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Production by solid-state and liquid fermentation and formulation of virulent strains of the fungal entomopathogens *Beauveria bassiana* and *Isaria fumosorosea* against whiteflies

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Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To God, for giving me faith and strength.

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Abraham Lincoln

SUMMARY

RESUMO

Produção por fermentação sólida e líquida e formulação de cepas virulentas dos fungos entomopatogênicos *Beauveria bassiana* e *Isaria fumosorosea* contra moscas-brancas

A mosca-branca, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) biótipo B, é uma praga cosmopolita e devastadora devido aos prejuízos oriundos dos seus danos diretos e transmissão de vírus. Fungos entomopatogênicos compreendem um grupo diversificado, que desempenha ação importante na regulaçãcao de populações de praga em agroecossistemas. Fungos ascomicetos anamórficos, como *Beauveria bassiana*, *Isaria fumosorosea* e *Lecanicillium* spp., constituem relevantes agentes de biocontrole de moscas-brancas. Avanços na pesquisa focando virulência, produção massal, formulação e estabilização de propágulos fúngicos são fundamentais para o desenvolvimento de micopesticidas eficientes contra moscas-brancas e outros insetos. Desta forma, este trabalho objetivou selecionar isolados fúngicos virulentos à mosca-branca; aumentar a eficácia mediante uso de surfactants não-iônicos em suspensões conidiais; desenvolver meios de cultura para produção rápida e estável por fermentação líquida submersa de células leveduriformes conhecidas por blastosporos. Na primeira etapa, isolados virulentos de *B. bassiana* e *I. fumosorosea* foram selecionados pela rápida e elevada atividade inseticida a ninfas e adultos de mosca-branca, bem como alto rendimento de conídios em arroz parboilizado. A adição de surfactantes organosiliconados permitiu a redução do volume de calda aplicado com resultados aditivos ou sinérgicos de controle. Foi ainda verificado que altos rendimentos de blastosporos tanto de *B. bassiana* como *I. fumosorosea* foram obtidos em curto tempo de fermentação líquida (3 dias de pré-cultivo e 2-3 dias de cultivo) usando nutrientes de baixo custo, como glucose e farelo de algodão. Esses blastosporos foram tolerantes à dessecação e mantiveram viabilidade por mais de um ano sob refrigeração (4 °C). Os blastosporos foram mais virulentos que conídios aéreos, o que coloca esta estrutura como a mais indicada como ingrediente ativo em bioinseticidas para moscas-brancas. Mediante manipulação nutricional e física do ambiente de fermentação, a produção de blastosporos de *B. bassiana* foi optimizada mediante aumento da aeração e pressão osmótica do meio líquido. Blastosporos produzidos em meio líquido altamente aerado e hiperosmótico (140 g glucose L^{-1}) mostraram-se mais virulentos à mosca-branca em relação àqueles produzidos em meio hipo-osmótico (40 g glucose L^{-1}). Esse processo foi reproduzido em escala piloto usando biorreator de 5 L resultando numa produção de 1-2 × 10^{12} blastosporos viáveis L^{-1} em apenas 3 dias a um custo de US$ 0,19 L^{-1}. Blastosporos de *B. bassiana* formulados com terra de diatomáceas e secados em fluxo de ar contínuo, ou secados em *spray dryer* tiveram estabilidade extendida por até 8 meses a 4 °C e superior em relação a 28 °C. Durante empacotamento, o uso de sachês absorventes de oxigênio e umidade prolongou consideravelmente a viabilidade de blastosporos armazenados a 28 °C por até 7 meses sem afetar sua eficiência contra mosca-branca. Em suma, esses resultados demonstram a viabilidade técnica e econômica de produção de blastosporos virulentos de *B. bassiana* e *I. fumosorosea*, tolerantes à dessecação e estáveis durante armazenamento. Esta tecnologia é uma nova opção que pode contribuir para expansão comercial de bioinseticidas à base de fungos entomopatogênicos.

Palavras-chave: Ascomycota; Conídios; Virulência; Produção massal; Crescimento dimórfico; Surfactantes; Formulação; Fermentação submersa; Blastosporos; *Bemisia tabaci*

ABSTRACT

Production by solid-state and liquid fermentation and formulation of virulent strains of the fungal entomopathogens *Beauveria bassiana* and *Isaria fumosorosea* against whiteflies

Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae) biotype B is a cosmopolitan, devastating insect pest due to their direct damages and transmission of plant viruses. Entomopathogenic fungi comprise the most diverse group of pathogens regulating arthropod pest populations in agroecosystems. Anamorphic fungal entomopathogens, including *Beauveria bassiana*, *Isaria fumosorosea*, and *Lecanicillium* spp., are among the main biocontrol agents of whitefly populations. Advances in research focusing on virulence, mass production, formulation, and storage stability of fungal propagules are imperative for the development of efficient mycopesticides toward whiteflies and other soft-bodied insects. Therefore, this study placed emphasis on screening for virulent fungal strains, enhancement of efficacy using nonionic surfactants in spray tank-mix, development of liquid culture conditions for rapid production and stabilization processes of single-yeast like cells known as blastospores. Firstly, we selected virulent strains of *B. bassiana* and *I. fumosorosea* displaying fastest speed of kill and inciting highest mortality levels of whitefly nymphs and adults along with their ability to produce high numbers of conidia on moistened parboiled rice. Secondly, insecticidal performance was enhanced by combining nonionic surfactants with spore suspensions rendering additive or synergistic effects. These surfactants also allowed reducing the volume application rate without altering fungal bioefficacy. Results from liquid fermentation studies using *B. bassiana* and *I. fumosorosea* revealed that appropriate amounts of inexpensive ingredients, such as cottonseed flour and glucose, are suitable for the rapid production of high yields of blastospores (3 days pre-culture and 2-3 days culture). The resultant blastospores of various strains survived well to desiccation and remained viable for more than one year under refrigeration. Moreover, these air-dried blastospores of both fungal species showed higher virulence against whitefly nymphs when compared with solid-substrate produced conidia. Optimized liquid culture production for *B. bassiana* blastospores was also achieved through the manipulation of oxygen rates and osmotic pressure in the liquid media. Furthermore, these blastospores produced in highly aerated and hyperosmotic liquid medium containing 140 g glucose L^{-1} were also more virulent to whitefly nymphs than those cells derived from low-osmotic medium amended with 40 g glucose L^{-1}. These optimal conditions were also scaled up in 5-L bioreactor that yielded 1-2 × 10^{12} viable blastospores L^{-1} in 6 days at a cost of US$ 0.19 L^{-1}. These blastospores were formulated with diatomaceous earth for air drying or for spray drying. Formulated blastospores of *B. bassiana* survived dehydration using both drying methods and showed improved shelf life when stored under vacuum-packaged at 4 °C rather than 28 °C. However, when these blastospores were actively packaged with dual action oxygen-moisture scavenging system, blastospores showed prolonged stability for up to 7 months at 28 °C and still remained virulent to whiteflies. Therefore, this low-cost production and stabilization method for the rapid production of shelf stable, virulent blastospores of *B. bassiana* and *I. fumosorosea* may expand the commercial use of mycopesticides for insect control in mainstream agriculture.

Keywords: Ascomycota; Aerial conidia; Virulence; Mass production; Dimorphic growth; Nonionic surfactants; Formulation; Liquid culture fermentation; Blastospores; *Bemisia tabaci*

1 INTRODUCTION

Discovery, production and formulation of microbial agents constitute key components in the development of effective and high quality biological control products. Biological control agents, more specifically the microbial pathogens of arthropods, are gaining expressive interest among scientists, society and private companies, as they offer an ecologically friendly alternative to chemical pesticides for incorporation in integrated pest management programs (IPM). Furthermore, current control strategies, heavily based on insecticide spraying, are threatened by the emergence of insecticide-resistant insect populations. Biocontrol agents are considered harmless to non-target organisms and play an important role in mitigating arthropod resistance to synthetic chemicals. The major catalyst aspects contributing to the expansion of this field of research stem from the fact that discovery of new chemical molecules to fight pests is facing serious challenges, while the organic agriculture keeps outstandingly growing year by year revealing a fertile turf for these biopesticides; and, second, agricultural products free of chemical residues are ruling the world exportation trade as part of the recent phytosanitary border policies of several countries. As a result, this set of conjunctures will undoubtedly promote the advance of biological control in the mainstream agriculture worldwide. Among these beneficial microorganisms, the interest in entomopathogenic fungi has increased recently, since they possess a unique route of infection through direct penetration to the host body, rather than being ingested like virus and bacteria, which results in the disease and eventual death (HUMBER, 2008). Most of them can be easily mass produced in artificial media allowing their exploitation in applied biological control (JARONSKI, 2010). The first step in developing mycopesticides consists in selecting the best fungal strain for controlling a susceptible insect target, and the attributes of selection usually involve tests of virulence, persistence, tolerance to abiotic stresses found associated with the target host and its habitat. Generally, this first stage is time-consuming and may demand tests of several strains before choosing the most proper candidate along with its type of infective propagule that meet the requirements of pest control. For instance, numerous fungal strains have been characterized based on their virulence, enzymatic activity profile, and tolerance to abiotic stresses, such as ultraviolet radiation, temperature and relative humidity (LACEY; FRANSEN; CARRUTHERS, 1996; WRAIGHT et al., 1998; WANG; TYPAS; BUTT, 2002; FERNANDES et al., 2007, 2008). The basic understanding of pathogen-host-environment interactions along with the epizootiological concepts consist one

of the keystones to design biorational strategies using entomopathogenic fungi for pest control.

Whiteflies, particularly *Bemisia tabaci* (Gennadius) biotype B (Hemiptera: Aleyrodidae), are considered the most global destructive pest of agricultural, ornamental and horticultural vegetable crops. Yield losses inherent due to virus transmission and direct damage by this insect pose a serious threat to world food safety. It has been noted that very few chemical insecticides still remain effective against whiteflies, since this species has the ability to develop resistance quickly due to its biological characteristics of high reproduction rate and genetic plasticity (De BARRO et al., 2011). Therefore, search for more environmentally sound and sustainable alternative control methods opens new avenues to explore microbial agents with potential for biological control. In light of this, the entomopathogenic fungi encompass a group of microorganisms that naturally infect and thus regulate populations of whiteflies (FARIA; WRAIGHT, 2001; LACEY; FRANSEN; CARRUTHERS, 1996). The high natural occurrence of these fungi in whiteflies makes them the most promising biocontrol agents to be explored in integrated pest management programs designed to battle this pest. Moreover, the broad genetic diversity among fungal entomopathogens coupled with their mode of action by direct contact make them suitable for the control of sap-sucking pests, such as whiteflies, that are not susceptible to other pathogens like viruses and bacteria. The most promising fungal candidates for whitefly biological control belong to Hypocreales (Ascomycota) and include *Beauveria bassiana* (Bals-Criv.) Vuill., *Isaria fumosorosea* (Wize) Brown & Smith (formerly *Paecilomyces fumosoroseus*), *Lecanicillium* spp. (formerly *Verticillium* spp.), and *Aschersonia* spp. Although *B. bassiana* is not considered epizootic to whiteflies, there have been virulent strains selected against nymphs and adults (WRAIGHT et al., 1998; RAMOS et al., 2004). *Isaria fumosorosea* and the recent identified *I. poprawskii* (CABANILLAS et al., 2013) are designated the best candidates for whitefly control due to their epizootic potential in suppressing this insect population. There is a wealthy body of literature that has dealt with strain selection for whitefly control (WRAIGHT et al., 1998; FARIA; WRAIGHT, 2001; CUTHBERTSON et al., 2010; RAMOS et al., 2004). Nonetheless, very little research has been conducted in Brazil to explore native fungal species and coupled with the lack of a commercial and efficient mycoinsecticide against this insect pest, additional research is encouraged.

After selecting virulent fungal candidates, the next effort is devoted for mass production of these biocontrol agents in which emphasis is taken place on their ability in producing efficiently the preferred propagule type for use in biological control. Three main techniques

are described to achieve different fungal biomasses and involve solid-state fermentation, submerged liquid fermentation, and biphasic fermentation. In all of them, emphasis is given to the optimization of solid or liquid culture media with the aim to maximize biomass yield and attain high survival after desiccation and during storage (JACKSON, 1997). The concept of mass production of fungal entomopathogens relies on the needs for use in the inundative biological control strategy that requires a high number of infective propagules to treat large areas in order to hit the insect or colonize its habitat in a way to achieve effective control (FARIA; WRAIGHT, 2001). This tactic is part of the applied biological control strategy that resembles in some ways the chemical principle of pest control, but with specific features related to the biological system. Therefore, to meet this expectation, this approach requires a cost-effective production and stabilization process that delivers viable, infective fungal propagules (JACKSON; DUNLAP; JARONSKI., 2010). To date, the most used active ingredient among mycopesticides throughout the world is the asexual spores known as aerial conidia (FARIA; WRAIGHT, 2007), which are naturally formed by the majority of the anamorphic entomopathogenic fungi, namely *Beauveria* spp., *Isaria* spp., *Metarhizium* spp., *Lecanicillium* spp., *Metarhizium* (formerly *Nomuraea) riley* (Farlow) Kepler, S.A. Rehner & Humber, *Aschersonia* spp., and *Hirsutella* spp., for dispersion, survival, and infection process. Typically, this infective propagule is commonly produced by solid-substrate cultivation and the scale-up process is performed by growing the fungus on moistened cereal grains for several weeks under controlled environmental conditions. This particular type of production method is classically practiced in developing countries, including Brazil and China, which possess several programs in microbial control based on fungal entomopathogens (LI et al., 2010). However, there are numerous evidences supporting advantages of submerged liquid fermentation over solid-state production (PAPAVIZAS et al., 1984; BIDOCHKA; PFEIFER; KHACHATOURIANS, 1987; JACKSON, 1997; JACKSON et al., 1997). The solid-substrate fermentation technique possesses several drawbacks related, for instance, to: (*i*) cost of the substrates, since they are mostly based on cereal grains; (*ii*) labor intensive, which demands manpower along the production line; (*iii*) nutritional and environmental conditions during the production phase is not homogenous and poorly controlled raising contamination issues in the process which, consequently, compromise quality assurance; (*iv*) low energy efficiency once it takes several days (> 10 days) to complete production. Conversely, the submerged liquid fermentation allows a better control of the nutritional and environmental conditions that leads to reduction of the fermentation time (30-72 hours), it is laborsaving and energy efficient, and ultimately it is less prone to contamination due to the rigorous quality control of the batches.

The liquid fermentation takes place in deep-tank bioreactors that are usually automatized and require less labor; furthermore, it can be also used to produce different microorganisms, since the sterilization part can be taken in place. Manipulation of the medium composition and physical characteristics, such as aeration rate, temperature, osmotic pressure and pH, make this process easy to evaluate proper nutritional and environmental conditions for growing rapidly and high amounts of active, stable fungal propagules (JACKSON, 1997). In liquid fermentation technology, there is a wide array of templates has been designed in food, bioenergy and biotechnology industries that can be adapted to the reality of fungal entomopathogens. Cost-effective media components are one the key elements for the success of liquid fermentation in downstream process and must be allied to the quality properties of the final product. To top it off, it has been shown that nutritional and physical manipulation of the growing environment in liquid culture can provide more fitted infective fungal propagules for use in biological control and more tolerant to environmental stresses, including ultraviolet (UV) radiation, heat, and low humidity (JACKSON; SCHISLER, 1992; JACKSON et al., 1997; JACKSON; DUNLAP; JARONSKI, 2010). Very little information regarding liquid fermentation techniques to produce entomopathogenic fungi has been conducted in Brazil. In the market, there are only *I. fumosorosea* and *L. longisporum* blastospore-based mycoinsecticides, although in Brazil there is none. The blastospore production ability regarding Brazilian isolates of *Isaria* and *Beauveria* is unknown and requires investigation. In addition, since *B. bassiana* is the most explored entomopathogenic fungus worldwide (FARIA; WRAIGHT, 2007), the liquid culture optimization for the rapid production of blastospores is nonexistent and hence its development would be of utmost importance for its broad adaptation in the mainstream agriculture.

Another critical step in the mycopesticide development concerns the formulation technology that offers a broad array of choices and processes, whereof ones more suitable than others depending on the type of fungal cell desired for multiplication. Formulation, as a final end, has as a role to aggregate value to the active ingredient by altering the chemical and physical attributes of a fungal propagule for improved insecticidal activity under varied environmental conditions. It is important to consider as part of the formulation process the stabilization method, which involves the drying technology and careful selection of compatible additives for the formulation *per se*. Compared with unformulated products, formulated fungal propagules are claimed to (*a*) enhance biological activity, (*b*) improve persistence in the field, (*c*) aid handling and application of the product, (*d*) provide biosafety, and (*e*) stabilize living cells during distribution and storage (BURGES, 1998; BRAR et al.,

2006). Therefore, these beneficial characteristics give “shape” to mycopesticides. The formulation costs are variable and will depend on a variety of factors, including ingredients, equipment, drying process, concentration and volume of the active ingredient. In order to reduce runoff and deleterious effects caused by UV exposure, high temperatures, and low relative humidities under field conditions, fungal cells can be formulated with exogenous protective agents, such as oils (ALVES et al., 1998; BATEMAN et al., 1993) and lignin (LELAND; BEHLE, 2004), or addition of nutrients or surfactants in the formulated product can also provide enhanced germination and speed of kill (JAMES, 2001). Toxicity of the formulation ingredients should also be carefully addressed for different fungal propagules, and usually blastospores and other vegetative fungal cells are likely more sensitive than aerial conidia to some chemical adjuvants, such as long-chain alkyl based surfactants (JACKSON; DUNLAP; JARONSKI, 2010). Therefore, a basic understanding on how fungal propagules interact with their host target or respond to their target environment forms an important guide for formulation development. Resistance to anhydrobiosis stress and the storage stability of fungal propagules have a great relevance for designing appropriate formulations. Some formulation components, such as exogenous nutrients, osmoprotectants, and oils can be added during growth or during drying to enhance desiccation tolerance and extend shelf life. Moreover, the drying method plays an important role in fungal viability and storage stability. Damages by dehydration can adversely affect fungal cell integrity and metabolism, hindering the maintenance of viability. These harmful effects occur at the membrane level as well as to their proteins (CROWE; CROWER; CHAPMAN., 1984) due to cell oxidation, rapid water loss, imbibitional damage (FARIA; HAJEK; WRAIGHT, 2009), and enzyme inactivation. Given that, the type of fungal cell and its nutritional status, speed of water removal, temperature, relative humidity, and water content in the final product are among the main factors that affect cell integrity, viability and stability. Another component in the formulation process stems from the packaging system. Generally, low water content (< 5% moisture), low temperature, and reduced oxygen levels are involved in prolonged storage stability of fungal propagules. Active packaging can be performed by using exogenous oxygen and moisture scavengers to prolong fungal survival in storage (FARIA; HOTCHKISS; WRAIGHT, 2012). This technology has been used for many years in food science and pharmaceutical products and now several examples of success are available and could be adapted for storage-sensitive fungal cells. Challenges in formulation still comprise a major constraint in biological control using fungal entomopathogens, but in recent years quite a few formulated products have appeared in the marketplace mainly based on oil dispersion of aerial conidia. However,

formulation for blastospores is still scarce and remains a research gap for its development. In downstream process, pre-existing techniques in food industry and biotechnology provide a standard template that can be adapted to mycopesticide production with modifications.

Mycopesticides are effective only if it has a major impact on the target pest and market size, despite how they are produced or formulated. Biological performance of these organisms is usually measured by virulence that encompasses speed of kill and lethal concentration. Also, side-effects are normally seen due to fungal infection and they can impair host development and reproduction; hence, they should take into account in bioassays, whenever it is possible to measure them. There are few reports comparing the virulence of different fungal propagules for pest control. It is known that blastospores, also referred to as single-yeast like cells, can germinate faster than aerial conidia and as a result it has been claimed more virulent against some arthropods. These blastospores seem to be more fitted for the control of soft-bodied insects, including whiteflies, aphids and psyllids, since they germinate and infect the host faster, which could be a great advantage for the management of insect vectors of plant pathogens and it would also confer less exposure to adverse environmental effects. The decision of which is the best fungal propagule to be developed as the active ingredient in a biological product will depend on numerous factors, including the cost of the production medium and formulation, production yields, virulence, persistence in the field, tolerance to environmental stresses, host target, delivery system, market size, and consistent performance under field conditions. Therefore, continued efforts in development more efficacious and stable fungal propagules are imperative for the widespread adoption of these biocontrol agents for pest control.

The present research was developed to explore different topics in microbial control and contributed to the development of efficient and consistent mycoinsecticides against whiteflies using entomopathogenic fungi. The specific objectives emphasized the following:

- Screening of virulent strains of *B. bassiana*, *I. fumosorosea*, and *Lecanicillium* spp. against different life stages of the whitefly *B. tabaci* biotype B and correlation with pathogenicity-transcripts of hydrolytic enzymes;
- Compatibility and biological activity of nonionic surfactants in combination with *B. bassiana* and *I. fumosorosea* with the aim to enhance bioefficacy by reducing effective dosage against whitefly;
- Potential of different *B. bassiana* and *I. fumosorosea* in producing blastospores by submerged liquid fermentation and assessment of desiccation tolerance, storage stability, and virulence against whitefly;

- Optimization of the liquid culture production method and media composition for enhanced production of desiccation tolerant blastospores of dimorphic entomopathogenic fungi with emphasis on *B. bassiana*;
- Impact of drying methods on desiccation tolerance and improvement of shelf life of *B. bassiana* blastospores through formulation and active packaging system.

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2 PRECISION MICRO-SPRAY TOWER FOR APPLICATION OF ENTOMOPATHOGENS[1]

Abstract

An inexpensive, portable and easy handling spraying device was developed to apply several entomopathogens in dose-response bioassays. The micro-sprayer was calibrated using different settings of pressure and time to apply a wide range of doses on a plain surface. Filter paper cards sprayed with dye solutions were used to examine the deposition uniformity. Also, the relationship between conidial suspensions of fungal entomopathogens and dose applied was determined. As a result, the combination of 10 PSI and 3 s provided the most uniform coverage when applied with a volume of 2.15 µL cm^{-2} (215 L ha^{-1}) delivering a dose of 150 conidia mm^{-2} (10^{12} conidia ha^{-1}) at a concentration of 1×10^7 conidia mL^{-1}.

Keywords: Biological control; Spray system; Entomopathogenic fungi; Bioassays

2.1 Introduction

Among entomopathogens, mitosporic fungi such as *Metarhizium anisopliae* sensu lato (s.l.), *Beauveria bassiana* s.l., *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*) and *Lecanicillium* spp. (formerly *Verticillium lecanii*) have been widely used as environmentally friendly biocontrol agents of a broad range of mites and insects for many decades worldwide (FARIA; WRAIGHT 2007). A key factor governing the success of biological control programs with these microbial agents is the accurate application of the inoculum onto the target insect (direct contact) or surfaces of insect habitat (e.g. crop leaves) for residual contact. For that reason, it is critical to determine the known amount of active ingredient (e.g. conidia or blastospores) applied per unit area to determine dose-mortality responses of the target insect under specific incubation conditions. For instance, the virulence of fungal entomopathogens is generally determined by lethal doses (LD) or lethal concentrations (LC) on basis of the known droplet dose deposited onto the insect or surface. Establishing the LD_{50} or LC_{50} of a fungal isolate allows comparisons with other isolates or different species and provides a starting point to establish appropriate doses for field testing. In addition, the estimation of median LD or LC for mycoinsecticides is valuable in the inundative application approach in which high number of infective propagules is required to kill the target under field conditions (JARONSKI, 2010). Many researchers establishing dose-responses of fungal suspensions under controlled conditions have used an air assisted spray tower (POTTER,

[1] MASCARIN, G.M.; QUINTELA, E.D.; SILVA, E.G.; ARTHURS, S.P. Precision micro-spray tower for application of entomopathogens. **BioAssay**, Piracicaba, v. 8, p. 1-4, 2013.

1952), which is now considered a standard device for this purpose. While effective, traditional spray towers are relatively non-portable and expensive for many workers in developing countries. Here, we described an inexpensive and portable precision spray tower that can be built using readily accessible materials and could be useful to microbial control researchers who do not currently have access to spray tower equipment.

2.2 Development

2.2.1 Materials and Methods

The portable micro-sprayer consists of a dual action gravity feed artist airbrush (Sagyma™ SW130K, Brazil; http://www.wkshop.com.br) with a 0.3-mm fine needle placed on the top of an cylindrical acrylic tower (inside dimensions 11.6 cm diameter by 23 cm height), which forms the spray tower (Figure 2.1). The airbrush was connected to a gas pressure regulator (Record S.A., R9-CO_2, São Paulo, Brazil), and a 1.6 L pressurized air tank (Figure 2.1). Once mounted, the micro-sprayer can be disassembled for cleaning or replacement of parts. The area covered by the micro-sprayer was 105.68 cm^2, large enough for standard size Petri-dishes used in many laboratory bioassays. We calibrated this device by spraying deionized water onto 7-cm-diameter pieces of round filter paper (1 Qualitative, Qualy™ - 14 µm) in order to evaluate the relationship between pressure (pounds per square inch, PSI) and spraying time. Each filter paper was weighed immediately before and after spraying to calculate microliters per square centimeter (µL cm^{-2}) at 5, 10, 15, 20, 25 and 30 PSI at intervals of 2, 3, 4 and 5 seconds. There were six replicates for each combination (N = 144 observations). The study was repeated using a cotton blue stain solution (10% v/v) to evaluate the uniformity of the spray deposition. We examined the spray patterns on stained filter papers. Images were transformed into simple 8-bit grayscale color bands and then subjected to the image analyzer software ImageJ™ in order to determine the frequency distribution curves of color reflectance (in pixels) (ABRAMOFF et al. 2004). According to this analysis, we selected 10 PSI to determine the relationship between the fungal concentrations and dose of conidia applied. Conidial suspensions of three fungal entomopathogens (*B. bassiana*, *I. fumosorosea* and *L. muscarium*) were prepared in an 0.01% v/v Tween 80 solution using nine concentrations ranging from 1×10^7 to 2.8×10^9 conidia/mL and sprayed for 3 s onto Petri plates (9 cm diameter) containing 6-8 glass coverslips (20 × 20 mm). We used 4 mL of these suspensions to load into the sprayer. Each fungal concentration was replicated four times and the whole experiment was repeated twice. After spraying, coverslips were transferred into 45-mL Falcon® centrifuged tubes containing 5 mL of 0.1% Tween 80 solution and vigorously

agitated on a vortex for 2 min, to dislodge conidia. Conidial counts of homogenized suspensions were made with an improved Neubauer (hemocytometer) chamber (New Optik®, Brazil) with 0.1 mm depth. The average number of conidia recorded from coverslips was used to quantify deposition rates (i.e. conidia cm^{-2}).

A non-linear model was used to examine the effect of pressure and spraying time on the dose response in PROC REG, while linear regression was used to describe the relationship between conidial dose deposited on a plain surface and fungal concentration (conidia mL^{-1}) (SAS INSTITUTE, 2008). Image analysis software was used to compare uniformity among different spray patterns according to pressure at a fixed spraying time of 3 s (ImageJ™, V1.47b, http://rsbweb.nih.gov/ij/notes.html).

2.2.2 Results and Discussion

A highly significant dose response relationship with pressure and spraying time was indicated by the non-linear model (Figure 2.2) (F= 713.2; df = 2, 141; P< 0.0001; adjusted R^2 = 0.91). We observed that the best coverage uniformity was achieved by the combination of 10 PSI and 3 s, which had the optimal distribution curve of reflectance (Figure 2.2). For higher volume settings the increased color intensity in the middle of sprayed cards translated into non-uniform distribution due to the high concentration of blue stain in the center (Figure 2.2). The lower pressure-time settings provided insufficient coverage on the paper disks. Generally, the standard pressure used in Potter spray tower varies from 10 to 15 PSI, and the selected pressure of our device fell within this range. Narrow reflectance curves correlate with spray uniformity, since wider bell curves, by definition, represent a greater range and variability of droplet deposition. The preferred setting (10 PSI for 3 s) provided a volume application rate of 2.15 µL cm^{-2} (= 215 L ha^{-1}), which is close to the standard recommended by the IOBC in many insecticide trials (i.e. 200 L ha^{-1}) (CANDOLFI; BLUMEL; FORSTER, 2000).

There was also a strong positive relationship between dose (conidia cm^{-2}) and concentration (conidia mL^{-1}) operating at 10 PSI for 3 s (Figure 2.2) (F = 116.63; df = 1, 19; P < 0.0001, adjusted R^2 = 0.85). Estimated doses for concentrations at 1×10^7, 5×10^7 and 1×10^8 conidial mL^{-1} were equivalent to 150, 501 and 1674 conidia mm^{-2}, respectively (Table 2.1). The tested combinations of pressure and time covered a wide range of field doses for commercial aerial and terrestrial applications of entomopathogenic fungi, where application rates fall within 1×10^{12} to 1×10^{14} conidia ha^{-1} (JARONSKI, 2010). However, due to the nozzle characteristics of the airbrush, the droplet size is likely to be smaller compared with

other types of sprayers such as conventional spray nozzles. According to Bateman (1991) the droplet size for optimum application to insects on foliage ranges from 30 to 80 µm.

In conclusion, we proposed an inexpensive spraying device for accurate application of mycopathogens as well as other microbial agents to portions of crop foliage or onto target arthropods. This device can be also used with various chemical pesticides. The portability of the system (e.g. requiring no electrical source) allows it to be used in remote locations where other types of spray towers cannot be used.

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Table 2.1 - Estimated doses of conidia applied after spraying different conidial suspensions at 10 PSI for 3 sec using the portable micro-spray tower

Concentration (conidia mL^{-1})	Dose (conidia mm^{-2})			Equivalent field dose (conidia ha^{-1})
	Mean	Minimum	Maximum	
1×10^{7}	149.9	5.50	4.08×10^{3}	1.5×10^{12}
5×10^{7}	500.8	16.4	1.53×10^{4}	5.0×10^{12}
1×10^{8}	1673.6	49.1	5.7×10^{4}	1.7×10^{13}

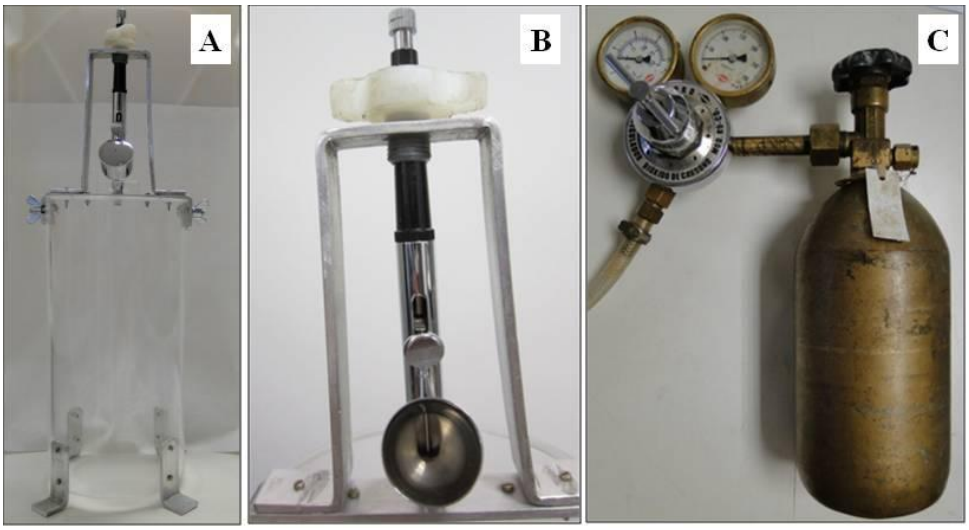

Figure 2.1 - Portable micro-sprayer. A) Artist airbrush connected to pressurized air and mounted on a cylindrical acrylic tower (11.6 inner diameter × 23 cm height); B) Close front view of the artist airbrush; C) CO_2 cylinder and regulator

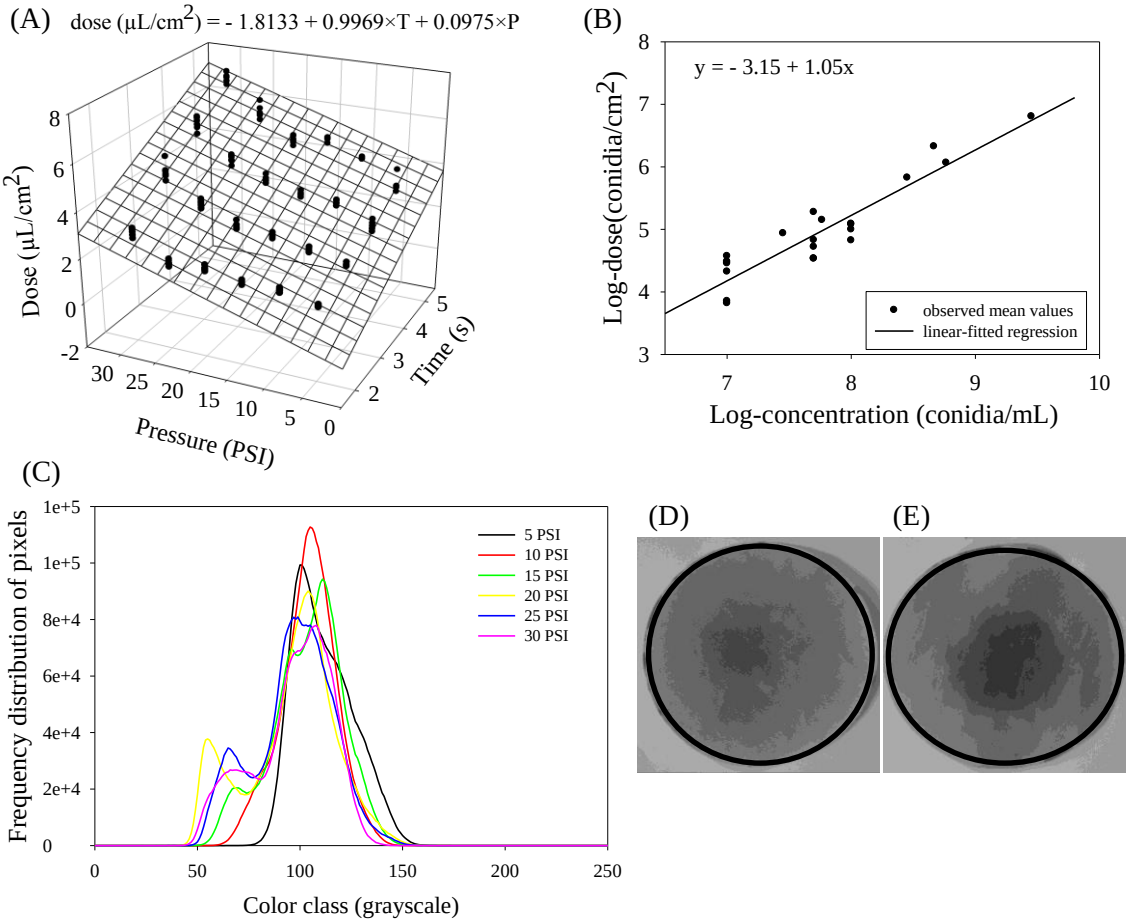

Figure 2.2 - Calibration of the micro-spray tower: (A) Linear regression for dose (µL cm^{-2}), spraying time (seconds) and pressure (PSI) as explanatory variables, (B) Linear regression of dose of conidia applied (conidia cm^{-2}) across concentration (conidia mL^{-1}) sprayed at 10 PSIfor3 s, and (C) spray patterns at different pressures at 3 s (fixed) expressed in frequency distribution of reflectance data from sprayed cards in grayscale (8-bit picture). Spray patterns of water application on filter paper cards in grayscale at 10 PSI/3 s (D) and 30 PSI/3 s (E)

3 THE VIRULENCE OF ENTOMOPATHOGENIC FUNGI AGAINST *BEMISIA TABACI* BIOTYPE B (HEMIPTERA: ALEYRODIDAE) AND PRODUCTIVITY IN SOLID-STATE FERMENTATION[2]

Abstract

The virulence of five isolates of *Beauveria bassiana*, five of *Isaria fumosorosea* and four of *Lecanicillium muscarium* from Brazil was determined on whitefly *Bemisia tabaci* biotype B lifestages on bean leaves under laboratory conditions. The conidial yield (on cadavers or parboiled rice), surface hydrophobicity and enzyme activity were also determined. The isolates of *B. bassiana* and *I. fumosorosea* were the most virulent against nymphs (71-86% mortality within 8 d), with LT_{50} values ranging from 3 to 4 d after treatment with 10^7 conidia mL^{-1} (150 conidia mm^{-2}). Spore production on nymph cadavers reached 4 to 8×10^5 conidia per insect. The *L. muscarium* isolates demonstrated low virulence toward nymphs. After spraying eggs with 1×10^8 conidia/mL (1674 conidia/mm^2) of *B. bassiana* and *I. fumosorosea*, most nymphs hatched, but then 40-70% of these nymphs were infected by indirect pick-up of conidia on the leaves. Adults exposed to treated leaf discs (150 conidia mm^{-2}) were more susceptible to *I. fumosorosea* than to *B. bassiana*. The enzyme activity (Pr1) and the relative conidial surface hydrophobicity were not correlated with any virulence parameter measured for *B. bassiana* or *I. fumosorosea*. In addition, the highest conidial yield on parboiled rice using solid-state fermentation (4.9-11.4 $\times 10^9$ conidia g^{-1}) was achieved by isolates of *I. fumosorosea* CG1228 and *B. bassiana* CG1229. *I. fumosorosea* CG1228 was highly virulent against whitefly nymphs and adults as well as attained high spore production on insect cadavers and parboiled rice. Our results indicate that *I. fumosorosea* CG1228 has desirable attributes for the development of a mycoinsecticide against *B. tabaci* biotype B.

Keywords: *Beauveria bassiana*; *Isaria fumosorosea*; *Lecanicillium muscarium*; Microbial control; *Phaseolus vulgaris;* Silverleaf whitefly

3.1 Introduction

Bemisia tabaci (Genn.) (Hemiptera: Aleyrodidae) is among the most devastating and widespread insect pest of a broad range of greenhouse and field crops worldwide. It is a serious threat to crop production due to direct damage and transmission of several plant viruses (OLIVEIRA; HENNEBERRY; ANDERSON, 2001; JONES, 2003). *Bemisia tabaci* is considered a cryptic species complex and was recently split into 11 groups encompassing 24 species (De BARRO et al., 2011).The biotype B of *B. tabaci* is widely distributed throughout Brazil and it can cause economic losses estimated in 714 million US$/year in crops such as beans, soybeans, cotton, tomatoes, and leafy vegetables (FONTES et al., 2012; OLIVERIA et al., 2013). The control of this insect relies primarily on the use of chemical insecticides, but this stand-alone approach has encountered difficulties due to the selection of resistant

[2] MASCARIN, G.M.; KOBORI, N.N.; QUINTELA, E.D.; DELALIBERA, I. The virulence of entomopathogenic fungi against *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) and their conidial production using solid substrate fermentation. **Biological Control,** Orlando, v. 66, p. 209-218, 2013.

individuals. In addition, the decreasing number of new registered insecticides, their harmful side effects to non-target organisms, the legal restrictions regarding their safe use and their environmental hazards have encouraged the adoption of additional control techniques, including biological control approaches.

Among several groups of biocontrol agents for whiteflies and other sap-sucking insects, entomopathogenic fungi possess the unique ability to infect their host directly through the integument. Moreover, they play a role in the natural mortality of whitefly populations (LACEY; FRANSEN; CARRUTHERS, 1996). The most promising mitosporic fungi include *Isaria fumosorosea*, *Lecanicillium* spp., *Beauveria bassiana* and *Aschersonia* spp. (Ascomycota: Hypocreales) (FARIA; WRAIGHT, 2001; WRAIGHT et al., 2007; LACEY; WRAIGHT; KIRK, 2008). *Lecanicillium* and *Aschersonia* species have been used to control whiteflies and related insects in greenhouses in Europe and Canada. These applications have been successful in cases where environmental conditions of high relative humidity and moderate temperatures are appropriate (FRANSEN WINKELMAN; LENTEREN, 1987; CHANDLER; HEALE; GILLESPIE., 1994).

Several researchers evaluated fungal pathogens for the control of *B. tabaci* biotype B in the 1990's (LACEY; WRAIGHT; KIRK, 2008). Despite considerable research in North America, Europe and Asia (FARIA; WRAIGHT, 2001; LACEY; WRAIGHT; KIRK, 2008), little recent information exists on this topic in South America. Furthermore, little research on fungal entomopathogens of whiteflies has been conducted in Brazil (SOSA-GÓMEZ; MOSCARDI; SANTOS, 1997; LOURENÇÃO; YUKI; ALVES, 1999). Currently, the authors are unaware of any commercial and registered mycoinsecticides available against whiteflies in Brazil.

The screening of fungal isolates for virulence characteristics is of paramount importance for the success of biocontrol strategies toward whiteflies and other insects (FARIA; WRAIGHT, 2001; LACEY; WRAIGHT; KIRK, 2008). Virulence can be indirectly measured with the activities of enzymes related to infection pathways, such as subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes and chitinases (SHAH, WANG; BUTT, 2005, SHAH et al., 2007; JACKSON; DUNLAP; JARONSKI, 2010). In addition to virulence, other important parameters should be considered for commercial development of mycoinsecticides. For example, the production of infective and stable propagules on inexpensive artificial substrates is a requirement for industrial-scale production with either solid-state or submerged liquid fermentation methods (JACKSON; DUNLAP; JARONSKI, 2010). In some cases the ability

of the fungus to sporulate on host cadavers may also be beneficial for secondary infections and extended control.

In this study, the virulence of isolates of three genera of hypocrealean anamorphic fungi (*Beauveria, Isaria,* and *Lecanicillium*) was determined on eggs, nymphs and adults of *B. tabaci* biotype B. The relationship between surface hydrophobicity, spore-bound Pr1 enzyme activity and the virulence factors of selected isolates was investigated. We also determined the yield of conidia obtained from whitefly cadavers and on parboiled rice using solid-state fermentation.

3.2 Development

3.2.1 Materials and Methods

3.2.1.1 Insect colony

The *B. tabaci* biotype B colony originated from bean, soybean and tomato crops at the EMBRAPA Rice and Beans Research Station in Santo Antônio de Goiás, GO, and Campinas, SP, Brazil. Whiteflies were mass-reared in a screenhouse (9 × 8 m) on butter bean (*Phaseolus lunatus* L.), common bean (*P. vulgaris* L., cv. Pérola), and soybean (*Glycine max* L., cv. Favorita). Biotype B was identified using a specific molecular marker (SCAR, Sequence Characterized Amplified Regions) (QUEIROZ DA SILVA, 2006).

3.2.1.2 Fungal preparations

Five isolates of *B. bassiana*, five of *I. fumosorosea* and four of *L. muscarium* originating from infected *B. tabaci* and other hosts from diverse sites of Brazil were obtained from the Invertebrate Fungal Collection at EMBRAPA Genetic Resources and Biotechnology (Brasília, Brazil) and the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) (Table 3.1). The identity of Brazilian isolates of *B. bassiana* and *I. fumosorosea* was confirmed by molecular analysis using the nucleotide sequence of the divergent domain (d1/d2) at the distal end of the 26S rRNA gene (LO CASCIO; LIGOZZI, 2011). Prior to use in studies, all fungal isolates were repeated passage through whitefly nymphs and subcultured on SDA + yeast extract (SDAY) no more than four times to avoid attenuation of virulence (BUTT; GOETTEL, 2000; SHAH et al., 2007). All assays utilized conidia inoculum harvested from 14-day-old cultures and suspended in 0.01% (v/v) surfactant (Tween 80®, Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil). Conidial viability was determined after 16 h on 1% (v/w) water agar at 400X. The conidial viability of all isolates exceeded 85% germination.

3.2.1.3 Bioassays against whitefly nymphs

Whiteflies were obtained from bean plants (cv. Pérola) in plastic pots (450 mL) filled with organic substrate amended with mineral nutrients (Tecnutri®, Itatiba, SP, Brazil) and maintained in a screenhouse (9 × 8 m). To generate infested leaves for bioassay, pest free plants (15-20 day old with two primary leaves) were placed near the adult-infested plants for 24 h. This procedure provided more than 40 eggs per leaf. The adult whiteflies were removed and newly infested plants moved to another screenhouse for 11-13 days, until nymphs reached the 2nd instar (0.30-0.44 mm in length and 0.18-0.36 mm in width) (QUINTELA, 2004). The nymph-infested leaves were excised and placed with ventral (abaxial) surface up on 14 mL of molten water agar (1% w/v) (Merck®) in polystyrene Petri plates (100 × 20 mm) with a lid containing a 4-cm diameter central hole covered with cheesecloth (30-µm pore mesh) to allow ventilation.

