggested.

I believe that the nature of delayed germination observed in my studies is fundamentally the same as that described in previous storage studies, the only difference being that while it took many months or even years to develop this effect

under low-temperature regimes, this effect was expressed after just a few hours of storage under conditions that boosted metabolic activity. Reactions in (hydrated) conidia are primarily enzymatic (Gottlieb, 1978), and increases in either temperature or water availability are expected to boost metabolic rates (Weber et al., 1999; Willcock and Magan, 2001). When both conidial a_w and temperatures are in the upper tolerable extreme, especially in the presence of O_2, onset of delayed germination would be greatly shortened. Other studies corroborate this hypothesis; for example, Fernandes et al. (2008) reported delayed germ tube emergence from Bb conidia following suspension in water and exposure to 45 ºC for 1 hour. Also, following exposure of *Metarhizium* sp. (formerly identified as *M. anisopliae*) conidia in aqueous suspension to 45 ºC for 30 minutes, germination percentages after 24 and 48 h on agar medium were 6 and 82%, respectively (Zimmermann, 1982). Likewise, Rangel et al. (2005) observed similar results with *M. robertsii* isolate ARSEF 2575 (segregated from *M. anisopliae* in a genomic reclassification by Bischoff et al., 2009) following aqueous exposure to 45-47 ºC for 3 hours, with germination percentages varying from < 10% after 24 h to about 50% after 48 h; for *M. acridum* the difference was less pronounced, ranging from about 80% to 95%. Finally, Luz and Fargues (1997) reported a considerable increase in the proportion of Bb conidia expressing delayed germination after storage at 35 ºC versus 20–30 ºC.

The germination patterns observed for Bb conidia following incubation in jars with different ERHs, in which increasing ERH led to lower viabilities (following both SR and FR), is in agreement with results previously reported for this fungus (Clerk and Madelin, 1965; Sandhu et al., 1993). Elevated release of CO_2 in jars was related to high conidial a_ws, demonstrating that respiration can be greatly stimulated under such conditions, resulting in increased occurrence of debilitated conidia (as indicated by pronounced differences in results between fast vs. slow rehydration protocols). When

CO_2 concentrations were assessed through storage under a high-temperature regime, no negative effects of this gas on germination percentages were observed. The benefits of replacing air with N_2 or CO_2 in terms of extended longevity under storage has been previously reported for beneficial fungi (Clerk and Madelin, 1965; Teshler et al., 2007). I have further demonstrated that conidial viability is prolonged in low-O_2 atmospheres, independent of CO_2 concentration. In this study, presence of even low concentrations of O_2 was detrimental to viability, and increasing O_2 concentrations yielded increasing proportions of debilitated conidia. The importance of O_2-free environments for enhancing mycopesticide shelf-life was highlighted by Jin et al. (1999), and I have now demonstrated a link between O_2-deprived atmospheres and reduced numbers of debilitated conidia. Due to methodological limitations, the lowest O_2 concentration tested in my study ranged from 0.2 to 0.5%, representing the residual O_2 in jars following prolonged flushing with pure nitrogen at a gentle flow rate. This value is in agreement with average residual O_2 of 0.26% following gas flushing of foil bags (Teshler et al., 2007). In all gas experiments, viabilities were higher and numbers of debilitated conidia were lower for the drier conidia (0.100 a_w or 4.3% moisture content vs. 0.143 a_w or 5.3% moisture content). The results reveal that even seemingly small variations in a_w can substantially impact conidial longevity. Optimal a_w for prolonged longevity of entomopathogenic fungi is reported to lie in the range of 0.11-0.14 (11–14% ERH) at room temperature, corresponding to ca. 5% moisture content (Hong et al., 1998; Hong et al., 2001). The fact that in my study 0.098-0.100 a_w provided better longevity than 0.143-0.147 a_w may be an indication that, under (quasi)anaerobic conditions, desirable a_w values for prolonged longevity may be lower than previously thought. Although the same procedure was used in both O_2 experiments, relative humidity conditions in the lab during transfer of samples to jars

just before gas flushing were not constant, resulting in variable conidial a_ws (and longevities). This fact highlights the potential importance of environmental conditions in production facilities during mycopesticide processing.