For bioassays, 40-60 nymphs on ventral surface of excised bean leaves were sprayed with conidia from each of the 14 isolates using a micro-spray tower (MASCARIN et al., 2013). Conidia were harvest from SDAY plates with a microbiological loop and immediately suspended in 10 mL of sterile aqueous solution of 0.01% (v/v) Tween 80 into 50-mL plastic centrifuge tubes. The suspension was vigorously agitated on vortex for 2 min and filtered through two layers of 30-µm pore-sized nylon cheesecloth. The filtered suspension (10 mL) was agitated again for 1 min before application and conidial concentrations were enumerated by hemocytometer (Brightline Improved Neubauer, New Optik®, Brazil) at 400X magnification. In each case a 3 mL aliquot of each suspension was applied at 1×10^7 conidia mL^{-1} applying resulting in a volume application rate of approximately 2.15 µL cm^{-2} (≈ 215 L ha^{-1}) and deposition rate of 150±34 conidia/mm^2 (95% confidence limits: 34–658 conidia/mm^2, n = 21) on the leaf surface. Controls consisted of nymphs sprayed with the carrier surfactant solution (0.01% Tween 80) and unsprayed nymphs (e.g., untreated control), which accounted for natural mortality. There were 5 replicates (plates) per fungal isolate (150-300 nymphs per treatment). After spraying, the leaves were allowed to air dry prior to incubation. The treated plates were placed upside down (inverted) on wire racks, so that the ventilation holes were not blocked, and held in a growth chamber at 26±0.5 °C, 80±11% relative humidity (RH) with a 14:10 (L:D) h photoperiod. Nymph mortality was monitored for 8 d under a dissecting stereoscope (40X). Dead nymphs became desiccated or developed yellowish (*L. muscarium* and *I. fumosorosea*) or reddish (*B. bassiana*) symptoms with mycelial or conidial growth. All non-sporulated nymphs were incubated on 1% water agar for

2-3 days to confirm infection status at 26±0.5 °C with a 12:12 (L:D) h photoperiod. All 14 fungal isolates were assayed together per experiment. The entire experiment was carried out three times on different days (randomized complete block design with days as block) using different batches of conidial suspensions and different insect cohorts per experiment.

3.2.1.4 *In vivo* sporulation

Comparisons of conidial yields were assessed from 4th instar nymphs. In each case, 60 freshly mycosed (non-sporulating) cadavers were randomly removed from each fungal treatment, surface sterilized with 70% alcohol for 1 min, rinsed twice in sterile deionized water for 30 s. Then cadavers were placed on wet filter paper and incubated inside a sealed Petri dish (90 × 15 cm) maintained at 26±0.5 °C, 14:10 (L:D) h photoperiod and 100% RH. To quantify yield, after 6 days four randomly selected cadavers were placed in 1.8-mL microcentrifuge tubes (Eppendorf®) containing 0.5 mL of 0.1% Tween 80 and vigorously vortexed for 1 min. The cadavers were macerated with a glass pestle for 30 s to enhance dislodgement of conidia. Conidial yields were calculated based on hemocytometer counts. Four replicate assays were used for each fungal isolate (minimum of 16 cadavers per treatment), and the experiment was repeated twice on different days (randomized complete block design with day as block). Sporulation was measured on a total of 448 cadavers (14 isolates × 8 replicates × 4 cadavers).

3.2.1.5 Bioassays with eggs and residual activity against hatched nymphs

Bean leaves (cv. Pérola) containing less than 48-h-old eggs were excised from plants and placed on 1% water agar plates. The surface of the leaf was marked with a permanent ink pen near to 40-60 eggs per leaf. The infested leaves were placed in ventilated incubation plates, as described above, and sprayed with suspensions of 1×10^8 conidia mL^{-1} of five *I. fumosorosea* and four *B. bassiana* isolates. The spray deposition on leaf surface delivered a final rate of 1674±261 conidia mm^{-2} (95% CL: 397–7060 conidia mm^{-2}, n = 21). The control eggs on leaves were sprayed with 0.05% solution of Tween 80. The excided leaves were incubated ventral side down on wire racks in a growth chamber at 26±0.5 °C at 87±11% RH with a 14:10 (L:D) h photoperiod. Each treatment comprised four to five replicate plates (minimum of 160 eggs per treatment). All treatments were tested in the same assay. The proportion of egg mortality was measured by comparing the number of unhatched eggs to the total number of initially marked eggs. Furthermore, the eggs with signs of mycosis were scored as sporulated eggs. At the same experiment, after 8 days incubation period, 40-50 late 1st and

early 2nd instar nymphs were selected and marked on the same treated leaves for all treatments. To determine the residual activity of the fungi against the hatched nymphs, three days later the number of dead nymphs was recorded. The experiment was repeated three times on different days (randomized complete block design with days as block).

3.2.1.6 Virulence of best fungal isolates against adults

Adults whitefly (≤ 5 days post-emergence) were exposed to leaf discs containing residues of leading fungal candidates. Leaf discs (3.8 cm, in diameter) were treated with 1×10^7 conidia mL^{-1} (= 150 conidia mm^{-2}) prepared in a sterile 0.05% Tween 80 solution for each fungal isolate using the micro-sprayer as explained above. Leaf arenas from the control groups were treated with a sterile solution of Tween 80 (0.05%). After spraying, leaf discs were allowed to air dry and then placed on 8 mL of 1% molten water agar in acrylic vials (77 × 38 mm). The adults were captured in glass vials, transferred to the treated leaf arenas and confined using cheesecloth tissue (30-µm pore mesh) affixed with a rubber band. Each vial contained approximately 25-40 individuals. The vials were placed top side down on wire racks and held in a growth chamber at 26±0.5 °C and 80±11% RH with a 14:10 (L:D) h photoperiod. Each fungal isolate had six leaf disks (replicates) and all isolates were tested at the same time per assay. The bioassay was conducted twice using a randomized complete block design with test date serving as block. The proportion of fungus-killed adults and sporulated cadavers were recorded 7 days post-inoculation. The remaining non-sporulated cadavers were also collected and transferred to humid chambers to confirm mycosis. The sporulated cadavers were recorded on the leaf arenas, on the vial walls, and in the humid chambers. All these records were pooled together to calculate the total percentage of sporulation. The entire bioassay was conducted twice over time.

3.2.1.7 Surface hydrophobicity measurements

The conidial surface hydrophobicity was examined using an aqueous-solvent partitioning assay that determines the ratio of conidia distributed in the aqueous or organic phases. The organic phase used was hexane (Sigma-Aldrich®). The conidia from nine sporulated fungal isolates grown for two weeks on SDAY plates were harvested and suspended in a 0.1 M KNO_3 solution and adjusted to a desired concentration of 1×10^7 conidia mL^{-1}, using a modification of Shah et al. (2007).Three mL of spore suspension was transferred to a glass vial, and 300 µL of hexane was added. Each isolate had three vials (replicates) prepared with different fungal stock cultures. The vials were agitated for 1 min. The suspension was left for

15 min to allow phase separation, after which the solvent phase was removed. The final conidial concentration in the aqueous phase (D_{aq}) was recorded. Percent relative hydrophobicity was determined as $100 \times [1 - (D_{aq}/D_{total})]$, where $D_{(total)}$ and $D_{(aq)}$ represent the values of the starting sample and resulting aqueous phase, respectively (SHAH et al., 2007).

3.2.1.8 Enzyme assay for spore-bound Pr1

The activity of spore-bound Pr1 was determined for preselected isolates of *I. fumosorosea* and *B. bassiana*. The procedure followed Shah et al. (2007) with slight modifications. Ten mg of freshly harvested conidia grown on SDAY plates was incubated in 1 mL 0.1 M Tris-Cl (pH 7.95) amended with 1 mM succinyl-ala-ala-pro-phe-p-nitroanilide ($C_{30}H_{36}N_6O_9$) (Sigma®) for 5 min at room temperature (26±1 °C). After incubation, conidia were separated from the aqueous phase by centrifugation at 12,000 *g* (Z324K, Hermle® centrifuge) for 10 min at 4°C. The yellowish aqueous phase (100 µL) located under the conidial mass was transferred to wells in a flat-bottom microtiter plate, and the absorbance at 405 nm was measured using a spectrophotometer (model Epocha, Biotek®). There were four replicates for each isolate. Buffered substrates were used as controls. The enzymatic cleavage of the 4-nitroanilide substrate yields 4-nitroaniline (yellow color under alkaline conditions). Therefore, to convert an absorbance value into Pr1 activity expressed as µmol mL^{-1} min^{-1}, we used the molar extinction coefficient of 8,800 $M^{-1}cm^{-1}$ for 4-nitroanilide, given by the manufacturer.

3.2.1.9 Conidial production using solid-state fermentation

Conidial yield of *B. bassiana*. and *I. fumosorosea* isolates grown on autoclaved parboiled rice were compared. Twenty grams of moistened and autoclaved rice were placed in a 125-mL conical Erlenmeyer flask, capped with a cotton ball and aluminum foil, and inoculated with 2 mL of conidial suspension, which delivered 10^7 conidia g^{-1} of rice based on wet weight. The wet basis moisture in the rice mass before inoculation was 44.0±0.3% (e.g., ~11.2 g of dried rice). Flasks were incubated for 11 days at 26 °C with a 14:10 (L:D) h photoperiod. There were three replicates per fungal isolate. Conidial yields were assessed through agitation of cultures with sterile 0.1% Tween 80 solutions at 200 rpm for 1 h in an incubator shaker (TE-420, Tecnal®, Brazil) at 27±1 °C. A 0.1-mL aliquot taken from the center of each conidial suspension was diluted (1000X) in a 0.1% Tween 80 solution. The amount of conidia produced per gram of wet weight of rice (20 g/flask) was determined through hemocytometer counts. Conidial viability was assessed from 200 conidia 16 h after inoculating 200 µL of 1×10^6 conidia mL^{-1} on 1% water agar plates (Rodak®, 60 × 15 mm) at 26 °C. All fungal isolates

were assayed together and the experiment was repeated twice on different times using different batches of fungal inoculums.

3.2.1.10 Statistical analysis

All the data sets were previously checked for normality and homoscedasticity with Shapiro-Wilk and Brown-Forsythe tests and with diagnostic residual plots. Proportional (categorical) data were analyzed using logistic regression in the generalized linear mixed model (GLMM) with binomial distribution and logit link function (JAEGER, 2008). On the other hand, the continuous or non-binomial data that did not match linear modeling assumptions were transformed to normalize variances prior to analysis of variance (ANOVA). All analyses were conducted in SAS software, version 9.2 (SAS INSTITUTE, 2008).

In all tests, fungal virulence was expressed and compared in terms of percent mortality at different life stages and survivorship. For experiments conducted over time, the test dates were considered as experimental blocks, but block interactions were not tested due to potential restriction error (SOKAL; ROHLF, 1995). The proportional (binomial) data of total nymph mortality, hatched nymph mortality, egg mortality, sporulated eggs, adult mortality and sporulated adults were fitted using logistic regression in GLMM with experimental blocks as a random effect in the mixed logit model (PROC GLIMMIX). If overdispersion was found in the data (e.g., residual deviance greater than 1), it was included in model as another random effect (JAEGER, 2008). *F*-statistics were derived from Wald-type III test for fixed effect in the mixed logit model (e.g., fungal isolates) and significantly different means were identified by Tukey's honestly significant difference (Tukey's HSD) test at $P < 0.05$. For non-binomial data, such as the number of conidia produced per cadaver and conidial production on parboiled rice, we used the square-root transformation prior to ANOVA. Then, treatment (e.g., fungal isolates) means were separated by Tukey's HSD test at $P \leq 0.05$. The survivorship analysis based on the Kaplan-Meier product-limit method calculated in the LIFETEST procedure in SAS was used to determine survival probability functions as well as to estimate the median survival time to death (ST_{50}, the time required to kill 50% of the treated insects) for whitefly nymphs exposed to different fungi with censored data for insects surviving beyond 8 days incubation period. The survival (function) curves for different fungal treatments were compared by the log-rank chi-squared test with *P*-values adjusted by Tukey's HSD and considering a significance level of 5% (ALLISON, 1995). Furthermore, estimated ST_{50} values were compared by the overlap of their 95% confidence intervals (95% CI). All data averages in the figures and tables are shown as untransformed values.

A principal component analysis (PCA) was used to identify the best isolates of *B. bassiana* and *I. fumosorosea* using seven out of the 11 measured response variables (categories) related to virulence factors, conidial production on cadavers and conidial production on rice. The seven preselected variables showed significant differences between the fungal isolates, whereas the others were not included in PCA (relative hydrophobicity, egg mortality, mycosed eggs, and Pr1 activity). The PCA was performed with package FactoMineR in R 2.15.2 (R DEVELOPMENT CORE TEAM, 2012) software. Since the variables were measured in different scales, the PCA was performed with standardized data (mean = 0, variance = 1). The principal axis method was used to extract the components, and this was followed by varimax (orthogonal) rotation to identify the observed variables that demonstrated high loading for each component retained in the analysis. The eigenvalues of the principal components represented a measure of their associated variance, and the contribution of each observable variable in these components was given by the loadings (LÊ; JOSSE; HUSSON, 2008). A Pearson's correlation coefficient was estimated to test for correlations between individual components and variables; hence, each component was described only by the significant variables at a significance threshold of $P < 0.15$. Biplot graphs were drawn based on the principal components that explained at least 70% of the total variance, to aid in the interpretation of the results. In addition, a cluster analysis was performed with the unweighted pair-group with arithmetic mean (e.g, average UPGMA) method to calculate the Euclidean distances of dissimilarity (EVERITT; HOTHORN, 2010). This method provided the number of clusters formed among the *B. bassiana* and *I. fumosorosea* isolates on the basis of the meaningful observed variables derived from PCA. Highest similarities between isolates were scored as having the shortest distances.

3.2.2 Results

3.2.2.1 Virulence of fungal entomopathogens to whitefly nymphs

All fungi were pathogenic to whitefly nymphs at 1×10^7 conidia mL^{-1} (150 conidia mm^{-2}) ($F_{15,209}$= 26.67, $P < 0.0001$) (Figure 3.1). Infected nymphs began to die 2 d after exposure to conidia. The average mortalities of the unsprayed insects and insects sprayed with Tween 80 (0.01%) were 2.0 and 6.2% after 8 days, respectively, and 88.4–90.7% of these nymphs turned into adults. Isolates ESALQ1409 and ESALQ1337 of *I. fumosorosea* and only CG1229 of *B. bassiana* were significantly more virulent to nymphs than all the *L. muscarium* isolates, causing nymphal mortalities > 77%; but these isolates were as virulent as the isolates ESALQ-PL63, ESALQ1432 and ESALQ447 of *B. bassiana* and ESALQ1296, ESALQ1364

and CG1228 of *I. fumosorosea* (Figure 1). The percentage of sporulated nymphal cadavers was > 90% for all fungal isolates (data not shown). Estimated ST_{50} values showed that most *B. bassiana* and *I. fumosorosea* isolates killed whiteflies faster (3–5 days) compared with *L. muscarium* isolates (≥ 6 d) (Table 2). *I. fumosorosea* ESALQ1364 attained the lowest ST_{50} value (3 days). All fungal treatments decreased survival rates of whitefly nymphs (Log-rank: $\chi^2_{15} = 2625.3$, $P < 0.0001$). *I. fumosorosea* and *B. bassiana* isolates also killed more quickly the nymphs than the *L. muscarium* isolates ($P < 0.05$), with the exception of *B. bassiana* ESALQ-CB66 (Figure 3.2).

3.2.2.2 *In vivo* sporulation

Quantitative estimates of in vivo sporulation varied among isolates ($F_{13,97} = 7.44$; $P < 0.0001$), ranging from 0.9 to 7.9×10^5 conidia cadaver^{-1} (Figure 3.3). Highest inoculum productions were achieved by *B. bassiana* ESALQ-CB66 and CG1229 although these isolates were not statistically different from those that produced $\geq 3.3 \times 10^5$ conidia cadaver^{-1}. From a practical viewpoint, it would be necessary from nine to 14 sporulated cadavers to provide inoculation similarly to the rates applied to the excised leaves (i.e., 150 conidia mm^{-2}). Three out of four *L. muscarium* isolates and *I. fumosorosea* (ESALQ1296) resulted in $\leq 1.8 \times 10^5$ conidia/cadaver. Interestingly, there was a positive relationship between the overall nymphal mortality and the conidial production on cadavers (Spearman's correlation: $r = 0.56$, $P = 0.039$).

3.2.2.3 Bioassays with eggs and residual activity against hatched nymphs

Whitefly eggs had low susceptibility to all fungal isolates, with > 91% eggs successfully emerging after being treated with the highest fungal dose (1674 conidia mm^{-2} = 1×10^8 conidia mL^{-1}), and no statistical treatment effect observed ($F_{10,194} = 0.61$, $P = 0.78$). However, significant mortality of 1st and 2nd instar nymphs originating from the treated eggs ($F_{10,142} = 18.50$, $P < 0.0001$), indicating a residual activity of fungal treatments on the leaf surface. All *B. bassiana* and *I. fumosorosea* isolates caused higher nymphal mortality, except for ESALQ1337 and ESALQ1409 (Figure 3.4). These results indicated that newly hatched nymphs probably acquired conidia from the eggs or leaf surfaces as secondary pick-up.

3.2.2.4 Virulence of best fungal isolates to adults

Adult *B. tabaci* were susceptible to all *I. fumosorosea* isolates while only *B. bassiana* CG1229 caused > 50% mortality ($F_{9,106} = 5.73$, $P < 0.0001$) (Figure 3.5). *I. fumosorosea*

CG1228 caused the highest mortality of adults (93% mortality). This result indicated that adults effectively acquired conidia via the surface of treated leaves. The sporulation on adults was affected by the fungal isolates ($F_{8,96}$ = 15.07, P < 0.0001) with higher levels (50–89.5% sporulation) achieved by *I. fumosorosea* compared with the *B. bassiana* isolates (0.9–21%) (Figure 3.5).

3.2.2.5 Conidial production using solid-state fermentation

B. bassiana and *I. fumosorosea* showed variability in spore production on rice ($F_{8,44}$ = 76.47; P < 0.0001). Isolate CG1228 of *I. fumosorosea* provided the highest conidial yield, followed by isolate CG1229 of *B. bassiana*, (Figure 3.6). The other isolates had a production range of 0.9–2.3 × 10^9 viable conidia g^{-1} rice based on wet weight. The conidial viabilities for all these isolates were > 95% in germination tests. Additional trials (data not shown, n = 3) showed high spore yields for CG1229 and CG1228 initially inoculated at 1 × 10^6 conidia g^{-1} parboiled rice and grown in plastic bags (250 g rice bag^{-1}). These isolates supported high yields of conidia after 7 days incubation at 26 °C (6.2±1.3 × 10^{10} and 14.2±1.7 × 10^{10} conidia g^{-1} of dry powder conidia with ~5% moisture content, respectively).

3.2.2.7 Surface hydrophobicity and spore-bound Pr1 activity

The majority of the *I. fumosorosea* and *B. bassiana* isolates showed high spore-bound Pr1 activity, except ESALQ1409 and ESALQ-PL63 that exhibited the lower enzyme activity ($F_{8,27}$ = 25.68, P < 0.0001) (Table 3.3). The conidia of the fungal isolates had similar relative hydrophobicity ranging from 84.7 to 99.6% ($F_{8,18}$ = 1.76; P = 0.153), though this parameter was not correlated with Pr1 activity or other virulence factors (Spearman's correlation: -0.23 ≤ r ≤ 0.25; 0.52 ≤ P ≤ 0.79).

3.2.2.8 Cluster and principal component analysis

The PCA provided important information about the variability among fungal isolates and it was useful to find linear relationships between the observed variables, i.e. the performance of isolates in virulence and conidial production. Components 1, 2 and 3 explained 84.54% of the total variance and displayed eigenvalues substantially greater than one (Table 3.4). However, the biplot graph was drawn using only the two major components (1 and 2), which together explained ~70% of the information contained in the data set and comprised all the seven variables. Together components 1 and 2 consisted of significant variables at P < 0.15, according to Pearson's correlation method (Table 3.4). The contribution of each variable on

each component axis in biplot graph was measured proportionally by the length of the arrows (Figure 3.7). The variables “adult mortality”, “sporulated adults”, “LT_{50} values”, “mortality of hatched nymphs” and “conidial production on rice” were the most important for the first principal component, which, in turn, explained the maximal amount of total variance (41.32%). The second component explained 27% of total variance and comprised the variables “total nymph mortality” and “conidial production on nymphal cadavers”. The biplot for individuals (Figure 3.7) indicates isolates with high virulence to adults and nymphs as well as with high conidial yields. Three out of four *B. bassiana* isolates resulted in higher mortality and faster kill of hatched nymphs. Conversely, all isolates of *I. fumosorosea* and *B. bassiana* (CG1229) were positively associated with other variables, indicating high virulence against nymphs and adults along with greater conidial production on rice and on cadavers. *I. fumosorosea* CG1228 had a distinctive performance than the other isolates since it achieved the highest mortality and sporulation on adults and conidial production on rice. Nonetheless, both CG1229 and CG1228 showed high virulence against nymphs, as they caused high mortality rates with lower ST_{50} values.

The cluster analysis suggested four distinct groups with similar profiles (Figure 3.8). The most closely related performances were identified by the shortest distances in the tree diagram. As a result, two single clusters comprised the isolates of *B. bassiana* (CG1229) and *I. fumosorosea* (CG1228). The other isolates of *B. bassiana* were grouped all together in a different cluster from *I. fumosorosea* isolates.

3.2.3 Discussion

We evaluated different fungal attributes to identify virulent isolates against whitefly biotype B. Important criteria for selecting fungal pathogens for commercial development are high virulence against the target, potential for conidial production on artificial substrates and production of inoculum on insect cadavers. In our work, *I. fumosorosea* and *B. bassiana* isolates were virulent against whitefly nymphs, providing > 70% mortality of 2^{nd} instar nymphs with $ST_{50} \leq 4$ days. As previously stated by Wraight et al. (1998) and Lacey et al. (2008), the most virulent isolates to *B. tabaci* biotype B required 50–150 spores/mm^2 to cause 50% nymphal mortality, hence the majority of the Brazilian *I. fumosorosea* and *B. bassiana* isolates fell within this range. *L. muscarium* and other species of *Lecanicillium* have been used commercially as bioinsecticides against whiteflies throughout the world with some degree of success (GINDIN et al., 2000; CUTHBERTSON; WALTERS; NORTHING, 2005; FARIA; WRAIGHT, 2007; GOETTEL et al., 2008), our results indicated that the Brazilian *L.*

muscarium isolates were comparatively less virulent towards *B. tabaci* biotype B. Cuthbertson et al. (2009) also verified a low efficacy (20–40% mortality) of a commercial isolate of *L. muscarium* (Mycotal®, Koppert Biological Systems Ltd., UK) against different life stages of *B. tabaci* on poinsettia.

We note that factors other than isolate, such as experimental method, host stage, environmental conditions and surfactant used, can affect the results of different bioassays. For example, Cabanillas and Jones (2009) prepared conidial suspensions with a 0.02% Silwet L-77® solution, whilst we suspended conidia in a 0.01% Tween 80 solution. Based on our previous studies, low concentrations of this organosilicone surfactant, as low as 0.01%, was toxic to 2nd instar whitefly nymphs inflicting up to 42% mortality after 7 days of exposure (MASCARIN et al., 2013). Thus, the type and concentration of chemical surfactants added in the conidial suspensions can influence insect susceptibility, as confirmed by previous studies (LIU; STANSLY, 2000; SRINIVASAN et al., 2008).

Several studies confirm the potential of *I. fumosorosea* and *B. bassiana* for the microbial control of whiteflies (WRAIGHT et al., 1998, 2000; VICENTINI et al., 2001; RAMOS et al., 2004; QUESADA-MORAGA et al., 2006; CABANILLAS; JONES, 2009). In Brazil, most registered mycopesticides are based on *M. anisopliae* sensu lato (LI et al., 2010), and no *Isaria* spp. product is currently available. Although *I. fumosorosea* has potential as a biological control agent for several insect pests (ZIMMERMANN, 2008), it is rarely included in experiments in Brazil compared to *B. bassiana* and *M. anisopliae*. According to our results, the best candidates based on virulence to *B. tabaci* nymphs and adults were CG1228, ESALQ1364, ESALQ1409, ESALQ1296, and ESALQ1337 of *I. fumosorosea* and CG1229 of *B. bassiana*. The best isolates of *I. fumosorosea* and *B. bassiana* can be employed in inundative biocontrol approach against whiteflies, as these fungi are easily mass cultured on artificial solid substrates and are compatible with different types of formulation (FARIA; WRAIGHT, 2001; JACKSON; DUNLAP; JARONSKI, 2010; MASCARIN; ALVES; LOPES, 2010).

Estimates of in vivo sporulation varied among fungal species, which might affect horizontal transmission and disease development in the field. We observed proportionally greater sporulation responses from *I. fumosorosea* compared with *B. bassiana* isolates. However, *B. bassiana* produced equivalent or higher quantities of conidia whereas only low spore production was achieved by *L. muscarium* isolates. It has been shown that *I. fumosorosea* can incite epizootics in whitefly populations under suitable weather conditions (LACEY; WRAIGHT; KIRK, 2008). This capability represents an advantage over *B.*

bassiana, but in practice the impact of transmission of *Isaria* on the host population in the field will be contingent on prolonged periods of very cloudy and wet weather. Conidiation on whitefly cadavers is very dependent on high relative humidity but also varies with temperature, fungal isolate, host species, host stage and incubation time (SOSA-GÓMEZ; ALVES, 2000).

We observed low mortality of whitefly eggs treated with fungi, confirming earlier reports that eggs have low susceptibility to entomopathogenic fungi (NEGASI; PARKER; BROWNBRIDGE, 1998; LACEY et al., 1999; GINDIN et al. 2000; RAMOS et al., 2000). However, our results demonstrated that conidia remained active and infected a substantial proportion of emerging nymphs, suggesting that application might be targeted against this stage. Fransen et al. (1987) also noted a better efficacy of *Aschersonia aleyrodis* to newly emerged nymphs from treated eggs.

The production of aerial conidia of fungal entomopathogens cultured on solid substrates also varied among species and strains. In Brazil, rice is most commonly used in solid-state fermentation of fungal entomopathogens (LI et al., 2010). The greatest conidial yield was attained by *I. fumosorosea* CG1228 cultured on parboiled rice. In contrast, Mascarin et al. (2010) reported the maximal yield of *I. fumosorosea* ESALQ1296 as 1.1×10^9 viable conidia g^{-1} grown on moisted whole rice in a biphasic fermentation process. Parboiled rice appears to be highly conducive to supporting higher yields of conidia in solid-state fermentation due to its better nutritional and physical properties. *B. bassiana* CG1229 was the second most productive fungus in terms of conidial production on rice.

Several studies show that enzymes and hydrophobicity correlate with fungal virulence (SHAH; WANG; BUTT, 2005, SHAH et al., 2007). We did not find significant positive or negative correlation of enzyme Pr1 and hydrophobicity with the virulence factors of *B. bassiana* or *I. fumosorosea.* This outcome might suggest that other virulence factors are involved in the infection process.

Principal component analysis (PCA) and cluster analysis suggested that median lethal time (LT_{50}), total nymph mortality, hatched nymph mortality, *in vitro* conidial production, *in vivo* sporulation on nymphal cadavers, adult mortality, and *in vivo* sporulation response amongst adult whiteflies were significant variables for virulence screening assays. In our investigation, the other variables such as egg mortality and in vivo sporulation on eggs along with relative hydrophobicity and Pr1 enzyme activity of conidia did not affect virulence of isolates and hence were not the best parameters associated with virulence of these isolates against whitefly. The isolate CG1228 of *I. fumosorosea* can be considered a promising

candidate for inundative biocontrol approach against *B. tabaci* biotype B based on our results, and will be selected for commercial development, while *B. bassiana* CG1229 was also promising.

Given the elevated pest status of *B. tabaci* biotype B in Brazil, the development of virulent entomopathogenic fungus is highly desirable. In crops such as beans and tomatoes, where *Bemisia* is a vector for geminivirus, population must be kept below control thresholds (FARIA; WRAIGHT, 2001). Therefore, further studies are underway to identify effective formulation techniques to optimize the efficacy and persistence of fungal isolates under field conditions, for use in integrated pest management system against this whitefly.

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Table 3.1 - Brazilian entomopathogenic fungal species, strain codes, hosts/substrates, sites of origin and collection dates

Fungus	Strain code[a]	Host/source	Collection site
Beauveria bassiana	ESALQ-PL63	*Atta* spp. (soil)	Piracicaba-SP
	ESALQ447	*Solenopsis invicta* (soil)	Cuiabá-MT
	ESALQ1432	*Diaphorina citri* (jasmine plant)	Piracicaba-SP
	ESALQ-CB66	*Hypothenemus hampei* (coffee)	São José do Rio Pardo-SP
	CG1229	*Rupela albinella* (rice)	Arari-MA
Lecanicillium muscarium	ESALQ 972	*Coccus viridis* (coffee)	Piracicaba-SP
	ESALQ 1351	*Orthezia praelonga* (citrus)	São Paulo state
	ESALQ 1410	*B. tabaci* (cucumber)	Piracicaba-SP
	ESALQ 1408	*B. tabaci* (cucumber)	Itapetininga-SP
Isaria fumosorosea	ESALQ 1296	*B. tabaci* (unknown)	Jaboticabal-SP
	ESALQ 1337	*B. tabaci* (greenhouse colony)	Piracicaba-SP
	ESALQ 1364	*Myzus persicae* (collard)	Piracicaba-SP
	ESALQ 1409	*B. tabaci* (cucumber)	Itapetininga-SP
	CG1228	*Rupela albinella* (rice)	Arari-MA

[a]ESALQ means that fungi are maintained in ESALQ-University of São Paulo (Piracicaba, SP, Brazil), whereas CG is the code for fungi preserved in EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil)

Table 3.2 - Virulence (median survival time, ST_{50}) of different fungal isolates to 2nd instar nymphs of *B. tabaci* biotype B

Fungus	Isolate	ST_{50} values [a] (± SE)	95% CI [b]	
			Lower	Upper
L. muscarium	ESALQ1408	6 ± 0.1	5.8	6.2
	ESALQ1410	8 ±0.2	7.7	8.3
	ESALQ972	8 ± 0.1	7.7	8.3
	ESALQ1351	NE[c]	–	–
I. fumosorosea	CG1228	4 ± 0.1	3.9	4.1
	ESALQ1296	4 ± 0.1	3.9	4.1
	ESALQ1337	4 ± 0.1	3.9	4.1
	ESALQ1364	3 ± 0.1	2.9	3.1
	ESALQ1409	4 ± 0.1	3.9	4.2
B. bassiana	CG1229	4 ± 0.1	3.9	4.1
	ESALQ1432	4 ± 0.2	3.7	4.3
	ESALQ447	6 ± 0.1	5.8	6.2
	ESALQ-PL63	5 ± 0.1	4.7	5.3
	ESALQ-CB66	6 ± 0.1	5.7	6.3

[a] ST_{50} values for mortality were estimated by survivorship analysis (Kaplan-Meier product-limit method) with censored data for insects surviving > 8 days of incubation period. [b] 95% confidence intervals that did not overlap indicated differences between ST_{50} values. [c] Nonestimable: nymphal survival exceeded 50% and, therefore, precluded calculation of ST_{50} value estimates

Table 3.3 - Relative hydrophobicity and spore-bound Pr1 enzyme activity for different isolates of *I. fumosorosea* and *B. bassiana* conidia produced on SDAY medium after 13 days of incubation at 26 °C with a 12 h photophase

Fungus	Isolate	Relative hydrophobicity (%)	Spore-bound Pr1 (µmol/mL/min)[a]
I. fumosorosea	ESALQ 1296	84.7±10.0 [ns]	0.193±0.002 a [b]
	ESALQ 1337	88.8±9.8	0.193±0.002 a
	ESALQ 1364	96.1±0.7	0.184±0.002 a
	ESALQ 1409	94.4±3.0	0.131±0.012 b
	CG1228	85.0±1.7	0.190±0.002 a
B. bassiana	ESALQ 447	86.0±1.7	0.190±0.002 a
	ESALQ 1432	99.6±0.1	0.196±0.024 a
	ESALQ-PL63	99.6±0.2	0.128±0.010 b
	CG1229	98.9±0.3	0.197±0.002 a

[a] Pr1 activity is expressed as µlmol NA $ml^{-1}min^{-1}$ released from succinyl-(Ala)2-Pro-Phe-NA. [b] Means (± SE) within a column followed by the same letter are not significantly different (Tukey's HSD test, $P < 0.05$)

Table 3.4 - Principal components analysis with eigenvalues and variation explained for each principal component

Component	Eigenvalue	Variation explained (%)	Cumulative variation (%)
1	2.91	41.53	41.53
2	1.98	28.28	69.81
3	1.07	15.23	85.04
4	0.76	10.88	95.92
5	0.25	3.62	99.54
6	0.03	0.39	99.93
7	0.005	0.07	100

Attribute loading for eigenvectors (rotated factor pattern) and correlations between variables and the three meaningful components retained [a]

Variable	Component		
	1	2	3
ST_{50}	-0.65 ($P = 0.06$)	–	0.59 ($P = 0.09$)
Total nymph mortality	–	0.79 ($P = 0.01$)	–
Hatched nymph mortality	-0.55 ($P = 0.12$)	–	–
Conidial production on cadavers	–	0.94 ($P < 0.01$)	–
Conidial production on rice	0.63 ($P = 0.07$)	–	0.687 ($P = 0.04$)
Adult mortality	0.89 ($P < 0.01$)	–	–
Sporulated adults	0.88 ($P < 0.01$)	–	–

[a] Eigenvectors are presented only for components 1, 2, and 3. There were just three principal components retained which account for 85.04% of the total variance in the data set. Correlations (r, Pearson's method) between variables and the principal components with a significance threshold at $P < 0.15$ are shown. Since the components 1 and 2 comprised all the meaningful variables, the component 3 was no longer important to build the biplot graph

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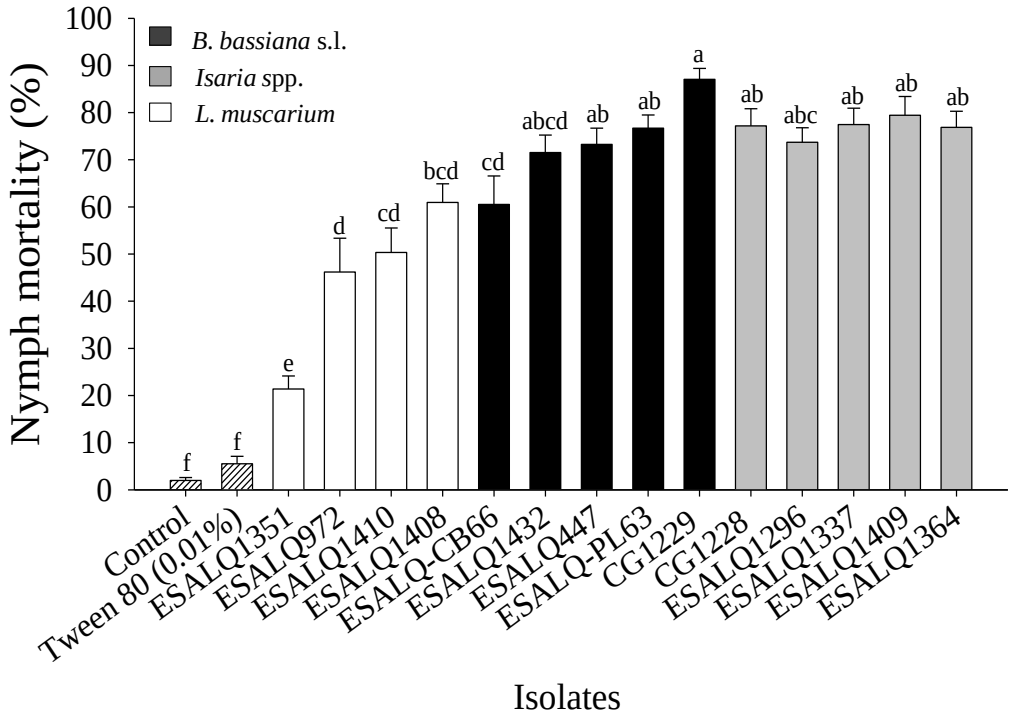

Figure 3.1 - Mortality of *B. tabaci* biotype B nymphs treated with 150 conidia mm^{-2} (1×10^7 conidia mL^{-1}) of *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates at 8 days post-inoculation. Error bars represent the standard error of the mean total mortality. Bars indicated by the same letters are not significantly different (Tukey's HSD, $P < 0.05$)

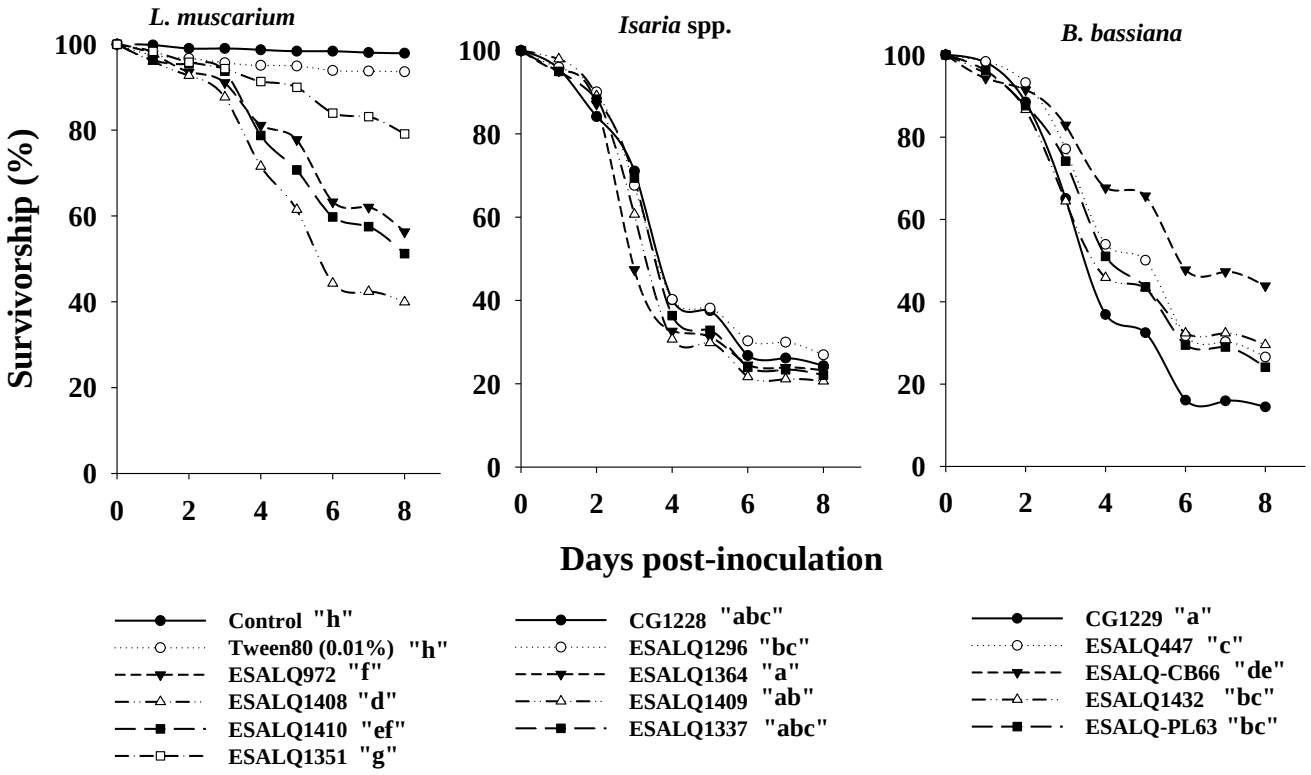

Figure 3.2 - Survival probability curves of 2nd instar nymphs of *B. tabaci* biotype B over time after exposure to *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates applied at a spray rate of 150 conidia mm^{-2} (1×10^7 conidia mL^{-1}). Survival curves followed by the same letters, in quotes, were not significantly different by log-rank test with *P*-values adjusted by Tukey's HSD ($P < 0.05$)

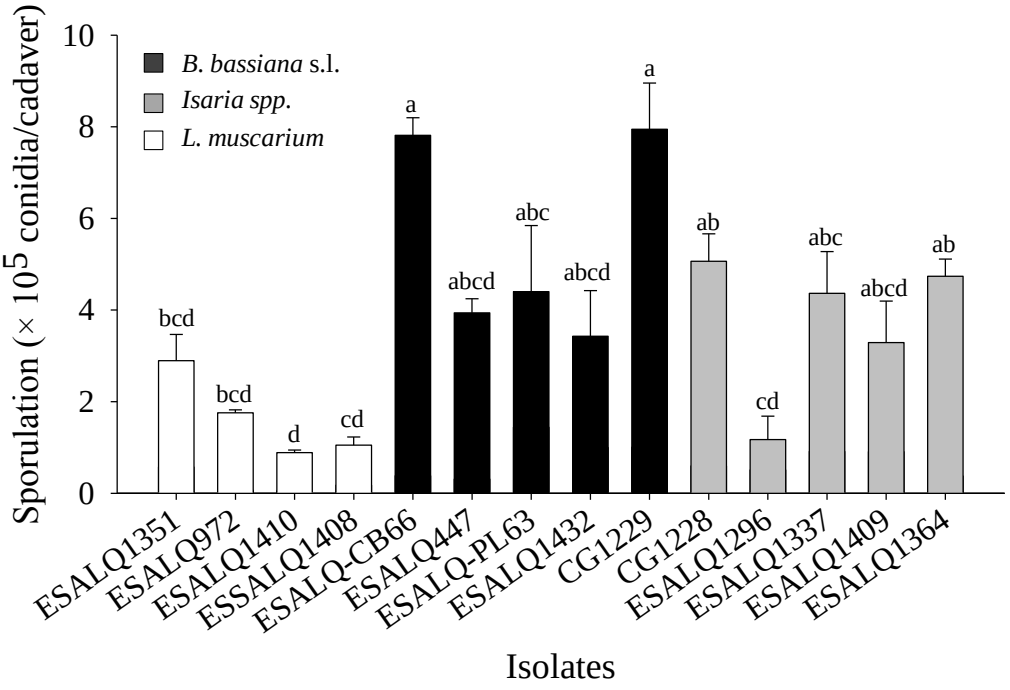

Figure 3.3 - Production of newly formed conidia by different *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates on mummified cadavers of *B. tabaci* biotype B nymphs incubated in a humid chamber. Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD test, $P < 0.05$)

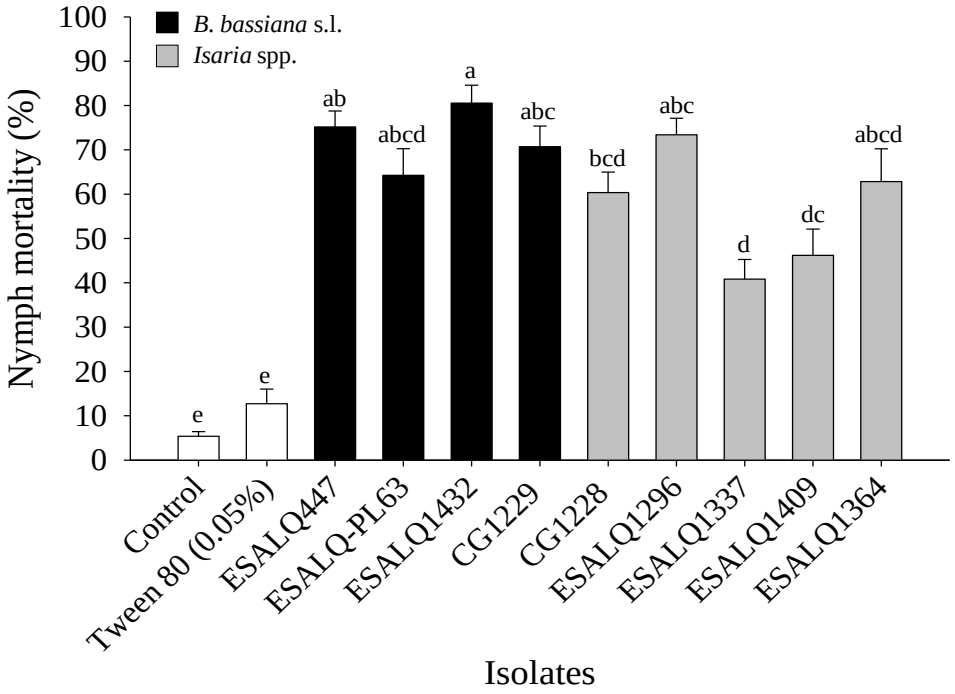

Figure 3.4 - Residual activity of *B. bassiana* and *I. fumosorosea* isolates on hatched nymphs by indirect uptake of conidia from treated leaves at 11 days after *B. tabaci* biotype B eggs were exposed to 1674 conidia mm^{-2} (1×10^8 conidia mL^{-1}). Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD test, $P < 0.05$)

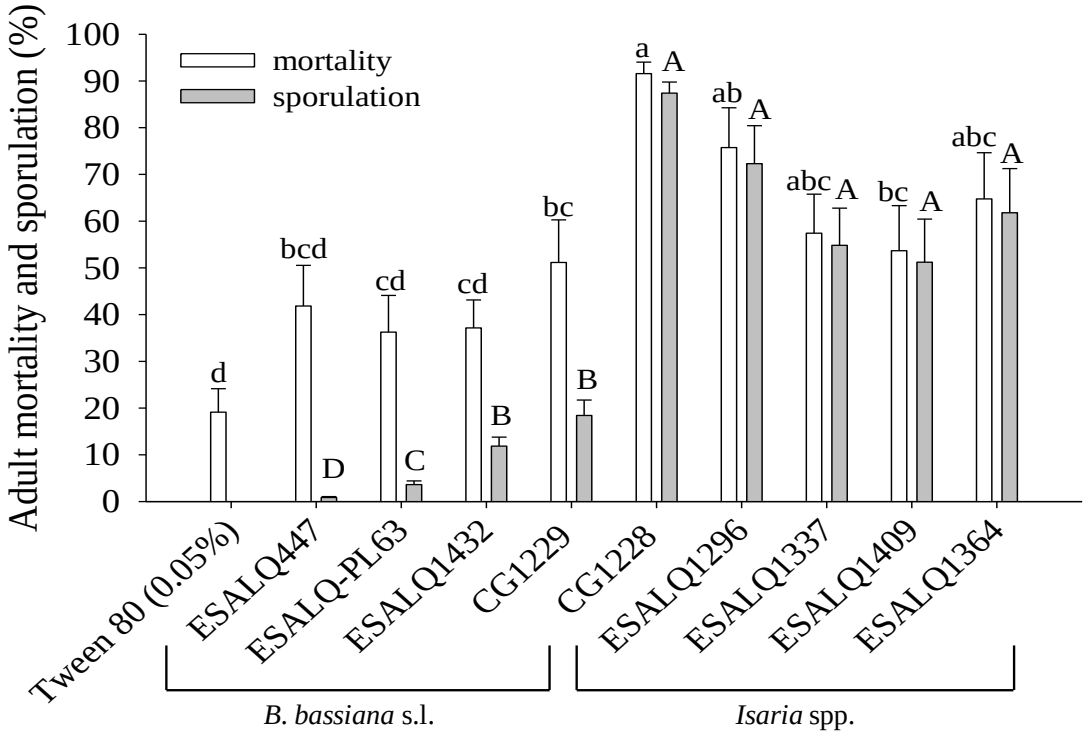

Figure 3.5 - Mortality and sporulation of *B. tabaci* biotype B adults at 7 days after exposure to different *B. bassiana* and *I. fumosorosea* isolates applied at the standard concentration of 150 conidia mm^{-2} (1×10^7 conidia mL^{-1}). Error bars represent the standard error of the mean. Bars indicated by the same letters, in lower case for mortality and capital letters for sporulation, are not significantly different (Tukey's HSD test, $P < 0.05$)