As previously discussed, the germination protocol based on slow rehydration in many cases led to substantially higher germination than the fast-rehydration protocol, as did extension of the incubation period beyond 24 h.; however, the higher counts from these methods included large numbers of debilitated conidia (those viable conidia unable to germinate within 24 h or exhibiting hyper-sensitivity to imbibitional damage). It is my belief that inclusion of debilitated conidia in counts could lead to misleading assessments of potential efficacy and that germination protocols based on fast rehydration (at a temperature that does not cause imbibitional damage in non-debilitated conidia) and short incubation times (≤ 24 h) are more likely to reflect the "true" quality of conidia. Indeed, preliminary bioassays with 2nd-instar *Spodoptera exigua* larvae indicated that debilitated conidia were significantly less virulent than non-debilitated conidia (unpublished data). It is well documented that rapid germination is an important virulence factor in conidia of entomopathogenic fungi, particularly when applied against rapidly developing (frequently molting) insect larvae or against insects in habitats where environmental conditions are only marginally or intermittently favorable for infection (Vey and Fargues, 1977; Al-Aidroos and Roberts, 1978; Hassan et al., 1989; Samuels et al., 1989; Vandenberg et al., 1998). Clearly, viability assessments using fast rehydration protocols should be performed with warm water when working with fungal species or strains whose healthy (non-debilitated) conidia are inherently susceptible to imbibitional damage (Chapter 2). But temperature of the suspending medium should not be so high as to permit germination of severely debilitated conidia. Further investigations are warranted. Finally, standardization of initial water activity would make germination estimates from

different laboratories more comparable. Drying of small amounts of conidia (0.6 g) in glass jars with ca. 30 g of Drierite for three days at 25 ºC has proven adequate for producing consistently low a_ws.

Only 2% of high-quality Bb conidia with very low water activity were susceptible to imbibitional damage at 34 ºC when not subjected to previous slow rehydration, and delayed germination was not recorded (Table 3.2), suggesting that non-stressed, dry Bb conidia are capable of rapid water uptake and germination in moist environments. The vast majority of non-debilitated Ma conidia were also able to germinate rapidly without prior slow rehydration when inoculated onto a moist substrate. The imbibitional damage (9.9%) recorded for the non-stressed Ma powder (Table 3.3) may have been caused by the use of a much older batch compared to a relatively fresh Bb powder.

James and Jaronski (2000) observed that Bb conidia in preparations with high versus low overall viabilities following storage were equally virulent against nymphs of *Bemisia tabaci* biotype B and concluded that the quality of the surviving propagules had not decreased with storage time. Doses had been adjusted for viability using germination counts recorded after fast rehydration and incubation for 18 h on an agar medium. These findings support my hypothesis that germination protocols based on fast rehydration and short incubation times may provide the best estimates of fungal quality.

Although the processes of conidial debilitation can be slowed by optimal drying, storage temperatures, and packaging systems (factors that extend shelf-life), virulence of debilitated conidia requires further investigation. Slow germination and hyper-sensitivity to imbibitional damage could severely compromise the ecological

fitness of fungal biocontrol agents. Developing methods that minimize expression of these negative traits and adopting germination protocols that allow for exclusion of severely debilitated conidia are of paramount importance.

APPENDIX 3

Standard germination protocol for quality assessment of hydrophobic conidia (exclusion of debilitated conidia)

Stressed (debilitated) conidia exhibit either hypersensitivity to imbibitional damage or delayed germination. These conidia are generally less virulent than their non-stressed counterparts, and in assessing the overall quality of a technical conidial powder or mycopesticide formulation, it may be desirable to exclude these conidia from viability counts. In order to exclude conidia hypersensitive to imbibitional damage, dry conidial samples should be directly plunged into a surfactant solution adjusted to a temperature that does not cause imbibitional damage in dry, non-stressed conidia. To exclude slow-germinating propagules, incubation time should be established as the minimal time required for non-stressed conidia of the test isolate to reach maximum germination. Although additional studies are needed, a basic protocol follows:

A) Standardization of initial water content

Conidia (0.6g or less) held in small plastic cups should be dried for three days at 25 ºC in 125-mL glass jar with sealed lid. Each jar should contain ca. 30 g of the desiccant calcium sulphate (eight-mesh indicating Drierite, W.A. Hammond Drierite Co., Xenia, OH, USA). This procedure has been shown to result in conidial water activity between 0.019-0.030 (in equilibrium with 1.9-3.0% relative humidity).

B) Suspension preparation

Dry conidial sample (0.15–0.2 mg picked up on the tip of spatula) should be added to 10mL of water containing any commonly used surfactant such as Tween 80. If the same suspension will be used for concentration determinations via hemacytometer

counts, use of a surfactant with a hydrophilic-lipophilic balance of 10, such as TDA (polyoxyethylene tridecyl ether) or Silwet L-77 is recommended, as surfactants with this balance produce optimal suspensions for counting. Vials with the surfactant solution and ca. 1 g of 2 mm glass beads, should be equilibrated at 33-34 ºC immediately prior to addition of conidia. Solutions equilibrated at lower temperatures may be used for fungi like *B. bassiana* strain GHA that are insensitive to imbibitional damage at room temperatures. Fresh surfactant solutions should be prepared for each viability assessment.