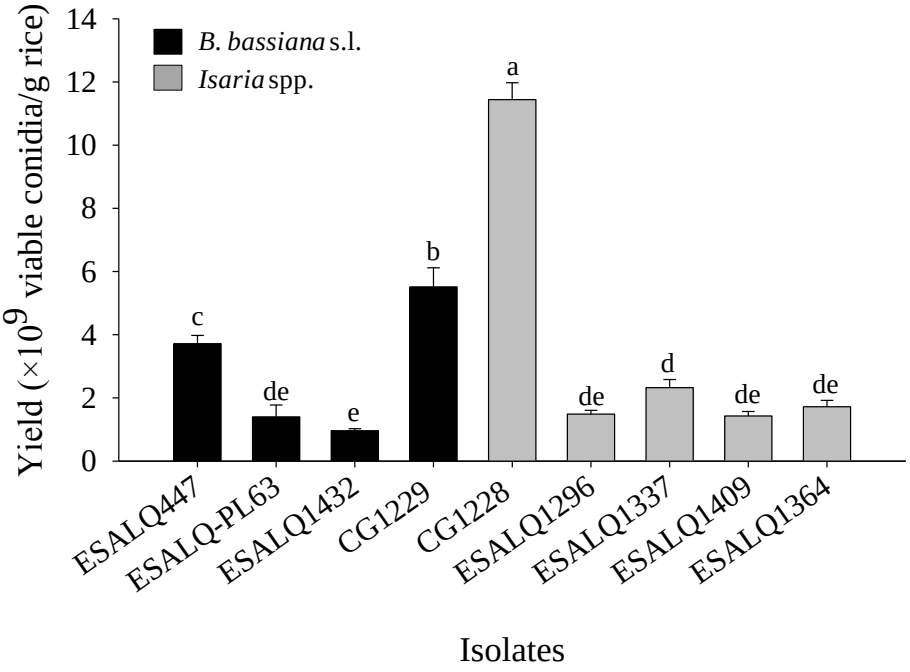

Figure 3.6 - Production of aerial conidia by different *B. bassiana* and *I. fumosorosea* isolates grown for 11 days on parboiled rice at 26 °C with a 14 h light regime. Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD test, $P < 0.05$)

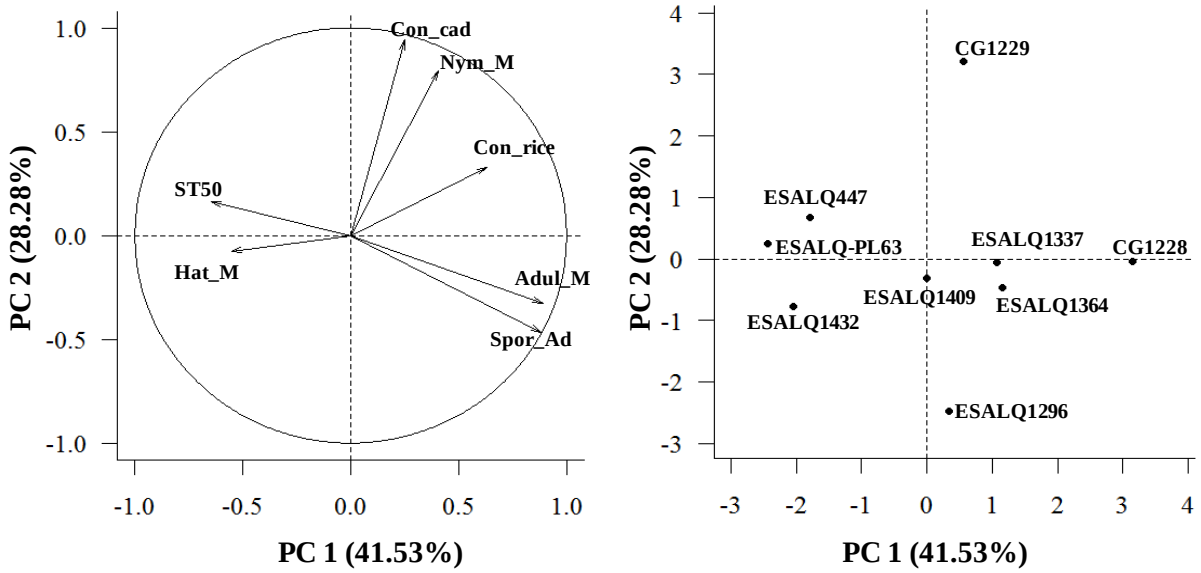

Figure 3.7 - Biplot pictures from PCA between components 1 and 2 which explained 69.81% of the total variance in the observed variables. (A) Variables' graph considering only active variables, in which the length of the arrows indicates the magnitude of its respective variable. (B) Individuals' graph where it shows the scores for the fungal isolates, according to the first two principal components. Component 1 comprises the variables "sporulated adults (Spor_Ad)", "adult mortality (Adult_M)", "total nymph mortality (Nym_M)", "ST_{50} values (ST50)", and "conidial production on rice (Con_rice)", while component 2 is described by "residual activity on hatched nymph (ResAct)" and "conidia production on cadavers (Con_cad)"

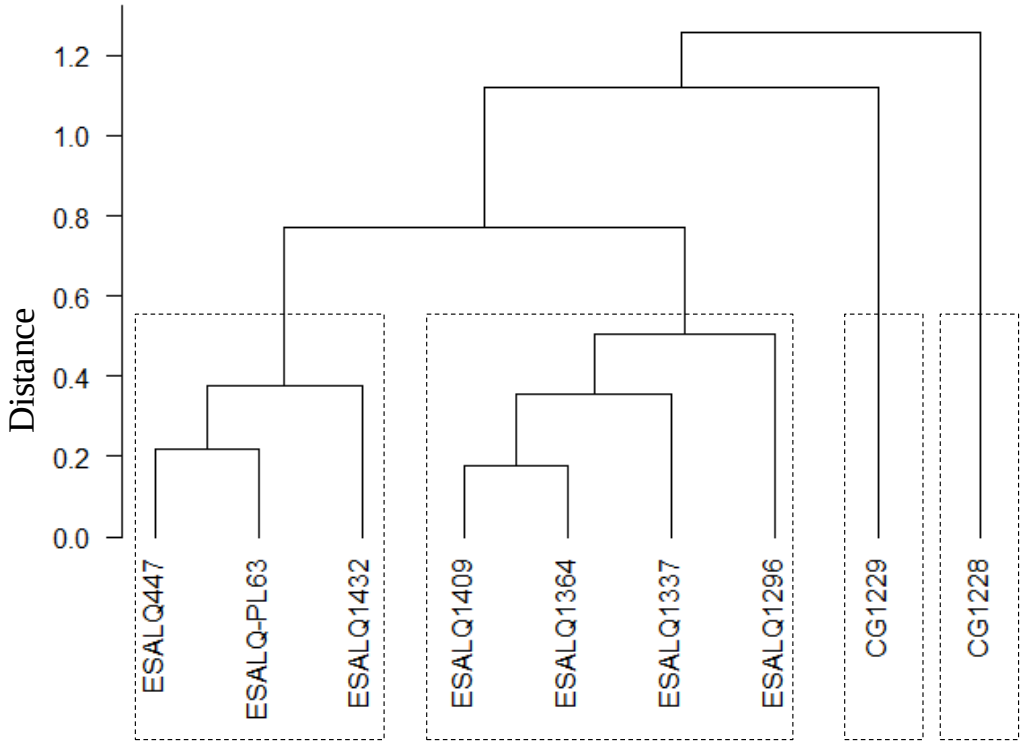

Figure 3.8 - Cluster dendogram showing four distinct groups (surrounded by dashed rectangles) formed with nine isolates of *B. bassiana* s.l. and *I. fumosorosea* taking into account the meaningful response variables retained by PCA and using the unweighted pair-group method with arithmetic mean (UPGMA) to calculate the distances

4 TOXICITY OF NON-IONIC SURFACTANTS AND INTERACTIONS WITH FUNGAL ENTOMOPATHOGENS TOWARD *BEMISIA TABACI* B BIOTYPE[3]

Abstract

A myriad of non-ionic surfactants can be used to enhance insecticidal performance of various bio-based products and agrochemicals. In this study, we described the toxicity of four commercial non-ionic surfactants against immature *Bemisia tabaci* (Gennadius) B biotype along with their spreading ability. Results revealed that trisiloxane-based surfactants (Break-thru® and Silwet L-77®) exhibited the highest toxicity to 1st–2nd (early) and 3rd–4th (late) instar nymphs as well as the greatest wetting performance. We also investigated the insecticidal efficacy of *Beauveria bassiana* (Balsamo) Vuillemin (strain CG1229) and *Isaria fumosorosea* Wise (strain CG1228) conidial suspensions prepared with Break-thru and Silwet L-77 against whitefly and compared with Tween 80®. Germination of hydrophobic conidia for both isolates were unaffected by these surfactants over the range 100–1000 ppm. The combinations of both fungi with trisiloxane carriers significantly increased nymphal mortality with mostly additive and synergistic effects on early and late instars, respectively. In screenhouse trials, both fungi at 10^7 conidia mL^{-1} mixed with 200 ppm of Silwet L-77 significantly improved effectiveness against early nymphs (72–74% mortality) compared with controls treated with water and Silwet L-77 alone. In addition, reduced volume rates of *I. fumosorosea* in Silwet L-77 (equivalent to 100 L ha^{-1}) were at least as effective against late nymphs as higher volume rates (200 L ha^{-1}) at equivalent conidial dosages. Our findings suggest that silicon-based surfactants are compatible with conidial suspensions of *B. bassiana* and *I. fumosorosea* for use in whitefly management programs.

Keywords: *Beauveria bassiana*; *Isaria fumosorosea*; Microbial control; Organosilicones; Spray adjuvants; Sweetpotato whitefly

4.1 Introduction

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a cryptic species complex that remains the most severe polyphagous and cosmopolitan pest of many economically important agronomical and horticultural crops (De BARRO et al., 2011). In Brazil, this invasive insect causes economic losses estimated in US$714 million annually in crops such as soybeans, cotton, beans, melon, tomatoes and various leafy vegetables (OLIVEIRA et al., 2013). The B biotype (also known as Middle East-Asia Minor 1 (De BARRO et al., 2011)) was recently confirmed the most abundant species throughout the Brazilian regions causing severe crop losses due to direct damage and transmitting several plant viruses (FONTES; COLOMBO; LOURENCÃO, 2012). Furthermore, populations of B biotype have developed resistance to several synthetic chemical insecticides in Brazil, which has been the major concern for farmers (SILVA et al. 2009).

[3] MASCARIN, G.M.; KOBORI, N.N.; QUINTELA, E.D.; ARTHURS, STEVEN S.P.; DELALIBERA JR., I. Toxicity of non-ionic surfactants and interactions with fungal entomopathogens toward *Bemisia tabaci* biotype B. **BioControl,** Dordrecht, v. 59, p. 111-123, 2014.

Horticultural oils, surfactants and entomopathogenic fungi provide alternatives to current synthetic pesticides for the sustainable management of whiteflies, because they are less harmful to the environment and can be included in strategies for the management of insecticide-resistant populations (CUTHBERTSON et al., 2012; LACEY; WRAIGHT; KIRK, 2008; LIU; STANSLY 2000; MASCARIN et al., 2013a). A wide range of agricultural surfactants are included in pesticide formulations and spray-mixes as spreaders and wetting agents with the aim to improve coverage of insecticides at the target surface. Although sometimes considered "inert", surfactants have exhibited direct toxicity to some soft-bodied arthropods, possibly through disrupting the respiratory systems (COWLES et al., 2000; SRINIVASAN et al., 2008). Moreover, recent studies highlight that various oil-based surfactants may impact both the viability and virulence of fungal entomopathogens in several ways (AKBAR et al., 2005; CUTHBERTSON et al., 2009, 2012; GATARAYIHA; LAING; MILLER, 2010; JIN et al., 2008; SANTOS et al., 2012). For instance, surfactants including vegetable oils, either applied alone or mixed with conidial suspensions of fungal entomopathogens, have been used against different life stages of *B. tabaci* in Europe (CUTHBERTSON et al., 2009, 2012). For these reasons, it is important to understand the effect of surfactants against whiteflies and their interaction with entomopathogenic fungi as part of a strategy for controlling whitefly populations.

Beauveria bassiana (Balsamo) Vuillemin and *Isaria fumosorosea* Wise within the anamorphic Hypocreales (Ascomycota: Cordycipitaceae) are promising insect pathogenic fungi for use against whiteflies and other leaf-feeding insect pests (LACEY; WRAIGHT; KIRK, 2008; WRAIGHT et al., 2000). Since these fungi can take several days to kill the target (LACEY; WRAIGHT; KIRK, 2008), insects or mites that molt frequently may shed conidia during ecdysis, thus preventing mycosis (VEY; FARGUES, 1977). One possible strategy to improve the speed of kill and effectiveness of entomopathogenic fungi is the addition of emulsifiable oils or other adjuvants in conidial suspensions (AKBAR et al., 2005; GATARAYIHA; LAING; MILLER, 2010). The goals of the current study were: i) investigate the wetting properties of commercial non-ionic surfactants and their toxicity to whitefly nymphs; ii) assess the compatibility in tank-mixing of surfactants with *B. bassiana* (strain CG1229) and *I. fumosorosea* (strain CG1228) against whitefly nymphs.

4.2 Development

4.2.1 Materials and Methods

4.2.1.1 Insect colony

Whitefly B biotype was reared on soybean (*Glycine max* L., cv. Favorita), dry common bean (*Phaseolus vulgaris* L., cv. Pérola), and butter bean (*P. vulgaris* L.) under ambient conditions in a screenhouse (8 × 9 m). The B biotype population was previously identified using molecular techniques such as the Sequence Characterized Amplified Regions (SCAR) (QUEIROZ et al., 2007) and mitochondrial cytochrome oxidase 1 (mtCO1) gene sequence (De BARRO et al., 2011).

4.2.1.2 Fungi and non-ionic surfactants

B. bassiana (strain CG1229) and *I. fumosorosea* (strain CG1228) were both isolated from *Rupela albinella* Stoll (Lepidoptera: Pyralidae) larvae collected from rice fields in Arari, State of Maranhão, Brazil, in 2011. Pure stock cultures of these fungal strains were stored at –80 °C in 10% glycerol solution after re-isolation from infected whitefly nymphs. Aerial conidia of both fungi harvested from 2-week-old cultures grown on Sabouraud dextrose agar amended with 2 g L^{-1} yeast extract (SDAY) served as the inoculum source to the bioassays. For screenhouse experiments, aerial conidia of both fungi were produced on autoclaved parboiled rice using the solid-stated fermentation technique. Briefly, fungus-inoculated rice was incubated into polypropylene bags at 26 °C with 12 h photophase and then conidia were harvested after 11 days to serve as inoculum. For all experiments, conidial viability of both fungi was determined by counting germ tubes produced on potato dextrose agar (PDA, Acumedia®) after 18 h incubation at 26 °C. Conidial viability reached > 95% in all cases.

Four commercial non-ionic surfactants were used in the experiments (Table 4.1). The first experiments with surfactants determined sublethal concentrations toward different nymphal stadia of whitefly. These parameters were then used in fungal compatibility studies.

4.2.1.3 Droplet spreading test

Solutions of each surfactant at different concentrations (i.e., 100, 200, 400, 600, 800, 1000 ppm v/v) were dropped onto a hydrophobic surface of paraffinic wax in Petri dishes (10 × 1.5 cm). The standard laboratory surfactant Tween 80® (polysorbate monooleate, HLB = 15, Vetec®, Rio de Janeiro, RJ, Brazil) was used for comparison. Droplets tested at two sizes (20 and 30 µL) were pipetted on the wax surface. Maximum droplet diameters (mm) attained within 5 min were recorded with a digital caliper rule under a stereoscope (10X

magnification). The control group consisted of deionized water. There were 8-10 replications (drops) for each surfactant concentration. The entire trial was repeated twice on different dates. The experiments were conducted at 27±1 °C and 50±10% relative humidity (RH).

4.2.1.4 Toxicity of non-ionic surfactants to whitefly nymphs

To obtain the nymphs, seedlings of common bean (cv. Pérola) at two-leaf stage were exposed to adult *B. tabaci* for 48 h oviposition. Excised leaves bearing 40–50 whitefly nymphs were placed abaxial side upward on 1% (w/v) water agar in Petri dishes (10 × 2 cm) following the method in Mascarin et al. (2013a). In the first experiment, early nymphs (1^{st}–2^{nd} instars) were sprayed with aqueous solutions of Agral®, Break-thru®, Silwet L-77® and Solub'oil® at 200, 400, 600, 800 and 1000 ppm (v/v). In the second experiment, late nymphs (3^{rd}–4^{th} instars) were sprayed with aqueous solutions of only Break-thru and Silwet L-77 at 100, 200, 300, 600 and 900 ppm (v/v). Agral and Solub'oil were not tested on late nymphs, as these products had lower toxicity to early nymphs and inferior spreading performance. Control group was sprayed with deionized water for all trials. There were 4–5 replicate leaves per treatment. Spraying was performed with a micro-sprayer tower equipped with a dual action gravity feed artist airbrush (Sagyma™ SW130K, Brazil; http://www.wkshop.com.br) fitted with a 0.3-mm fine needle, and calibrated at 10 psi (MASCARIN et al., 2013b). Treatments were applied for 3 s which provided a deposition rate of 2.15 µL cm^{-2} of leaf surface (equivalent to 215 L ha^{-1} of a planar surface). After air-drying, the plates were covered with a ventilated lid having a 4-cm diameter central hole covered with fine nylon cheesecloth (30-µm hole size) and incubated in a growth chamber set to 26±0.2 °C, 91 ± 7% RH with 14:10 L:D photophase. Nymphal mortality was recorded daily for 1 week. Shriveled, discolored or dried nymphs were considered dead by the surfactants (LIU; STANSLY, 2000). The study was repeated twice over time for both nymphal stages and surfactant products.

4.2.1.5 Effect of surfactants on conidial germination

The effect of Break-thru and Silwet L-77 on conidial germination of *B. bassiana* and *I. fumosorosea* was assessed. Briefly, original fungal suspensions consisted of harvested conidia grown on SDAY plates. Through serial dilutions, a 10 mL suspension containing approximately 10^6 conidia mL^{-1} was prepared with 100, 500 and 1000 ppm (v/v) of either surfactant in sterile deionized water. Tween 80, at a standard 100 ppm concentration, was included as surfactant control. Since the hydrophobic conidia did not suspend readily in water, conidia in water only treatments were not included. The fungal-surfactant mixtures were

placed in 45-ml centrifuge Falcon® tubes and agitated for 3 h in a rotary incubator shaker at 25±2 °C and 200 rpm. Fungal germination was determined on 1% water agar plates (6 × 1.5 cm) by inoculating 200 µL of a conidial suspension containing approximately 10^5 conidia mL^{-1}. Conidia were considered germinated with a germ tube longer than the conidial diameter after 16 h incubation at 26±0.5 °C with 12 h photophase.

4.2.1.6 Combinations of surfactants and fungi against whitefly nymphs

Experiments were carried out to assess the toxicity of *B. bassiana* and *I. fumosorosea* applied with non-ionic surfactants. The bioassay protocol followed the procedure mentioned above using ventilated 1% water agar plates. In the first experiment, we tested 150 ppm (v/v) of each surfactant (Break-thru, Silwet L-77 or Tween 80) with *B. bassiana* and *I. fumosorosea* suspensions containing 10^6 conidia mL^{-1}. Test materials were sprayed on bean leaves bearing 40–60 early nymphs at a deposition rate of ≈ 1342 conidia cm^{-2} for each fungus. Controls were sprayed with sterile deionized water and surfactants alone. Therefore, a total of 10 treatments were sprayed and each one had 3 replicates.

In the second study, sublethal concentrations (LC_{25}) of surfactants (Break-thru at 100 ppm and Silwet L-77 at 82 ppm), derived from the previous study, were added to fungal suspensions (10^6 conidia mL^{-1}) and assayed against early nymphs as described above. Aqueous solutions of Tween 80 at 100 ppm alone and mixed with fungal suspensions at 10^6 conidia mL^{-1} were included for comparisons. Controls were sprayed with sterile deionized water. Each treatment was replicated 3 times comprising different excised leaves.

A third experiment was conducted against late nymphs sprayed with fungal suspensions at concentrations of 10^6 and 10^7 conidia mL^{-1} (≈ 1.3×10^3 and 1.5×10^4 conidia cm^{-2}, respectively) prepared in aqueous solutions with 200 ppm of Break-thru, Silwet L-77 or Tween 80. Control for study 3 consisted of late nymphs sprayed with sterile deionized water. There were 16 treatments with 4 replicates each. All experiments were repeated twice on different dates with time as a block effect. After spraying, leaves were air dried at ambient temperature prior to incubation at 26±0.2 °C with 14 h photophase and 85–95% RH. Dead nymphs were recorded daily for 1 week. The cadavers bearing conidiophores after incubation in humid chambers for 3 days were counted and scored as percent mycosis.

4.2.1.7 Whitefly screenhouse experiments

The screenhouse (9 × 18 m) trials used bean plants (cv. Pérola) grown in 2-L potted mix soil with fertilizer (NPK 5-30-15 with 0.5% Zinc (2.86 g kg^{-1} soil)). In the first

experiment, 12-day-old potted bean plants (cv. Pérola) with a pair of primary leaves and one trifoliolate leaf were exposed for 6 h to whitefly adults for oviposition. As a result, bean plants had > 50 early nymphs per leaf 8 days post-infestation. Treatments comprised fungi (*B. beauveria* and *I. fumosorosea*) tested at 10^6 and 10^7 conidia mL^{-1} prepared with Silwet L-77 at 200 ppm and Silwet L-77 alone, while sterile water was the control. Conidial suspensions were applied at 200 µL $leaf^{-1}$ (depositing 100±14 conidia mm^{-2}) using a portable airbrush (Sagyma® SW130K) operating at 10 psi. The study was a randomized block design with 5 replicate plants. Assessments were based on the percent dead nymphs 7 days post-inoculation. The experiment was repeated on different dates (total of 10 plants per treatment), with time considered a block effect.

In the second experiment, we assessed fungal concentrations and volume application rates with *I. fumosorosea* against late nymphs using the procedure mentioned above. In this case, however, plants were transferred to another whitefly-free screenhouse for an additional 13 days to reach 3rd instar nymph. Bean leaves were sprayed on the abaxial face with two concentrations of *I. fumosorosea* (10^6 and 10^7 conidia mL^{-1}) prepared with Silwet L-77 (200 ppm) at a volume rate of 167 µL $leaf^{-1}$, corresponding to 200 L ha^{-1} (bean crop standard assuming 240,000 plants ha^{-1}). Fungal deposition rates were 1.7×10^5 and 1.7×10^6 conidia $leaf^{-1}$ for the lower and higher concentration, respectively. In addition, these same fungal concentrations were doubled when applied at a low volume application rate of 100 L ha^{-1} in order to maintain an equivalent fungal deposition per leaf. Control plants were treated with sterile water and Silwet L-77 alone. Each treatment was replicated with 5 bean plants. Evaluations of whitefly mortality and mycosis in cadavers were recorded (40X magnification) from three leaflets per plant 7 days post-application. The experiment was repeated twice on different occasions (total of 10 plants per treatment) with time considered a block effect.

Daily temperature and RH were monitored with a data logger (AZ8829®, AZ Instrument Corp. Ltd., Hong Kong). Environmental conditions inside the screenhouse averaged 23.7 °C (range 18.4–34.3) and 91.4% RH (range 49.5–99.9) for the first tests, while for the second tests averaged 24.1 °C (range 19.2–32.8) and 78.3% RH (range 40.9–95.8).

4.2.1.8 Statistical analyses

The data from droplet spreading experiments were fitted by the two-parameter exponential rise to a maximum function, $f(x) = a*(1-\exp(-b*x))$, using SigmaPlot V11.0 (SYSTAT SOTWARE, 2008) . Data from mortality-concentration bioassays with test surfactants were corrected by Abbott's equation prior to logit analysis with (PROC PROBIT,

SAS INSTITUTE, 2008) (ROBERTSON; PREISLER, 1992). Estimates of LC_{50} and LC_{90} values were compared on the basis of overlapping 95% confidence intervals. The impact of surfactants on conidial germination and the whitefly mortality bioassays were submitted to one-way analysis of variance (ANOVA, PROC GLM, SAS INSTITUTE, 2008). Percent mortality data from laboratory and screenhouse assays were submitted to the mixed model ANOVA with treatments as a fixed effect and the experimental replicate as a random blocking effect. Significant differences between treatment means were separated through Fisher's protected LSD test at 5% level of significance. Where appropriate, mortality data were previously transformed by square-root to meet the assumptions of homoscedasticity and normality of the residuals. The type of interaction of surfactants with fungi in combination treatments were based on the t-Student ($\alpha = 0.05$) test (FARENHORST et al., 2010), which compares observed and expected mortality.

4.2.3 Results

4.2.3.1 Droplet spreading test on hydrophobic surface

Non-linear regression model accurately described the relationship between surfactant concentration and spreading distance for both droplet size (Figure 4.1). The spreading capability of surfactants diverged at concentrations higher than 100 ppm. Spreading curves depicted the same trend across droplet sizes, but larger droplets with surfactants spread relatively farther than smaller ones. The maximum spreading was given by coefficient *a*, which is inversely proportion to the spreading capability. Hence, the best spreading capability was achieved by Silwet L-77 followed by Break-thru, whereas Tween 80, Agral and Solub'oil had the lowest spreading rates.

4.2.3.2 Effect of non-ionic surfactants on whitefly nymphs

The relationship of surfactant concentration and mortality of early and late nymphs was well described by the sigmoid model (Figure 4.2). Early nymphs were more sensitive to Silwet L-77, Solub'oil and Break-thru compared with Agral. While Silwet L-77 and Break-thru had similar toxicity toward late nymphs based on logit analysis (Table 4.2). Slopes indicated that Break-thru and Silwet L-77 killed early nymphs more quickly than Agral and Solub-oil. Higher LC_{50} and LC_{90} values and lower logit slopes (Table 4.2) indicated that late nymphs were less susceptible to Silwet L-77 and Break-thru compared with early nymphs. Early nymphs killed by surfactants became dehydrated and reddish and sometimes

disintegrated on leaf surface, whilst late nymphs became firstly opaque or yellowish and then reddish and desiccated.

4.2.3.3 Effect of surfactants on conidial germination

Overall, surfactant concentration did not affect the conidial viability of *I. fumosorosea* ($F_{6, 21}$ = 2.0; P = 0.113), which remained > 95% in all treatments. The viability of *B. bassiana* was slightly affected by surfactant treatments ($F_{6, 21}$ = 3.1; P = 0.0243), which post hoc tests confirmed was due to reduced viability when mixed with Silwet L-77 at the highest concentration (1000 ppm). Numerically germination was still high (95%) in comparison to the control prepared with Tween 80 (98% viability) (Table 4.3).

4.2.3.4 Combinations of surfactants and fungi against whitefly nymphs

In the first experiment, there was a significant effect of surfactant-conidia mixtures on whitefly mortality ($F_{9, 50}$ = 60.8, P < 0.0001). The addition of sublethal concentrations of either Break-thru or Silwet L-77 to conidial suspensions enhanced the pathogenicity of *B. bassiana* toward early nymphs in comparison with fungus mixed with Tween 80 (Figure 4.3). The same trend did not hold for *I. fumosorosea*, where none of the trisiloxane surfactants significantly enhanced nymphal mortality compared with Tween 80 preparation (P > 0.05). The surfactants also had a significant effect on the proportion of dead whiteflies that sporulated from fungal treatments ($F_{5, 30}$ = 3.9, P = 0.0075). Percent mycosis was higher in the Silwet L-77 + *B. bassiana* compared with the standard treatment (*B. bassiana* + Tween 80); however, no significant differences in mycosis were observed with Break-thru as well as with any *I. fumosorosea* treatments (Figure 4.3).

There was also a significant overall impact of surfactants and fungal treatments on whitefly mortality in the second study toward early nymphs in relation to unsprayed control ($F_{9, 50}$ = 34.9, P < 0.0001). With respect to *B. bassiana*, only Break-thru added to conidial suspension instead of Silwet L-77 provided higher nymphal mortality in relation to Tween 80 preparation (Figure 4.4). Regarding *I. fumosorosea*, either Break-thru or Silwet L-77 added to conidial suspension increased mortality of early nymphs relative to Tween 80 preparation. The high mortality levels of early nymphs exposed to 150 ppm of these trisiloxane surfactants may have prevented synergism with fungi. Both trisiloxane compounds did not reduce mycosis in whitefly cadavers compared with Tween 80 treatments. Percent mycosis was higher for *I. fumosorosea* prepared with Break-thru when compared with Silwet L-77 and

Tween 80 ($F_{5, 30}$ = 4.8; P < 0.0025). Mycosis caused by *B. bassiana* did not vary among tested surfactants (Figure 4.4).

In the third experiment, adding Silwet L-77 and Break-thru (200 ppm) to conidial suspensions enhanced whitefly mortality (64–73%) compared with Tween 80 added to fungal preparations (8–16.5%) and controls (0.5%) ($F_{15, 112}$ = 67.9; P < 0.0001) (Figure 4.5). No differences in whitefly mortality were found between fungal concentrations when suspended in trisiloxane surfactants. Percent mycosis in whitefly cadavers was also higher for conidial suspensions prepared in either Silwet L-77 or Break-thru compared with fungi mixed with Tween 80 ($F_{11, 84}$ = 36.0; P < 0.0001) (Figure 4.5). Therefore, according to the pairwise t-test, the interactions of surfactants in tank-mixing with fungi were mostly additive and synergistic toward early and late nymphs, respectively (Table 4.4).

4.2.3.5 Whitefly screenhouse experiment

Seven days post-treatment, both *B. bassiana* and *I. fumosorosea* combined with Silwet L-77 (200 ppm) killed more early nymphs (72–74% mortality) relative to their lower concentrations and control ($F_{5, 53}$ = 49.5; P < 0.0001) (Figure 4.6).

Higher rates of *I. fumosorosea* + Silwet L-77 directly sprayed on late whitefly nymphs significantly increased mortality in comparison with lower fungal rates and controls ($F_{6, 62}$ = 123.4; P < 0.0001). Furthermore, the lower volume application rate (100 L ha^{-1}) of fungal treatment at higher concentration incited higher nymphal mortality in relation to the standard volume application rate (200 L ha^{-1}) (Figure 4.7). Nevertheless, mortality rates for late nymphs were lower (maximum 41.5%) compared with previous studies under controlled environmental conditions. Percentage of nymphal cadavers with mycosis increased with fungal concentration, and was also enhanced by the lower volume application rate (100 L ha^{-1}) ($F_{3, 35}$ = 105.7; P < 0.0001) (Figure 4.7). In contrast to the laboratory results, Silwet L-77 solution (200 ppm) applied alone induced relatively low toxicity (15% mortality), regardless the spray volume rate.

4.2.3 Discussion

Soft-bodied arthropods are susceptible to trisiloxane surfactants, a subclass of non-ionic organosilicones, and some of these products can be more toxic than horticultural oils at equivalent rates (COWLES et al., 2000; LIU; STANSLY, 2000; CUTHBERTSON et al., 2009). Surfactants are typically used to enhance foliage deposition of pesticides (HOLLOWAY et al., 2000), but their effect on target pests has received comparatively little

attention. Our results indicate that the direct insecticidal activity of most surfactants against whitefly nymphs was closely related to their spreading properties. An exception, the oil solubilizer Solub'oil showed poor spreading performance, although it was comparatively toxic to early nymphs. We also demonstrated that surfactant toxicity depended on the nymphal stage, with older (late) nymphs becoming slightly less susceptible.

Possible mechanisms on the insecticidal toxicity of surfactants include disturbing integument integrity or metabolism. In our studies whitefly nymphs were killed within 24 h of exposure. Srinivasan et al. (2008) also observed insecticidal effect of organosilicone surfactants towards Asian citrus psyllid (*Diaphorina citri* Kuwayama). The authors reported that Silwet L-77 showed a LC_{50} ranging from 7 to 15 ppm for psyllid nymphs and at 500 ppm all nymphs were killed in laboratory assays, which indicate that immatures of *D. citri* are more sensitive to Silwet L-77 than whitefly nymphs. Other studies show that trisiloxane surfactants can affect the survivorship of other insects and mites (COWLES et al., 2000; GATARAYIHA; LAING; MILLER, 2010). These authors propose suffocation as one of the main causes of death, although toxicity may also result from these materials entering the body and disrupting cell membrane functions (Puritch 1981).

We were interested in the compatibility of trisiloxane surfactants with hydrophobic aerial conidia of some fungal pathogens used for whitefly management. Our studies found that Break-thru and Silwet L-77 (rate range 100–1000 ppm) were compatible to *B. bassiana* and *I. fumosorosea* and resulted mostly in additive and synergistic insecticidal activity against whitefly nymphs. Although a variety of surfactants may be used with entomopathogenic fungi, previous reports on their compatibility against insect pests are variable. In some cases, non-ionic surfactants such as TDA (polyoxyethylene tridecyl ether), Triton-X, Span and Tween 80 did not provide improvement in fungal efficacy, but promoted rapid germination rates and required less wetting time to suspend hydrophobic conidia (JIN et al., 2008). Santos et al. (2012) also reported no significant effect of different surfactants on the effectiveness of *B. bassiana* against the scale *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae). We observed that early (1^{st}–2^{nd} instars) whitefly nymphs were relatively more susceptible to trisiloxane surfactants and to fungal pathogens compared with late (3^{rd}–4^{th} instars) nymphs. Previous authors also report that late nymphs of *B. tabaci* and *Trialeurodes vaporariorum* (Westwood) are less susceptible than early nymphs to synthetic chemical and botanical (i.e. neem) insecticides (PINHEIRO et al., 2009; WANG et al., 2003) as well as to fungal pathogens (CABANILLAS; JONES, 2009; CUTHBERTSO; WALTERS; NORTHING, 2005). We have found a good efficiency (72–74% mortality at day 7) toward early rather than

late whitefly nymphs by single application of either *B. bassiana* or *I. fumosorosea* (1×10^7 conidia mL^{-1}) amended with Silwet L-77 (200 ppm) in screenhouse trials.

The usefulness surfactants for whitefly control will depend on achieving good spray coverage on the underside leaf surface, where whiteflies are located. The relatively poor control of late whitefly nymphs by *I. fumosorosea* under screenhouse conditions (maximum of 42% mortality) may have been influenced by poor coverage compared with other studies that applied insecticides to 'run off' on the whole plant (COWLES et al., 2000; GATARAYIHA; LAING; MILLER, 2010; LIU; STANSLY, 2000) or due to lower RH or other possible causes not clear here.

There are several biotic and abiotic factors affecting the persistence, growth and insecticidal activity of fungal entomopathogens, including temperature, relative humidity, solar radiation, effect of original insect host, host plant, method of application, type of propagule, effect of exogenous nutrients on germination, and exposure to certain allelochemicals (LACEY; WRAIGHT; KIRK, 2008), and their interactions warrant more studies. Environmental conditions, especially ambient RH, may also influence the performance of surfactants (IMAI; TSUCHIYA; FUJIMORI, 1995; LIU; STANSLY, 2000). Different environmental conditions might explain the inconsistent results between our screenhouse and laboratory experiments. The role of ambient RH in fungal infection is unclear, as some investigators did not report significant effects (WRAIGHT et al., 2000), whereas others claimed high ambient RH is critical to promote germination and infection (FARGUES; LUZ, 2000; MARCANDIER; KHACHATOURIANS, 1987).

In conclusion, based on their low cost (e.g. $2 per acre for Silwet L-77 at 500 ppm) (SRINIVASAN et al., 2008), the use of organosilicones as adjuvants or substitutes for synthetic insecticides could result in substantial savings in managing whitefly populations. Also, spray applications using organosilicones as tank mixture additives with mycoinsecticides could be included in insecticidal rotation strategies for whiteflies.

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Table 4.1 - Summary of agricultural non-ionic surfactants used in our experiments

Trade name	Formulation [a]	Chemical group	Active ingredient	HLB [b]	CMC [c]	Label rates	Manufacturer
Silwet L-77®	EC	Trisiloxane ethoxylate	Polyalkyleneoxide modified heptamethyltrisiloxane (99.5 % w/v)	7.0	0.007	0.025–0.1%	Momentive Performance Materials Ltd.
Solub'oil®	EC	Oil solubilizer	- [d]	-	-	5 L/200 L oil	General Chemicals Brazil Ltd.
Agral®	SL	Alkylphenol ethoxylate	Nonyl phenoxy poly (ethyleneoxy) ethanol (20% w/v)	-		0.03–0.05%	Syngenta Ltd.
Break-thru S 240®	EC	Organo-modified trisiloxane	Oxirane, methyl-, polymer with oxirane, mono[3-[1,3,3,3-tetramethyl-1-[(trimethylsilyl)oxy]disiloxanyl] propyl] ether (≥ 75%)	8.2	0.01	0.025–0.1%	Evonik Ltd. (formerly Degussa)

[a] EC = emulsifiable concentrate, SL = soluble concentrate. [b] Hydrophilic-lipophilic balance. [c] Critical micelle concentration (% by wt).[d] Not found neither indicated by the manufacturer (-)

Table 4.2 - Toxicity of non-ionic surfactants applied at 2.15 µl cm^{-2} toward early (1^{st}–2^{nd} instars) and late (3^{rd}–4^{th} instars) nymphs of *B. tabaci* biotype B on bean leaves

Surfactant	*n* [a]	Parameter estimates [b]			df	χ^2 (*P* value) [c]	LC_{50} (ppm) (95% FL) [d]	LC_{90} (ppm) (95% FL)
		a	*b* (slope)	*C*				
Early nymphs (1^{st}-2^{nd} instars)								
Agral	2453	-11.8±2.0	3.8±0.7	0.05±0.01	38	57.2 (0.023)	1225 (1002–1823)	4582 (2680–14709)
Break-thru	2718	-16.2±1.4	7.5±0.6	0.03±0.01	40	122.1 (<0.0001)	149.9 (136.5–163.2)	296 (265–340.2)
Silwet L-77	2499	-13.8±1.0	6.6±0.4	0.05±0.01	38	56.5 (0.027)	120.1 (110.5–129.3)	258 (235.5–287.9)
Solub'oil	2541	-9.9±0.8	4.7±0.4	0.05±0.01	38	76.4 (0.0002)	124.3 (108.9–138.9)	364 (322–425)
Late nymphs (3^{st}-4^{nd} instars)								
Break-thru	2060	-7.7±0.9	3.3±0.4	0.002±0.004	34	124.31 (< 0.0001)	289 (234–354)	1032 (766.5–1829)
Silwet L77	2140	-9.8±1.5	3.9±0.6	0.002±0.006	34	291,32 (< 0.0001)	220 (185–257)	1017 (233.5–1556)

[a] Number of nymphs tested. [b] Logistic model equation: f(x) = *C* + (1 - *C*)*(1 / (1 + exp(- *a* + *b**log(x)))), where f(x) is the proportion mortality function, *a* is the intercept (±SE), *b* is the slope (±SE), and *C* is the natural mortality (±SE).[c] Chi-squared value (with *df*) indicates the goodness-of-fit of binary logistic model fitted for each data set. [d] FL, fiducial limits at 95% probability

Table 4.3 - Effect of two organosilicone (non-ionic surfactants Break-thru and Silwet L-77) on conidial viability of *Beauveria bassiana* (CG1229) and *Isaria fumosorosea* (CG1228) after 3 h exposure under constant agitation at 25±2 °C and 200 rpm

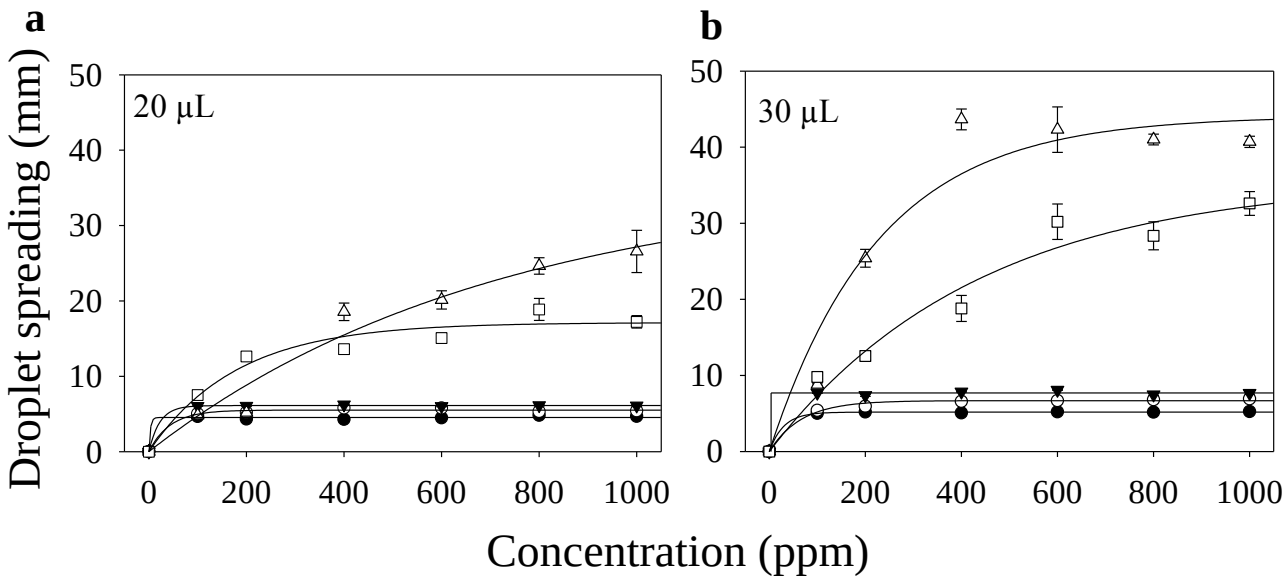
Surfactant	Concentration (ppm)	Germination (%)	
		B. bassiana	*I. fumosorosea*
Tween 80 [a]	100	97.9 ± 0.3 a [b]	99.8 ± 0.3 [ns]
Break-thru	100	98.1 ± 0.9 a	99.5 ± 0.2
	500	97.6 ± 0.4 a	99.8 ± 0.1
	1000	97.3 ± 0.6 a	99.7 ± 0.2
Silwet L-77	100	96.2 ± 1.3 a	99.7 ± 0.1
	500	96.2 ± 0.5 ab	98.7 ± 0.3
	1000	95.2 ± 0.7 b	98.1 ± 0.8

[a] Tween 80 at 100 ppm was used as control. [b] Means (± SE) followed by the same letters within columns are not significantly different at $P < 0.05$(LSD test). [ns] Not significant ($P > 0.05$)

Table 4.4 - Interactions of fungi with trisiloxane surfactants based on laboratory bioassays

Target instar	Conidial preparations		Mortality (%)		t-test [b]	*P* value	Response
	Fungus (conidia/ml)	Surfactant (ppm)	Observed	Expected [a]			
1st–2nd	Ifr (10^6)	Break-thru® (100)	61.5	57.5	0.41	0.537	Additive
	Ifr (10^6)	Silwet® (82)	61.7	53.2	2.23	0.166	Additive
	Bb (10^6)	Break-thru® (100)	63.1	60.5	0.25	0.626	Additive
	Bb (10^6)	Silwet® (82)	65.3	60.1	0.67	0.431	Additive
3rd–4th	Ifr (10^6)	Break-thru® (200)	65.1	56.1	2.25	0.156	Additive
	Ifr (10^6)	Silwet® (200)	65.5	53.9	4.77	0.046	Synergism
	Bb (10^6)	Break-thru® (200)	57.5	54.8	0.12	0.740	Additive
	Bb (10^6)	Silwet® (200)	72.5	52.2	13.60	0.002	Synergism
	Ifr (10^7)	Break-thru® (200)	64.5	57.7	0.64	0.437	Additive
	Ifr (10^7)	Silwet® (200)	71.8	56.2	5.57	0.033	Synergism
	Bb (10^7)	Break-thru® (200)	76.4	57.4	11.47	0.004	Synergism
	Bb (10^7)	Silwet® (200)	73.3	55.5	11.14	0.005	Synergism

[a] The expected mortality was given by Me = Mf + Ms (1 – Mf/100), where Mf and Ms were % observed mortality caused by the fungus (Bb = *B. bassiana* and Ifr = *I. fumosorosea* both suspended in Tween 80 solution) and surfactant alone, respectively. When t-test is significant and Mix – Me > 0 = synergism. If t-test is significant and Mix – Me < 0 = antagonistic; if t-test is not significant and the observed mortality for combined treatment is higher than each single agent, thus the effect can be considered additive. [b] Results show outcomes of paired-samples t-test comparisons of observed and expected cumulative mortality. No interaction effect is indicated by (-)