C) Plating technique

After agitation for a few minutes, 10µl droplets of conidial suspension (approximately 10^5 or 10^6 conidia/ml) should be immediately applied onto a 1.0x1.0 cm blocks of the desired agar-based benomyl-free germination medium on microscope slides (1 droplet/block, no spreading necessary). The microscope slides with the inoculated agar blocks should then be placed in Petri dishes, sealed with parafilm, and incubated in darkness at 25 ºC. Alternatively, droplets of conidial suspension may be applied to agar medium in standard Petri dishes. Commonly used germination media include potato dextrose agar and Sabouraud dextrose agar supplemented with 1% yeast extract. Addition of gentamycin or other antibiotics may be required when working with contaminated conidial preparations. Optimal incubation time will be fungal species- and strain-specific. Recommended incubation time is 16-18 h for isolate GHA of *Beauveria bassiana* and ca. 20 h for isolate CB-10 of *Metarhizium anisopliae*.

D) Counting

After the desired incubation time, a coverslip is placed over each agar block and at least 300 conidia counted through phase-contrast microscopy, although other microscopic techniques and staining (other than vital staining) can be used.

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CHAPTER 4

APPLICATION OF MODIFIED ATMOSPHERE PACKAGING (GAS FLUSHING AND ACTIVE PACKAGING) FOR EXTENDING THE SHELF LIFE OF *BEAUVERIA BASSIANA* CONIDIA AT HIGH TEMPERATURES

Abstract

Shelf life determinations under non-refrigerated conditions, especially high temperature regimes characteristic of tropical regions, deserve growing attention. In this study, I investigated effects of modified atmosphere packaging (MAP) on longevity of *Beauveria bassiana* (Bb) strain GHA conidia. In gas flushing experiments, pure CO_2, N_2, H_2, or He provided similar conidial longevities, whereas O_2-rich atmospheres (21 and 100%) were detrimental to viability when conidia were stored in atmospheres with 20% CO_2. Times for initial viability to drop to 80% at 40 and 50 ºC were > 91 and > 15 days, respectively, whereas viability was still 87% after a 16-month storage period at 25 ºC. Residual O_2 in vials flushed with 20% CO_2/80% N_2 ranged from 0.3% at the start to as high as 12.4% at the end of experiments. When active packaging (truly hermetic packages with O_2/moisture scavengers) was tested, shelf lives were substantially improved. Viabilities in the 80-89% range after six months at 40 ºC or 2 months at 50 ºC were consistently recorded when a dual O_2/moisture absorber (RP series) or a combination of sachets (dual O_2absorber/CO_2generator + desiccant) were used. Best water activities (a_w) were ≤ 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, I 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 ambient temperatures. To my knowledge, these are the longest survival times (retention of ≥ 80% viability) for Bb conidia under tropical conditions reported to this date, representing more than two- and four-fold increases over previous best results at 40 and 50 ºC, respectively.

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 30s to 50 ºC for extended periods, particularly during transport or on-farm storage in the tropics (Stathers et al., 1993, Alves et al., 1996; Hong et al., 1997; Burges, 1998; Jin et al., 1999; Roberts and St. Leger, 2004). Shelf life may vary according to species (Hong et al., 1997), isolates (Aregger, 1992; Hong et al., 2001), and propagule types (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 Jr 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 *B. bassiana* (Bb) conidia at high ambient temperatures (≥ 30 ºC). Marques and Alves (1996) reported that viability of conidia with 15.5% moisture content (MC) stored at 30 ºC 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 ºC 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 ºC (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 ºC 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 ≥ 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 ºC, respectively (Hong et al., 2001).

To my 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 tropical temperature regimes.

Material and Methods

2.1 Conidial powders

Two batches of technical Bb powder based on aerial conidia of strain GHA, acquired from Laverlam International Corp. (Butte, MT, USA), were used in the experiments. Conidia were stored at -20 ºC 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), and 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 plated on 1x1x0.3cm blocks (1 droplet/block) of a yeast extract agar-based solid medium (Meikle et al., 2003) amended with 0.005 g L^{-1} of benomyl (Bonide Chemical Co., Yorkville, NY, USA). In the second germination protocol (fast rehydration protocol), viability was determined by directly suspending dry conidia in the water/surfactant solution and plating on the yeast extract agar/benomyl medium (YEA). As it was previously shown that dry Bb conidia with high viability (high quality) are not susceptible to imbibitional damage at moderate temperatures (Chapter 2), solutions used for preparing suspensions

(water/surfactant) were equilibrated at room temperature. Following each of the rehydration protocols, the inoculated agar blocks (on glass slides) were incubated in parafilmed Petri dishes at 25 ºC in darkness, and germination counts were performed 24 h post-inoculation (p.i.). 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.

In all experiments of the present study, the fast and slow rehydration protocols estimated initial viabilities at ≥ 96.1% and ≥ 96.8%, respectively.