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Droplet size	Surfactant	Parameter estimates		
		a	b	R^2
20 µl	Agral	5.5±0.1	0.023±0.005	0.99
	Break-thru	17.1±0.9	0.006±0.001	0.96
	Silwet L-77	36.0±8.4	0.0014±0.0006	0.97
	Solub'oil	6.1±0.03	0.045±0.01	0.99
	Tween 80	4.5±1.3	0.33±0.0	0.99
30 µl	Agral	6.7±0.1	0.015±0.002	0.99
	Break-thru	35.8±4.1	0.0023±0.0006	0.97
	Silwet L-77	44.1±3.7	0.0044±0.0012	0.94
	Solub'oil	7.7±0.0	9.19±0.0	0.99
	Tween 80	5.2±0.03	0.034±0.004	0.99

Figure 4.1 - Wetting distance (mm, ±SE) of different concentrations of non-ionic surfactants after application of 20 µl (a) and 30 µl (b) droplets on paraffinic wax. Estimated parameters (c) for this model are presented for Silwet L-77 (Δ), Break-thru (□), Agral (○), Tween 80 (●), Solub'oil (▼). Lines are fitted curves by the nonlinear regression model: $f(x)=a(1-\exp(-bx))$, where f(x) is the maximum wetting distance (mm), x is the surfactant concentration, *a* is the maximum theoretical distance, and *b* denotes how quickly the function rises to the maximum

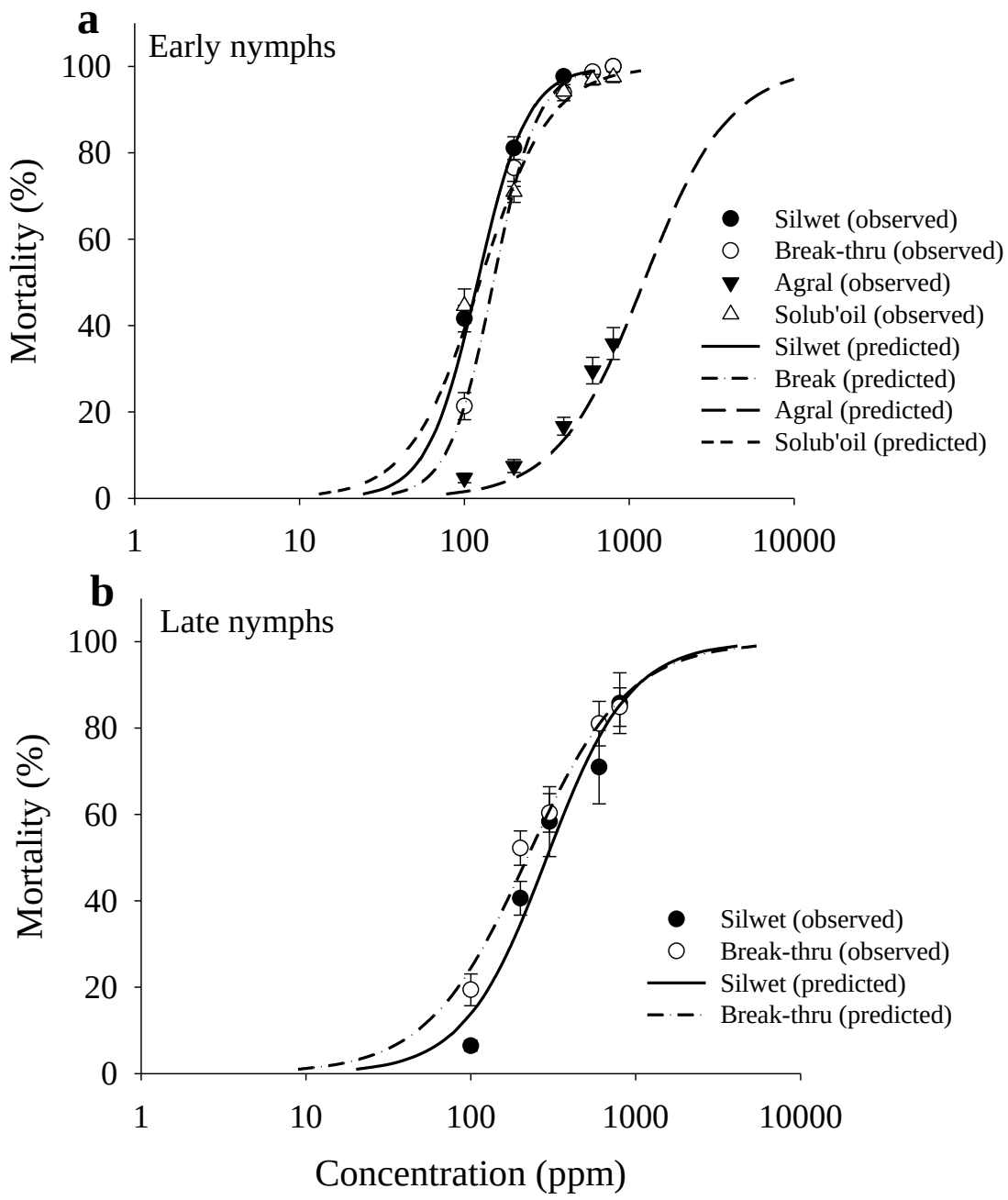

Figure 4.2 - Logistic model describing mortality of whitefly nymphs against different non-ionic surfactants. Susceptibility of early nymphs [1st–2nd instars] (a) and late nymphs [3rd–4th instars] (b) of *B. tabaci* biotype B. Circles refer to mean values (±SE) of observed mortality (%, ±SE). Curves (lines) were corrected by Abbott's equation

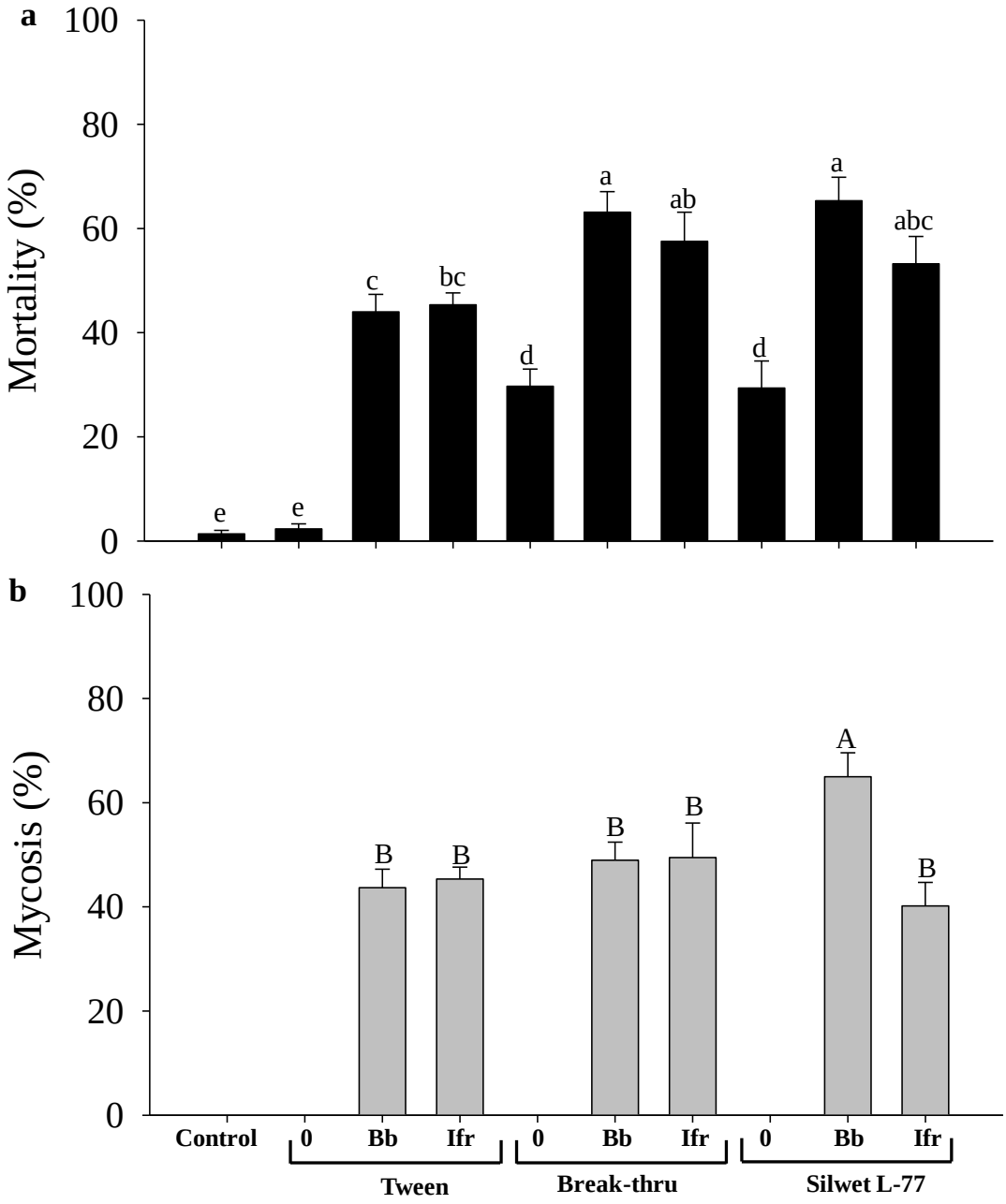

Figure 4.3 - Mortality (a) and mycosis (b) of early nymphs (1^{st}–2^{nd} instars) of *B. tabaci* biotype B 7 days after exposure to sublethal concentrations (LC_{25}) of surfactants (Silwet L-77 at 82 ppm, Break-thru at 100 ppm) added to a single conidial suspension of each fungus (Bb = *B. bassiana* CG1229 and Ifr = *I. fumosorosea* CG1228 at 10^6 conidia mL^{-1}). Tween 80 at 100 ppm was used as control for surfactants. Means (±SE) followed by the same letters are not significantly different at $P < 0.05$ (LSD test)

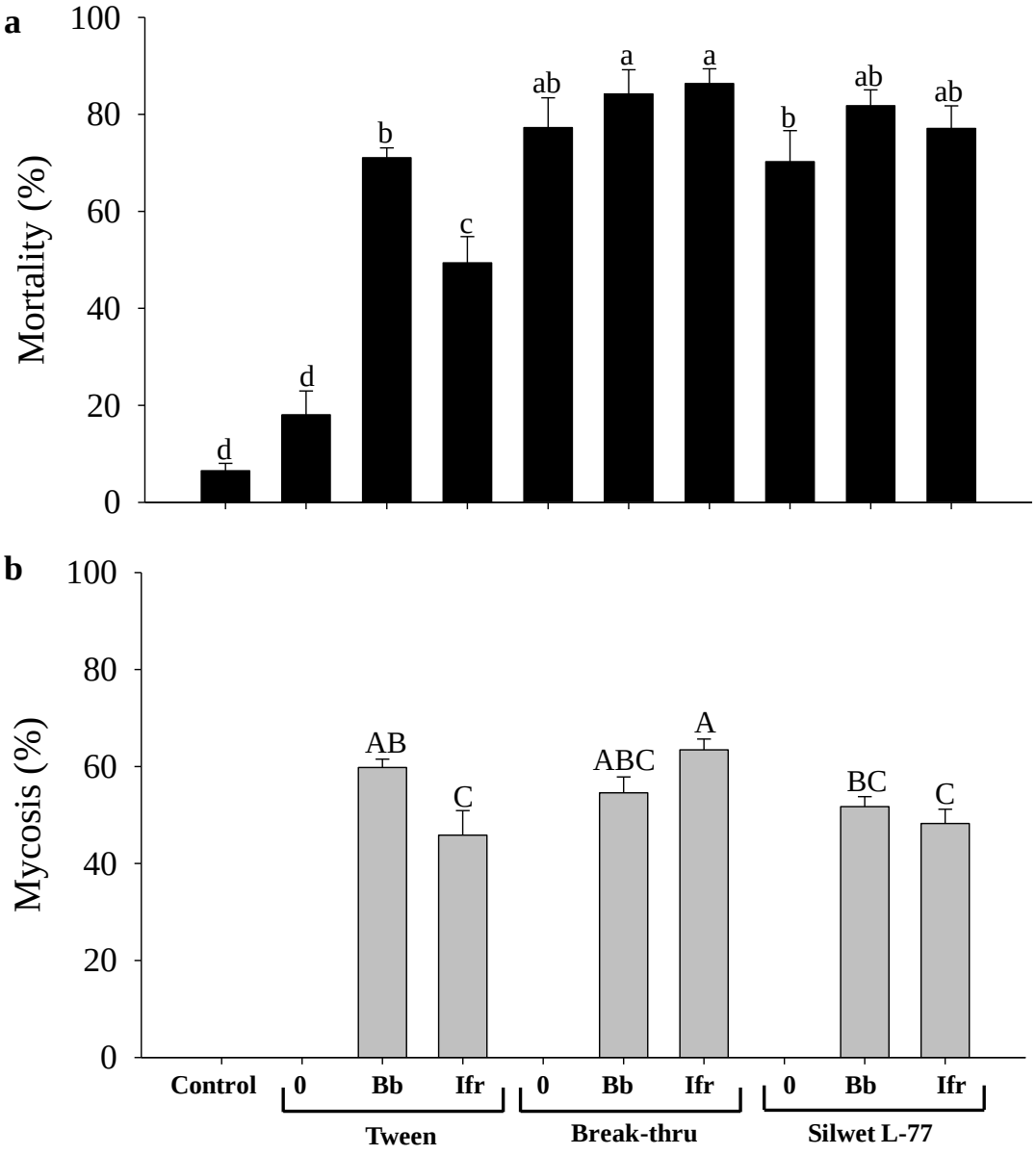

Figure 4.4 - Mortality (a) and mycosis (b) of early nymphs (1^{st}–2^{nd} instars) of *B. tabaci* biotype B 7 days after exposure to different combinations of surfactants at 150 ppm added to a single conidial suspension of each fungus (Bb = *B. bassiana* CG1229 and Ifr = *I. fumosorosea* CG1228 at 1×10^6 conidia mL^{-1}). Tween 80 at 150 ppm was used as control for surfactants. Means (±SE) followed by the same letters are not significantly different at $P < 0.05$ (LSD test)

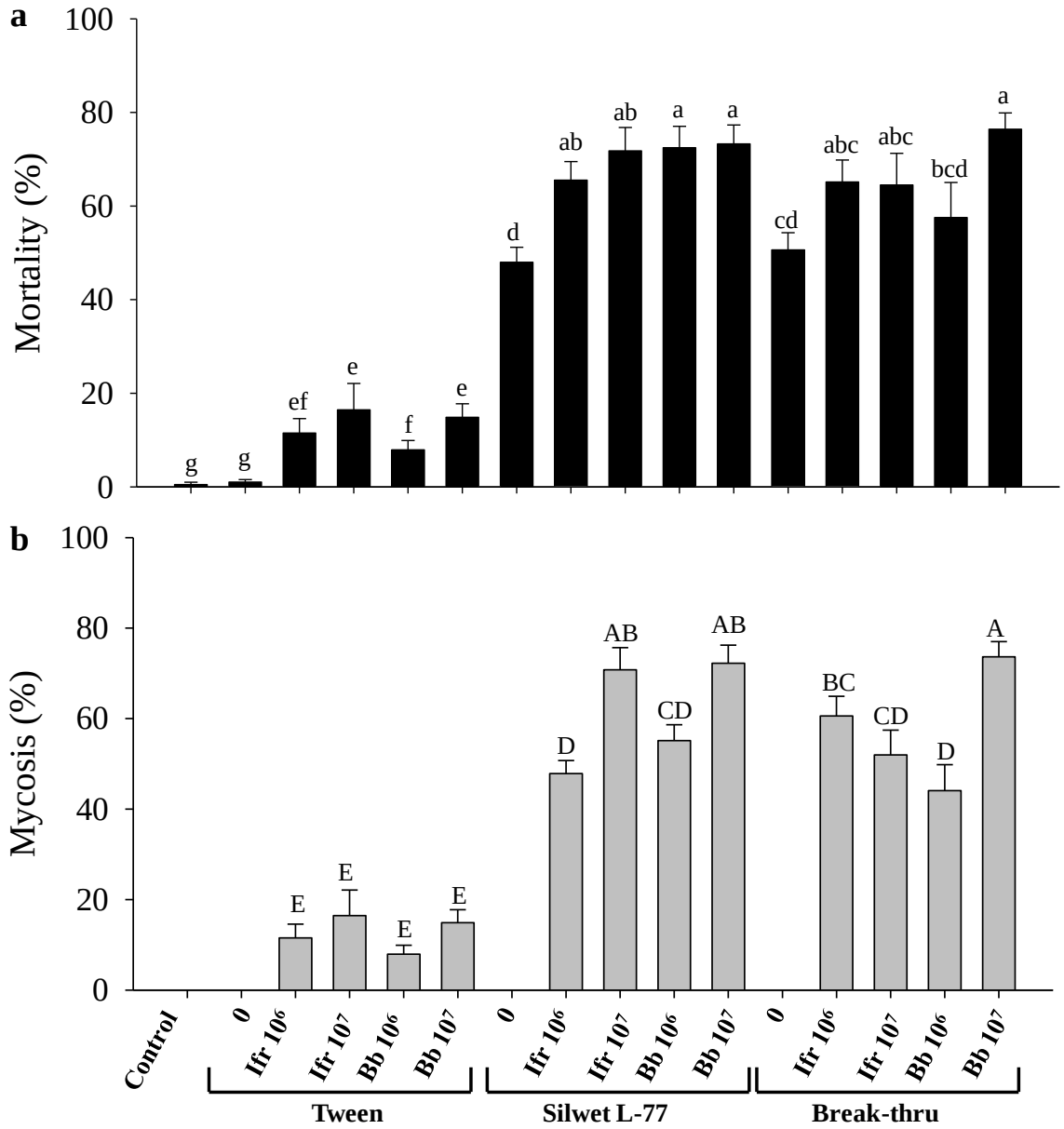

Figure 4.5 - Mortality (a) and mycosis (b) of late nymphs (3^{rd}–4^{th} instars) of *B. tabaci* biotype B 7 days after exposure to conidial suspensions (Bb = *B. bassiana* CG1229 and Ifr = *I. fumosorosea* CG1228 at 10^6 and 10^7 conidia mL^{-1}) prepared with different non-ionic surfactants (Silwet L-77, Break-thru and Tween 80 at 200 ppm). Tween 80 at 200 ppm was used as control for surfactants. Means (±SE) followed by the same letters are not significantly different at $P < 0.05$ (LSD test)

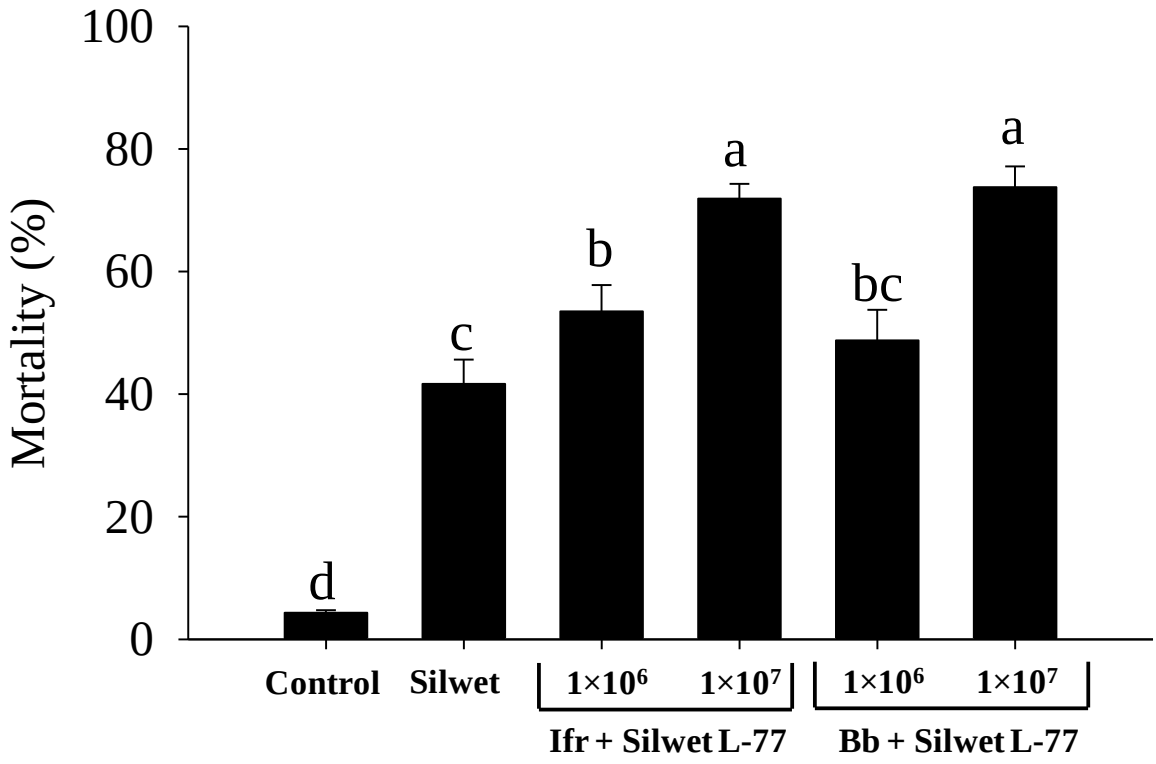

Figure 4.6 - Mortality of *B. tabaci* biotype B early nymphs (1^{st}–2^{nd} instars) 7 days after exposure to direct spray of *B. bassiana* (Bb) and *I. fumosorosea* (Ifr) (10^6 and 10^7 conidia mL^{-1}) amended with Silwet L-77 (200 ppm) on bean plants, under screenhouse conditions. Means (±SE) followed by the same letters are not significantly different at $P < 0.05$ (LSD test)

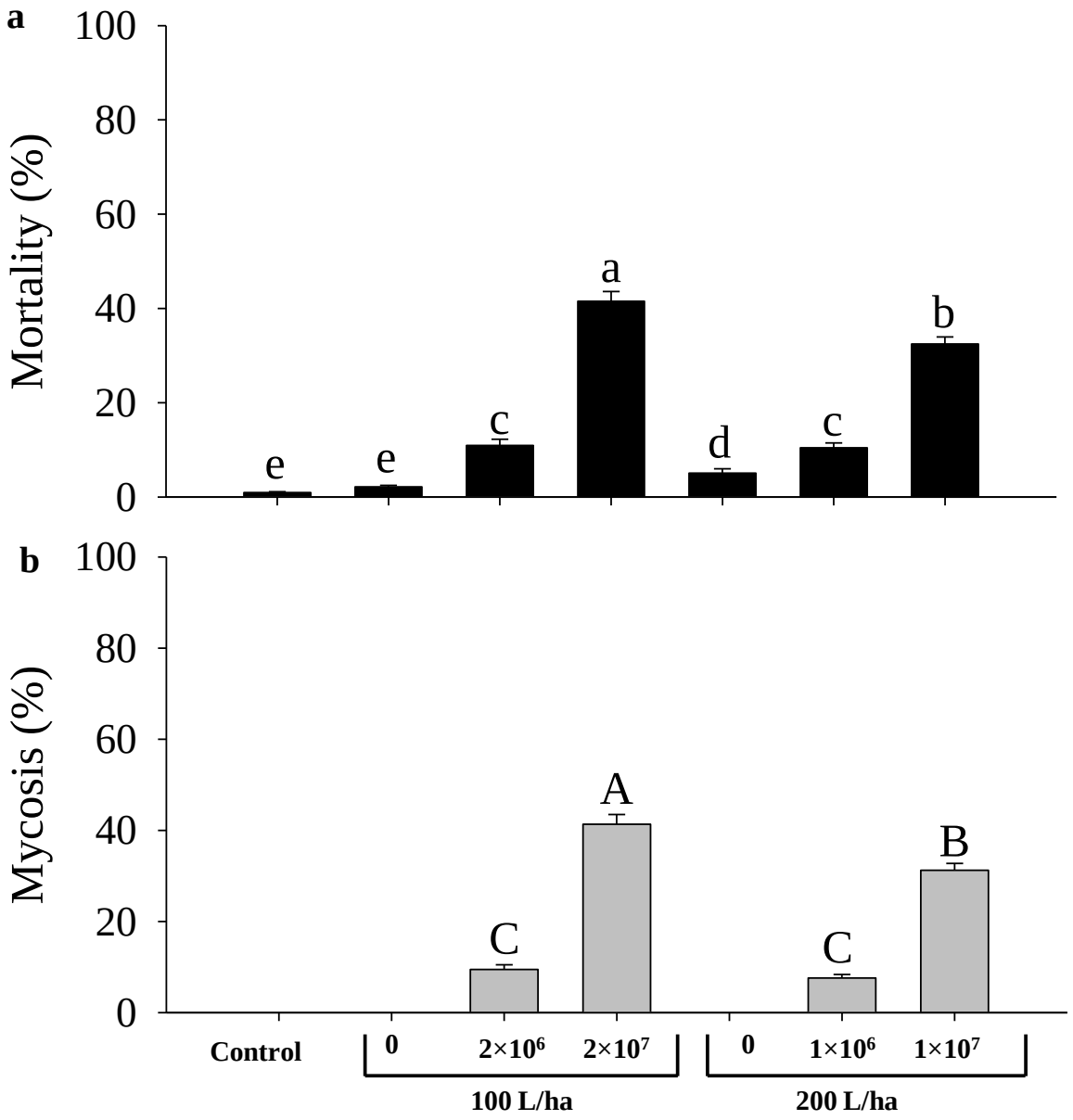

Figure 4.7 - Percentage of mortality (a) and mycosis (b) of late nymphs (3^{rd}–4^{th} instars) of *B. tabaci* biotype B reared on dry bean plants 7 days after exposure to different direct spray treatments of 10^6 and 10^7 conidia mL^{-1} of *I. fumosorosea* prepared with Silwet L-77 (200 ppm) and applied at 200 L ha^{-1}, or containing 2×10^6 and 2×10^7 conidia mL^{-1} applied at 100 l/ha, under screenhouse conditions. Conidial concentrations were doubled in order to spray the volume rate of 100 L ha^{-1} and maintain an equivalent deposition rate per leaf. Means (±SE) followed by the same letters are not significantly different at $P < 0.05$ (LSD test)

5 LIQUID CULTURE FERMENTATION FOR RAPID PRODUCTION OF DESICCATION TOLERANT BLASTOSPORES OF VARIOUS *BEAUVERIA BASSIANA* AND *ISARIA FUMOSOROSEA* STRAINS [4]

Abstract

A major constraint to the commercial use of fungal biocontrol agents is the availability of low-cost production media and processes. Previous attempts in producing *Beauveria* blastospores using liquid culture fermentation processes required long fermentation times (6–8 days) and produced cells that had poor survival after desiccation and storage. In this study, isolates of *Beauveria bassiana* and *Isaria fumosorosea* were evaluated for blastospore yield, desiccation tolerance, storage stability, and biocontrol efficacy using fermentation media containing acid hydrolyzed casein or cottonseed flour as the nitrogen source. Cultures of *B. bassiana* and *I. fumosorosea* grown in media containing cottonseed flour produced high blastospore concentrations (> 1×10^9 mL^{-1}) after 3 days which is comparatively less expensive nitrogen source than acid hydrolyzed casein. The resultant air-dried blastospores (< 3% moisture) of all fungal isolates survived drying (61–86% viability), irrespective of the nitrogen source tested. Storage stability at 4 °C varied with nitrogen source and fungal strain. Air-dried blastospores of *B. bassiana* strains showed half-lives > 13 months in contrast to 9.2–13.1 months for *I. fumosorosea.* Blastospores of *B. bassiana* and *I. fumosorosea* killed *Bemisia tabaci* whitefly nymphs faster and required lower concentrations compared with aerial conidia. Our findings support the use of liquid culture fermentation as a cost-effective process to rapidly produce high yields of stable and infective blastospores of either *B. bassiana* or *I. fumosorosea*. These results support further evaluation of blastospore sprayable formulations for the control of soft-bodied insects.

Keywords: Mycopesticides; Dimorphic growth; Submerged fermentation; Desiccation tolerance; Silverleaf whitefly; Storage stability

5.1 Introduction

Insect fungal pathogens are useful as biological control agents due to their ability to infect a wide range of insect pests and potential for mass-production. Production and formulation are key components to their success as commercial products. There are different methods for mass production, including solid substrate fermentation (SSF) for aerial conidia and liquid culture fermentation (LCF) for yeast-like blastospores, microcycle conidia, and microsclerotial (HEGEDUS et al., 1992; JACKSON et al., 1997; JARONSKI; JACKSON, 2012; LI et al., 2010). To date, the majority of the ascomycete fungal entomopathogens (Ascomycota: Hypocreales) deployed in inundative biocontrol strategies include *Beauveria bassiana* sensu lato (Bals.) Vuill., *B. brongniartii* (Sacc.) Petch, *Isaria fumosorosea* Wise

[4] MASCARIN, G.M.; JACKSON, M.A.; KOBORI, N.N.; BEHLE, R.W.; DELALIBERA JR., I. Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. **Journal of Invertebrate Pathology** (doi:10.1016/j.jip.2014.12.001).

(formerly *Paecilomyces fumosoroseus*), *Lecanicillium longisporum* and *L. muscarium* (Petch) R. Zare and W. Gams (formerly *Verticillium lecanii*), and *Metarhizium anisopliae* sensu lato (Metsch.) Sorokin. Aerial conidia comprise the main active ingredient of these mycoinsecticides that are mostly produced using solid culture techniques (FARIA; WRAIGHT, 2007; JARONSKI; JACKSON, 2012). Unfortunately, fermentation time for sporulation on solid substrates generally requires weeks and the process is labor-intensive with a high risk of contamination, resulting in high production costs. Liquid fermentation technology, on the other hand, can overcome these production drawbacks by providing more economical scale-up capabilities to produce different fungal propagules under controlled nutritional and environmental conditions (JACKSON; CLIQUET; ITEN, 2003). Due to the short fermentation time of a few days, the ease of product recovery, the automation of the process, and the availability of inexpensive media components, liquid fermentation is considered the most cost-effective method to produce fungal biocontrol agents (JACKSON, 1997). Since fungal entomopathogens possess a wide genetic variability and respond differently when growing in liquid media, suitable strain-specific parameters must be considered while evaluating and optimizing liquid culture production parameters (KLEESPIES; ZIMMERMANN, 1992). Previous works with *I. fumosorosea* have defined nutritional and environmental conditions that support the rapid production of high concentrations of desiccation tolerant blastospores (JACKSON, 1999, 2012; JACKSON et al., 1997, 2003). However, the commercial use of blastospores of *B. bassiana* is nonexistent due to long fermentation times (> 5 days), poor desiccation tolerance and shelf life of its blastospore formulations.

After carbon and oxygen, nitrogen is the most abundant element in fungal cells and is one of the most expensive nutrients in the fermentation media (ZABRISKIE et al., 2008). Identifying low-cost sources of nitrogen is critical in developing a suitable biopesticide production medium. Inexpensive nitrogen sources, such as cottonseed and soy flours, are generally unrefined containing mainly proteins and oligopeptides. These agricultural and food processing by-products are less expensive than more highly refined nitrogen sources such as acid or enzymatically hydrolyzed casein, soy hydrolysates, or meat proteins, which contain high amounts of free amino acids. Previous studies have demonstrated the feasibility of producing blastospores of *I. fumosorosea* in short fermentation times (< 3 days) with good desiccation tolerance using more refined nitrogen sources such as acid hydrolyzed casein (JACKSON, 2012; JACKSON; CLIQUET; ITEN, 2003; MASCARIN; ALVES; LOPES, 2010). However, the impact of less-refined nitrogen sources, such as cottonseed flour, on

culture growth and blastospore production, desiccation tolerance, storage stability, and bioefficacy of *B. bassiana* and *I. fumosorosea* needs to be investigated.

Fungal entomopathogens are considered the frontline of biorational tools to manage populations of whiteflies with the potential to be integrated with application of synthetic chemical insecticides in order to mitigate development of insect resistance to chemical insecticides. A wealthy body of literature has focused on the use of aerial conidia of *B. bassiana* and *I. fumosorosea* deployed as contact bioinsecticides for the control of whiteflies (FARIA; WRAIGHT, 2001; MASCARIN et al., 2013; WRAIGHT et al., 1998). Several studies demonstrated that the yeast phase (blastospore) of *I. fumosorosea* was more effective than aerial conidia in controlling whiteflies, subterranean termites, planthoppers, aphids and beetles (BEHLE et al., 2006; JACKSON, 1997; POPRAWSKI; JACKSON, 1999; SHAPIRO-ILAN et al., 2008). In our recent whitefly control study using aerial conidia, we identified virulent Brazilian isolates of *B. bassiana* and *I. fumosorosea* that were efficacious in infecting and killing various life stages of the silverleaf whitefly *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) (MASCARIN et al., 2013). At the present time, neither *B. bassiana* nor *I. fumosorosea* blastospores have been commercially developed in Brazil for use as insect biocontrol agents. Moreover, due to the economic importance of *B. tabaci* biotype B to food security in Brazil and throughout the world, the development of a cost effective liquid fermentation production process for rapidly producing stable and infective blastospores of these entomopathogens warrants investigation.

The goal of this study was to determine the blastospore production potential of selected Brazilian isolates of *B. bassiana* and *I. fumosorosea* by evaluating nutritional and environmental conditions for the rapid production of high concentrations of blastospores. General nutritional and environmental conditions shown that support rapid blastospore production by *I. fumosorosea* using liquid culture fermentation were used to compare two different nitrogen sources. Biomass accumulation and blastospore concentration were used as measures of growth, and blastospores produced under these conditions were analyzed for desiccation tolerance, storage stability, and biocontrol efficacy against *B. tabaci* biotype B.

5.2 Development

5.2.1 Materials and Methods

5.2.1.1 Fungi and inoculum preparation

Five isolates of *B. bassiana* and five isolates of *I. fumosorosea* were tested in this study. The majority of the fungal isolates originated from Brazil with details given in Table 5.1. The isolates ARSEF 3581 of *I. fumosorosea* and GHA (ARSEF 6444) of *B. bassiana*, currently designated the active ingredient of the commercial bioinsecticide known as Mycotrol® (Laverlam International Corp., Butte, MT, USA), served as standards for the liquid culture studies. Brazilian fungal isolates were previously identified using a molecular technique based on gene sequencing.[14] Stock cultures of these fungi were grown on potato dextrose agar ([PDA] Difco®, Detroit, MI, USA) in Petri dishes for 2–3 weeks at 22 ± 2 °C with a 12:12 h (L:D) photoperiod until sporulation, cut into 1 mm^2 agar plugs and stored in 10 % glycerol in sterile cryovials at –80 °C. To produce conidial inoculum, frozen stock cultures were used to inoculate PDA plates that were incubated for 2–3 weeks until cultures sporulated on the plates.

5.2.1.2 Media and culture conditions

Liquid media used for pre-cultures and blastospore production contained the following basal salts per liter: KH_2PO_4, 2.0 g; $CaCl_2.2H_2O$, 0.4 g; $MgSO_4.7H_2O$, 0.3 g; $CoCl_2.6H_2O$, 37 mg; $FeSO_4.7H_2O$, 50 mg; $MnSO_4.H_2O$, 16 mg; $ZnSO_4.7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500 mg each; folic acid, biotin, vitamin B12, 50 mg each (JACKSON et al., 1997). All chemicals used were obtained from Sigma® (St. Louis, MO, USA) unless otherwise stated. The pre-culture basal salts medium was supplemented with glucose (Fisher Scientific®) at 80 g L^{-1} (40% carbon [C]) and acid hydrolyzed casein (derived from bovine milk, Hy-case™ MSF, Kerry Bioscience, New York, NY, USA) at 25 g L^{-1} (8.5% nitrogen [N] and 53% C), which produced a medium with a carbon-to-nitrogen ratio (C:N) of 23:1. The pre-culture medium had an initial pH of 5.8 and pH was not adjusted during cultivation. Glucose stock solutions (20% w/v) were autoclaved separately and added prior to inoculation. Sterilization of liquid cultures and glucose stock solutions were performed at 123 °C for 20 min. All media were prepared with double-deionized water (ddH_2O). Conidial inocula for pre-cultures were harvested by scraping plates with 10 mL of sterile aqueous solution of 0.04% polyoxyethylene sorbitan mono-oleate (Tween® 80, Sigma®, St. Louis, US). Pre-cultures were inoculated with conidial suspensions to deliver a final concentration of 5×10^5 conidia mL^{-1} for *I. fumosorosea* and 1×10^6 conidia

mL^{-1} for *B. bassiana* in the liquid culture medium. One hundred mL pre-cultures of all fungi were grown in 250-mL baffled, Erlenmeyer flasks (Bellco Glass, Vineland, NJ, USA) for 3 days at 28 °C and 350 rpm using a rotary incubator shaker (INNOVA 4000, New Brunswick Scientific, Edison, NJ). Conidial and blastospore concentrations were measured microscopically using a hemacytometer (400X magnification) with a phase-contrast microscope with DIC optics (BH2, Olympus America, Center Valley, PA, USA).

5.2.1.3 Experimental design for liquid fermentation studies

A factorial experimental design was used to investigate the impact of nitrogen source on different *B. bassiana* and *I. fumosorosea* isolates grown in submerged liquid cultures. *Isaria* and *Beauveria* cultures were grown in 50 mL or 100 mL medium volume in 250-mL Erlenmeyer flasks and incubated at 28 °C and 350 rpm in a rotary shaker incubator. The culture volume was altered to increase aeration rates, where 50 mL would give more aeration than 100 mL medium (JACKSON, 2012). The blastospore production medium contained the previously described basal salts medium supplemented with 100 g glucose L^{-1} and acid hydrolyzed casein or cottonseed flour (9.4% N and 40% C; Pharmamedia®, Traders Protein, Memphis, TN, USA) tested at a concentration of 25 g L^{-1}. The medium had an initial pH ~ 5.5 and a C:N ratio of 21:1. Blastospore inocula (eq. 10% total volume) were obtained from 3-day-old pre-cultures (exponential growth phase) providing a final inoculum concentration of 5×10^6 blastospores mL^{-1}. Flasks were hand-shaken frequently during the fermentation process to minimize mycelial growth and sporulation on the flask walls. In all experiments, pH was uncontrolled during culture growth.

During culture broth sampling and dilution, blastospore suspensions were constantly vortexed to ensure homogeneity. Dry weight was used as a measure of biomass accumulation. Duplicate 1-mL culture broth samples were collected from flasks, the biomass separated from the spent medium by vacuum filtration (model 1225, Millipore®) onto pre-weighed 2.4-cm glass fiber filter disks (G6, Fisher Scientific®, Pittsburgh, PA, USA), and then dried at 80 °C for 24 h until constant weight prior to measurement. Additionally, final pH from all culture broths was recorded. All shake-flask culture experiments were run in duplicates and experiments were conducted at least three times on different dates.

5.2.1.4 Harvesting, drying and storage studies

After growing *B. bassiana* and *I. fumosorosea* for 3 days in 100 mL liquid media, the whole culture was mixed with 7.5% (w/v) diatomaceous earth [DE (HYFLO®, Celite Corp., Lompoc, CA, USA)]. The blastospore-DE mixtures were vacuum-filtered using a Buchner funnel with 12.5-cm filter paper disks (Whatman No. 1, Maidstone, England). The resulting filter cake from each replicate flask was crumbled, placed on 10-cm Petri dish, and dehydrated overnight at ~22 °C using an air drying chamber with lateral air inflow and controlled RH atmosphere (RH ~ 50–60%) for 16–20 h to achieve moisture less than 4% (w/w) (JACKSON; PAYNE, 2007). Dried blastospore preparations were broken up by pulsing in a blender (Mini Prep® Plus, Cuisinart, East Windsor, NJ, USA), vacuum packaged at 999 mbar (eq. < 0.021% oxygen) (Multivac Inc., Kansas City, MO, USA) in nylon polyethylene bags (15.3 × 21.8 cm), and stored at 4 °C. The whole methodological procedure is illustrated in Figure 5.1. After drying, the moisture content (on wet basis [w.b.]) and water activity (a_w) of these formulations were measured before storage with a moisture analyzer (Mark II, Denver Instruments, Arvada, CO, USA) and a water activity analyzer (AquaLab 4TEV, Decagon Devices, Inc., Pullman, WA, USA), respectively, and considered as potential covariates.

At least 4 packages for each fungal isolate were obtained from different fermentation batches and monitored over time to assess the survivorship of air-dried blastospores. The viability of all air-dried blastospore preparations was determined immediately after drying and during storage using a previously described germination assay (JACKSON et al., 1997). Briefly, we employed a fast-rehydration assay to assess germination by adding ~25 mg of air-dried blastospore preparation into 25 mL of potato dextrose broth (PDB, Difco®) in a 125-mL baffled, Erlenmeyer flask. After 6 h incubation for *I. fumosorosea* and 7 h incubation for *B. bassiana* at 28 °C and 300 rpm in a rotary shaker incubator, percentage viability was determined microscopically by examining 200 discrete blastospores per replicate flask for germ tube formation. Blastospores were considered to have germinated when germ tube was any size visible at 400X magnification. Stability studies were conducted on samples stored under refrigerated conditions (4 °C) and blastospore viability monitored monthly over a period of 13 months using the previously described germination protocol upon fast-rehydration.

To determine the relationship between a_w and moisture content, sorption isotherms were established for *B. bassiana* and *I. fumosorosea* formulated with 7.5% diatomaceous earth (w/v). Different saturated salt solutions were prepared to create different equilibrium relative humidities (ERH) using the following salts (WINSTON; BATES, 1960): sodium

hydroxide (NaOH), lithium chloride (LiCl), magnesium chloride ($MgCl_2.6H_2O$), potassium carbonate (K_2CO_3), sodium chloride (NaCl), potassium chloride (KCl), and potassium sulfate (K_2SO_4) that corresponded to a_w values of 0.082, 0.113, 0.328, 0.432, 0.753, 0.843, and 0.973, respectively. All salts were purchased from Sigma®. To achieve very low ERH, Drierite® (anhydrous calcium sulfate, 8 mesh, W.A. Hammond Drierite Company, Xenia, OH, USA) was used as a standard desiccant agent and provided 0.0221 a_w. Salt solutions were added to the bottom of vacuum desiccators (206 mm height × 149 mm inside diameter) (Bel-Art Products®, Wayne, NJ, USA), and samples were incubated at 25 °C upon vacuum for seven days before readings.

5.2.1.5 Insects

Founders of *B. tabaci* biotype B colony was originally obtained from Apopka, FL, USA, in 2013 and raised on cabbage cv. 'Bravo' (*Brassica oleracea* L.; Harris Seeds, Rochester, NY, USA) and blue lake bush bean (*Phaseolus vulgaris* L. [Kelly Seed Co., Peoria, IL, USA]) with plants confined in 0.6 m^3 PVC-frame cages covered with fine screen fabric (off-white dracon chiffon material, BioQuip Products Inc., Rancho Dominguez, CA, USA) under greenhouse conditions (temperature range: 25–35 °C). All plants were grown in a soil potting medium prepared with 77.5 L of pasteurized Redi Earth® growing mix (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) amended with 60 g micromax granular and 400 g osmocote 14-14-14 (N-P-K). Untreated seeds were used and plants were grown free of chemical pesticides.

5.2.1.6 Virulence of fungal infective propagules against whitefly

To compare the virulence between blastospores and aerial conidia of *B. bassiana* and *I. fumosorosea*, dosage-response bioassays were conducted against newly emerged second-instar *B. tabaci* biotype B nymphs following a previous protocol (MASCARIN et al., 2013). Laboratory bioassays were carried out with *B. bassiana* (ESALQ1432) and *I. fumosorosea* (CG1228). Blastospores were produced in a liquid culture medium, as previously described, containing 100 g glucose L^{-1} and 25 g cottonseed flour L^{-1}, and harvested after 3 days incubation at 28 °C and 350 rpm. Blastospore preparations (size range 3–11 µm) were formulated with 7.5% diatomaceous earth and then subjected to air drying until < 4% moisture, while fresh aerial conidia (size range 1.8–5.0 µm) were grown on PDA plates for 10–14 days at 22 °C and 12:12 (L:D) h photoperiod prior to use in bioassays. Original

suspensions of both spores were prepared with a solution of Tween 80 at 0.01% and filtered through a sterile double layer of cheesecloth to remove clumps and most of the diatomaceous earth from blastospore preparations. Desired concentrations were adjusted through serial dilutions using Tween 80 (0.01%) and then adjusted to 1×10^5, 5×10^5, 2.5×10^6, 1.25×10^7, and 6.25×10^7 spores mL^{-1}, which corresponded to applied dosages of 1.26×10^2, 6.82×10^2, 3.7×10^3, 2.0×10^4, and 1.09×10^5 spores cm^{-2}, respectively. Controls consisted of nymphs sprayed with 0.01% Tween 80 solution. The viability of blastospores used in all bioassays was > 75% after 6 h (for *I. fumosorosea*) and 7 h (for *B. bassiana*) incubation in PDB, while aerial conidia retained > 90% viability after 17 h incubation on PDA at 25 °C. Briefly, individual bean leaves were placed in polystyrene Petri dishes (Falcon®, 100 × 15 mm) lined with 20 mL of water agar (2%, w/v), hereafter referred to as 'ventilated plates'. Each bean leaf, previously infested with 50–70 early second-instar nymphs, was sprayed using a micro-sprayer tower set to 10 PSI and 3 sec of application time. There were five replicates per fungal-dosage and all dosages were assayed at the same time. The entire experiment was conducted at least twice on different dates using different fungal batches and insect cohorts. After treatment, ventilated plates were inverted so that the abaxial side of the leaf faced down with the adaxial side touching the agar and then incubated in a growth chamber at 27 ± 1 °C, 70% (48–78%) RH and 14:10 (L:D) h photoperiod for six days before assessing mortality. Only nymphs showing signs of infection or symptoms from fungal disease (i.e., mycosis) were scored as dead individuals six days after application to estimate the median lethal concentration (LC_{50}), expressed as spores cm^{-2}. To compare the speed of kill between blastospores and aerial conidia based on median survival time (ST_{50}), the same protocol mentioned above was used, but instead performing a single-dosage bioassay at a concentration of 1.25×10^7 spores mL^{-1} (i.e., 2.7×10^4 spores cm^{-2}). Nymphal mortality was recorded daily up to six days after treatment.

5.2.1.8 Statistical analysis

Experiments were carried out with a completely randomized design and repeated two to three times to ensure reproducibility. Linear mixed models were used to fit data on blastospore concentration and biomass accumulation with Gaussian (normal) distribution from the fermentation studies using the SAS macro PROC MIXED. The dependent variable "blastospore concentration" was log_{10}-transformed prior to analysis to meet homoscedasticity assumptions. Fungal isolate, nitrogen source, and fermentation day (time) were implemented as fixed factors, while shake flasks (i.e., repeated measure over time) and experimental

repetitions were declared as random effects in these models. Proportion (binary) data on blastospore viability from desiccation tolerance assays were fitted to a generalized linear mixed model with binomial distribution for errors (PROC GLIMMIX), in which experimental repetition was included in the random term, and fungal isolate along with nitrogen source comprised the fixed factors. Statistics for fixed effects and their interaction terms were also determined by Wald type III *F*-test. Treatment means were separate by post-hoc pair-wise multiple comparisons using Tukey's honestly significant difference (HSD) test at $P < 0.05$. Time-course data on blastospore survival at 4 °C storage were computed as % relative germination [(% germination treatment – germination control) / (100 – % germination control)] and then fitted to a logistic, 4-parameter nonlinear model to estimate the half-lives ($t_{1/2}$) of air-dried blastospores produced with different nitrogen sources. The model had the following notation: $S = S_0 + (\alpha/(1 + (t/t_0)^{\beta}))$, where S is the blastospore survival (% germination), t is the storage time (in months) and α, β (slope), S_0 and t_0 are the best fit constants estimated by the interactive analysis performed in SAS macro PROC NLIN. To test the hypothesis that nitrogen source could affect the storage stability of air-dried blastospores across incubation time, the sum-of-squares reduction test was employed to compare their nonlinear regressions. The relationship between a_w and moisture content for air-dried DE-formulated blastospores was explained by fitting the experimental results to the GAB (Guggenheim-Anderson-de Boer) model to draw the moisture sorption isotherm curves (SCHÄR; RÜEGG, 1985). We also tested whether a_w and moisture content could have any influence on the viability data for blastospores based on Spearman's correlation (PROC CORR). Abbott's formula (ABBOTT, 1925) was applied to correct percentage mortality before estimating dosage-response mortality relationships between different fungal spores and second-instar nymphs using a logistic model (PROC PROBIT). The logistic model was chosen as it provided the best fit due to its lowest deviance. Median lethal concentration (LC_{50}) with its corresponding confidence limits (95% CL) and slope were consecutively calculated for each spore type. Virulence of spore type was compared through the pair-wise ratio test at $\alpha = 5\%$ applied to LC_{50} (WHEELER et al., 2007). Median survival times (ST_{50}) of whitefly nymphs exposed to different fungal treatments were estimated by the survival analysis with Kaplan-Meier method (PROC LIFETEST). Differences in speed of kill between blastospores and aerial conidia were considered significant based on the non-overlapping of 95% confidence limits for ST_{50} values. All analyses were performed in the Statistical Analysis System v.9.2 (SAS Institute Inc., Cary, NC).

5.2.2 Results

5.2.2.1 Blastospore yields at different nitrogen sources and aeration rates

Fungal isolates responded significantly different in terms of yeast-like growth when cultured in liquid media supplemented with different sources of nitrogen (i.e., acid hydrolyzed casein or cottonseed flour at 25 g L^{-1}) and altering the culture volumes to improve aeration. Blastospore production increased for some *B. bassiana* isolates mainly by day 3 of growth ($F_{4,84}$ = 18.55, P < 0.0001), and yields were generally more expressive when grown with cottonseed flour ($F_{4,84}$ = 10.96, P < 0.0001) (Figures 5.2). When examining *I. fumosorosea*, the nitrogen source by itself was not the major factor contributing to blastospore production ($F_{1,62}$ = 2.93, P = 0.0918), but rather yields were markedly influenced by culture volume and nitrogen ($F_{1,62}$ = 27.57, P < 0.0001), although significant variation was found among isolates grown with different nitrogen compounds ($F_{4,62}$ = 2.69, P = 0.0394) (Figure 5.3). Blastospore production increased for most *B. bassiana* isolates by day 3 of growth ($F_{4,84}$ = 18.55, P < 0.0001), whereas most *I. fumosorosea* isolates did not show improvement in production with the increase of fermentation time ($F_{4,62}$ = 1.2, P = 0.32). Blastospore counts usually reached greater numbers by day 3 of growth for both *B. bassiana* ($F_{1,84}$ = 361.79, P < 0.0001) and *I. fumosorosea* ($F_{1,62}$ = 156.52, P < 0.0001), although few isolates did not produce significantly more blastospores at day 3 compared with day 2. Overall, cottonseed flour promoted better yeast-like growth than acid hydrolyzed casein for *B. bassiana* isolates ($F_{1,84}$ = 48.86, P < 0.0001). Comparisons among *Isaria* isolates revealed that CG1228 attained the highest concentration of blastospores with maximum yield achieved at day 3 (2.6–2.8 × 10^9 blastospores mL^{-1}), regardless of the nitrogen source tested. Under the same fermentation conditions, the best blastospore-producing isolates of *B. bassiana* were CG1229, GHA and ESALQ1432 grown in liquid media with cottonseed flour rather than acid hydrolyzed casein by producing 0.9–1.2 × 10^9 blastospores mL^{-1} in 3 days growth. All isolates of *I. fumosorosea* were able to produce more than 1 × 10^9 blastospores mL^{-1} by day 3, whereas 3/5 of the *B. bassiana* isolates reached this high concentration. In addition, *I. fumosorosea* isolates depicted a faster yeast-like growth pattern when compared with *B. bassiana* isolates, considering that these fungi were inoculated at the same initial inoculum density. Among all fungal isolates tested, ESALQ-PL63 showed the poorest yeast-like growth, as the hyphal growth was predominantly induced during fermentation resulting in thicker culture broth. Finally, the final pH of *B. bassiana* and *I. fumosorosea* culture broths had increased acidity and were within the range of 3.8–4.8 and 3.6–4.9, respectively.

5.2.2.2 Desiccation tolerance

The resultant blastospores in the dry form mixed with diatomaceous earth of all fungal isolates retrieved metabolic activity upon fast-rehydration when incubated for 6–7 h in PDB with retained spore viabilities higher than 61%. Drying 3-day-old blastospores to < 4% moisture content (a_w < 0.3) revealed a significant interaction effect between nitrogen source and fungal isolate on blastospore viability for *I. fumosorosea* ($F_{4,29}$ = 4.90, P = 0.0038) and *B. bassiana* ($F_{4,56}$ = 5.26, P = 0.0011). Blastospore survival after air-drying significantly varied across isolates within *B. bassiana* ($F_{4,56}$ = 16.5, P < 0.0001) as well as within *I. fumosorosea* ($F_{4,29}$ = 12.96, P < 0.0001). When examining desiccation tolerance among *B. bassiana* isolates, blastospores of ESALQ1432 and ESALQ447 exhibited better desiccation tolerance by retaining greater viability (70–86% survival) when produced with cottonseed flour rather than acid hydrolyzed casein ($F_{1,56}$ = 14.06, P = 0.0004) (Table 5.2). By contrast, most the *I. fumosorosea* isolates retained greater blastospore viability (74–80% survival) when grown in media containing acid hydrolyzed casein rather than cottonseed flour (65–74% survival) ($F_{1,29}$ = 8.37, P = 0.0072), while others showed better cell survival when cultured with cottonseed flour (e.g., ESALQ1409) or was not affected by the nitrogen source (e.g., ARSEF3581). These results suggest that stress tolerance caused by convective air drying process to liquid produced blastospores varied with the type of nitrogen used in the liquid production media and with the isolate within each fungal species. After air-drying, the moisture sorption isotherms were determined at 25 °C to describe the relationship between a_w of fungal biomass (blastospores + mycelium) formulated with 7.5% DE and moisture content (%, w/w). Experimental data were significantly explained by the GAB model (*B. bassiana*: R^2 = 0.99, $F_{3,28}$ = 3666.55, P < 0.0001; *I. fumosorosea*: R^2 = 1.00, $F_{3,21}$ = 2738.01, P < 0.0001) that assumed a sigmoidal shape curve (Figure 5.4). Water activities (a_w) of air-dried blastospores of *B. bassiana* isolates ranged from 0.251–0.364 with corresponding moisture contents of 1.38–2.70%, while for *I. fumosorosea* isolates the a_w varied from 0.270–0.323 that corresponded to 1.76–2.63% water content. Neither water activity nor moisture content was significantly correlated with initial blastospore survival rates after drying for either fungal species (Spearman correlation: $-0.02 \leq r \leq 0.30$, $0.06 \leq P \leq 0.86$).

5.2.2.3 Storage

The long-term storage stability of air-dried blastospores under refrigerated conditions varied with the fungal isolate and nitrogen source (Figures 5.5, 5.6). The logistic model with

four parameters fitted well (R^2 = 0.74–0.99, $P < 0.01$) the experimental data on blastospore survival across storage time for all isolates of *B. bassiana* and *I. fumosorosea* produced with either nitrogen source. According to the sum-of-squares reduction test used to compare the nonlinear regressions for blastospore survival curves, it was found that the nitrogen source did not influence the survivorship of air-dried blastospores of *B. bassiana* over the period of 13 months under refrigerated storage conditions, except the isolates CG1229 and GHA that survived considerably longer when produced with cottonseed flour and acid hydrolyzed casein, respectively (Table 5.3, Figure 5.5). Particularly to *I. fumosorosea*, blastospores of isolates ARSEF3581, ESALQ1296, and ESALQ1364 remained viable for a longer period of time when grown in cottonseed flour, whereas the long-term viability of the other isolates was not influenced by the nitrogen source (Table 5.3, Figure 5.6). Generally, survival curves of *I. fumosorosea* blastospores depicted a faster decline pattern compare with *B. bassiana*, regardless of the nitrogen source. Estimates of half-lives for *B. bassiana* air-dried blastospores stored at 4 °C exhibited a minimum time of 14.1 months for GHA grown in cottonseed flour, while most isolates retained a half-life longer than 14 months (Table 5.3). By contrast, *I. fumosorosea* air-dried blastospores showed the shortest half-life (9.2 months) with ESALQ1296 grown in acid hydrolyzed casein. While the longest half-life (13.1 months) was achieved by *I. fumosorosea* ARSEF3581 grown in cottonseed flour. In 3/5 *I. fumosorosea* isolates, cottonseed flour supported greater half-lives.

5.2.2.5 Virulence against whitefly

According to the LC_{50} values, *B. bassiana* blastospores were 78% more virulent toward whitefly nymphs compared with aerial conidia and this result was statistically significant as the 95% confidence limits for the ratio test did not include "1" (Table 5.4). In addition, the median survival time of nymphs (ST_{50}, time where 50% of insects survive) exposed to aerial conidia of *B. bassiana* was significantly longer than that calculated for blastospores indicating an enhanced speed of kill (25% faster) for blastospores (Table 5.5). Blastospores of *I. fumosorosea* were also 41% more lethal as well as 25% faster in killing whitefly nymphs in comparison to conidia. Overall, all fungal treatments reduced the survival rates of whitefly nymphs compared with the controls (Log-rank test: χ^2 = 1312.44, df = 4, $P <$ 0.0001) (data not shown). All nymphal cadavers infected by both types of propagules supported fungal outgrowth that subsequently sporulated confirming mycosis. No differences were observed concerning the proportion of mycosed cadavers between blastospores- and conidia-treated nymphs.

5.2.3 Discussion

In the present study, we demonstrated that liquid fermentation can be used to rapidly produce high concentrations of stable, efficacious blastospores of various isolates of *B. bassiana* and *I. fumosorosea* isolates using a low cost medium containing glucose and cottonseed flour. While the basic medium composition using acid hydrolyzed casein as the nitrogen source coupled with highly aerated culture conditions had been shown in previous studies to support high numbers of blastospores ($> 1 \times 10^9$ mL^{-1}) by cultures of *I. fumosorosea* ARSEF 3581 (JACKSON et al., 2003; JACKSON, 2012), this is the first report of the rapid production (2–3 day fermentation) of high blastospore yields by Brazilian isolates of *I. fumosorosea* or by any *B. bassiana* isolate (Tables 5.2, 5.3). Comparison of these two nitrogen sources for blastospore production revealed that either cottonseed flour or acid hydrolyzed casein used at 25 g L^{-1} and amended with 100 g L^{-1} glucose supported high blastospore concentrations ($> 1 \times 10^9$ blastospores mL^{-1}) for different isolates of *B. bassiana* and *I. fumosorosea* within a short fermentation time. Cottonseed flour, which is an unrefined nitrogen source and therefore significantly less expensive than hydrolyzed casein, worked as well and sometimes better than acid hydrolyzed casein for blastospore production. Relative to the initial inoculum concentration, the best producing isolates of both insect fungal pathogens had an increase of 160–560 times in blastospore concentration in 3 day fermentation. Therefore, we documented significant yield efficiency gains in our fermentation process over earlier published reports of *Beauveria* blastospores using liquid culture fermentation processes, which required longer fermentation times (6–8 days) that resulted in lower yields and produced cells that had poor survival after desiccation and storage (CHONG-RODRIGUEZ et al., 2011; HUMPHREYS; MATEWELE; TRINCI, 1989; ROMBACH, 1989; SAMSINAKOVA, 1966; VEGA et al., 2003, VIDAL et al., 1998).

Fungal dimorphic growth in submerged liquid cultures are mediated by a number of factors, including length of incubation time, inherent features of the isolate, media composition, C:N ratio, environmental conditions during fermentation (pH, osmotic pressure, temperature, dissolved oxygen), and inoculum size (RUIZ-HERRERA, 1985). Among the environmental factors, yeast-like growth of various fungal genera has shown to be profoundly affected by gaseous environment, including *I. fumosorosea* and *Metarhizium flavoviridae* that require higher aerobic conditions to switch from mycelium to blastospores (ISSALY et al., 2005; JACKSON, 2012). Likewise, our data indicate that *B. bassiana* is also responsive to

elevated oxygen levels in the liquid medium that was promoted by the higher surface to volume ratio for the low volume cultures. Our results indicate that the oxygen level in liquid cultures of *B. bassiana* and *I. fumosorosea* plays an important role in promoting rapidly production of blastospores. Therefore, dimorphic entomopathogenic fungi will require elevated oxygen levels to grow 'yeast-like' in submerged cultures.

The ability of liquid culture produced blastospores to survive drying and remain viable during storage is critical requirements for their use as a microbial biocontrol agent (FARIA et al., 2011; HONG et al., 2000). In our study, 61–86% of the blastospores of *I. fumosorosea* or *B. bassiana* produced with either acid hydrolyzed casein or cottonseed flour survived air-drying to less than 3% moisture as measured using our stringent 6 or 7 h blastospore germination assay. The desiccation tolerance of blastospores during convective air-drying varied greatly among isolates within and between fungal species as well as between nitrogen sources, suggesting that desiccation tolerance is intrinsic to the isolate and influenced by nutritional aspects in the liquid production medium. Long-term storage experiments under refrigerated conditions showed that *I. fumosorosea* blastospores retained half-lives of 9–13 months, whereas half-lives of *B. bassiana* isolates were greater than 14 months. These results have shown that, in general, dehydrated blastospores of *B. bassiana* are more stable as dried formulations of *I. fumosorosea* and may be better candidates for commercial development. Interestingly, blastospores produced in liquid cultures supplemented with the low-cost nitrogen source, cottonseed flour, tended to have similar or even better shelf life compared with blastospores harvested from medium supplemented with acid hydrolyzed casein. Despite the intra-specific variation among fungal isolates, cottonseed flour was the preferred nitrogen source for blastospore production, desiccation tolerance, and storage stability.

Improved shelf life and stabilization of fungal propagules during storage are among the major determinant aspects in the development of commercial biopesticides. In the present study, low a_w (< 0.35) or moisture content (< 3% moisture) were achieved by using convective air-drying process to gently dehydrate blastospores with controlled RH atmosphere (> 50% RH) in shorter period of time (16–24 h). We also described for the first time the isotherm sorption at 25 °C between a_w and moisture for blastospores of both fungi formulated with diatomaceous earth, which comprises a relevant feature in shelf life studies with microbial insecticides. The combination of cool temperature and low oxygen level in the vacuum sealed polyethylene packages containing air-dried blastospores during the storage conditions resulted in substantial extension of shelf life for all fungal isolates tested, with half-lives above 9 months for *I. fumosorosea* and 14 months for *B. bassiana*. This

demonstrates that dehydrated blastospores of *B. bassiana* appear to be more tolerant to anhydrobiosis imposed by convective air-drying process than *I. fumosorosea*. However, the storage stability of these blastospores showed a clear fungal isolate and nitrogen source dependent decline in survivorship upon fast-rehydration over the monitoring period. Interestingly, blastospores produced in liquid cultures supplemented with cottonseed flour tended to have similar or even better shelf life compared with blastospores harvested from medium amended with acid hydrolyzed casein. These results corroborate with those obtained for *I. fumosorosea* blastospores in which drying air above 50% RH improved both desiccation tolerance and shelf life under cool conditions (JACKSON; PAYNE, 2007). The storage stability of solid-substrate produced conidia of *M. flavoviridae* and *B. bassiana* were improved by drying spores to low moisture content (< 5%) along with anaerobic storage (FARIA; HOTCHKISS; WRAIGHT, 2012; HONG; JENKINS; ELLIS, 2000). According to the shelf life data, intrinsic blastospore longevity under optimal storage conditions can be variable among fungal species and isolates, hence each fungal strain has to be examined separately.

The isolates ESALQ1432 of *B. bassiana* and CG1228 of *I. fumosorosea* were chosen for the bioassays against whitefly nymphs due to their high blastospore production and excellent desiccation tolerance. Bioassay data indicated that air-dried blastospores of *B. bassiana* and *I. fumosorosea,* once rehydrated and sprayed directly onto whitefly nymphs, exhibited greater virulence than aerial conidia by requiring lower lethal spore dosages and reduced time to cause death. The faster bioinsecticidal activity obtained using air-dried blastospores is likely related to their quicker germination rate and ability to evade the host immune system better than aerial conidia (HEGEDUS et al., 1992; VEGA et al., 1999). Supporting these observations, earlier studies have pointed out that *I. fumosorosea* blastospores were more efficient at infecting several soft-bodied insects compared with aerial conidia (VEGA et al., 1999; BEHLE et al., 2006; POPRAWSKI; JACKSON, 1999; SHAPIRO-ILAN et al., 2008). Although diatomaceous earth is well known to possess insecticidal activity against some insects, we tested filtered blastospore suspensions to exclude the effect of this carrier in the virulence bioassays. Additionally, early evidence also showed that this carrier by itself had no influence on whitefly nymphal survival in relation to untreated controls (JACKSON, 1999; JACKSON et al., 1997). In contrast to these findings, there have been some reports showing no significant differences in virulence between blastospores and aerial conidia against insects (KIM et al., 2013; LAN; TRINCI, GILLESPIE,

1991). Results from different studies may not be directly comparable, since fungal isolates, insect populations and experimental protocols are variable. Fast-killing fungal propagules, such as blastospores, are more desirable for the control of insects that vector plant pathogens, such as the silverleaf whitefly. Further studies would be worthwhile emphasizing the virulence mechanisms of blastospores toward different insect hosts as well as their performance under field conditions. Field application rates for conidia of *Beauveria* or *Isaria* will depend on the insect target as well as the foliar area of the crop, but sprays are generally practiced in the range of $1 \times 10^{12\text{-}13}$ conidia per hectare (ha) in Brazil, whilst in the USA the label rate can reach up to 2.5×10^{13} ha^{-1}. Depending on the fungal isolate and species tested, our production and stabilization process can yield $1–3 \times 10^{12}$ blastospores L^{-1} fermentation broth, which is very similar to yields of aerial conidia per kg of rice by solid-state fermentation (MASCARIN; ALVES; LOPES, 2010, MASCARIN et al., 2013). Based on these data and depending on the insect target as well as leaf area of the crop, 0.5 to 10 L of fermentation broth would be required to produce enough blastospores to treat one hectare.

Concerning the costs of using liquid culturing technology *versus* solid-substrate fermentation, the described liquid medium contained inexpensive ingredients, including glucose as the main carbon and energy source and cottonseed flour as the nitrogen source. Although acid hydrolyzed casein worked very well as a nitrogen source, it is relatively expensive (i.e., \$13 kg^{-1}) for use in the mass-production of insect pathogenic fungi. This study demonstrated that the use of cottonseed flour (\$1.6 kg^{-1}) would lower production costs (\$0.18 L^{-1}) while supporting the rapid production of high numbers of infective blastospores with good desiccation tolerance and extended shelf life under refrigerated conditions. In Brazil, the standard solid-substrate fermentation process for conidia of *B. bassiana* is practiced with moistened parboiled rice whose average cost is \$0.63 kg^{-1}. Therefore, despite the high initial capital investment for equipment (i.e., bioreactor), liquid fermentation technology allows large-scale production and reduced production costs in relation to solid-substrate fermentation.

Our results highlight the potential for the commercial production of *Isaria* and *Beauveria* blastospores using appropriate liquid culture fermentation processes. We have identified low cost nutritional substrates that support the formation of high concentrations of *B. bassiana* and *I fumosorosea* blastospores in a short 2–3 day fermentation. A high percentage of the blastospores of *B. bassiana* and *I fumosorosea* survived drying, remained viable for extended periods of time when stored at 4 °C, and were more effective than conidia in infecting and killing whitefly nymphs. In particular, the development of a liquid culture

production method for stable, effective blastospores of *B. bassiana* using liquid culture fermentation should facilitate the commercial development of this mycoinsecticides by reducing production costs and enabling large-scale production of a superior bioinsecticidal propagule.

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Table 5.1 - Isolates of *B. bassiana* and *I. fumosorosea* used in liquid production studies

Fungus[a]	Isolate code	Host/source	Location of collection
B. bassiana	ESALQ-PL63	*Atta* sp.	Brazil: Piracicaba-SP
	ESALQ447	*Solenopsis invicta*	Brazil: Cuiabá-MT
	ESALQ1432	*Diaphorina citri*	Brazil: Piracicaba-SP
	CG1229	*Rupela albinella*	Brazil: Arari-MA
	GHA (ARSEF6444)[b]	*Diabrotica undecimpunctata*	California, USA
I. fumosorosea	ESALQ1296	*Bemisia tabaci*	Brazil: Jaboticabal-SP
	ESALQ1364	*Myzus persicae*	Brazil: Piracicaba-SP
	ESALQ1409	*B. tabaci*	Brazil: Itapetininga-SP
	CG1228	*R. albinella*	Brazil: Arari-MA
	ARSEF3581	*B. tabaci*	USA: Mission-TX

[a] ESALQ means that isolates are maintained by ESALQ-University of São Paulo (Piracicaba, SP, Brazil), CG is the code for isolates preserved in EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil), while ARSEF designates isolates stored in ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, USA.

[b] GHA is the designation for the isolate within the commercial product Mycotrol® (Laverlam Inc., Butte, MT, USA)

Table 5.2 - Desiccation tolerance of blastospores after air-drying (< 4% moisture content) for various isolates of *B. bassiana* and *I. fumosorosea* grown in 100 mL liquid cultures supplemented with either acid hydrolyzed casein or cottonseed flour at 25 g/L as the nitrogen source and 100 g/L glucose after 3 days of incubation at 28 °C and 350 rpm

Fungal isolates	Desiccation tolerance (% Blastospore survival) [a]		Moisture content (%)	
	Acid hydrolyzed casein	Cottonseed flour	Acid hydrolyzed casein	Cottonseed flour
B. bassiana				
CG1229	80.8 ± 2.1 a, A [b]	78.2 ± 2.3 c, A	2.0 ± 0.07	2.4 ± 0.19
ESALQ1432	73.0 ± 2.8 b, B	79.2 ± 2.4 bc, A	1.4 ± 0.07	2.3 ± 0.18
ESALQ447	73.9 ± 2.7 b, B	84.0 ± 2.0 ab, A	1.6 ± 0.08	2.1 ± 0.19
ESALQ-PL63	85.3 ± 2.0 a, A	86.5 ± 1.9 a, A	1.4 ± 0.21	2.0 ± 0.14
GHA	70.9 ± 3.0 b, A	75.5 ± 2.8 c, A	2.2 ± 0.06	2.7 ± 0.33
I. fumosorosea				
CG1228	80.3 ± 2.5 a, A	74.1 ± 2.9 ab, B	1.8 ± 0.12	2.6 ± 0.2
ESALQ1296	77.3 ± 2.7 a, A	68.6 ± 3.1 b, B	1.7 ± 0.11	2.2 ± 0.2
ESALQ1364	74.1 ± 2.9 ab, A	65.3 ± 3.3 b, B	2.0 ± 0.14	2.0 ± 0.2
ESALQ1409	62.3 ± 3.4 b, B	70.6 ± 3.1 ab, A	1.8 ± 0.23	2.5 ± 0.1
ARSEF3581	82.4 ± 2.4 a, A	79.1 ± 2.6 a, A	2.1 ± 0.13	2.1 ± 0.24

[a] Blastospore desiccation tolerance was assessed by measuring blastospore germination by air-dried blastospores rehydrated in potato dextrose broth and incubated for 7 h and 6 h at 28 °C and 300 rpm for *B. bassiana* and *I. fumosorosea*, respectively. [b] Means (± SE) with non-corresponding letters denote statistical difference (Tukey test, $P < 0.05$). Small letters refer to comparison between isolates within each nitrogen source (in columns), while capital letters refer to comparisons between nitrogen sources (in rows) for each isolate. Not significant (ns)

Table 5.3 - Logistic model parameters fitted to survival data for air-dried blastospores stored at 4 °C over 13 months for various isolates of *B. bassiana* and *I. fumosorosea* produced with different nitrogen sources

Fungus	Isolate	Nitrogen source	Logistic parameters (estimate ± SE)					Half-life (months) [b]	Comparison between survival curves ($F_{calculated}$) [d]
			α	β	S_0	T_0	R^2	$t_{1/2}$	
B. bassiana									
	CG1229	AHC [a]	45.4	2.8	10.7	50.5	0.91	>14 [c]	**3.19**
		CSF	15.3	13.8	9.5	77.6	0.88	>14	
	ESALQ1432	AHC	12.9	18.7	10.3	83.5	0.89	>14	0.59
		CSF	13.2	12.1	9.7	84.5	0.88	>14	
	ESALQ447	AHC	303.8	3.1	32.3	-205.2	0.84	>14	2.68
		CSF	15.6	9.8	8.6	79.7	0.86	>14	
	ESAL-PL63	AHC	32.8	4.0	13.3	63.5	0.74	>14	0.93
		CSF	34.3	3.4	15.1	64.3	0.90	>14	
	GHA	AHC	17.8	23.9	9.3	78.4	0.97	>14	**4.33**
		CSF	652.0	2.8	37.0	-561.0	0.89	14.1	
I. fumosorosea									
	CG1228	AHC	1107.7	2.7	37.4	-1012.3	0.97	11.73	2.75
		CSF	119.7	4.9	13.9	-34.3	0.96	11.63	
	ESALQ1296	AHC	98.6	3.2	10.0	-5.6	0.97	9.22	**4.0**
		CSF	65.6	5.5	11.0	24.6	0.93	11.97	

ESALQ1364	AHC	148.5	3.4	11.8	-52.3	0.97	9.35	**4.1**
	CSF	935.2	2.7	32.6	-837.8	0.99	10.93	
ESALQ1409	AHC	4738.6	1.7	151.4	-4638.6	0.95	9.82	2.1
	CSF	196.0	3.9	15.7	-108.8	0.90	10.77	
ARSEF3581	AHC	92.6	5.0	10.5	6.0	0.98	10.67	**4.12**
	CSF	136.8	4.5	15.4	-41.6	0.92	13.11	

[a] Nitrogen sources used to produce the fungi: AHC = acid hydrolyzed casein; CSF = cottonseed flour

[b] Half-life: The time taken for 50% degradation (i.e., loss in viability) of air-dried blastospores was given by $t_{1/2} = t_0 ((\alpha / (S_{50} - S_0)) - 1)^{1/\beta})$, where S_{50} is the 50% viability and β is the steepness (slope) of the curve.

[c] Half-life longer than 14 months of storage was indicated when viability did not reach 50%.

[d] Survival curves for air-dried blastospores were compared between nitrogen sources. Bold indicates statistical significant at $P < 0.05$ when $F_{calculated} > F_{table}$

Table 5.4 - Bioefficacy (LC_{50}) of air-dried blastospores and aerial conidia of *B. bassiana* (isolate ESALQ-1432) and *I. fumosorosea* (isolate CG1228) against the second-instar nymph of *Bemisia tabaci* biotype B through direct spray application

Fungus	Infective form	n [a]	Slope ± SE [b]	χ^2 (*P*-value) [c]	LC_{50} (propagules cm^{-2}) [d]	(95% CL) [e]		RP_{50} [f]	(95% CL)	
						Lower	Upper		Lower	Upper
B. bassiana	Blastospores	3113	2.05 ± 0.17	109.35 (< 0.0001)	485	353	643	4.6	3.25	6.50
	Conidia	3059	1.85 ± 0.22	72.22 (< 0.0001)	2230	1362	3393	-	-	-
I. fumosorosea	Blastospores	3102	2.00 ± 0.16	166.29 (< 0.0001)	350	261	454	1.7	1.21	2.38
	Conidia	3190	2.02 ± 0.18	132.76 (< 0.0001)	594	426	798	-	-	-

[a] Total number of whitefly nymphs tested (10 replicates per fungal-concentration)

[b] Slope for mortality represents regression of proportion of nymph mortality versus log of spores cm^{-2}

[c] χ^2 and *P* values represent the probability of slope $\neq 0$, rather than fit to logistic model

[d] Delivered median lethal concentration (LC_{50}) expressed by infective propagules cm^{-2} and estimated by the logistic model. Cumulative mortality censored up to day 6 post-application. Control mortality averaged 3.7 ± 1.3%

[e] Confidence limits (CL95%)

[f] Relative potency is the measure of relative efficacy of blastospores to aerial conidia within each fungal species: (LC_{50} conidia / LC_{50} blastospores). Comparisons were undertaken within each fungal species and if the confidence limit for the LC ratio does not contain 1, hence it is concluded that the LC_{50} values are significantly different (WHEELER et al., 2007)

Table 5.5 - Survival times for *B. tabaci* biotype B nymphs following spray application with a concentration of 2.0×10^4 spores cm^{-2} (eq., 1.25×10^7 spores mL^{-1}) of either blastospores or aerial conidia of *B. bassiana* (ESALQ1432) and *I. fumosorosea* (CG1228)

Fungus	Infective form	n [a]	Median survival time (days)	95% CL (days) [b]	
				Lower	Upper
B. bassiana	Blastospores	512	3.0 ± 0.03 b [c]	2.95	3.05
	Conidia	561	4.0 ± 0.05 a	3.91	4.10
I. fumosorosea	Blastospores	545	3.0 ± 0.05 b	2.91	3.09
	Conidia	511	4.0 ± 0.02 a	3.96	4.04

[a] Number of insects censored throughout the 6-day-incubation period (n = 10 replicates per treatment)

[b] Confidence limits (95%)

[c] Median lethal time (ST_{50} ± SE) values with different letters within each fungus indicate statistical difference (*t*-test, $P < 0.05$). Control insects were sprayed with a Tween 80 solution at 0.01% and mortality averaged 11.5 ± 1.6%

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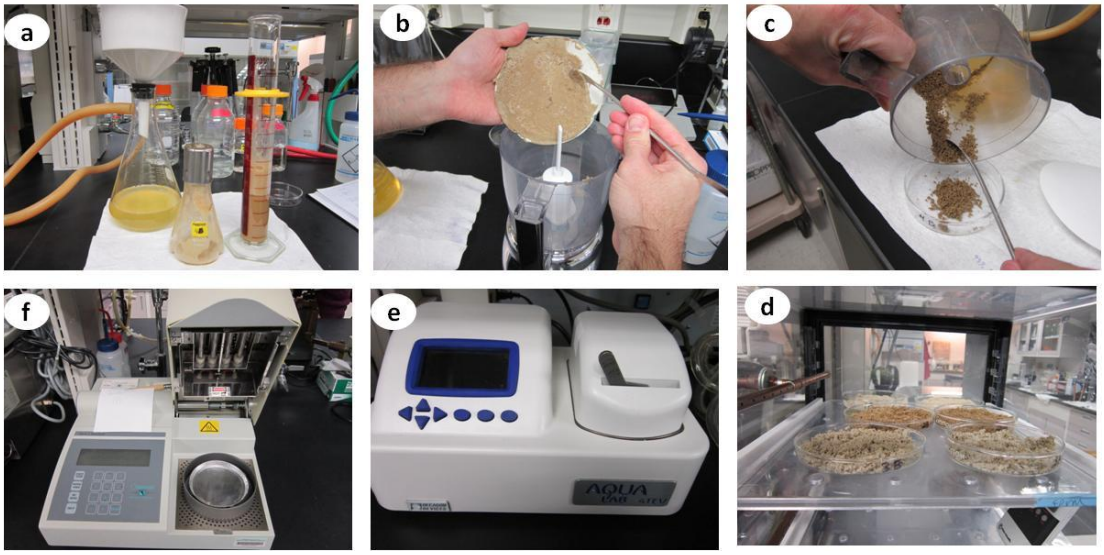

Figure 5.1 - Harvesting and drying process for blastospores produced in liquid cultures: a) blastospores were harvested by day 3 and vacuum filtered with 7.5% diatomaceous earth through a Buchner funnel; b) Blastospore + DE mixture was ground in a blender; c) fine particles of blastospores + DE was placed on glass Petri dishes for drying; d) Blastospore + DE preparations were dehydrated in an air drying chamber with controlled RH air (> 50%) for 16–20 h; e) water activity device and f) moisture analyzer were used to monitor the dryness in blastospore formulation

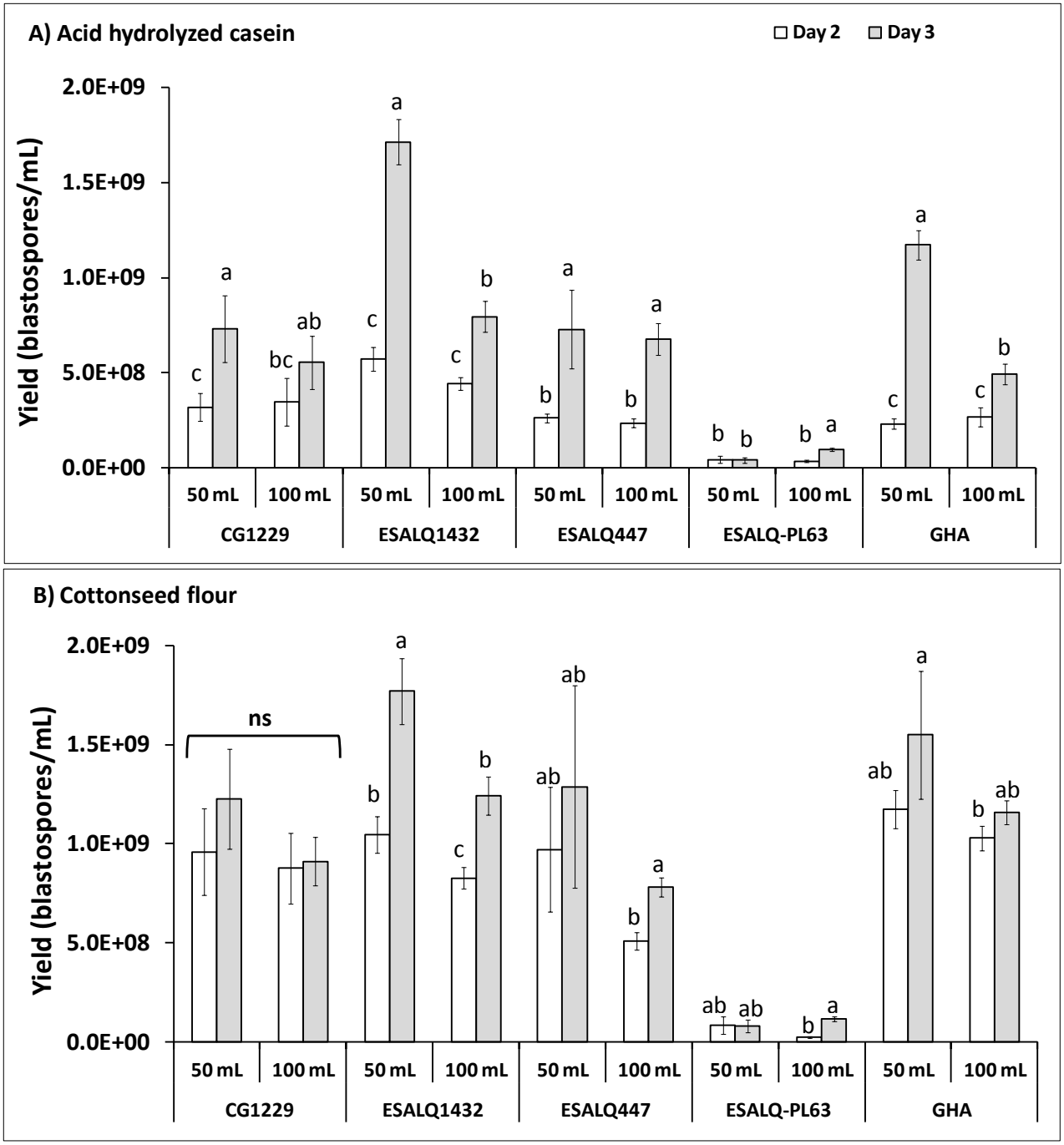

Figure 5.2 - Liquid culture production of blastospores by various isolates of *Beauveria bassiana* grown in 100 mL or 50 mL liquid medium supplemented with basal salts, 100 g L^{-1} glucose, and 25 g L^{-1} acid hydrolyzed casein (Hy-Case®) or cottonseed flour (Pharmamedia®). Cultures were grown in 250 mL baffled, Erlenmeyer flasks in a shaker incubator for up to 3 days at 28 °C and 350 rpm. Culture volumes were altered from 100 mL to 50 mL to increase aeration. Means (± SE) followed by the same letters, within each fungal isolate, denote statistical difference (Tukey test, $P < 0.05$)

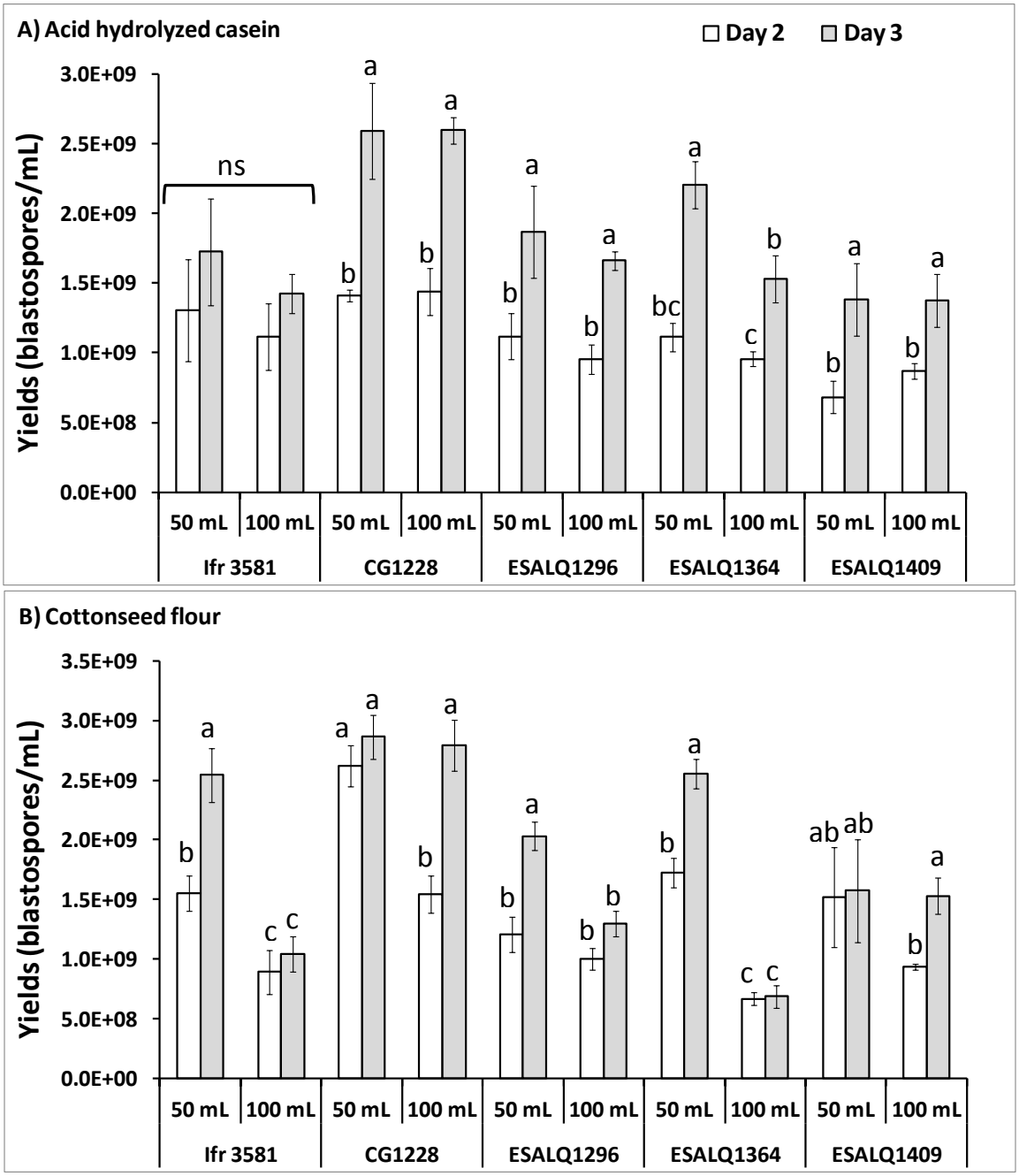

Figure 5.3 - Liquid culture production of blastospores by various isolates of *Isaria fumosorosea* grown in 100 mL or 50 mL liquid medium supplemented with basal salts, 100 g L^{-1} glucose, and 25 g L^{-1} acid hydrolyzed casein (Hy-Case®) or cottonseed flour (Pharmamedia®). Cultures were grown in 250 mL baffled, Erlenmeyer flasks in a shaker incubator for up to 3 days at 28 °C and 350 rpm. Culture volumes were altered from 100 mL to 50 mL to increase aeration. Means (± SE) followed by the same letters, within each fungal isolate, denote statistical difference (Tukey test, $P < 0.05$)

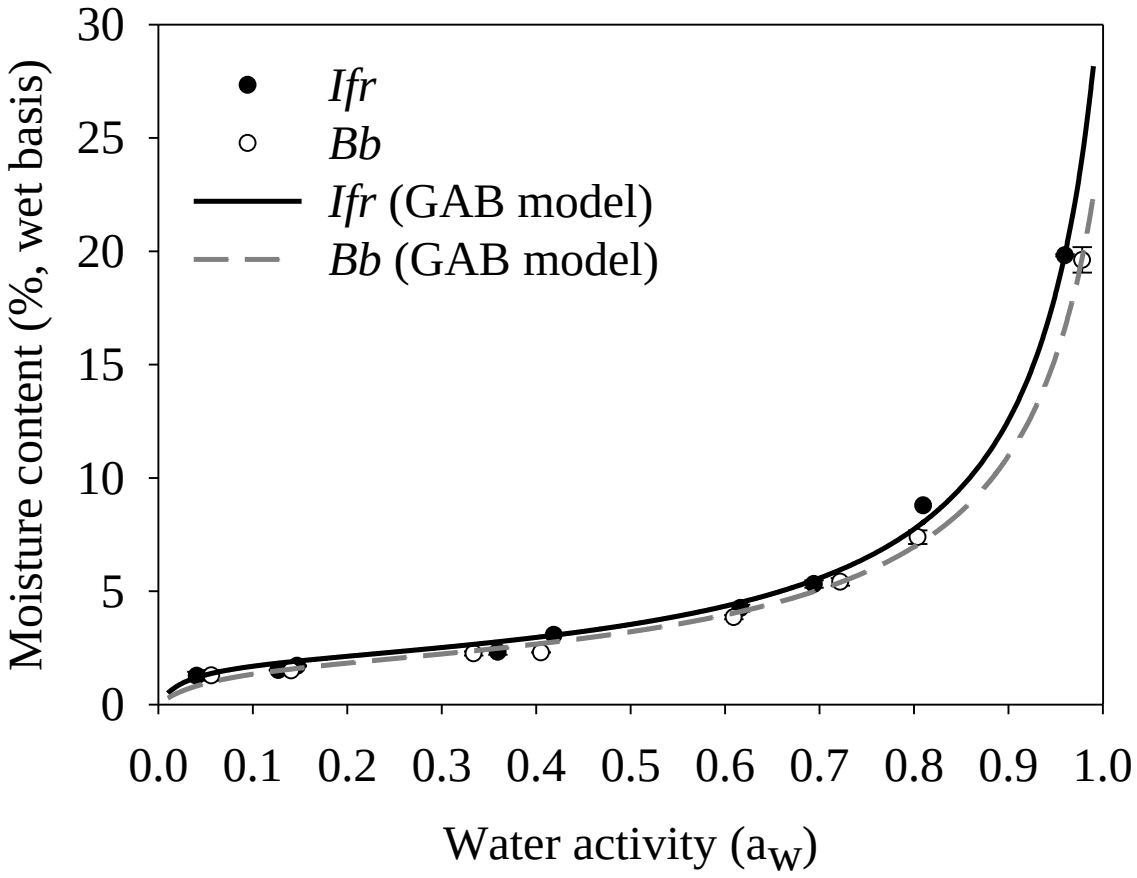

Figure 5.4 - Sorption isotherm, determined at 25 °C, of *B. bassiana* (*Bb*) and *I. fumosorosea* (*Ifr*) blastospores formulated with 7.5% (w/v) diatomaceous earth. Sorption isotherm curves were determined by the Guggenheim-Anderson-de Boer (GAB) model fitted to experimental data. Circles and triangles represent mean values (± SE) of experimental data. Fitted equations *Bb*:

$MC\ (\%) = 1.82\left(\frac{19.36a_w}{(1-0.93a_w)-(1-0.93a_w+19.36a_w)}\right)$;

Ifr: $MC\ (\%) = 2\left(\frac{17.99a_w}{(1-0.94a_w)-(1-0.94a_w+17.99a_w)}\right)$.