2.3 Gas flushing

2.3.1 Flushing with different gases

Conidial samples (0.6 g) were added to 3.4 cm diam. x 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 minutes 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-fold. 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 ºC 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 25ºC 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 ± 1ºC, as determined from continuous monitored 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, conidial samples were flushed with 20% CO_2 (+80% N_2). Four replicate samples for each treatment were checked for residual O_2 and final a_w after storage for 45, 91, 180 and 240 days at 40 ºC. The experiment was repeated, beginning on a different date. Additionally, separate experiments were conducted investigating effects of storage at 25 ºC (assessments at 46, 120, 180, 365, and 480 d) and 50 ºC (assessments at 15, 30, 47, 75, and 90 d). In all cases, viability was also assessed at 0 d. Different jars were sampled on each occasion, so this study did not use repeated measures design.

2.4 Active packaging of conidia

2.4.1 Comparison between aluminized films

In order to select an aluminized film for the active-packaging experiments, conidial samples were held over $MgCl_2.6H_2O$ for 2 d at 25 ºC, resulting in 0.321±0.0006 (SE) a_w. Conidia were then transferred to pouches (10 x 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-laminate material composed of 12 μm polyethylene terephthalate, 8 μm foil, and an 85 μm crystallized polypropylene heat-seal layer (manufacturer unknown). One dual-action O_2/moisture absorbing sachet (RP-3A, Mitsubishi Gas Chemical Co., Japan) was enclosed loose in each pouch before heat sealing and incubation at 40 ºC for 5 mo. Each treatment was replicated four-fold. After 5 months at 40 ºC, final conidial a_ws and viabilities for samples stored with two films were virtually identical (Table 4.1), and both materials were employed in the subsequent tests (one material used for all treatments within a test).

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 ºC, 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 (8x8.5 cm) with either one RP-3A O_2/moisture absorbing sachet, one O_2 absorbing film (code M-0034, lot 19208A, 88.9x63.5x0.3 mm; CSP Technologies, Auburn, AL, USA) plus one moisture absorbing film (CSP Technologies, code M-0026, lot 02208A, 63.5x38.1x0.6 mm), or one O_2/moisture absorbing film (CSP Technologies, code M-0033, lot 10808A, 76.2x76.2x0.6 mm). As a control, conidia were kept in pouches made of 30 μm thick polyethylene (code P827-2.1.2; Empac Agroindustrial de Plásticos Ltda, Brasilia, Brazil) with one RP-3A O_2/moisture absorbing sachet.

Table 4.1. Comparison between two aluminized pouches with respect to water activity (a_w) and germination counts (fast- vs. slow-rehydration protocols) of *Beauveria bassiana* conidia stored for 5 months at 40 ºC with addition of O_2/moisture absorbing sachet.

Pouch type[1]	Final a_w[2]	Fast rehydration	Slow rehydration
A	0.027±0.0000	90.9±0.13% a	98.0±0.35% a
B	0.027±0.0000	89.9±2.00% a	97.6±0.63% a

[1]Pouch types A and B were a material of unknown composition provided by a mycopesticide company (overall thickness 114 µm) or a three-laminate material composed of 12 µm polyethylene terephthalate, 8 µm foil, and an 85 µm crystallized polypropylene heat-seal layer, respectively.
[2]For both treatments, initial a_w was 0.321±0.0006.
[3]Within each column, germination means (±SE) followed by the same letter are not significantly different (t-test, α= 0.05).

Following preparation, all containers with conidia were pre-incubated at 25 ºC for 5 d and then transferred to 50 ºC. Residual O_2 in glass jars flushed with N_2 was 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 ºC. Conidia were incubated at 50 ºC 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, so this study was not designed for repeated measure analysis.

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 ºC. 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 ºC, which resulted in 0.738±0.0007 a_w prior to packaging. Samples were then transferred to foil pouches (10x12 cm) containing one of the following AP sachets: RP-3A O_2/moisture 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 moisture absorber (56.7 g). As a control, foil pouches without any AP sachet were employed. Pouches were incubated at 50 ºC 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-fold.

2.4.4 Combination of AP sachets for shelf-life extension

Conidial samples were dried over drierite for 2 d at 25 ºC (resulting in 0.020±0.0008 a_w) and then transferred to 16x20 cm foil pouches with different sachets: one RP-5A O_2/moisture 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-fold and conidial a_w and germination assessments were performed following 148 and 180 d post-storage at 40 ºC.

2.4.5 Moisture absorbing capacity of a O_2/moisture absorbing sachet

Initial a_w of conidial samples was adjusted to 0.321±0.0022 by storage over saturated $MgCl_2.6H_2O$ solutions. Two different amounts of conidia (0.6 vs. 30 g) were then transferred to 16x20-cm foil pouches, each provisioned with one RP-5A O_2/moisture absorbing sachet. Half of the pouches containing 30 g of conidia were also each provided with one sachet of drierite (56.7 g). Pouches were transferred to 40 ºC without a pre-incubation equilibration period. Each treatment was replicated four-fold, and a_w and germination counts were determined after storage for 6 mo.

2.4.6 Effect of a pre-incubation regime on shelf-life

Due to lower than expected viabilities after a 6-mo period at 40 ºC in the previous experiment, I 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 ºC, 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/moisture absorber sachet and pre-incubated for an additional 5-d period at 25 ºC before storage at target temperatures (25, 40, and 50 ºC). Alternatively, samples were kept over

drierite or NaCl for 7 d at 25 ºC, transferred to foil pouches with RP-3A sachets and immediately stored at target temperatures without a 5-d pre-incubation period at 25 ºC. Each treatment was replicated four-fold and assessment of conidial a_w and germinations were carried out after 60 d at 50 ºC, and 180 d at either 25 or 40 ºC.

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 residual $O_2 > 3.5\%$) were discarded. In gas flushing experiments with 20% CO_2 at different temperatures, all glass jars were considered in statistical analyses independently of O_2 ingress. Percentage data 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).

Results

Actual temperatures in closed, equilibrated incubators set at 25, 40, and 50 ºC were ±0.5, ±1, and ±1 ºC, respectively (in the 40 and 50 ºC treatments, temperatures momentarily dropped 2-3 ºC and 4-6 ºC, respectively, when incubator were opened). Germination percentages determined through fast and slow rehydration are shown in all figures and tables, but I regard estimates from the fast rehydration protocol as being better indicators of conidial quality (Chapter 3). Thus, unless otherwise stated, the use of the terms "viability" and "germination" refer to germination percentages measured following fast rehydration

3.1 Flushing with different gases

Final a_w for conidia in the first assay did not vary with gas treatment (P= 0.4150, $F_{[5,13]}$= 1.1); overall mean a_w was 0.099±0.0248. Significant germination differences were recorded after 60 d at 50 ºC (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 (Figure 4.1A). Residual 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 0.3-1.1%.

In the second assay, 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%) (Figure 4.1B). These germination rates were markedly lower than the range of 49–51% observed in the first assay. Residual 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 ºC storage experiment, a significant drop in viability was observed (P= 0.0002, $F_{[5,18]}$= 8.8), but the decrease was gradual and small, from 96% to 90% within 365 d and to 87% within 480 d (Figure 4.2A). Conidial a_w increased from 0.104 at 46 d post-storage to 0.204 at the end of the experiment (P< 0.0001, $F_{[4,15]}$= 36.3), and residual O_2 increased from an average of 0.5% to 12.4% (P< 0.0001, $F_{[4,15]}$= 38.0).