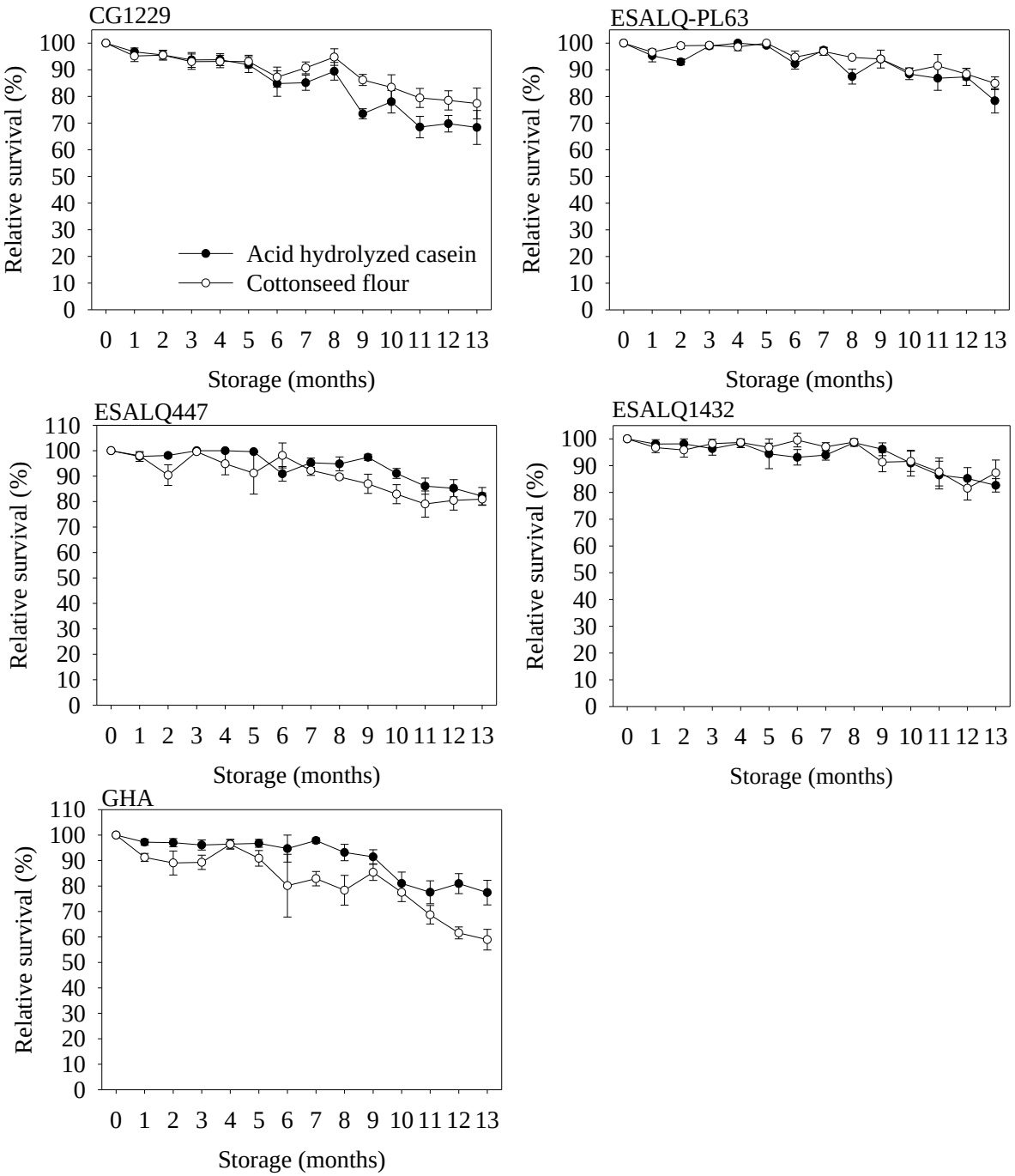

Figure 5.5 - Storage stability of air-dried blastospores for various isolates of *B. bassiana* grown with either acid hydrolyzed casein or cottonseed flour as the nitrogen source and stored at refrigerated conditions (4 °C) in vacuum-sealed polyethylene bags. Spore viability determined after 7 h incubation in potato dextrose broth at 28 °C and 300 rpm. Relative survival was calculated based on 100% viable blastospores (those that survived drying) after desiccation (time 0)

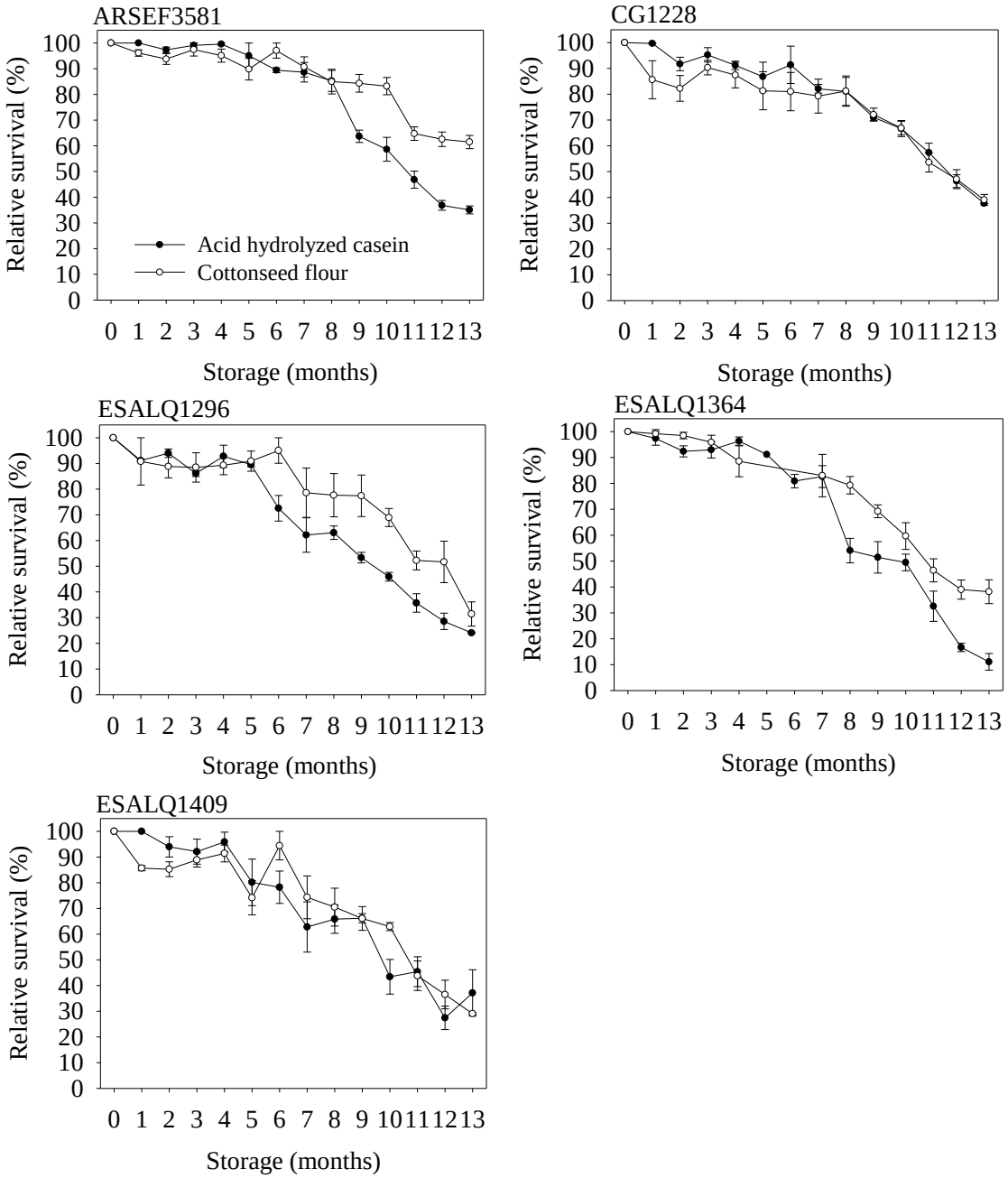

Figure 5.6 - Storage stability of air-dried blastospores for various isolates of *I. fumosorosea* grown with either acid hydrolyzed casein or cottonseed flour as the nitrogen source and stored at refrigerated conditions (4 °C) in vacuum-sealed polyethylene bags. Spore viability determined after 6 h incubation in potato dextrose broth at 28 °C and 300 rpm. Relative survival was calculated based on 100% viable blastospores (those that survived drying) after desiccation (time 0)

6 OSMOTIC PRESSURE AND DISSOLVED OXYGEN LEVELS AFFECT DIMORPHIC GROWTH IN *BEAUVERIA BASSIANA* AND ENHANCE ITS VIRULENCE[5]

Abstract

Beauveria bassiana is the most used entomopathogenic fungus against numerous insect pests worldwide. The present study placed emphasis on the optimization of a submerged liquid fermentation process to improve production of *B. bassiana* blastospores. To accomplish this goal, we examined the effects of oxygen level and osmotic pressure on two strains of *B. bassiana* for blastospore production, desiccation tolerance, and insecticidal activity. We demonstrated that high aeration (oxygen) rates in *B. bassiana* liquid cultures enhanced blastospore concentration (0.9–1.4 × 10^9 blastospores mL^{-1}) and biomass (30.7–35.6 g L^{-1}) in short fermentation time (2–3 days). In oxygen-rich cultures, high osmotic pressures (> 0.7–1.2 MPa) imposed by non-ionic osmolytes such as glucose or polyethylene glycol (PEG 200) in liquid media rendered greater numbers of blastospores within 3 day growth (> 2.0 × 10^9 blastospores mL^{-1}). Osmotic stress imposed by higher glucose concentrations (> 120 g L^{-1}) during fermentation did not affect blastospore resistance to dehydration with survival range of 64–73% (ESALQ1432) and 54–67% (GHA) based on a stringent 7 h germination assay. Likewise, PEG 200-amended cultures supplemented with 100 g glucose L^{-1} affected metabolism and yeast-like growth. Interestingly, blastospore germination rate was delayed in 3 h when produced in PEG-amended media. Adaptation of blastospores to hyperosmotic media was translated into lower cell size when compared with unstressed cells. Whitefly bioassays revealed that smaller blastospores from hyperosmotic medium (140 g glucose L^{-1}) showed enhanced virulence against nymphs than larger blastospores from low osmotic medium (40 g L^{-1}). Collectively, these results strongly indicate that osmotic pressure coupled with high aeration rates improve yeast-like growth of *B. bassiana* by supporting higher yields of active desiccation tolerant blastospores. This finding comprises the first report on the optimal nutritional and environmental growing conditions for feasible production of *Beauveria* blastospores by liquid fermentation.

Keywords: Mycoinsecticides; Blastospore; *Beauveria bassiana*; Desiccation tolerance; Aeration; Virulence; *Bemisia tabaci*

6.1 Introduction

Unlike bacteria and viruses that need to be ingested to cause disease, entomopathogenic fungi such as *Beauveria bassiana* sensu lato (Bals.) Vuill. kill insects by direct penetration of the cuticle followed by multiplication in the hemocoel (HUMBER, 2008). Filamentous entomopathogenic fungi (Ascomycota: Hypocreales), including *B. bassiana*, typically possess dimorphic growth when grown in liquid culture, which is characterized by the yeast and mycelium morphologies regulated by chemical and

[5] MASCARIN, G.M.; JACKSON, M.A.; KOBORI, N.N.; BEHLE, R.W.; DUNLAP, C.A.; DELALIBERA JR., I. Glucose and dissolved oxygen levels during liquid fermentation affect dimorphic growth and virulence of *Beauveria bassiana*. **Applied Microbiology and Biotechnology** (submitted).

environmental stimuli. In the insect hemolymph, which constitutes a high osmotic pressure microenvironment, these fungi differentiate into thin-walled, single-celled hyphal bodies also known as blastospores that are specialized to rapid exploit the host nutrients and tissues (WAN; DUAN; ST LEGER, 2008). Earlier studies conducted with *Isaria fumosorosea* Wise (formerly *Paecilomyces fumosoroseus*) and *B. bassiana* have shown that nutrient-rich media supported mostly yeast-like growth in submerged liquid cultures (SAMSINAKOVA, 1966; ROMBACH, 1988; BIDOCHKA; PFEIFER; KHACHATOURIANS, 1987; JACKSON et al., 1997; MASCARIN et al., 2015). To date, *B. bassiana* is the most widely used entomopathogenic fungus for biological control of arthropod pests throughout the world (FARIA; WRAIGHT, 2007). Previous works have reported concentrations of *B. bassiana* up to 1×10^9 blastospores mL^{-1} achieved after 5–10 days fermentation and these blastospores were generally not desiccation resistant or they survived poorly under storage (CHONG-RODRIGUEZ et al., 2011; HUMPHREYS; MATEWELE; TRINCI, 1989; LANE; TRINCI; GILLESPIE, 1991; PHAM; KIM; KIM, 2009; ROMBACH, 1988; SAMSINAKOVA, 1966; VIDAL et al., 1998; VEGA et al., 2003;). The lack of cost-effective liquid production methods for blastospores has constrained the commercial interest of the biopesticide industry and thus impaired the scale-up production of this fungus in deep-tank fermentors. Consequently, the mass-production of *Beauveria* still relies primarily on solid culture substrates, in which the aerial conidia comprise the most used active ingredient for pest control. However, solid-substrate fermentation has proven costly and very labor intensive with high risk of contamination (JACKSON, 1997; JACKSON; DUNLAP; JARONSKI, 2010). To overcome these bottlenecks, liquid culture techniques are laborsaving and cost-effective for the rapid production of yeast-like blastospores that survive well after drying and storage and with excellent insecticidal activity when proper nutritional and environmental conditions are provided during culture growth (JARONSKI; JACKSON, 2012; JACKSON et al., 1997). A recent study provided a baseline for blastospore yields through liquid fermentation of different strains of *B. bassiana* and *I. fumosorosea*, which survived well to air drying, remained stable for several months under cool storage and presented high virulence against whiteflies (MASCARIN et al., 2015).

Osmotic pressure and oxygen supply are crucial parameters for industrial fermentation of filamentous fungi by changing their metabolism, growth and fitness. In light of this, osmotic and oxidative stress responses in true yeasts such as *Saccharomyces cerevisiae* are well understood and documented (HOHMANN, 2002), yet among entomopathogenic fungi there is a lack of information underlying the yeast-mycelium morphogenesis in submerged

liquid cultures mediated by these two factors. Osmoregulation in artificial media has been shown to affect microcycle conidiation in liquid cultures of *M. anisopliae* with additional impact on its resistance to dehydration and virulence toward grasshoppers (LELAND et al., 2005). Elevated oxygen rates during liquid culture growth of *I. fumosorosea* have proven to be critical for enhanced blastospore production, desiccation tolerance, and shelf life (JACKSON, 2012). Therefore, it is imperative to optimize fermentation parameters for feasible blastospore production using economic media components.

In the present study, we investigated nutritional and environmental factors that could lead to a rapid production of high concentrations of *B. bassiana* blastospores by liquid culture techniques, which to the authors' knowledge this was never documented so far. Aeration, measured as dissolved oxygen level, and initial osmotic stress of the liquid media imposed by compatible solutes (i.e., osmolytes) are two major factors addressed here to investigate the dimorphic growth of *B. bassiana* in submerged culture conditions. This study provides new discovery on rapid fermentation process for producing high numbers of blastospores that are desiccation tolerant and more fitted for the control of soft-bodied insects. To accomplish this goal, we investigated through a series of experiments how the osmotic pressure coupled with aeration influenced the oxygen availability and its relation to yeast-mycelium dimorphism in submerged liquid cultures of *B. bassiana.* These findings are catalyst for improving productivity and fitness of blastospore-based bioinsecticides.

6.2 Development

6.2.1 Materials and Methods

In the following subsections, we describe various experiments to study the influence of osmotic pressure generated by a monosaccharide sugar along with oxygen availability (aeration) on different fermentation parameters of *B. bassiana* in the form of shorter fermentation times, higher product yields, tolerance to dehydration, and bioefficacy against a target insect pest. We also evaluated the impact of osmotic pressure on these growth parameters during fermentation using a non-ionic osmolyte (polyethylene glycol, PEG) which is not metabolized by these fungi in order to confirm the hypothesis that osmotic pressure could be by itself driving dimorphic growth in *B. bassiana* along with aeration. To accomplish these assignments, we studied two strains of *B. bassiana* as a tractable model.

6.2.1.1 Culture maintenance and inoculum preparation

Pure cultures of two *B. bassiana* strains, ESALQ1432 isolated from the citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), in Piracicaba-SP, Brazil, and GHA (ARSEF6444) isolated from *Diabrotica undecimpunctata* in California, USA, and the active ingredient of the commercial product Mycotrol® (Laverlam International Cop., Butte, MT, USA), were maintained in 10% glycerol solution and stored at –80 °C in cryovial tubes. Conidial inocula were obtained by growing the frozen stock cultures on potato dextrose agar (PDA, Difco®) in Petri dishes (Falcon®, 100 × 15 mm) at 22 °C with 12:12 (L:D) h photoperiod. To produce the pre-cultures, conidia were harvested by washing off 2–3 weeks old, sporulated PDA plates with 10 mL of 0.04% (v/v) Tween 80 (Sigma® Chemical, St. Louis, MO) solution, and added to basal medium to provide a final concentration of 1×10^6 conidia mL^{-1}. Each liter of basal medium contained the following salts and vitamins: KH_2PO_4, 2.0 g; $CaCl_2.2H_2O$, 0.4 g; $MgSO_4.7H_2O$, 0.3 g; $CoCl_2.6H_2O$, 37 mg; $FeSO_4.7H_2O$, 50 mg; $MnSO_4.H_2O$, 16 mg; $ZnSO_4.7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500 mg each; folic acid, biotin, vitamin B12, 50 mg each. Glucose (Sigma®) at 80 g L^{-1} (40% carbon [C]) and acid hydrolyzed casein (derived from bovine milk, Hy-case™ MSF, Kerry Bioscience, New York, NY, USA) at 25 g L^{-1} (8.5% nitrogen [N] and 53% C) were added to the basal medium to provide a carbon-to-nitrogen (C:N) ratio of 23:1. The stock solution of glucose was autoclaved separately, while the nitrogen ingredient was autoclaved with the basal salts medium. Pre-cultures were grown in 100 mL of this semi-defined liquid medium using 250-mL baffled, Erlenmeyer shake flasks (Bellco Glass, Vineland, NJ, USA) at 28 °C and 350 revolutions per minute (rpm) in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ, USA). Blastospores from 3-day-old pre-cultures (exponential growth phase) were used to inoculate 250-mL shake-flasks containing 50 or 100 mL liquid medium by delivering a final inoculum concentration of 5×10^6 blastospores mL^{-1}. Conidia and blastospores were measured microscopically using a hemacytometer. The pH was not adjusted during fermentation.

6.2.1.2 Effect of aeration on dimorphic growth

To evaluate the influence of dissolved oxygen rates on growth and blastospore yield of *B. bassiana* cultures (ESALQ1432 and GHA), these strains were grown in different liquid culture volumes (50 or 100 mL) in modified 250-mL baffled Erlenmeyer flasks. Oxygen sensors (PSt3, PreSens) provided continuous monitoring the oxygen saturation in shake flask cultures and were recorded *via* Bluetooth at 15-min intervals for each flask through the SFR

v2 system (PreSens, Regensburg, Germany) to generate a cumulated graph of time-coursed measurements, as previously described in Jackson (2012). The oxygen concentration ($C_{O2,L}$) was measured as O_2 molecules in the liquid phase, which can be expressed as % saturation or mol m^{-3}. Cultures were incubated at 28 °C in a rotary shaker incubator for up to 3 days. All liquid media consisted of the same basal salts medium but amended with cottonseed flour at 25 g L^{-1} (9.4% N and 40% C, Pharmamedia®, Traders Protein, Memphis, TN, USA) as the nitrogen source in replace of acid hydrolyzed casein, and initial glucose concentration at 100 g L^{-1}. This media composition provided a C:N ratio of 21:1 and all media were inoculated with 3-day-old blastospores from liquid pre-cultures to deliver a final concentration of 5×10^6 blastospores mL^{-1}. Carbon and nitrogen concentrations were calculated based on the information provided by manufacturers. The shaker was stopped to obtain samples from culture flasks at 48 and 72 h for biomass and blastospore measurements. In order to prevent fungal conidiation on the flask walls, shake-flasks were hand shaken at least twice a day. Each experimental treatment was carried out in duplicates and the entire experiment performed twice providing a total of 4 repetitions.

6.2.1.3 Effect of glucose concentration on dimorphic growth and osmotic pressure

The first part of this study focused on the effect of osmotic pressure imposed by glucose concentration on *B. bassiana* dimorphic growth. Glucose is considered one of the most inexpensive carbon sources in liquid fermentation and thus it was tested here as a non-ionic compatible osmolyte (solute) to generate different osmotic pressures in the medium. Stock solutions of glucose were prepared using double-deionized water (ddH_2O) and autoclaved separately from the other medium components. Glucose solutions were added in the liquid cultures at different concentrations with intervals at 20 g L^{-1} within the range 20–200 g L^{-1}. These media and the same glucose concentrations (not in media) were evaluated for water activity (a_w) using the AquaLab series 4TEV (Decagon Devices, Inc., Pullman, WA, USA) equilibrated at 25 °C. Preliminary measurements showed that a_w of the liquid media with different water-stress treatments imposed by glucose rendered very similar values compared with glucose solutions alone. Water activity measurements were used to calculate the expected osmotic pressure in the glucose-modified liquid media based on Norrish's equation (NORRISH, 1966), since both parameters are directly related: $\Pi = -\frac{RT \ln a_w}{V_w}$, where Π is the osmotic pressure in Megapascal (MPa), R is the perfect gas constant (8.3144720 mL MPa mol^{-1} K^{-1}), T is the temperature (25 °C = 298.15 K), and V_w is the partial

molar volume of water (18 mL mol^{-1}). Both *B. bassiana* strains (ESALQ1432 and GHA) were grown in liquid media supplemented with basal salts, as previously indicated, supplemented with cottonseed flour at 25 g L^{-1}. Liquid cultures were inoculated with pre-cultures to provide a final concentration of 5×10^6 blastospores mL^{-1} and then grown in 50-mL volume of liquid medium using 250-mL baffled, Erlenmeyer flasks. Fermentation was carried out in a rotary incubator shaker set to 28 °C and 350 rpm. These conditions were characterized as the highest aeration rate that provided elevated oxygen availability to support yeast-like growth. Each treatment was duplicated and the experiment conducted three times on different dates for a total of six replications.

6.2.1.4 Impact of osmotic pressure and oxygenation on culture dimorphic growth

In attempt to understand whether the osmotic pressure was affecting oxygenation through raising dissolved oxygen levels in liquid cultures, we studied two extreme osmotic pressures (4% glucose [0.13 MPa] and 20% glucose [2.82 MPa]) imposed by glucose solutions with two agitation speeds (350 and 175 rpm) to examine the dissolved oxygen profile along with blastospore concentration and dry weight in shake flask cultures of both *B. bassiana* strains. This experiment followed the same procedure described in the previous section (6.2.1.3). Cultures were grown in a liquid medium composed of basal salts and cottonseed flour (nitrogen source) at 25 g L^{-1}, as previously mentioned. These liquid cultures contained 50 mL liquid medium and were inoculated with a final concentration of 5×10^6 blastospores mL^{-1} using 3-day-old pre-cultures. Two shake flasks (replicates) per experimental treatment were used and the whole experiment was conducted at least three times for each agitation speed for a total of six replications.

6.2.1.5 Effect of polyethylene glycol (PEG) on dimorphic growth and osmotic pressure

To confirm the hypothesis that the osmotic pressure induced yeast-like growth in *B. bassiana* cultures grown under water-stress conditions imposed by glucose in the aforementioned experiments, we altered osmotic pressures using polyethylene glycol 200 (PEG 200, mw = 200 g mol^{-1}; Sigma®), which is a non-ionic osmolyte that is not metabolized by these fungi. Osmotic pressures were measured based on a_w values, as previously mentioned for glucose concentrations. Media contained 100 g glucose L^{-1}, which comprised our previous standard medium composition to support normal fungal growth (MASCARIN et al., 2015). Stock aqueous solutions of PEG 200 at 50% (w/v) and glucose at 40% (w/v) were autoclaved separately and then added to liquid media containing basal salts amended with

cottonseed flour at 25 g L^{-1}. The modified liquid media contained 0, 20, 40 and 60 g L^{-1} PEG 200 with a fixed concentration of glucose (100 g L^{-1}). The growth conditions were the same for the aforementioned experiments in section 6.2.1.3. Each treatment was carried out in duplicates and the entire experiment conducted three times for a total of six replications.

6.2.1.6 Analysis of growth parameters

Blastospore yield, biomass accumulation, glucose utilization and pH were determined in all experiments. After 2 and 3 days of growth, a 3-mL sample was taken from each flask to determine biomass accumulation and blastospore concentration. Biomass of the whole culture was expressed as dry weight accumulation by filtering duplicate 1-mL culture samples through a dried and pre-weighed 2.4-cm glass filter paper (G6, Fisher Scientific, Pittsburgh, PA, USA). Thereafter, the total mass was dried at 80 °C for 24 h until constant weight. Blastospore concentrations were enumerated microscopically with a Neubauer hemacytometer under phase-contrast microscope (BH2, Olympus America, Center Valley, PA, USA) with Nomarski optics at 400X magnification. To determine the glucose utilization by *B. bassiana* cultures, the final glucose concentration in the spent medium after 3 days growth was recorded by a glucose meter (GlucCell®, CESCO, Atlanta, GA, USA) and then consumption was computed by the difference between initial and final concentration. To determine blastospore size produced in different water-stress media, we examined at least 50 randomly blastospores from different experimental flasks of each treatment by measuring their length in an ocular micrometer (WHB10x-H/20, Olympus) at 400X magnification.

6.2.1.7 Harvesting and drying blastospores

After 3 days of fermentation, blastospores from each liquid culture were harvested and subjected to dehydration to determine their desiccation tolerance. Briefly, each culture broth was mixed with 7.5% (w/v) diatomaceous earth (DE; HYFLO®, Celite Corp., Lompoc, CA, USA) and then vacuum-filtered through Whatman No. 1 filter paper in a Buchner funnel. The filter cake was broken up by pulsing in a blender (Mini Prep® Plus, Cuisinart, Stamford, CT, USA), layered in glass Petri dishes (9 × 1.5 cm), and gently air-dried overnight in a horizontal air flow chamber with controlled RH atmosphere (RH ≈ 50–60%) (JACKSON; PAYNE, 2007). The moisture content and a_w of the blastospores–DE preparations were determined with a moisture analyzer (Mark II, Denver Instruments, Arvada, CO, USA) and using the AquaLab water activity meter, respectively. When these formulations were dried to a

moisture content < 3%, they were vacuum packed in nylon polyethylene bags (15.3 × 21.8 cm) with a vacuum packer set to 999 mbar (eq. < 0.021% oxygen) (Multivac Inc., Kansas City, MO, USA) and stored at refrigerated conditions (4 °C). Three days after packing, a sample (~ 0.05 g) from each replicate per treatment was transferred into 25 mL potato-dextrose (PD) broth (Difco®) in 125-mL baffled Erlenmeyer flasks (Bellco Glass Inc., USA) and incubated at 28 °C with 300 rpm in a rotary shaker incubator to assess the germination of blastospores. After 7 h or 10 h (for PEG-produced blastospores) incubation, two subsamples per flask were transferred onto slides with coverslips to microscopically assess the germination of blastospores by enumerating a minimum total of 200 blastospores. Blastospores were considered viable (germinated) when a germ tube of any size was visible at 400X magnification. This germination test represented a fast-rehydration method to assess anhydrobiosis in blastospores .

6.2.1.8 Effect of glucose concentration on blastospore virulence against whitefly

The virulences of blastospores produced under different osmotic stress conditions of liquid medium were evaluated against the silverleaf whitefly (biotype B), *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). Insect colony was originally provided by University of Florida, Apopka-FL, and reared on cabbage cv. 'Bravo' (*Brassica oleracea* L.; Harris Seeds, Rochester, NY, USA) and blue lake bush bean (*Phaseolus vulgaris* L.; Kelly Seeds Co., Peoria, IL, USA) plants in the greenhouse of USDA (Peoria, IL). Air-dried blastospores were harvested from highly aerated liquid cultures grown in 25 g L^{-1} of cottonseed flour at different initial glucose concentrations (40, 100, and 140 g L^{-1}), and then formulated with 7.5% DE (w/v) prior to air-drying, as described below. These blastospore preparations were assayed against 2nd instar nymphs hosted on the underside of primary bean leaves (approx. 14 days after seeding). Individual bean leaf was placed ventral side up on water agar (2% w/v) in ventilated plastic Petri dishes and 60–70 nymphs per leaf were marked nearby with a blue ink, as described in Mascarin et al. (2013). Briefly, four aqueous suspensions of blastospores (1.25×10^7, 2.5×10^6, 5×10^5, and 2.5×10^5 blastospores mL^{-1}) were prepared in 0.01% (v/v) Tween 80 solution and sprayed on the insects, which delivered the applied concentrations on leaf surface: 2.7×10^4, 5.4×10^3, 1.1×10^3, and 2.2×10^2 blastospores cm^{-2}. Spraying was performed using a micro-sprayer device set to 10 PSI and 3 sec application time (MASCARIN et al., 2013). Control insects were sprayed only with 0.01% Tween 80 solution. Four infested leaves were treated with each blastospore concentration. Treated leaves were incubated in a growth chamber set to 27±1 °C, 60–70% RH and 14:10 h L:D photophase for 6

days until mortality was assessed. In another experiment, we also compared the speed of kill (median survival time, ST_{50}) for blastospores produced under different osmotic pressures through a single-dosage assay based on the highest concentration (2.7×10^4 blastospores cm^{-2}) to monitor mortality daily up to 6 days. Multiple dosage-response and single-dosage experiments were conducted three times using different insect cohorts and fungal batches.

6.2.1.9 Statistical analysis

Each experiment was conducted with a completely randomized design. A linear mixed model was employed to fit experimental data for the response variables blastospore concentration and biomass (dry weight). Blastospore concentration was log-transformed prior to analysis of variance to meet the normality assumptions of the linear mixed model. In the model, the fixed effects consisted of culture medium composition, speed of agitation, culture medium volume or a combination of these factors, while the experimental repetitions and shake flasks (experimental units repeatedly measured over time) were included in the random term. Statistics for fixed effects and their interaction terms were also determined by Wald type III *F*-test. Post-hoc pair-wise multiple comparisons were performed using Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$ for fixed effects and their interaction terms, while *t*-Student test was employed to compare only two groups of means. These statistical analyses were performed using the procedure MIXED in the Statistical Analysis System v.9.2 (SAS Institute Inc., Cary, NC). To explain the impact of osmotic pressure imposed by glucose levels on blastospore yields, empirical data were fitted to a logistic growth model (sigmoidal with four parameters) according to the equation: $y = y_0 \frac{a}{1+e^{-\frac{(x-x_0)}{b}}}$, where y is the concentration (blastospore mL^{-1}), x is the glucose concentration (g L^{-1}), and a, b, y_0 and x_0 are constants estimated by the interactive analysis using in the software SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). Proportion mortality data for whitefly nymphs were recorded 6 days post-inoculation and fitted to a logistic model using the PROBIT procedure of SAS to compute the median lethal concentration (LC_{50}) for each treatment. Dosage responses were corrected for control mortality automatically in this analysis. To compare statistically the LC_{50} values at 5% probability, the potency ratio test was used (WHEELER et al., 2007). Survival analysis and median survival times (ST_{50}) obtained from single-concentration response bioassays were determined by Kaplan-Meier method, and survival curves were compared with log-rank test ($P < 0.05$) with *P*-values adjusted by Bonferroni's

test. Particularly, median lethal times were considered significantly different based on non-overlapping of 95% confidence intervals.

6.2.2 Results

6.2.2.1 Effect of dissolved oxygen on dimorphic growth

Higher aeration was achieved by lower volume (50 ml) or higher agitation speed (350 rpm) compared with lower aeration provided by 100 ml volume or 175 rpm agitation (Figure 6.1). Culture volume and agitation speed affected the dissolved oxygen profile of *B. bassiana* cultures as well as their growth parameters. Irrespective of the *B. bassiana* strain, higher shaking frequencies (350 rpm) and lower culture volumes (50 mL) sustained higher dissolved oxygen levels. Dissolved oxygen levels remained as high as 50% (DO_{50}) for 6.8–9.6 h longer when cultures of *B. bassiana* were grown at a higher agitation speed (350 rpm) and 4 h longer when grown in a smaller culture volume (50 mL) in comparison to cultures grown at 175 rpm or 100 mL culture volume (Figure 6.1). Dissolved oxygen profiles remained low (< 10% oxygen level) after 24 h growth and throughout the remaining three days of fermentation when *B. bassiana* cultures were incubated at 350 rpm suggesting that these conditions promoted maximum oxygen consumption as shown by increased biomass production. In particular, greater oxygen availability remarkably increased fungal biomass (dry weight) over the fermentation time when *B. bassiana* strains were grown in oxygen-rich cultures (50-mL liquid medium at 350 rpm) (Table 6.1). The main effects of agitation speed (ESALQ1432: $F_{1,24}$ = 185.65, P < 0.0001; GHA: $F_{1,24}$ = 75.68, P < 0.0001) and culture volume (ESALQ1432: $F_{1,24}$ = 39.19, P < 0.0001; GHA: $F_{1,24}$ = 32.88, P < 0.0001) substantially supported more yeast-like growth measured as blastospore concentration for both *B. bassiana* strains (Table 6.1). Blastospore production increased over the fermentation time for ESALQ1432 (ESALQ1432: $F_{1,24}$ = 5.08, P = 0.034), although no significant change was observed for GHA ($F_{1,24}$ = 0.97, P = 0.334) which had reached its maximum production by day 2, indicating that strains responded differently under these fermentation conditions. No interactions between the main factors were significant on blastospore yield for ESALQ1432 (P > 0.05). Only the interaction of agitation speed by culture volume was significant for GHA ($F_{1,24}$ = 33.46, P < 0.0001). Typically, limited-oxygen cultures exhibited more viscous (thicker) appearance from day 2 to day 3 growth due to the formation of more hyphae. These results demonstrate that media with better aeration improved yeast-like growth.

Biomass accumulation was significantly greater over fermentation time for ESALQ1432 ($F_{1,24}$ = 73.87, P < 0.0001), but not for GHA ($F_{1,24}$ = 2.07, P = 0.163). Agitation

speed (ESALQ1432: $F_{1,24}$ = 146.27, P < 0.0001; GHA: $F_{1,24}$ = 17.08, P < 0.0001) and culture volume (ESALQ1432: $F_{1,24}$ = 62.29, P < 0.0001; GHA: $F_{1,24}$ = 13.31, P < 0.0001) increased the biomass accumulation of both *B. bassiana* strains. Interaction between culture volume and agitation speed revealed that ESALQ1432 cultures under high agitation speed (350 rpm) supported more biomass accumulation ($F_{1,24}$ = 8.56, P = 0.0074). The poorest blastospore yields were achieved with oxygen-limited growth conditions (i.e., 100 mL / 175 rpm), which also resulted in lower dry weights (Table 6.1). Strain ESALQ1432 was more responsive than GHA to oxygen availability showing greater biomass accumulation when cultured in oxygen-rich medium. During initial fermentation (< 24 h), the oxygen level was high (100% saturation) corresponding to a lag growth phase. Then, dissolved oxygen declined as the biomass increased after 24 h growth. Comparatively, GHA exhibited higher metabolism measured by glucose consumption than ESALQ1432, and final pH in the final broth was moderately acid for both strains. Concerning the morphological aspects, it was noticed that the highest speed of agitation (350 rpm) combined with the lowest volume rate (50 mL) rendered less viscous cultures of *B. bassiana* in relation to poorly aerated cultures. By contrast, more viscous cultures resulted in more mycelial growth due to deficiency in oxygen diffusion.

6.2.2.2 Effect of glucose concentration on dimorphic growth

Water activity in artificial liquid media was manipulated through the addition of non-ionic compatible osmolytes such as glucose and PEG 200. Adding these osmolytes increased osmotic pressure which, in turn, reduced water availability in liquid culture (Appendix A). Examining the impact of increased osmotic pressure by increasing concentrations of glucose revealed a progressive, dose-dependent increase in blastospore concentration over time (Figure 6.2). Hyperosmotic growing media yielded maximum blastospore concentrations at ≥ 14 g L^{-1} glucose (≥ 0.9886 ± 0.0007 a_w or ≥ 1.579 ± 0.095 MPa) and ≥ 20 g L^{-1} glucose (≥ 0.9797 ± 0.0003 a_w or ≥ 2.82 ± 0.049 MPa) for ESALQ1432 and GHA by day 3 fermentation, with yields as high as 2.93×10^9 and 2.94×10^9 blastospores mL^{-1}, respectively. The dose-response relation between blastospore production and glucose concentration was significantly explained by a sigmoidal growth model (R^2 ≥ 85%, P < 0.001) for days 2 and 3 of fermentation, yet growth rate at day 3 progressed significantly faster than day 2 (Figure 6.2, Table 6.2). Residual glucose (Figure 6.3) increased with the initial glucose concentration and this trend was accompanied with blastospore yield increase, suggesting that the residual

glucose also contributed to the osmotic pressure in liquid media. Similarly, total fungal biomass responded positively to the increase of initial glucose concentration up to 100 g L^{-1} at day 2 and 180 g L^{-1} at day 3 growth for ESALQ1432 ($F_{9,117}$ = 12.78, P < 0.0001). By contrast, GHA cultures accumulated greater biomass when grown with 160 g glucose L^{-1} by day 2 and 60 g L^{-1} by day 3 ($F_{10,129}$ = 6.61, P < 0.0001) (Figure 6.3). The growth rate of *B. bassiana* cultures was significantly higher by day 3 than 2 (ESALQ1432: $F_{9,117}$ = 8.76, P < 0.0001; GHA: $F_{10,129}$ = 4.44, P < 0.0001). Consistent with these results, the glucose consumption increased with the increase of initial glucose concentration, indicating that growth cultures utilized only 50–60 g L^{-1} of the total initial glucose available (Figure 6.3).

Desiccation tolerance was measured as air dried blastospore surviving fast-rehydration in PDB and incubated for 7 h. Osmotic pressure imposed by initial glucose concentration did not affect tolerance of blastospores of *B. bassiana* ESALQ1432 to dehydration ($F_{9,48}$ = 2.09, P = 0.05) (Table 6.3). By contrast, air-dried GHA blastospores produced with either 60 or 80 g glucose L^{-1} had significantly lower germination when compared with blastospores grown with 140 or 160 g glucose L^{-1} ($F_{10,59}$ = 4.34, P = 0.0001). Liquid cultures became less viscous and thinner when grown in medium amended with higher glucose concentrations (> 120 g glucose L^{-1}). This observation suggests that less hyphae was produced while more biomass was converted into blastospores. When examining the potential for a significant relationship between drying parameters and spore germination, neither a_w nor moisture content measured in the final dried samples of blastospores influenced their resistance to anhydrobiosis (Spearman correlation: $0.27 \leq r \leq 0.03$, n = 40–68, P > 0.05), except the negative correlation between a_w and viability found for ESALQ1432 (Spearman correlation: r = -0.37, n = 40, P = 0.02). In addition, the small variation across samples indicates that both a_w and moisture were strictly controlled relative during drying process.

6.2.2.3 Impact of osmotic pressure and oxygenation on culture dimorphic growth

Agitation speed and glucose concentration both affected the dissolved oxygen profile for cultures of *B. bassiana* grown in baffled shake flasks. Regardless of agitation rate or fungal strain, *B. bassiana* cultures with an initial glucose concentration of 200 g glucose L^{-1} [osmotic pressure 2.82 MPa] maintained dissolved oxygen up to 50% for up to 8–11.3 h longer in comparison to cultures grown in media with a lower glucose concentration (40 g glucose L^{-1} [0.13 MPa]) (Figure 6.4). Increasing the glucose concentration of the medium and the rate of agitation increased dissolved oxygen levels corresponding to 9.8–16.3 h longer

oxygen availability (DO_{50}) compared to cultures with lower glucose concentrations and those grown at a slower agitation rate (Figure 6.4).

Increasing agitation speed by itself promoted higher dissolved oxygen rates and consequently yielded greater numbers of blastospores up to 3 days growth irrespective of the fungal strain ($F_{1,76}$ = 94.94, P < 0.0001) (Table 6.4). In particular, osmotic pressure also promoted better yeast-like growth regardless of agitation or fungal strain ($F_{1,76}$ = 437.15, P < 0.0001). Higher glucose concentration in the culture medium enhanced dissolved oxygen levels and supported better yeast-like growth even under low agitation rate for both *B. bassiana* strains ($F_{1,76}$ = 437.15, P < 0.0001). There was a synergistic effect between glucose concentration and agitation rate on blastospore yields ($F_{1,76}$ = 7.58, P = 0.0074) with more blastospores being produced by day 3 when compared with day 2 of fermentation ($F_{1,76}$ = 84.29, P < 0.0001). Significantly greater dry weights were attributed to higher agitation rates ($F_{1,78}$ = 39.58, P < 0.0001), whereas glucose concentration alone did not have a significant effect on this growth parameter ($F_{1,78}$ = 0.76, P = 0.3849) (Table 6.4). The final pH in the culture broth was moderately acidic (4.86 ± 0.2 for ESALQ1432 and 5.22 ± 0.14 for GHA) and was similar among all cultures. At the end of the fermentation, culture broths cultivated in high osmotic pressure coupled with high agitation speed tended to be less viscous due to the fact that more blastospores were formed rather than hyphae.

6.2.2.4 Effect of PEG-amended media on dimorphic growth

For PEG-amended cultures, ESALQ1432 produced more blastospores after 3 days of fermentation when compared with 2 days of fermentation ($F_{3,39}$ = 3.24, P = 0.0323), and these higher yields were attained with increasing PEG concentrations ($F_{3,39}$ = 8.06, P = 0.0003) (Figure 6.5). By contrast, adding PEG 200 to GHA cultures neither enhanced blastospore yield with increased PEG concentration (day 2: $F_{3,19}$ = 2.89, P = 0.063; day 3: $F_{3,19}$ = 2.45, P = 0.095) nor with increased fermentation time ($F_{3,39}$ = 2.17, P = 0.1076) (Figure 6.5). Therefore, *B. bassiana* strains responded differently to PEG 200 added to the liquid medium. When examining PEG-amended cultures of ESALQ1432, similar blastospore yields were achieved when mixing 100 g glucose L^{-1} with 20–60 g PEG L^{-1} in comparison with media supplemented only with 120–160 g glucose L^{-1}. In contrast with blastospore yields, increasing PEG 200 in cultures of ESALQ1432 resulted in significant reductions of dry weight ($F_{3,39}$ = 8.12, P = 0.0003) (Table 6.5). Although biomass increased between day 2 and day 3 ($F_{1,39}$ = 22.6, P = 0.0323), ESALQ1432 cultures amended with 40 and 60 g PEG L^{-1} showed the

lowest dry weights ($F_{3,39}$ = 16.56, P < 0.0001). GHA cultures amended with 20–60 g PEG L^{-1} showed no significant variation in dry weight compared with cultures grown without PEG 200 ($F_{3,39}$ = 0.25, P = 0.864). When air-dried blastospores were rehydrated, there was a significant increase in germination rate from 7 h to 10 h incubation for both *B. bassiana* strains (ESALQ1432: $F_{3,39}$ = 5.85, P = 0.0021; GHA: $F_{3,39}$ = 8.0, P = 0.0003) (Figure 6.6). There was a significant decrease in blastospore germination when cultures were grown in liquid media with increased concentrations of PEG 200 after 7 h incubation for GHA ($F_{3,39}$ = 18.56, P < 0.0001) and both 7 h and 10 h incubation for ESALQ1432 ($F_{3,39}$ = 15.12, P < 0.0001). This outcome suggests that modified media with higher rates of PEG 200 induce delayed germination of air-dried blastospores. Blastospores cultivated in PEG-amended cultures were dehydrated as low as 0.2038–0.2308 a_w (1.92–2.49% moisture) and 0.1970–0.2128 aw (1.59–2.25% moisture) using a convective air drying system for ESALQ1432 and GHA, respectively. Spore germination of ESALQ1432 at 7 h incubation was 52% (P = 0.009) and 74% (P < 0.0001) correlated with a_w and moisture content, respectively, and only 64% correlation (P = 0.0008) with moisture at 10 h incubation. These drying parameters were not significantly correlated with spore germination at either incubation time for GHA (P > 0.05).