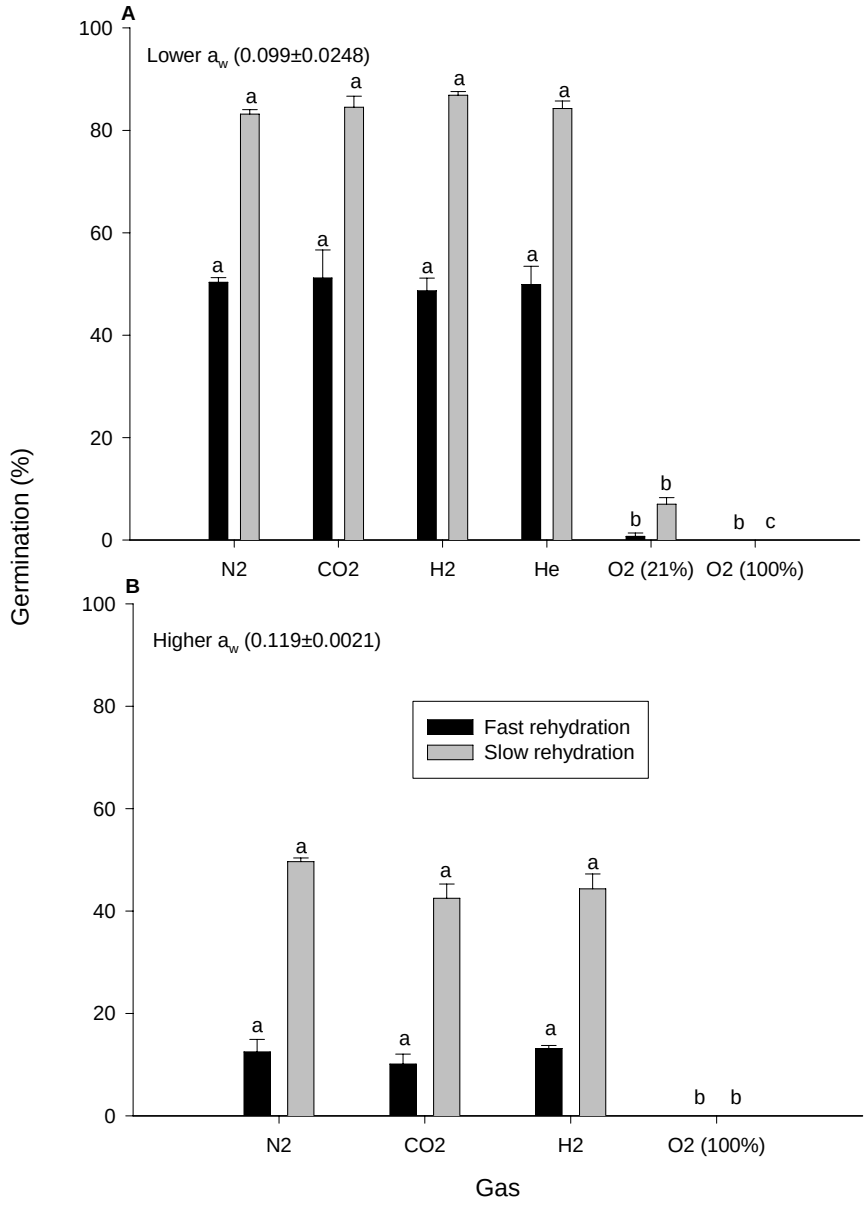

Figure 4.1. Effect of different gases on percent viabilities (mean±SE) after fast vs. slow rehydration protocols for *Beauveria bassiana* conidia after storage at 50 ºC for 60 days. In both experiments, viabilities for the 100% O_2 treatment were 0%. Within each germination protocol, bars (±SE) with the same letter are not significantly different (Tukey HSD, α= 0.05).

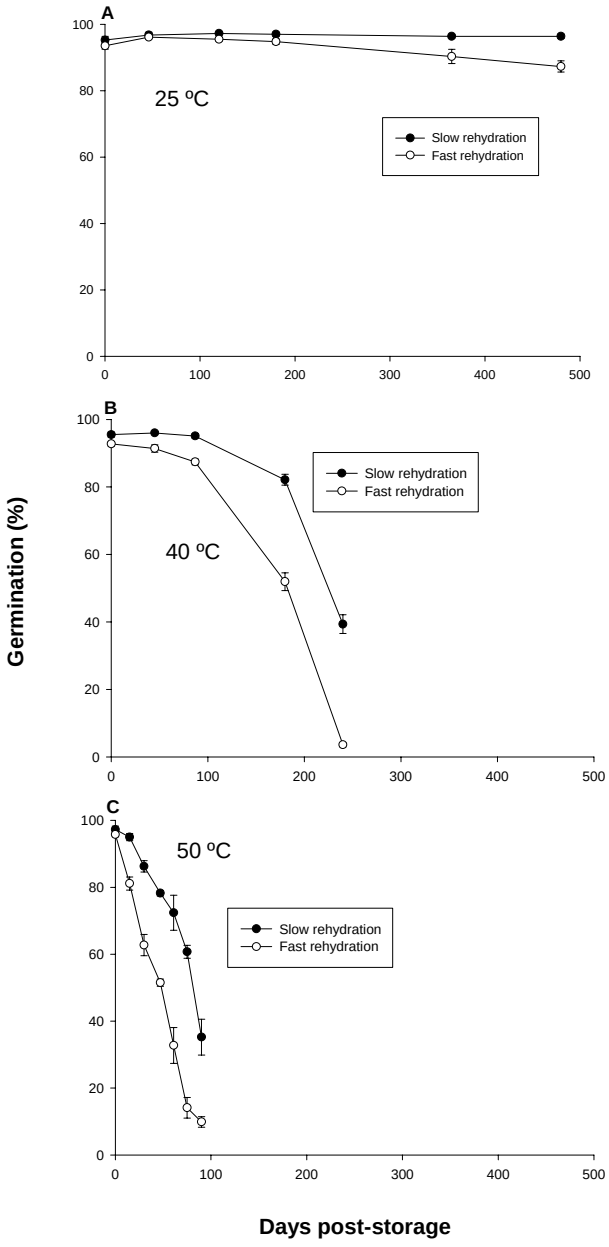

Figure 4.2. Viabilities after fast- vs. slow-rehydration protocols of *Beauveria bassiana* conidia flushed with 20% CO_2 (+80% N_2) after storage at either 25 (A), 40 (B), or 50 ºC (C).

In the experiment at 40 ºC (Figure 4.2B), 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 following fast rehydration (overall ANOVA $P < 0.0001$, $F_{[4,34]}= 361.7$). During the interval between 45 and 240 days, mean residual 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 ºC, initial viability dropped quickly, from 96 to 81% within the first 15 d to 10% after just 90 d of storage ($P < 0.0001$, $F_{[6,21}= 129.1$) (Figure 4.2C). Residual O_2 increased from an average 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 aluminized foil with one O_2/moisture absorbing RP-3A sachet significantly lowered conidial a_w to 0.059 following a 5-d equilibration period at 25 ºC ($P= 0.016$, $F_{[4,10]}= 5.2$) (Table 4.2). 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 ºC ($P< 0.0001$, $F_{[4,15]}= 427.9$). The combination of two absorbing films provided satisfactory viabilities up to 56 d post-storage, but results after 129 d were not as good as the use of RP-3A. Residual O_2 in glass jars flushed with N_2 was 0.2% just before incubation at 50 ºC. Gas flushing or use of a polyethylene pouch (+ RP-3A) resulted in poor viabilities compared to active packaging with a dual O_2/moisture absorber.

Table 4.2. Water activity (a_w) and viabilities (fast- vs. slow-rehydration protocols) of *Beauveria bassiana* conidia stored at 50 ºC in gas flushed jars or pouches with absorbing sachets/films.

Treatments	Day 0[1]			Day 56			Day 129		
	Initial a_w	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration
Flushing with 100% 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 + O_2/moisture 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 + O_2/moisture 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 + O_2 absorbing film + moisture 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 + O_2/moisture 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]Following a 5-day pre-incubation (equilibrium) period at 25 ºC, and immediately before transference to 50 ºC.
[2]Within each column, means (±SE) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

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 4.3). 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/moisture 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 4.4). The combo approach was as good as the use of a dual O_2/moisture absorber (RP-5A), both at 148 d ($P< 0.0001$, $F_{[2,6]}= 309.0$) and 178 d post-storage at 40 ºC ($P< 0.0001$, $F_{[2,6]}= 2,035$).

3.6 Moisture absorbing capacity of a O_2/moisture absorbing sachet

Although initial a_w was the same for all treatments (0.321±0.0022), final a_ws were different as intended ($P< 0.0001$, $F_{[2,6]}= 248.5$) (Table 4.5). Final conidial a_ws for two of the treatments (0.6 g conidia+RP-5A, or 30 g+RP-5A+drierite sachet) were essentially equivalent (0.026 vs. 0.029), but a_w was higher (0.066) in the third treatment (30 g+RP-5A and no drierite sachet). Based on initial and final a_ws for the latter treatment, and their respective moisture contents (8.2 and 3.4%, respectively, according to isotherms reported in Chapter 2), it was determined that each RP-5A sachet removed ca. 1.5 ± 0.01 g H_2O from conidial powders (including an

Table 4.3. Percent germination for fast vs. slow rehydration protocols of *Beauveria bassiana* conidia stored for 45 days at 50 ºC in pouches with different absorbing and/or generating sachets.

AP Sachet	Low initial a_w (0.019)			High initial a_w (0.738)		
	Final a_w	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration
Ageless (O_2 absorber)	0.807± 0.0012 a	0.0% c	0.0% c	0.819± 0.0015 a	0.0% c	0.0% c
Drierite (moisture 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/moisture 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
504 A (O_2 and CO_2 absorber)	0.704± 0.0003 c	0.0% c	0.0% c	0.729± 0.0009 c	0.0% c	0.0% c
504 E (O_2 absorber and CO_2 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 (±SE) followed by the same letter are not significantly different (Tukey HSD, α= 0.05).

Table 4.4. Effect of an O_2absorber/CO_2generator, with or without a desiccant sachet, in water activity (a_w) and viabilities (fast- vs. slow-rehydration protocols) of *Beauveria bassiana* conidia stored at 40 ºC for either 5 or 6 months.

Sachet type	Day 148			Day 178		
	Final a_w[1]	Fast rehydration	Slow rehydration	Final a_w[1]	Fast rehydration	Slow rehydration
504 E (O_2absorber/CO_2 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 (moisture 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/moisture 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 not pre-incubated at moderate temperature before exposure to 40 ºC.
[2]Within each column, means (±SE) followed by the same letter are not significantly different (Tukey HSD, α= 0.05).

Table 4.5. Effect of high initial a_w (0.321) of conidia on germination of *Beauveria bassiana* conidia following storage at 40 ºC for 6 months, without previous pre-incubation.

Condition	Final a_w	Fast rehydration	Slow rehydration
Small conidial volume (0.6g) + O_2/H_20 absorber	0.029± 0.0016 b	65.3±2.0% a	92.5±1.1% a
Large conidial volume (30.0 g) + O_2/H_20 absorber + drierite sachet	0.026±0.0003 b	70.5±2.3% a	91.3±0.8% a
Large conidial volume (30.0 g) + O_2/H_20 absorber	0.066±0.0013 a	69.0±1.8% a	89.7±1.6% a

[1]Means (±SE) followed by the same letter are not significantly different (Tukey HSD, $\alpha = 0.05$).

undetermined, but small amount of moisture from the air). For the other treatments the final a_w was considerably lower (≤ 0.029), and quantification of moisture removal was not attempted because it could not be determined if the AP sachets were fully saturated (it is possible that the sachets could have absorbed more moisture if put into a high ERH atmosphere). Despite the higher final a_w in the third treatment, viabilities did not differ from other treatments after exposure to 40 ºC for 6 mo (P= 0.3510, $F_{[2,6]}$= 1.6).