Hyperosmotic cultures significantly reduced blastospore length when cultured with 140–200 g glucose L^{-1} or with PEG at 40–60 g L^{-1} resulting in different phenotypes. These blastospores became significantly smaller (up to 53%) and exhibited ovoid shape rather than oblong when compared with blastospores produced with glucose levels at 40–100 g L^{-1} for both *B. bassiana* strains (ESALQ1432-glucose: $F_{4,342}$ = 42.66, P < 0.0001 and -PEG: $F_{3,216}$ = 9.19, P < 0.0001; GHA-glucose: $F_{4,415}$ = 68.11, P < 0.0001 and -PEG: $F_{3,202}$ = 13.94, P < 0.0001) (Figure 6.7). These results suggest that blastospore size was inversely related to the osmotic pressure in the medium.

6.2.2.5 Effect of glucose concentration on blastospore virulence against whitefly

Bioassays performed against second-instar whiteflies revealed that ESALQ1432 blastospores produced in media with higher glucose concentrations (140 g L^{-1}) resulted in higher virulence when compared with blastospores produced in media with 40 g L^{-1} glucose (Table 6.6). The ratio test comparing LC_{50} values showed that blastospores harvested from media supplemented with 140 g glucose L^{-1} yielded a twofold increase in insecticidal activity compared with blastospores produced with 40 g glucose L^{-1}. Instead, LC_{50} value for blastospores grown in 100 g glucose L^{-1} was not statistically different from the one estimated for blastospores derived from 40 g glucose L^{-1}. The proportion of whiteflies surviving all

blastospore-treatments was significantly lower when compared with the control group ($\chi^2_{(3)}$ = 990.59, $P < 0.0001$). Moreover, the survival analysis suggested a faster insecticidal activity for blastospores produced in medium with 140 g glucose L^{-1} in comparison to those cultured in media with either 40 g glucose L^{-1} ($\chi^2_{(1)}$ = 16.16, $P < 0.0001$) or 100 g glucose L^{-1} ($\chi^2_{(1)}$ = 5.99, $P = 0.0143$); however, there was no significant difference in survival rates of these two treatments in relation to blastospores from media containing 100 g glucose L^{-1}. Based on the LT_{50} values, blastospores obtained from high-glucose media (100 and 140 g glucose L^{-1}) incited 50% infection in whitefly nymphs one day earlier (25% faster) than blastospores produced in a medium amended with 40 g glucose L^{-1} (Table 6.6). Concentrations as high as 2 × 10^4 blastospores cm^{-2} (eq. 1.25 × 10^7 blastospores mL^{-1}) inflicted > 95% mortality in second-instar whiteflies, regardless of the glucose concentration of the blastospore production medium.

6.2.3 Discussion

Numerous nutritional and environmental variables mediate dimorphic growth in fungi (RUIZ-HERRERA, 1985). Among them, the ability of fungal cells to adapt to altered availability of free water and oxygen availability play key roles in regulating yeast-mycelium dimorphism, yet little is known how these factors influence the filamentous growth in anamorphic hypocrealean entomopathogenic fungi. Based on our previous studies with *B. bassiana* and *I. fumosorosea* (JACKSON, 2012; MASCARIN et al., 2015), our hypothesis was that osmotic pressure and aeration could be critical components for blastospore productivity during liquid fermentation of *B. bassiana*. In this study, we confirmed this hypothesis by demonstrating that the combination of osmotic stress, agitation speed (shaker rotation), and ratio surface-area (culture volumes) changes dramatically the oxygen availability and fungal metabolism, and induces profound effects in dimorphic growth of *B. bassiana* cultivated in liquid media. We have found that elevated dissolved oxygen rates generated by oxygen-rich environment combined with external hyperosmotic conditions synergistically stimulate more production of blastospores over hyphal growth in liquid cultures of *B. bassiana*. Moreover, significant greater numbers of blastospores (> 2 × 10^9 blastospores mL^{-1}) were produced in shorter fermentation times (2–3 days) under this growing environment. Oxygen-rich cultures with high osmolarity yielded less viscous cultivation broth that subsequently allowed better oxygen transfer in the liquid phase during the fermentation process, and thus enhancing blastospore production. In agreement with these results, it was

recently shown that maximum oxygen transfer in liquid films on shake flask walls was dependent on culture volume, shaker rotation, and initial viscosity of the liquid phase (GIESE et al., 2013). Previous submerged fermentation studies on blastospore production of *B. bassiana*, *I. fumosorosea* and *Metarhizium* spp. have been conducted with low glucose levels ($\leq$ 50 g L^{-1}) and low agitation speed (< 200 rpm) resulting in fewer blastospores produced in longer fermentation times ($\geq$ 5 days), which were characterized to have poor desiccation tolerance or short storage stability (CHONG-RODRIGUEZ et al., 2011; HUMPHREYS; MATEWELE; TRINCI, 1989; INCH; TRINCI, 1987; ISSALY et al., 2005; KLEESPIES; ZIMMERMANN, 1992; LANE; TRINCI; GILLESPIE, 1991; LELAND et al., 2005; LOHSE et al., 2014; PHAM et al., 2009; ROMBACH, 1989; SAMSONIKOVA, 1966; YPSILOS; MAGAN, 2005; VEGA et al., 2003; VIDAL et al., 1998). In contrast to these results, in the present study, we have used inoculum of blastospores to shorten the fermentation time by accelerating the exponential growth phase associated with highly aerobic and osmotic growing environment.

Although previous studies have shown that yeast-like growth of entomopathogenic fungi can be associated with water activity, some authors have cultured them in deficient-oxygen environment leading to with low yields with no survival or poor desiccation tolerance (INCH; TRINCI, 1987; HUMPHREYS; MATEWELE; TRINCI, 1989; LOHSE et al., 2014; PHAM et al., 2009). In other studies, authors verified reduction in mycelial-pellet growth by modified liquid media containing both ionic (salts) and non-ionic solutes (PEG) to increase osmolarity that also supported maximum spore yields of 2–4 × 10^8 blastospores + submerged conidia mL^{-1} for *M. anisopliae* in 3 days growth (HUMPHREYS; MATEWELE; TRINCI, 1989; KLEESPIES; ZIMMERMANN, 1992; YPSILOS; MAGAN, 2005). Leland et al. (2005) improved concentrations up to 6.9–8.4 × 10^8 submerged conidia mL^{-1} of *Metarhizium acridum* after 4 days incubation when grown in modified Adamek's medium supplemented with 50–150 g PEG L^{-1} and low glucose concentration (40 g L^{-1}). Conversely, high levels of PEG 200 ($\geq$ 100 g L^{-1}) were considered deleterious to yeast-like growth in some *M. anisopliae* strains (KLEESPIES; ZIMMERMANN, 1992). We also noted that increased concentrations of PEG 200 did not improve blastospore production or biomass accumulation in strain GHA of *B. bassiana*, thus implying that the effect of osmolytes on fungal dimorphic growth might be strain-dependent. On the other hand, *B. bassiana* ESALQ1432 showed enhanced blastospore production with increased PEG titer likewise when this fungus was grown only with glucose. It is important to mention that fungal isolates are variably

osmotolerant which implies that the osmolyte compound added to the medium has to be selected carefully according to the isolate.

It is noteworthy that external hyperosmotic conditions ($\Pi > 1.0$ MPa) in the growing media have changed the configuration of blastospores by inducing the formation of smaller cells with spherical shape in response to this osmoadptation. These cells were assumed to be phenotypically different from those produced under lower osmotic pressure. Besides its great cell density, insect bioassays revealed that these smaller blastospores harvested from hyperosmotic media (i.e., high glucose levels 100–140 g L^{-1}) displayed better insecticidal activity when compared with those produced in media with a lower glucose concentration (40 g L^{-1}). This is consistent with the evidence that osmoregulation and carbon sources in some fungi such as *M. anisopliae* and the human pathogen *Candida albicans* play important role in the virulence process (ALONSO-MONGE et al., 1999; WANG; DUAN; ST LEGER, 2009). In support to our findings on the smaller spherical blastospores, it was proposed that cellular volume reduction in true yeasts imposed by osmotic stress is mainly due to water loss and subsequently increase in the concentration of all molecules inside the cell, particularly glycerol (BABAZADEH et al., 2013). To our knowledge, this is the first time that direct evidence has been obtained for cell size reduction and enhanced virulence related to blastospores of an entomopathogenic fungus in response to hyperosmotic stress environment using liquid culture fermentation.

The yeast-like growth of various filamentous fungi has shown to be profoundly affected by oxygen supply, including *I. fumosorosea* and *M. flavoviridae* that required aerobic conditions to switch from mycelium to blastospore (JACKSON, 2012; ISSALY et al., 2005). Likewise, our data indicate that *B. bassiana* is highly responsive to elevated oxygen levels in liquid culture due to higher surface to volume ratio provided by low culture volume along with high agitation speed. Our results underpin the importance of elevated oxygen supply by itself to promoting yeast-like growth translated into high blastospore densities in *B. bassiana* shake-flask liquid cultures. Apart from the osmotic pressure, this rich oxygen environment might have also contributed to improved virulence of blastospores of *B. bassiana* against whitefly nymphs, which is in comply with previous data demonstrating a markedly insecticidal activity of aerial conidia of *I. fumosorosea* strains induced by oxidative stress conditions during cultivation (MIRANDA-HERNÁNDEZ et al., 2014). Therefore, the mechanisms underlying this superior virulence associated with oxygen-rich and osmostress-induced blastospores need further investigation.

The ability of blastospores to withstand dehydration and fast-rehydration stress using a stringent 7-h germination assay was not influenced by the osmotic pressure imposed by either the initial glucose concentration or the residual glucose in the spent medium. Instead, enhanced desiccation tolerance of *I. fumosorosea* and blastospores was firstly shown to be associated with the initial nitrogen concentration in the culture medium and the residual glucose concentration in the spent medium (JACKSON et al., 1997; CLIQUET; JACKSON, 2005). To our knowledge, this is the first report of high numbers of desiccation tolerant blastospores being produced in shorter fermentation times (≤ 3 days) using the combination of hyperosmotic and oxygen-rich cultures that yielded up to 580 times increase in blastospore density over the initial inoculum concentration in the medium. This also conveys significant information for the optimization process towards combinatorial high oxygen supply plus hyperosmotic stress for blastospore production of *B. bassiana* that resulted in tolerance to dehydration-rehydration stress as well as enhanced virulence against whiteflies. This concept also applies to other filamentous entomopathogenic fungi, such as *I. fumosorosea* (strain CG1228) that profusely grew yeast-like form attaining a production of up to 5×10^9 blastospores mL^{-1} within 3 days when cultivated in oxygen-rich and hyperosmotic culture environment (data not shown).

Nutritional and environmental conditions in liquid culture fermentation ubiquitously affect cell viability and resistance to anhydrobiosis. We have observed that cultures produced in 100 g L^{-1} glucose amended with PEG 200 (20–60 g L^{-1}) delayed blastospore germination rate between 7 h and 10 h incubation. A reasonable explanation for the lower viability acquired with air-dried blastospores of *B. bassiana* in water-stress cultures containing PEG 200 might be related to osmotic effect caused by the water of rehydration rather than the extreme dehydration itself (i.e., hypo-osmotic stress), or blastospores could be coated by PEG during dehydration that led to delaying water imbibition by the cells. Fungal cells produced in high-osmolarity media apparently contain higher concentrations of solutes in the cytoplasm and this might slow rehydration. Although not showed here, we speculate that PEG must have coated the blastospores during the air drying process resulting in slower germination. As a response to the osmotic-stress growing media caused by increased concentrations of either glucose or PEG (Π > 1.0 MPa), blastospores of *B. bassiana* were smaller in size when compared with those unstressed cells produced in culture with higher water availability (Π < 0.8 MPa). In agreement with these observations, it was shown that hyperosmotic conditions decrease the cell volume of bakery's yeasts, which is considered an osmoadaptation with a

resultant increase of the concentrations in all substances present in the cytoplasm (HOHMANN, 2002; BABAZADEH et al., 2013).

After penetrating the insect cuticle during the infection process, entomopathogenic fungi adapt to the high osmotic pressure of the hemolymph and multiply. Typically, the majority of the fungi maintain cytoplasmatic osmotic pressures between 0.1 and 1.0 MPa higher than their surroundings with the aim to maintain water influx and turgor pressure (MONEY, 1997). Once in the insect hemocoel, certain osmosensing proteins found in fungal cells (hyphal bodies or blastospores), including the newly identified MOS1 osmosensor of *M. anisopliae*, are required for osmoadaptation to the water-stress conditions of the insect hemolymph (WANG; DUAN; ST LEGER, 2008). Recent research has shown that osmotic stress response in true yeasts, such as *Saccaromyces cerevisiae*, is regulated by mitogen-activated protein (MAP) kinase cascade and high-osmolarity glycerol pathway (HOHMANN, 2002), which are most likely involved in osmoregulation response among entomopathogenic fungi, though very little information has been documented. Insect hemolymph is a solute-rich environment and hence it is characterized by a high osmotic pressure in the range of 0.7 to 1.2 MPa (CHAPMAN, 2013). In principle, this fact support our findings on artificial high-osmotic-pressure conditions imposed by non-ionic osmolytes added to liquid cultures of *B. bassiana* enabled to mimic the water-stress conditions in insect hemolymph leading to a shift from mycelium to blastospore growth. Although the mechanisms involved in osmotic and oxidative stress responses inducing morphogenesis in liquid cultures of anamorphic filamentous fungi are not well understood, it is clear based on the present results that these factors are to a very large degree responsible for triggering yeast-like growth. Furthermore, this information is of direct relevance for mass-production of these cells in industrial fermentors.

In closing, the information gathered in this study emphasizes the role of osmotic pressure and oxygen availability in yeast-like growth of *B. bassiana* cultivated in liquid fermentation. These findings comprise a significant advance for producing high concentrations of desiccation tolerant blastospores of *Beauveria* in short fermentation times and with notorious virulence against soft-bodied insects, which is ultimately important for the industrial fermentation of these mycoinsectides. Therefore, oxidative and osmotic stresses along with nutritional requirements play concomitantly a key role in industrial aerobic fermentation bioprocesses of filamentous fungi, and understanding these factors could potentially contribute to the productivity and fitness of blastospores.

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Table 6.1 - Effect of aeration rates on blastospore yield, biomass accumulation, glucose utilization and final pH by *B. bassiana* (ESALQ1432 and GHA) grown in different liquid culture volumes (50 and 100 mL) and agitation speeds (175 and 350 rpm) using 250-mL baffled Erlenmeyer flasks incubated at 28 °C in a rotary shaker incubator

Beauveria bassiana Strain	Agitation speed (rpm)	Culture volume (mL) [a]	Spore concentration ($\times 10^8$ blastospores mL^{-1})		Dry weight ($mg\ mL^{-1}$)		Glucose utilization ($g\ L^{-1}$)	Final pH
			Day 2	Day 3	Day 2	Day 3		
ESALQ1432	350	100	7.6 ± 1.0 b [b]	9.9 ± 1.1 a	25.0 ± 0.4 b	33.1 ± 0.9 a	41.8±3.7	4.6
		50	11.0 ± 0.6 a	14.0 ± 1.5 b	31.8 ± 1.0 a	35.6 ± 1.0 a	44.0±0.9	4.6
	175	100	3.3 ± 0.1 c	3.3 ± 0.3 c	22.1 ± 0.3 c	24.9 ± 0.6 c	37.5±1.6	4.9
		50	4.9 ± 0.2 c	5.7 ± 0.5 c	25.3 ± 0.1 b	28.4 ± 0.5 b	44.0±2.1	4.9
GHA	350	100	8.3 ± 0.9 a	9.6 ± 1.8 a	28.8 ± 2.7 ab	25.4 ± 0.6 b	52.3±3.4	5.1
		50	8.9 ± 0.7 a	9.0 ± 0.2 a	30.1 ± 2.0 a	30.7 ± 0.9 a	60.0±3.2	4.8
	175	100	3.1 ± 0.1 b	3.2 ± 0.3 b	22.3 ± 0.6 c	22.6 ± 0.3 c	40.5±4.7	4.8
		50	7.2 ± 0.4 a	7.2 ± 0.3 a	23.0 ± 0.4 bc	30.1 ± 1.0 a	50.3±3.1	4.5

[a] Culture volumes and agitation speeds were altered to increase (50 mL or 350 rpm) or decrease (100 mL or 175 rpm) aeration. [b]For each strain within each sample day, mean values followed by different letters are significantly different (Tukey test, $P < 0.05$)

Table 6.2 - Parameters of the exponential growth model describing the impact of glucose concentration on blastospore yield of *B. bassiana* grown in liquid cultures

B. bassiana Strain	Fermentation time	Parameters [a]				R^2	*F*	*P*
		y_0	x_0	*a*	*b*			
ESALQ1432	Day 2	2.6×10^8	90.5	1.5×10^9	19.0	0.95	40.33	0.0002
	Day 3	6.0×10^8	100.3	2.2×10^9	9.3	0.99	473.48	<0.0001
GHA	Day 2	4.8×10^8	91.3	1.1×10^9	27.1	0.86	14.40	0.0022
	Day 3	6.0×10^8	152.8	2.2×10^9	25.8	0.95	48.71	<0.0001

[a] Growth model: $y = y_0\ (a\ /\ (1 + \exp(x - x_0)\ /\ b))$

Table 6.3 - Effect of glucose concentration on desiccation tolerance of *B. bassiana* blastospores (ESALQ1432 and GHA) after air-drying to < 3% moisture content (i.e., $a_w < 0.3$)

Glucose concentration (g L^{-1})	Osmotic pressure (MPa)	ESALQ1432			GHA		
		Survival (%)	a_w	Moisture (%)	Survival (%)	a_w	Moisture (%)
20	-0.2201	73.3 ± 1.0 [ns]	0.118 ± 0.005	1.86 ± 0.21	56.4 ± 1.9 ab [b]	0.209 ± 0.052	2.28 ± 0.76
40	0.1332	69.7 ± 3.0	0.134 ± 0.013	1.76 ± 0.14	57.5 ± 5.2 ab	0.229 ± 0.066	2.54 ± 0.58
60	0.2528	70.7 ± 1.4	0.164 ± 0.002	1.70 ± 0.12	49.6 ± 1.7 b	0.174 ± 0.057	2.21 ± 0.26
80	0.5704	71.8 ± 1.1	0.198 ± 0.002	2.06 ± 0.14	53.3 ± 2.2 b	0.132 ± 0.025	2.33 ± 0.27
100	0.7873	70.5 ± 1.8	0.192 ± 0.027	1.80 ± 0.01	54.6 ± 4.1 ab	0.122 ± 0.006	2.53 ± 0.09
120	1.1803	69.6 ± 3.2	0.212 ± 0.021	2.30 ± 0.12	54.6 ± 1.0 ab	0.184 ± 0.008	2.20 ± 0.15
140	1.5791	68.9 ± 2.5	0.176 ± 0.016	2.29 ± 0.12	66.6 ± 1.8 a	0.172 ± 0.015	1.96 ± 0.17
160	1.8486	64.2 ± 0.6	0.246 ± 0.007	2.25 ± 0.15	64.3 ± 1.0 a	0.197 ± 0.004	2.02 ± 0.05
180	2.5156	65.9 ± 0.8	0.239 ± 0.009	2.31 ± 0.10	61.7 ± 1.1 ab	0.178 ± 0.005	1.93 ± 0.05
200	2.8198	66.2 ± 2.1	0.242 ± 0.013	2.36 ± 0.16	58.6 ± 2.2 ab	0.183 ± 0.005	1.84 ± 0.08
220	2.9980	-	-	-	55.5 ± 2.9 ab	0.238 ± 0.003	2.05 ± 0.05

[a] Blastospore desiccation tolerance (% survival) was assessed by measuring blastospore germination by air-dried blastospores rehydrated in potato dextrose broth and incubated for 7 h at 28 °C and 300 rpm. [b] Means (± SE) followed by the different letters within columns are significantly different using (Tukey test, $P < 0.05$). Not significant (ns). Not performed (-)

Table 6.4 - Effect of osmotic pressure and agitation speed on blastospore concentration and dry weight accumulation by *Beauveria bassiana* (ESALQ1432 and GHA) grown in 50 mL culture culture volume at 28±0.5 °C.

Beauveria bassiana Strain	Agitation speed (rpm)	Initial glucose (g L^{-1}) [a]	Spore concentration (× 10^8 blastospores mL^{-1})		Dry weight (mg mL^{-1})	
			Day 2	Day 3	Day 2	Day 3
ESALQ1432	350	200	17.7 ± 0.8 a [b]	31.3 ± 1.3 a	27.5 ± 0.8 b	38.3 ± 1.0 a
		40	4.6 ± 0.3 c	6.1 ± 0.5 c	32.1 ± 1.0 a	35.6 ± 0.8 a
	175	200	9.2 ± 0.7 b	14.2 ± 1.2 b	26.1 ± 0.5 b	28.4 ± 0.6 b
		40	2.8 ± 0.2 d	3.8 ± 0.5 d	28.77 ± 0.8 b	29.6 ± 0.8 b
GHA	350	200	15.8 ± 1.0 a	25.3 ± 2.1 a	29.0 ± 0.7 a	33.2 ± 0.9 a
		40	6.2 ± 0.5 b	6.6 ± 0.7 c	29.5 ± 0.5 a	30.3 ± 0.4 a
	175	200	8.0 ± 0.8 b	12.6 ± 1.6 b	24.3 ± 0.6 b	32.1 ± 2.8 a
		40	3.9 ± 0.5 c	5.5 ± 0.7 c	24.7 ± 0.5 b	25.2 ± 0.6 b

[a] Glucose rates at 200 and 40 g L^{-1} generated low (0.13 MPa) and high (2.82 MPa) osmotic pressures, and agitation speed was altered to increase (350 rpm) or decrease (175 rpm) aeration. [b] For each strain within each sample day, mean values followed by different letters are significantly different (Tukey test, $P < 0.05$)

Table 6.5 - Effect of media containing glucose amended with polyethylene glycol (PEG 200) on total biomass, percentage of blastospore mass, glucose utilization, and pH of *B. bassiana* grown in liquid cultures at 28±0.5 °C and 350 rpm

Osmolyte concentration (g L^{-1})		Dry weight (mg mL^{-1})			
		ESALQ1432		GHA	
Glucose	PEG 200	Day 2	Day 3	D ay 2	Day 3
100	-	36.5 ± 0.5 a [a]	42.3 ± 2.1 a	33.4 ± 1.6 [ns]	33.1 ± 1.5 [ns]
100	20	41.9 ± 1.5 a	39.1±2.1 ab	33.6 ± 0.7	32.5 ± 1.3
100	40	29.6 ± 0.6 b	35.3±0.4 b	35.9 ± 1.3	31.5 ± 1.2
100	60	30.1 ± 1.4 b	34.4 ± 1.1 b	31.6 ± 0.7	33.5 ± 1.6

[a] Means (± SE) followed by different letters within columns are significantly different (Tukey test, $P < 0.05$). Not significant (ns)

Table 6.6 - Virulence bioassay using 2nd instar whitefly nymphs (*B. tabaci* biotype B) to assess the impact of blastospores (*B. bassiana* ESALQ1432) produced in liquid media containing 25 g L^{-1} cottonseed flour at different initial glucose concentrations

Glucose (g L^{-1})	n [a]	Intercept ± SE	Slope ± SE [b]	χ^2 (*P*-value) [c]	LC_{50} (spores cm^{-2}) [d]	95% CL (spores cm^{-2}) [e]		RP_{50} [f]	95% CL		ST_{50} (days) [g]	95% CL (days)	
						Lower	Upper		Lower	Upper		Lower	Upper
40	2042	-4.25±0.68	1.44±0.2	49.4 (< 0.0001)	899.6	484.9	1492	-	-	-	4.0	3.86	4.14
100	1944	-3.44±0.52	1.26±0.16	60.3 (< 0.0001)	536.8	294.0	869.0	1.68	0.97	2.9	3.0	2.85	3.14
140	1908	-3.79±0.56	1.44±0.18	63.2 (< 0.0001)	429.6	237.7	683.7	2.09	1.21	3.63	3.0	2.87	3.14

[a] Total number of whitefly nymphs tested (two independent assays with four replicates per fungal-concentration)

[b] Slope for mortality represents regression of proportion of nymph mortality versus log of blastospores cm^{-2}

[c] χ^2 and *P* values represent the probability of slope $\neq 0$, rather than fit to logistic model

[d] Delivered median lethal dose (LC_{50}) expressed by blastospores cm^{-2} and estimated by the logistic model. Cumulative mortality censored up to day 6 post-application. Control mortality averaged 5.7 ± 1.5%

[e] Confidence limits

[f] Relative potency is the measure of relative efficacy of blastospores produced in medium with 4% glucose (baseline) to blastospores produced in higher glucose concentrations. Comparisons were undertaken within each fungal species and if the confidence limit for the LC ratio does not contain 1, hence it is concluded that the LC values are significantly different (WHEELER et al., 2007)

[g] Median survival time (ST_{50}) estimated by Kaplan-Meier product limit method from a single-concentration assay based on concentration at 1.25×10^7 blastospores mL^{-1}

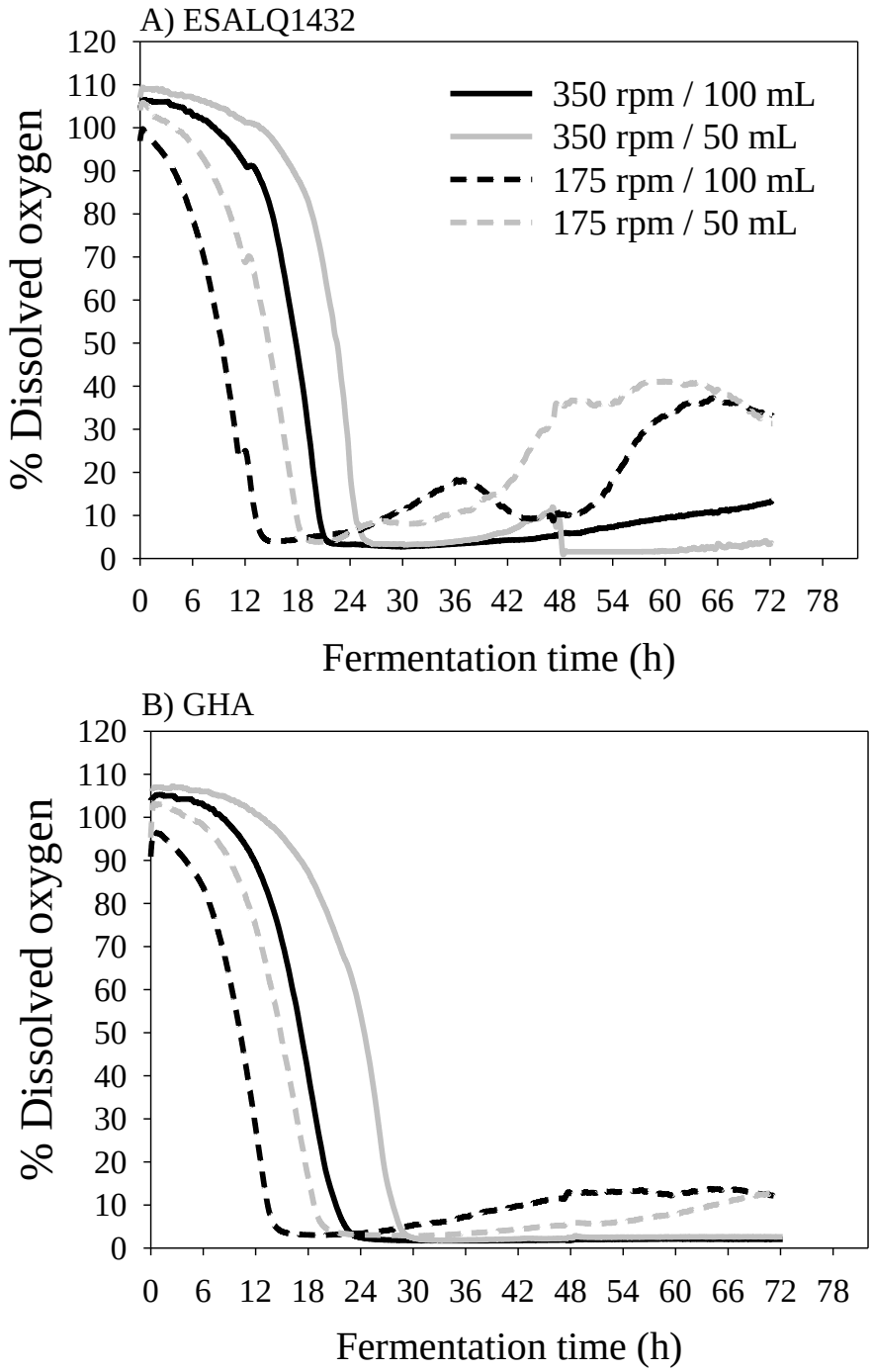


Figure 6.1 - Dissolved oxygen (% O_2 concentration in the liquid medium) profiles for cultures of *B. bassiana* grown in different liquid culture volumes (50 and 100 mL) and agitation speeds (350 and 175 rpm) using 250-mL baffled Erlenmeyer flasks incubated at 28±0.5 °C in a rotary shaker incubator. Results combined from different trials. (The shaker was stopped to obtain samples from culture flasks at 24 and 48 h)

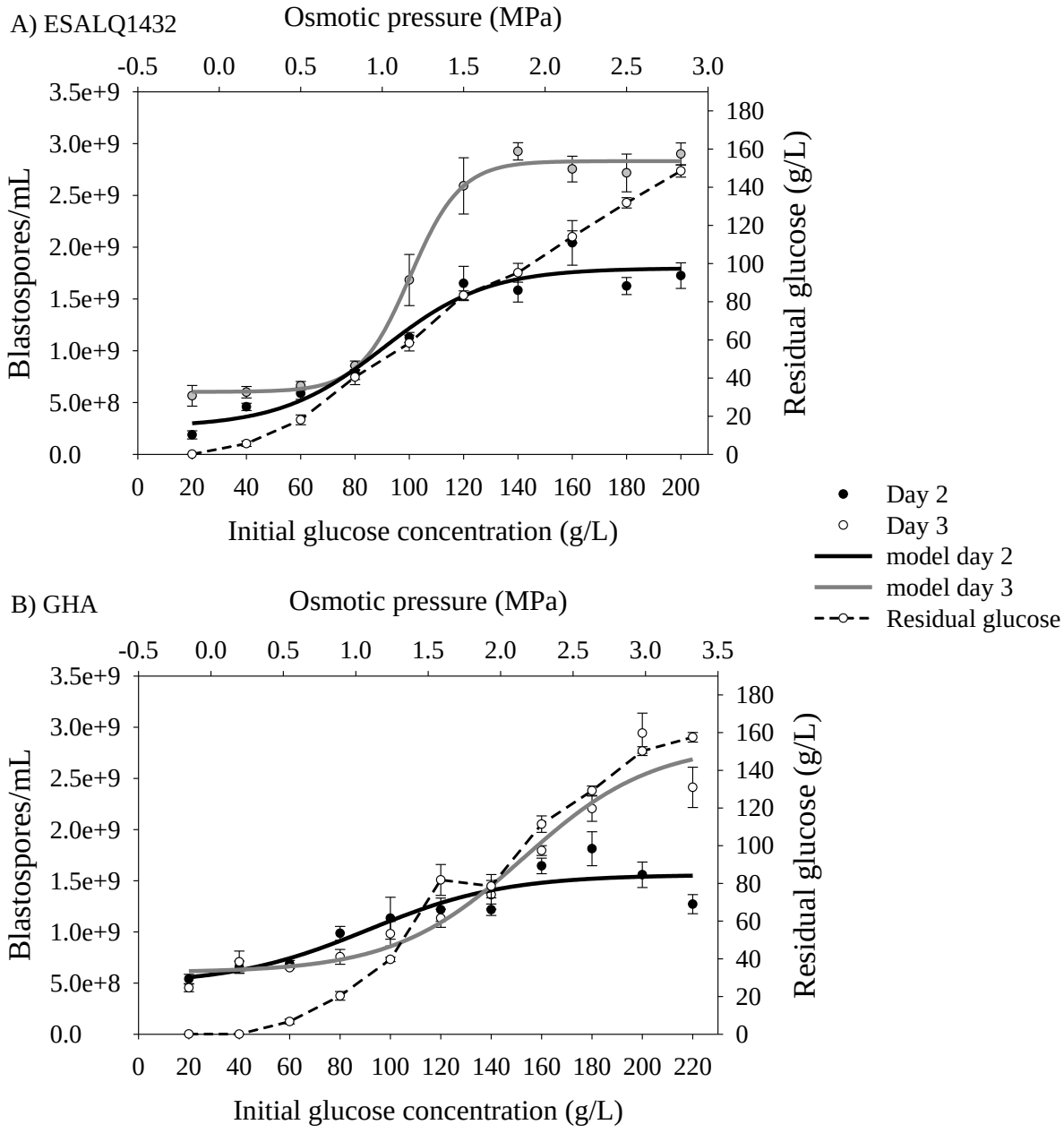


Figure 6.2 - Effect of glucose concentration on osmotic pressure and blastospore production by *B. bassiana* (ESALQ1432 [A] and GHA [B]) cultures grown in 50 mL liquid media containing basal salts with 25 g L^{-1} cottonseed flour at 28±0.5 °C and 350 rpm. Solid lines (blue and red) represent the fitted exponential growth model (see Table 6.2), while circles designate means values (± SE)

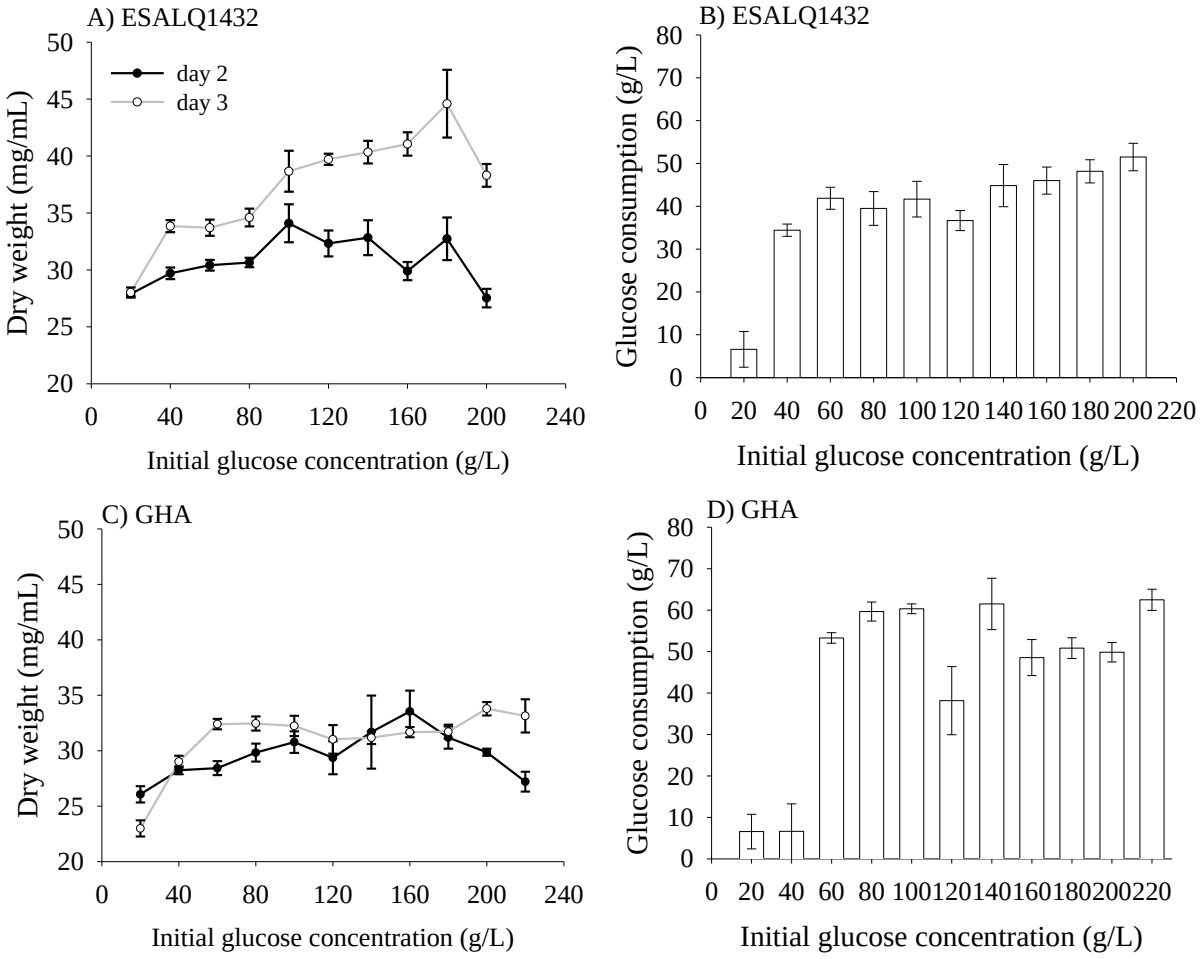


Figure 6.3 - Effect of initial glucose concentration on biomass accumulation and glucose utilization by *B. bassiana* (ESALQ1432 [A,B] and GHA [C,D]) grown in liquid cultures at 28±0.5 °C and 350 rpm. Lines and bars represent observed mean values (± SE)

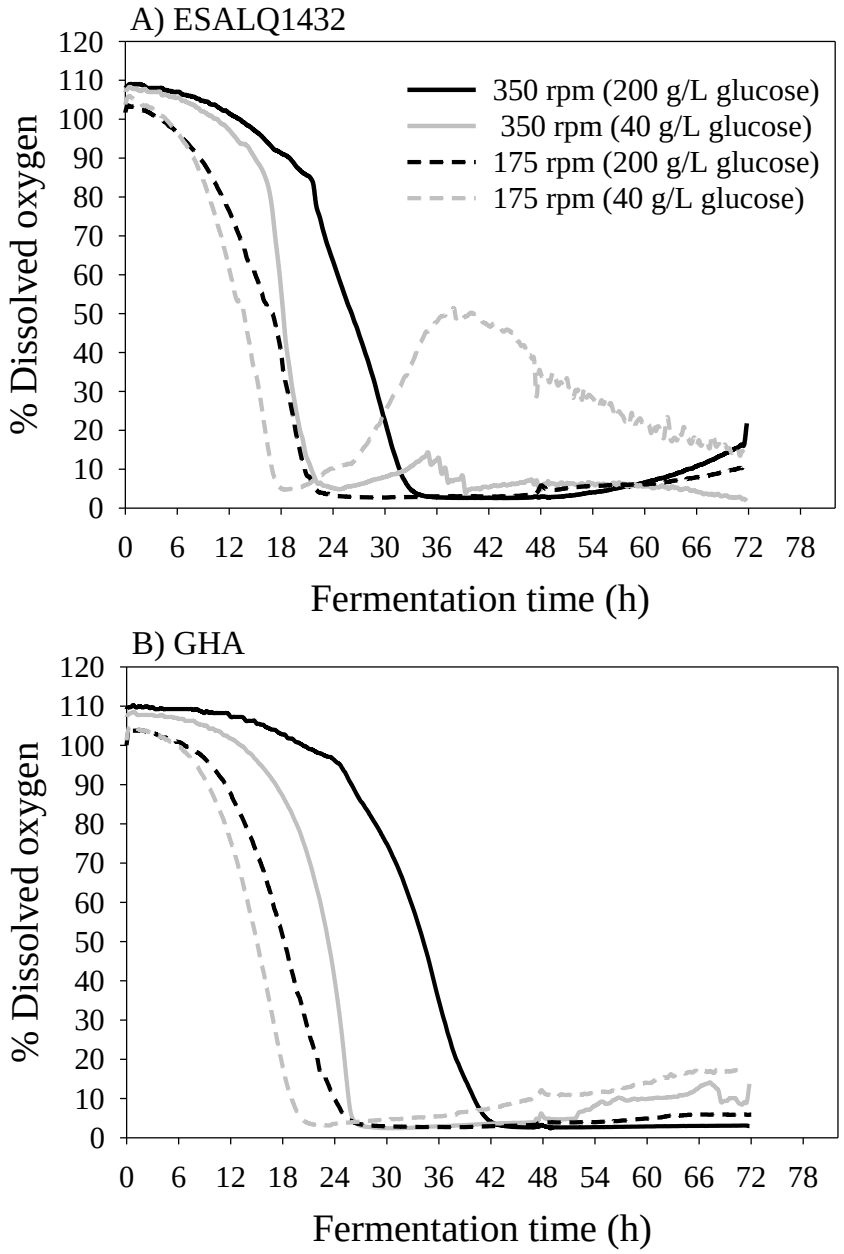


Figure 6.4 - Effect of osmotic pressure imposed by initial glucose concentrations (40 and 200 g L^{-1}) on dissolved oxygen (% O_2 concentration in the liquid medium) profiles for cultures of *Beauveria bassiana* grown in 50 mL liquid media at different shaking frequencies (350 rpm [A] and 175 rpm [B]), using 250-mL baffled Erlenmeyer flasks incubated at 28±0.5 °C in a rotary shaker incubator

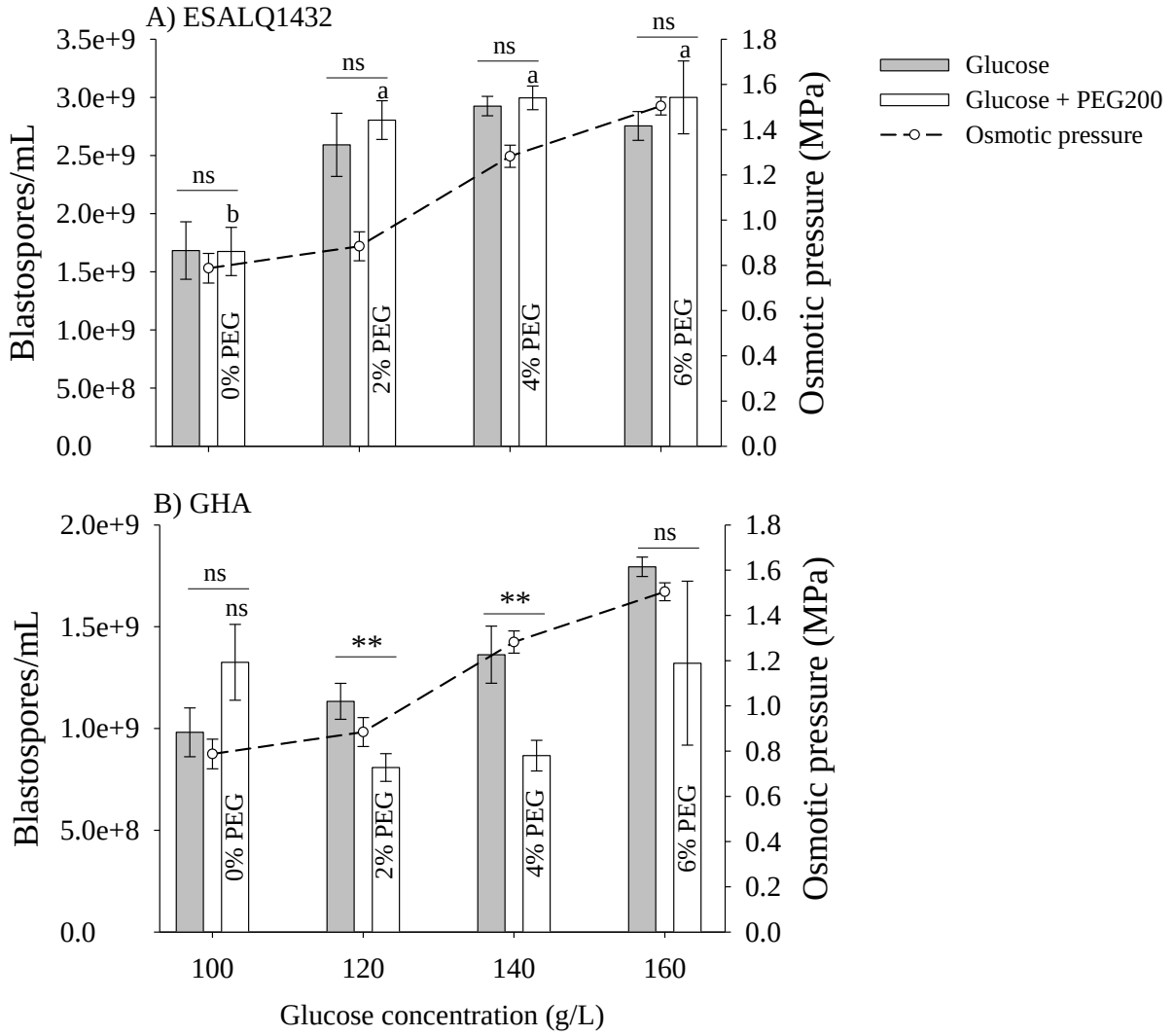


Figure 6.5 - Effect of osmotic pressure generated by glucose alone (filled bars) or amended with polyethylene glycol (PEG 200) (empty bars) on blastospore production of *Beauveria bassiana* (ESALQ1432 [A] and GHA [B]) cultures grown in 50 mL liquid medium containing basal salts with 25 g L^{-1} cottonseed flour and incubated for 3 days at 28±0.5 °C and 350 rpm. PEG-amended cultures contained 100 g L^{-1} glucose. Means (± SE) followed by the different letters are significantly different (Tukey test, $P \leq 0.05$). Capital letters designate comparisons within day 2 or germination by 7 h, while lowercase letters designate comparisons within day 3 or germination by 10 h. Comparisons according to *t*-Student test are indicated by $P < 0.05$ (*), $P < 0.01$ (**), or not significant (ns)