3.7 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 4.6). Percent germinations after 180 d at 25 ºC was 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 4.6). After 180 d at 40 ºC, 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 ºC 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).

Discussion

To my knowledge, shelf lives observed in the present study are longer than data 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 ºC. When an atmosphere with 20% CO_2 (+ 80% N_2) was tested in jars, times for conidial viability to drop to 80% were > 91 and > 15 d at 40 and 50 ºC, respectively. These times are similar to estimates calculable from data published by

Table 4.6. Effect of initial a_w and pre-incubation at a moderate temperature (25ºC) on germination of *Beauveria bassiana* conidia stored in foil pouches with an O_2/moisture absorber sachet.

Condition	Day 180 at 25 ºC			Day 180 at 40 ºC			Day 60 at 50 ºC		
	Final a_w[2]	Fast rehydration	Slow rehydration	Final a_w	Fast rehydration	Slow rehydration	Final a_w	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	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	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	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	60.0±3.0% b	94.7±1.0% b

[1]Within each column, means (±SE) 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.

Hong et al. (2001), indicating that 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 ºC, 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 mo at 40 ºC or 2 mo at 50 ºC.

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. I recently demonstrated that atmospheres in which air was replaced by CO_2/N_2 increased longevity of Bb conidia (Chapter 3). 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 pouches showed 74% viability after 2 months at 37 ºC, whereas 0% viability was reported in 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 mo at 23 ºC by use of Ageless and silica gel also proved successful (Leite et al., 2002), but I am not aware of additional studies on modified atmosphere packaging of entomopathogenic fungi.

Gas flushing (for 40 min) 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. 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 I 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 (Chapter 3). 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, I 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 300cc 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 most 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. Each sachet absorbed about 1.5 g of water from the conidial powder, which was enough to bring a_w close to the apparently optimal range. Preliminary measurement demonstrated that each drierite sachet (56.7 g) removes about 4 g of water from saturated atmospheres within 5 d, and an additional 4 g within the following 15 d (results not shown). For larger 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 to 6 mo under 'ambient storage' for products supplied by contract for application at a specific time, and 18 mo otherwise (Couch & Ignoffo, 1981). Shelf life (which I define as the time during which ≥ 80% viability is retained under certain temperature) of ca. one year has been recorded for the relatively thermotolerant *M. acridum* with 6.2% MC (but not 7.0% MC) at 27-32 ºC stored under vacuum (Hong et al., 1999), and paraffinic oil formulations of Bb at 25 ºC (Wraight et al., 2001). Longevities observed in my study at 25 and 40 ºC 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 mo at 25 ºC, and it is very likely that 80% viability would have been retained for at least 18 mo. I also achieved a shelf life of at least 6 mo at 40 ºC 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 ºC. Even longer shelf life could be expected in more commonly encountered tropical climates where conditions are less extreme. Experiments were also carried out tests at 50 ºC. Temperatures as high as or higher than 50 ºC can be reached in some regions (Hong et al., 1997) or during transportation (Ostrem and Godshall, 1979), but the main reason for testing such a high temperature was to accelerate most preliminary experiments. The use of this strategy was successful in order to test the effect of different gases, compare different packaging strategies (gas flushing vs. truly hermetic

packaging and packaging materials), and select AP sachets more fit for shelf-life extension of Bb conidia, as subsequently demonstrated in experiments carried out at 40 ºC.

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 Grzymacz (2000) it should be greater than 85%, although in this work I 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 ºC (refrigeration) and, for example, 35 ºC (for products commercialized in regions or seasons with effective mean temperatures around this value) would be very useful.

In addition, 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 impact on longevity. I 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, may result in misleading viabilities if not performed appropriately (Chapter 3). 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 slow rehydration counts were adopted. However, the biological/microbial control significance of this improvement is questionable, and I have shown that germination counts following slow rehydration are inflated by the presence of debilitated conidia, including those that have lost the capacity for rapid germination or are hypersensitive to imbibitional damage (Chapter 3).

Water activity 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). The term was initially proposed by microbiologists (Troller, 1980) and 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 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. In my study, best conidial a_ws were consistently associated with drierite, which is considerably < 5% MC (see also Chapter 3). It is possible that under anaerobic conditions (< 0.03% O_2) optimal water activities for storage are lower than under aerobic atmospheres. In the experiment with different amounts of conidia (0.6 or 30 g per pouch) with high initial a_w, the lowest a_w ranges recorded following a long-term storage were within the "desiccant range" (0.019-0.030), even in pouches with only 0.6 gr of conidia and one RP-5A sachet that, as discussed before, can absorb ca. 1.5 g H_2O. This suggests that further depression of conidial a_w by use of AP sachets would be a challenge, and it is unknown if conidia can tolerate storage at a_ws significantly lower than 0.019.

A great number of mycoinsecticides are commercialized worldwide (Chapter 1) 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. I 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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