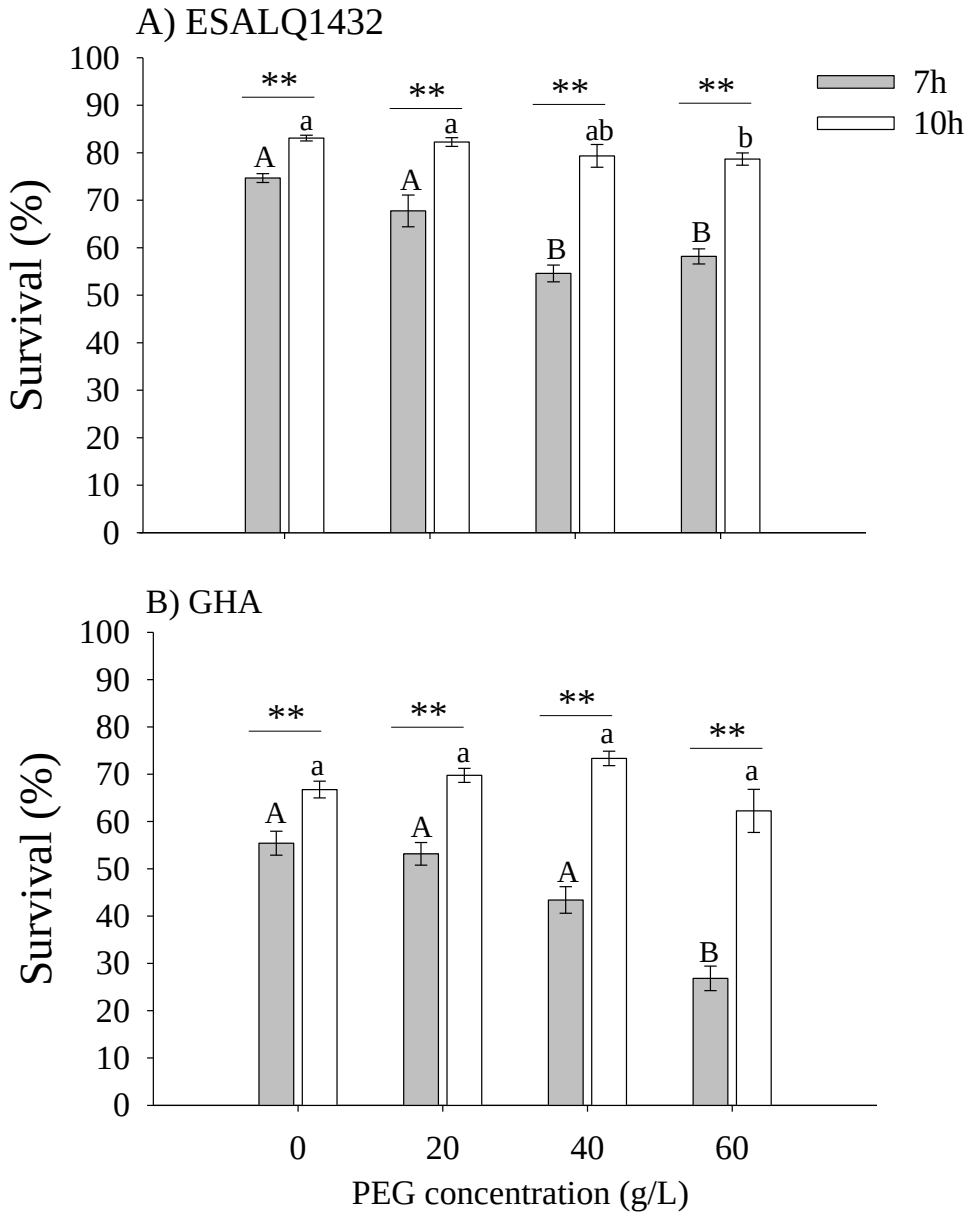


Figure 6.6 - Effect of osmotic pressure generated polyethylene glycol (PEG 200) on desiccation tolerance (air-drying) of *Beauveria bassiana* (ESALQ1432 [A] and GHA [B]) cultures grown under high oxygen rates (50 mL culture volume and 350 rpm). PEG-amended cultures contained 100 g glucose L^{-1}. Blastospore desiccation tolerance was assessed by measuring blastospore germination by air-dried blastospores rehydrated in PD broth and incubated for 7 h (filled bars) and 10 h (empty bars) at 28±0.5 °C and 300 rpm. Means (± SE) followed by the different letters are significantly different (Tukey test, $P < 0.05$). Capital letters designate comparisons within 7 h incubation, while lowercase letters designate comparisons within 10 h incubation. Comparisons according to *t*-Student test are indicated by $P < 0.05$ (*), $P < 0.01$ (**), or not significant (ns)

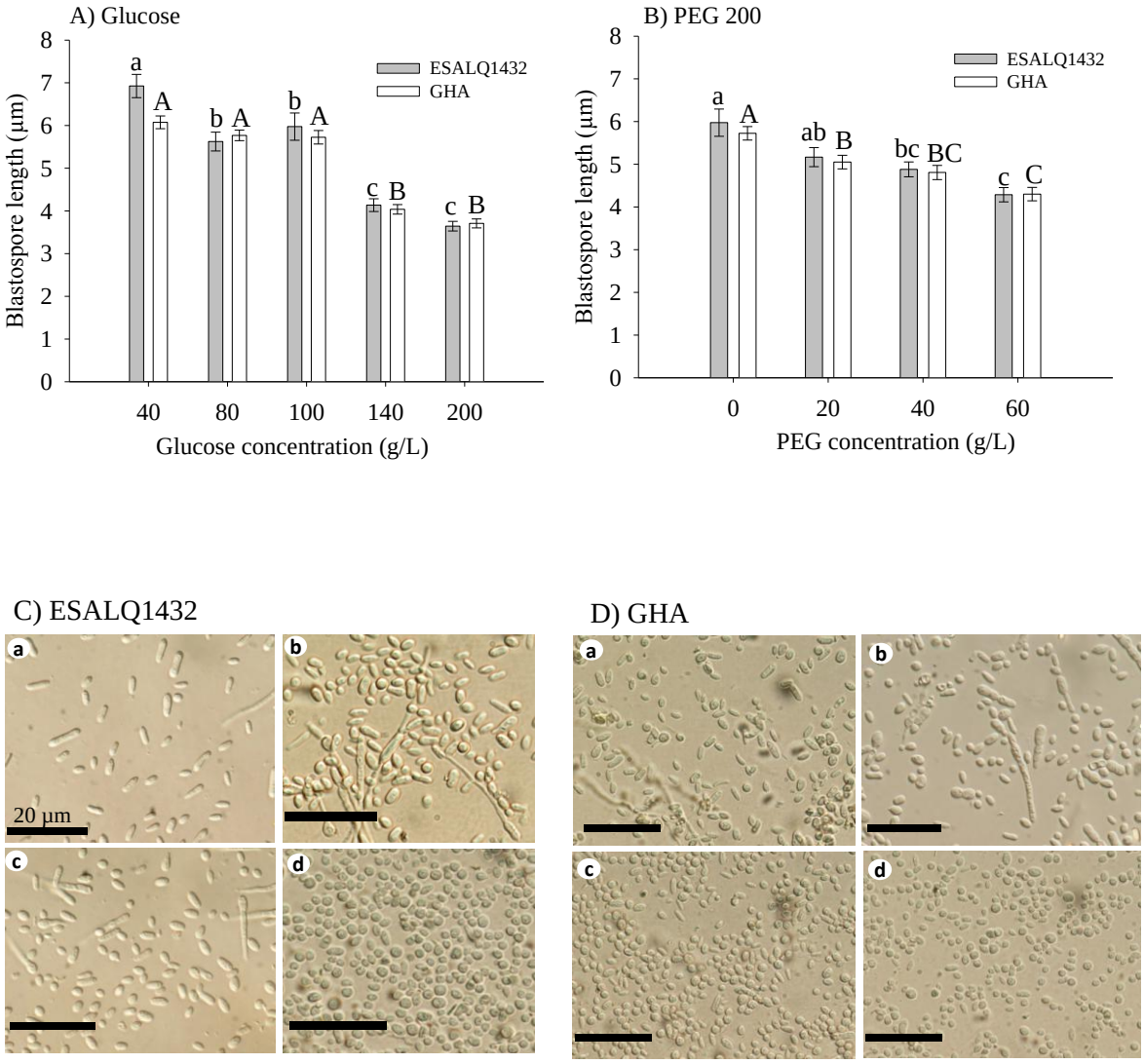
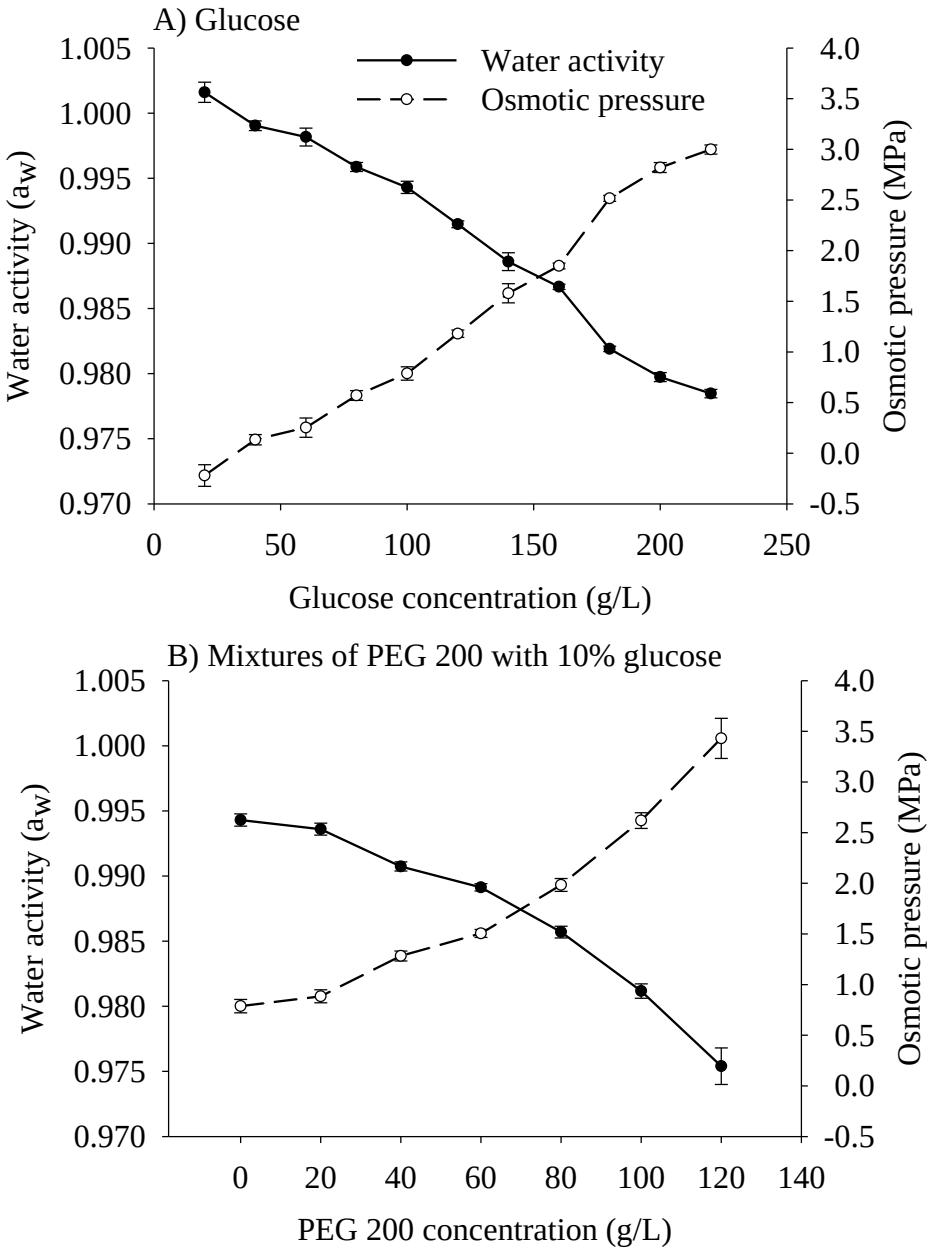


Figure 6.7 - Effect of osmotic pressure imposed by glucose (A) or by polyethylene glycol (PEG 200) amended with glucose (100 g L^{-1}) (B) on blastospore size of *Beauveria bassiana* ESALQ1432 and GHA after 3 days growth in media supplemented with 25 g L^{-1} cottonseed flour. Blastospores decreased in size as glucose concentration increased (C, D): (a) 40 g L^{-1} = 0.1332 MPa, (b) 80 g L^{-1} = 0.5704 MPa, (c) 140 g L^{-1} = 1.5791 MPa, (d) 200 g L^{-1} = 2.8198 MPa. Scale bar = 20 µm. Means (± SE) followed by non-corresponding letters, within each fungal strain, are significantly different (Tukey test, $P < 0.05$)

Appendix



Appendix A - Relationship between concentration, water activity and osmotic pressure imposed by glucose concentrations alone or in combination with PEG 200 at 25 °C. Each data point represents the mean value (± SE) of osmotic pressure and water activity

7 IMPROVED SHELF LIFE OF *BEAUVERIA BASSIANA* BLASTOSPORES BY CONVECTIVE DRYING METHODS AND ACTIVE PACKAGING[6]

Abstract

Desiccation/rehydration and aging stresses in fungal cells are constraints for the development of active, stable biocontrol products. The effects of drying methods (spray drying or air drying), modified atmosphere packaging (reduced oxygen and/or humidity), and storage temperature (4 or 28 °C) on viability and virulence of blastospores of the insect pathogenic fungus *Beauveria bassiana* were evaluated against whitefly (*Bemisia tabaci*). All formulations were dried to < 5% water content or < 0.3 water activity. Blastospores were encapsulated by spray drying in skim milk (SM) matrix (20% w/v) with or without ascorbic acid (2.5% w/v) and retained 82–83% viability upon fast-rehydration. Air dried blastospores were formulated with diatomaceous earth as a wettable powder formulation and retained 77% viability. Blastospores from both dry formulations that were vacuum-packed in aluminized oxygen-moisture proof bags had shelf life of at least 9 months at 4 °C. Viability decreased faster for spray-dried cells when stored at 28 °C. Adding oxygen-moisture scavengers in aluminized bags without vacuum significantly lengthened shelf life of both air dried and spray dried blastospores, remaining > 60% viable for at least 7 months at 28 °C. Bioassays revealed that both spray and air dried blastospores were similarly virulent toward whitefly nymphs confirming that bioefficacy was not affected by either drying method. Furthermore, 4-month-old air and spray-dried blastospores stored at 28 °C were highly effective against whitefly nymphs, with the former formulation displaying better performance. These results provide novel information for improvement of stability of *B. bassiana* blastospore using cost-effective drying techniques coupled with modified atmosphere packaging based on dual action enzymatic O_2/moisture scavenging system for storage at room temperature.

Keywords: *Beauveria bassiana*; Submerge liquid fermentation; Air drying; Spray drying; Storage stability; Oxygen scavengers; Sprayable formulations

7.1 Introduction

Entomopathogenic fungi are useful as bioinsecticides for control of a wide range of insect pests. However, adoption of microbial biological control of insects has been slow for many reasons, including inadequate knowledge regarding methodology for mass production and formulation (SUNDH; GOETTEL, 2013). *Beauveria bassiana* (Bals.) Vuill. is one of the most common commercialized microbial agents for use in insect control worldwide (FARIA; WRAIGHT, 2007). Most fungal-based products contain mainly aerial conidia (asexual spores) as the active form. Conidia are typically produced using solid substrates (DHAR; KAUR, 2011), and are amenable to a variety of liquid and powder formulations that provide reasonable storage stability and pest control efficacy (FARIA; WRAIGHT, 2007). Yet, there are significant disadvantages associated with production of conidia. Specifically, solid

[6] MASCARIN, G.M.; JACKSON, M.A.; BEHLE, R.W.; KOBORI, N.N.; DELALIBERA JR., I. Improved shelf life of *Beauveria bassiana* blastospores by convective drying methods and active packaging. **Applied Microbiology and Biotechnology** (submitted).

substrate production of conidia using grains is expensive, labor intensive and most commercial processes operate on relatively small scales; furthermore, scale-up is very difficult using solid-substrate fermentation bioreactors. Production costs have been estimated at greater than US$ 27 per hectare (KRAUSS et al., 2002). Also, solid substrate production has a relatively high risk of undesirable contamination, which results in production losses and contributes to higher production costs.

Other fungal cells such as the 'yeast-like' blastospores can be as effective as conidia to control insect pests (JACKSON et al., 1997; VANDENBERG et al., 1998). Recent advances in blastospores production in liquid media make this propagule an attractive alternative to conidia as the active propagule in mycopesticides. Although productivities vary among fungal strains (MASCARIN et al., 2015), spore yields from submerged liquid cultures have substantially increased from early cultures that provided 1×10^9 spores mL^{-1} after 5 days fermentation (BIDOCHKA et al., 1987; CHONG-RODRIGUES et al., 2011). Long fermentation times coupled to poor desiccation tolerance and short shelf life have limited the adoption of blastospore-based mycoinsecticides worldwide. Economies of scale for liquid fermentation combined with higher spore production and shorter fermentation duration result in high production capacity. In our recent discovery, we found that oxygen-rich and hyperosmotic growing environment is markedly conducive to yield high concentrations of desiccant tolerant blastospores of *B. bassiana* as well as *Isaria fumosorosea* ($> 2 \times 10^9$ viable blastospores mL^{-1}) in shorter cultivation time (≤ 3 days) and with improved virulence against whiteflies (unpublished results).

Improvements in production necessitate improvements in subsequent processes needed to produce commercially viable products. Drying of entomopathogenic fungal cells poses a challenge for the maintenance of viability and biological activity. Although blastospores have been generally considered more sensitive to anhydrobiosis stress than aerial conidia, research has shown fungal blastospores to be desiccation tolerant under laboratory conditions consisting of gentle air drying by controlling relative humidity (RH) atmosphere (JACKSON et al., 1997; MASCARIN et al., 2015). Laboratory drying techniques tend to be labor intensive and slow when compared with established commercial processes. Particularly, spray drying is a commonly used technique for commercial drying of tolerant microbes. Initial trials to spray dry blastospores demonstrated the ability of the spores to survive drying (STEPHAN; ZIMMERMANN, 1998), but subsequently had short viability when stored above refrigerated temperatures.

Furthermore, active packaging through the use of oxygen and moisture scavengers are becoming increasingly attractive for biopesticide formulation due to their potential for extending shelf life of dry preparations, especially of fungal entomopathogens. Overall, oxygen absorbing technology is based on oxidation or combination of one of the following components: iron powder, ascorbic acid, photosensitive polymers, enzymes. These compounds are able to reduce the levels of oxygen to below 0.01%, which is lower than the levels typically found (0.3–3%) in the conventional systems of modified atmosphere, vacuum or substitution of internal atmosphere for inert gas (CRUZ et al., 2007). Concerning moisture absorbers, the most economical and common desiccants rely on silica gel and calcium sulfate (drierite). Improved shelf life was previously achieved by using dual action O_2/moisture scavenger for stored hydrophobic conidiospores of *B. bassiana* at temperatures as high as 50 °C (FARIA et al., 2011). Thus, these technologies would be suitable for prolonged storage stability of blastospores at room temperatures in tropical and subtropical countries.

The development of blastospores as a commercially viable product requires downstream processing and formulation to provide sufficient storage stability, ease of application, and adequate pest control efficacy. These properties are of paramount importance for the successful development of fungal-based insecticides. To address this phase of development, we evaluated two distinct drying techniques to produce dry formulations of *B. bassiana* blastospores for desiccation tolerance, storage stability, and virulence against whiteflies.

7.2. Development

7.2.1 Materials and Methods

7.2.1.1 Liquid culture production for blastospores

Stock cultures of *Beauveria bassiana* strain ESALQ1432, originally isolated from adults of the citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), in Piracicaba, SP, Brazil) were grown on potato dextrose agar ([PDA] Difco®, Detroit, MI, USA) in Petri dishes for 2–3 weeks at 22 ± 2 °C with a 12:12 h (L:D) photoperiod until sporulation, then cut into 1 mm^2 agar plugs and stored in 10 % glycerol in sterile cryovials at –80 °C. These frozen stock cultures were used to inoculate PDA plates that were incubated for 2–3 weeks until sporulation. Conidia from these plates were used for inoculation of the pre-cultures to produce blastospores. Conidia and blastospore concentrations were measured microscopically using a

hemacytometer (400X magnification) with a light microscope with DIC optics (BH2, Olympus America, Center Valley, PA, USA).

Pre-cultures of *B. bassiana* blastospores were produced in 100 mL of a semi-defined synthetic liquid medium using 250-mL baffled, Erlenmeyer flasks in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific, Edison, NJ) at 28 °C and 350 rpm. The liquid medium for the pre-cultures consisted of 80 g glucose L^{-1} (Fisher Scientific®, 40% carbon), 25 g acid hydrolyzed casein L^{-1} (Hy-Case™ MSF, Kerry Bioscience, New York, NY, USA; 53% carbon and 8.5% nitrogen), and basal salts supplemented with trace metals (basal salts medium) containing per liter: KH_2PO_4, 2.0 g; $CaCl_2.2H_2O$, 0.4 g; $MgSO_4.7H_2O$, 0.3 g; $CoCl_2.6H_2O$, 37 mg; $FeSO_4.7H_2O$, 50 mg; $MnSO_4.H_2O$, 16 mg; $ZnSO_4.7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500 mg each; folic acid, biotin, vitamin B12, 50 mg each (JACKSON et al., 1997). These pre-cultures were used to inoculate 4 L liquid medium in a 5-L benchtop bioreactor (BIOSTAT® B, Braun Sartorius, San Diego, CA, USA) to produce *B. bassiana* blastospores. The fermentation medium consisted of 140 g glucose L^{-1} and 25 g cottonseed flour L^{-1} supplemented with basal salts + vitamins, as previously described for the pre-cultures. This fermentation medium provided a carbon-to-nitrogen (C:N) ratio of 28:1. Three-day-old pre-cultures were used to inoculate the 4-L liquid medium by delivering a final concentration of 5×10^6 blastospores mL^{-1} with inoculum volume of 10% (v/v). Fermentation conditions were set to 28 °C, 2.0 L min^{-1} of air flow and 500 rpm stirred speed with three impellers and conducted in a 5-L glass tank vessel. The initial pH was 5.3 and uncontrolled throughout fermentation. This fermentation study was repeated at least six times to generate enough material for further experiments on desiccation tolerance, storage stability, and virulence. This fermentation procedure produced $1–3 \times 10^9$ blastospores mL^{-1} and 40 g L^{-1} of dry biomass after 3 days growth.

7.2.1.2 Air drying

Before air or spray drying, three-day-old culture broth was cleaned. Broth was filtered through 80-mesh sieve to remove most of hyphae and collected only blastospores. The filtered blastospore suspensions were centrifuged at 10,000 rpm (= 15,182 *g*) (Sorvall SLA-1500 RC-5 Plus Series rotor, Thermo Scientific®, USA) for 10 min at 10 °C to remove the spent medium, and then the blastospores were re-suspended in sterile double-deionized water at a minimum concentration of 2×10^9 blastospores mL^{-1}. This blastospore suspension was used to create the feed stock material for drying.

For air drying, blastospores were mixed with diatomaceous earth (DE; HYFLO®, Celite Corp., Lompoc, CA, USA) at a ratio of 1×10^{10} blastospores g^{-1}, and then preliminary water removal was performed using vacuum-filtration in a Buchner funnel through a Whatman No. 1 filter paper. The blastospore-DE preparation was crumbled using a blender, placed on aluminum trays, then placed in an air drying chamber with controlled relative humidity (RH) ~ 50–60% for 15–20 h and then decreased to 15–25% for 1 h until a final moisture content of < 4% (w/w) was achieved (JACKSON; PAYNE, 2007).

7.2.1.3 Spray drying

For the spray drying, the cleaned blastospore suspension was mixed with dissolved skim milk powder (SMP, Great Value, Wallmart, USA) to provide final concentrations of 2×10^{11} blastospores and 200 g SMP L^{-1}. The final dried product was expected to contain 1×10^{9} blastospores g^{-1}. In another formulation, ascorbic acid (Sigma®, St. Louis, MO, USA) at 25 g L^{-1} was added to the blastospore suspension with 175 g L^{-1} SMP. The spray drying conditions were based on those previously reported for blastospores (STEPHAN; ZIMMERMANN, 1998). Briefly, these two formulations were spray dried using a Niro Atomizer Spray Dryer (Niro Atomizer, Inc., Columbia, MD, USA) with a spinning disc atomizer. Drying conditions were 95±2 °C inlet temperature, 48±2 °C outlet temperature, 20 mL min^{-1} feed rate, and 5.7–5.9 bar air pressure. The feed stock volume varied from 100–300 mL according to the blastospore produced by various fermentations. The final concentration in dried formulation had a minimum of 1×10^{9} blastospores g^{-1}. Production yield after spray drying was calculated based on the weight of product collected and the theoretical solids in the feed stock.

7.2.1.4 Packaging, desiccation tolerance, and shelf life

The general procedure is illustrated in Figure 7.1. The formulations were prepared as indicated above and spray or air dried to powders for use in desiccation tolerance and shelf life experiments. In the first experiment, formulations comprising air-dried and spray-dried (only with SPM) blastospores were vacuum-sealed (999 mbar; Multivac Inc., Kansas City, MO, USA) to reduce atmospheric oxygen (< 0.021% O_2) in packaging and then stored under refrigerated (4 °C) and high room temperature conditions. Each formulation consisted of three independent packages containing 11 g dry material. Packages consisted of 4.3 mil Mylar bags (volume ~165 cm^3 in 5” × 8” bags [PAJVF4, Mylar®, Impak Corp., Los Angeles, CA, USA)

which are gas and moisture proof (O_2 transmission rate <0.001/cc/m^2 24 h) to create a hermetic seal.

In the second experiment, we evaluated the influence of active packaging using different types of oxygen and moisture scavengers on the storage stability of dry preparations of blastospores. All absorbers were provided by Mitsubish Gas Chemical America (New York, NY, USA). Pharmakeep® RP-3A is a dual action O_2 and moisture absorber which has organic composition. Ageless® ZM-1 is a new iron type oxygen absorber that transfers very little moisture to low water activity products designed for use with pharmaceuticals. Ageless® ZPT-50 is a standard iron type oxygen absorber with a very wide range of applications. Packages were sealed but contained atmospheric oxygen (21% O_2) inside. Spray or air dried formulations of *B. bassiana* blastospore with water activity < 0.3 and moisture < 5% were packed and sealed in 15 × 22 cm aluminized Mylar bags (PAKVF4, IMPAK Corporation, Los Angeles, CA, USA) and kept at high room temperature (28 °C) or under refrigeration (4 °C). Each bag contained 7–10 g of formulated, dried blastospores.

In another experiment, we tested the single effect and combined effect of oxygen scavengers (ZM-1 and ZPT-50) and a desiccant (silica gel) following a full factorial design. Single treatments contained a sachet of each absorber and combinations were performed adding both oxygen and silica sachet in the same package. The tolerance of blastospores to dehydration and survival during storage conditions was evaluated by measuring the percent spore viability using a fast-rehydration protocol, whereof a sample of 0.05 g dried blastospores was incubated for 7 h in 25 mL potato-dextrose broth (PDB) using 150-mL baffled Erlenmeyer flasks at 28 °C and 300 rpm in a rotary shaker incubator. Sampling was performed on the same package characterizing a repeated measure assay. Independent experiments were repeated at least twice using different fermentation batches.

7.2.1.5 Comparison of drying methods for virulence against whitefly

Insect colony consisted of silverleaf whitefly, *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) to serve as a target host for virulence studies with blastospores of entomopathogenic fungi. Founders for the colony were originally provided by University of Florida, Apopka, FL, and reared in the USDA (Peoria, IL) on cabbage cv. 'Bravo' (*Brassica oleracea* L.; Harris Seeds, Rochester, NY, USA) and blue lake bush bean (*Phaseolus vulgaris* L. [Kelly Seed Co., Peoria, IL, USA]) plants in 0.6 m^3 PVC-frame cages covered with fine screen fabric under greenhouse conditions (temperature range: 25–35 °C). The bioassay procedure with whitefly nymphs was previously described in Mascarin et al. (2013, 2015).

Formulations of spray-dried and air-dried blastospores were tested against second instar whiteflies on bean leaves. Spray application to simulate topical (direct) contact was carried out with a micro-sprayer device set to 10 PSI and 3 sec application time (MASCARIN et al., 2013). Spores were mixed with 0.01 % Tween 80 and suspensions were sprayed at concentrations of 1×10^5, 5×10^5, 2.5×10^6, and 1.25×10^7 blastospores mL^{-1}, which corresponded to application dosages of 2.2×10^2, 1.1×10^3, 5.4×10^3, and 2.7×10^4 blastospores cm^{-2} of leaf area. Controls were sprayed with only 0.01% Tween 80 solution. Four leaves were treated with each concentration. Subsequently, sprayed leaves were air dried at room temperature (~ 22 °C) for about 10 min prior to incubation in a growth chamber at 27±1 °C, 65±3% RH and 14:10 h L:D photophase. After 6 days incubation, nymphal mortality was assessed visually using a dissecting stereo-microscope (10–50X magnification) and the median lethal concentration (LC_{50}) of these blastospore formulations was calculated using probit analysis (see below). This bioassay was repeated three times using different batches of fungal fermentations and insect cohorts. All formulation batches of blastospores exhibited higher than 75% (range: 76–89%) germination in 7 h incubation in PDB.

An additional study was carried out to evaluate the impact of storage on virulence. After 4 months of storage at 28 °C, a single-dosage assay was performed to verify insecticidal activity of air and spray-dried blastospores based on the LC_{80} (, as determined by the dosage-response model, using the same protocol above. Dry preparations of blastospores attained 73–85% viability after 7 h incubation in PD broth. Each experimental dosage had 5 replication leaves containing 60–70 second instars and the whole experiment was conducted three times with different production batches. Control insects were sprayed only with 0.01% Tween 80 solution. Mortality caused by 4-month-old blastospores was compared with initial results for dry blastospore preparations prior to storage. To ensure reproducibility, we tested three different batches of each formulation with a control group for each batch.

7.2.1.6 Statistical analysis

Experiments were carried out with a completely randomized design and conducted independently at least two times. Generalized linear mixed models (GLMM) were used to fit proportional data, such as spore survival, with repeated measures for time using the SAS macro PROC GLIMMIX. Spore survival data were computed based on the relative germination formula given by y = (% germination treatment – germination control) / (100 – % germination control). After that, data were normalized by logit transform (i.e., $\log_{10}([y + 0.01]$

/ [1 – y + 0.01])) prior to analysis, whenever necessary (WARTON; HUI, 2011). Survival rates (slopes) from the time-course curves of various treatments were compared and considered significant at $P < 0.05$. For storage studies, half-life ($t_{½}$), in weeks, was estimated for each treatment according to the formula $t_{½} = \ln(2)/b$, where a is the initial survival and b the slope or decay rate. This formula was derived from the exponential decay model written as $y = ae^{(-bx)}$. However, storage data that did not follow the previous model were fitted to 2nd order polynomial regression: $y = a + bx + cx^2$, where y is the relative spore survival (%), x is time in weeks, while a, b, and c, the model parameters. For the bioassay data, virulence of dried formulations of blastospores were measured as nymphal mortality after 6 days and mortality data were subjected to Probit analysis (PROBIT procedure) with extreme value (Gompertz) distribution, which provided the best fit to experimental data. Median lethal concentrations (LC_{50}) were estimated from the fitted models and then compared statistically based on the potency ratio test, in which the assumption of equality means that the confidence interval of the ratio contains the number 1 (WHEELER et al., 2007). Persistence of 4-month-old air and spray dried blastospores against whitefly nymphs was compared based on χ^2 analysis with binomial distribution and logit as the link function (PROC GENMOD). All analyses were performed in the Statistical Analysis System v.9.2 (SAS Institute Inc., Cary, NC).

7.2.2 Results

7.2.2.1 Tolerance to dehydration-rehydration

Upon rehydration in PDB medium, spray and air dried blastospores of *B. bassiana* exhibited 77–81% germination after 7 h incubation and neither drying method affected desiccation tolerance of blastospores ($F = 1.73$, df = 2, 43, $P = 0.1902$). These yeast cells were dried less than 5% moisture content, and water activity values were lower than 0.26. The air dried DE formulation had lower moisture and water activity values when compared with the spray dried SMP formulations (Table 7.1).

7.2.2.2 Effect of vacuum packaging and temperatures on shelf life

Air dried and spray dried blastospores were vacuum packaged (≤ 0.021% O_2) and stored for 35 weeks at different temperatures without employing active packaging. Examining the survival curves, blastospores kept under refrigerated conditions remained notably viable for a longer period of time ($t_{½}$ > 35 weeks) rather than when they were stored at high room temperature at 28 °C, regardless of the drying method employed ($F = 46.79$, df =1, 216, $P <$

0.0001) (Figure 7.2). Regards to the drying system and storage at 28 °C, blastospores survived 7 times longer (13.3 weeks half-life) after air drying and formulated with diatomaceous earth rather than encapsulated with skim milk and spray dried (2.0 weeks half-life) (F = 26.52, df = 1, 216, P < 0.0001) (Table 7.2). Overall, refrigerated conditions significantly extended blastospore viability in storage independent on the formulation used (F = 14.69, df = 1, 216, P = 0.0002).

7.2.2.3 Effect of active packaging on shelf life

Evaluating the active packaging in the presence of atmospheric oxygen for stability of air dried and spray dried blastospores of *B. bassiana* stored at high room temperature (28 °C), cell survival was remarkably extended by using the dual action O_2/moisture absorber (RP-3A) in all storage studies performed here (Air dried: F = 18.57, df = 4, 141, P < 0.0001; Spray dried: F = 83.11, df = 4, 350, P < 0.0001) (Figures 7.2,7.3). Air dried formulation stored with silica gel particularly enhanced spore survival rate by prolonging at least 5 to 12-fold the half-life in comparison to the iron-based scavengers and control (Table 7.3, Figure 7.3). Comparing with the control groups, most of the active packaging treatments worked well, except for the O_2 absorber ZPT-50. When examining the spray drying formulations, the addition of ascorbic acid (ASA) to the skim milk powder in the formulation matrix did not enhance shelf stability of blastospores compared with only skim milk based product irrespective of the absorber used (F = 0.93, df = 1, 70, P = 0.338) (Figure 7.4). For all tested formulations, the O_2 absorber ZPT-50 exhibited the poorest performance, sometimes resulting in even lower survival rates or half-lives (≤ 2.2 weeks) in relation to the control groups. This ZPT-50 absorber generated 44.1–64.3% more moisture in the formulations that led to an increased cell metabolism and thus poor shelf life (Table 7.3). After RP-3A, the second best performance was achieved by ZM-1 or the moisture absorber (silica gel), which when combined in the same package these absorbers provided an additive increase in blastospore survival (5 weeks half-life) than their counterparts (F = 3.01, df = 5, 138, P = 0.0132), although the survival rate decay (i.e., slope) was not significantly different among the treatments (F = 0.67, df = 5, 138, P = 0.6479) (Figure 7.5). Furthermore, silica gel by itself significantly extended spore survival for air dried formulation rather than spray dried.

In additional, water availability of dried blastospore formulations was altered throughout the storage period by the absorbers used here (Table 7.3). Controls (without any scavenger) naturally produced moisture at 28 °C due to metabolic activity throughout storage.

In addition to maintaining high spore viability, the dual action O_2/moisture absorber RP-3A was the most effective among all active scavengers attributed to its notorious ability in reducing moisture in all formulations of blastospores ($0.02 < a_w < 0.18$). By contrast, ZTP-50 increased a_w over time up to a range of 0.38–0.68, followed by ZM-1 which also produced moisture similarly to what was found in the control samples ($0.34 < a_w < 0.51$). Silica gel alone at a ratio of 1 g to 7–10 g formulated product neither reduced dryness nor increased moisture in comparison to the initial a_w values.

7.2.2.4 Comparison of drying methods for virulence against whitefly

Bioassays against whitefly nymphs, spray and air dried blastospores yielded LC_{50} values of 1530 and 1141 blastospores cm^{-2}, respectively, and the potency ratio revealed no consistent difference between these concentrations (Table 7.4). However, when comparing LC_{80} values, the potency ratio test indicated that spray dried formulation required twofold more blastospores than the air dried form. This suggests that air dried formulation displayed better biological activity relative to spray dried formulate at higher dosages tested. Nymphal mortality significantly increased with the increase in blastospore concentration, where concentrations higher than 5400 propagules cm^{-2} incited > 78% mortality after 6 days post-treatment (Figure 7.6). Dead nymphs showed mycosis with a typical pink color symptom that characterized the production of oosporein, one of the mycotoxins commonly produced by *B. bassiana*. Mortality in controls was lower than 5%.

When analyzing the persistence of insecticidal activity for these dry formulations of blastospores, whitefly bioassays indicated higher bioefficacy attained by air-dried blastospores than spray-dried form after 4 months of storage at 28 °C using active packaging with RP-3A absorber (Figure 7.7). Interestingly, the air dried formulation exhibited higher insecticidal activity in relation to the spray dried formulation, although both formulations caused significantly higher nymphal mortality compared with the control ($\chi^2 = 263.34$, df = 2, $P < 0.0001$). Overall, these results indicated that both 4-month-old blastospore formulations were still effective in killing and causing disease in whitefly nymphs.

7.2.3 Discussion

Formulation, drying, and packaging are critical steps for the development of a downstream process to retain desiccation tolerance, shelf-life, and bioefficacy of fungal biopesticides (BURGES, 1998). Our results demonstrate that blastospores of *B. bassiana* resist to removal of more than 95% water by air or spray drying and still reverting to its original virulence against whitefly nymphs after rehydration. The blastospores remained

viable under refrigeration after vacuum-packaging (≤ 0.021% O_2) at refrigerated temperature for up to 35 months, or through active packaging using dual O_2/moisture scavenger at 28 °C over 7 months of storage. Blastospores have been thought to be sensitive to drying, especially for spray drying, which is a quick and relatively harsh method of water removal. Spray drying is widely employed in industrial scale to dehydrate cells of some beneficial microbes. Here, we demonstrated that *Beauveria* blastospores produced under optimum nutritional conditions can survive to fast dehydration process by spray drying and then still remain stable at high room temperature when actively packaged under very low moisture and oxygen levels. It is worth noting that repeated measure sampling by opening and resealing the same packages over the storage period likely underestimates the storage stability of our blastospore formulations. However, this methodology is comparable to real conditions where users would open and close the packages of blastospore formulations containing an oxygen/moisture absorber. This is the first report of prolonged shelf life of blastospores from an entomopathogenic fungus under storage at room temperature.

Spray drying is known to likely harm various living microbial cells, including blastospores. In this work, *Beauveria bassiana* blastospores produced under optimal fermentation conditions survived well to spray drying when subjected to a stringent fast-rehydration protocol for germination assessment. The high viability after spray drying *B. bassiana* blastospores can be attributed to its encapsulation in 20% instant skim milk. Skim milk powder is composed mainly of lactose and soluble proteins that can function as osmoprotectants by maintaining cell integrity after dehydration. These results are consistent with early studies in which skim milk powder effectively afforded spray drying of *Metarhizium acridum* blastospores as well as *Lactobacillus acidophilus* cells (STEPHAN; ZIMMERMANN, 1998; SOUKOULIS et al., 2014). Supporting this evidence, exogenous lactose was reported to increase the stability of *Saccharomyces cerevisae* cells during dehydration (RAPOPORT et al., 2009). The ascorbic acid (2.5% w/w) was used as an antioxidant for scavenging the reactive oxygen during drying and storage of blastospores. Interestingly, this agent seemed to prolong the storage stability of blastospores in comparison to skim milk alone (Figure 7.4). Ascorbic acid at 0.7% (w/w) along with milk-derivative protectants (35% w/w) also improved survival of the bacterium *Lactococcus lactic* during spray drying, but no shelf life was evaluated (GHANDI et al., 2012). Osmoprotectants, including oils and sugars, used as part of microbial formulations combined with the manipulation of endogenous reserves in fungal cells have been proposed as practical

alternatives to produce shelf stable microbial cells (FARIA et al., 2009; JIN; CUSTIS, 2010; YPSILOS; MAGAN, 2004). Relative to endogenous reserves, protein content in blastospore cells of *I. fumosorosea* was found to be critical for resistance to anhydrobiosis stress (JACKSON et al., 1997, 2003), while alcohol sugars and disaccharides have been typically associated with stress tolerance to dryness in aerial conidia of some entomopathogenic fungi (HALLSWORTH; MAGAN, 1994). Taking into account the present results, these concepts need to be examined more closely to understand their benefits in improving desiccation tolerance and shelf stability of blastospores.

Active packaging has been developed in food industry with the aim to ameliorate shelf-life of perishable products due to oxidation and microbial contamination (ROONEY, 1995). Oxygen and moisture scavengers of different types and compositions are currently available for use in a variety of packaging conditions. According to Cruz et al. (2007), the ZPT and ZM types consist of iron-based scavengers that require free moisture ($0.4 < a_w < 0.6$) to absorb oxygen. Since these sachets were used under lower a_w (< 0.3), it was expected their inferior performance in scavenging oxygen in comparison to RP-3A. Furthermore, ZPT and ZM, differently from RP-3A, have no moisture scavenger and release moisture once react with oxygen, which explain higher a_w achieved with these former sachets and lower water activity obtained with the latter (Table 7.3). Unlike iron based absorbers, the RP-3A is a special organic-based oxygen absorber which can work under extremely dry conditions. The superior performance of RP-3A at extending shelf life of blastospores can be associated with both mechanisms of protecting the cells from degradation by oxidation and by removing free water from the cells resulting in lower a_w values (< 0.18). This moisture scavenger component is contained in the sachet and it is suitable for moisture sensitive materials such as dry forms of fungal propagules. The efficient O_2 scavenging in RP-3A, although not specified by the manufacturer, it is probably mediated by an enzymatic system based on glucose oxidase/catalase (AITKEN; MARSHALL; PUGLISI, 2008; CRUZ et al., 2007). In another study, Faria et al. (2011) showed that such oxygen and moisture scavengers prolonged shelf life of hydrophobic conidia of *B. bassiana* (strain GHA) at high room temperatures and also demonstrated that drier conidia had longer storage stability. In this work, we have applied for the first time this technology to drying-sensitive cells (blastospores) that resulted in excellent storage stability under room temperature using the dual O_2/moisture action scavenger RP-3A. Additional studies, however, are needed to determine the optimum dimensions for package size, amount of dry material, and sachet size. In order to reduce the costs related to packaging, other brands and types of O_2 and moisture absorbers should also be investigated. RP-3A was the most expensive absorber tested here, as it is designed for pharmaceutical products. The RP-3A absorber can work under extremely dry conditions,

whereas iron based absorbers, such as ZPT-50 and ZM-1, release water to absorb oxygen (CRUZ et al., 2007). This probably explains the inferior performance of these iron-based O_2 absorbers which in the end increased moisture to the formulations (Table 7.3). The addition of silica gel to the formulations or in combination with O_2 scavengers provided longer stability than the iron-based O_2 absorbers alone, although none of these treatments had better performance than RP-3A. In contrast to these results, the RP-3A had the advantage of not producing moisture when reacts with oxygen and thus there is no concern of hydroxylation.

The drying method should produce a uniform product with minimal damage to cell integrity and viability and should be easy to scale up with acceptable processing costs (RATHORE et al., 2013). Both drying systems employed here are cost effective technologies for fungal desiccation and suitable for downstream industrial operation. The main advantage of spray drying over air drying is that the former involves the combination of particle formation and fast drying rate in a single step. Stephan and Zimmermann (1998) showed that *M. acridum* blastospores when encapsulated in skim milk matrix survived well spray drying only at high moisture content (15% w/w) after drying, yet no shelf life was reported. By contrast, our data highlight the key role of lowering the water content (< 5% moisture) to achieve extended shelf life in *B. bassiana* blastospores after spray drying or air drying. Another essential feature in spray drying is the outlet air temperature. It has been shown that survival of fungal propagules subjected to spray drying process was inversely proportional to the outlet air temperature used in the spray dryer (JIN; CUSTIS, 2010; STEPHAN; ZIMMERMANN, 1998). Furthermore, it has been shown that spray drying is used to produce formulations with extended shelf-life of the fungus of *Trichoderma harzianum* (MUÑOZ-CELAYA et al., 2012). With respect to the air drying, this technique consists of a slow drying process (16–20 h) which allows the control of the atmospheric RH. It is also feasible for industrial scale since there is similar equipment with controlled RH air for use in food industry that could be easily adapted to dehydrate fungal propagules. This method provides gentle drying and thus is highly recommended for drying-sensitive microbial cells, such as blastospores. In agreement with our results, Jackson and Payne (2007) reported that blastospores of *I. fumosorosea* survived well and were storage stable when dried with RH air greater than 50%. Other studies also have noted good shelf life when fungal propagules were dried more slowly at higher relative humidity (HONG et al., 2000; SHAH et al., 2000). Moreover, in the air dried formulation, diatomaceous earth has the advantage of not holding water which helps to keep the water activity low during storage.

Few studies have investigated the influence of drying techniques and formulations on storage stability, bioefficacy, and persistence of blastospores. Here we provide the first evidence of air and spray dried formulations of *B. bassiana* blastospores effectively killing

whitefly nymphs and remaining viable (persistent) after 4 months of storage at 28 °C. Leland and Behle (2004) microencapsulated aerial conidia of *B. bassiana* with lignin-based matrix using a spray-dryer to provide UV protection, but this formulation exhibited less virulence against the plant bug *Lygus lineolaris* (Heteroptera: Miridae) compared with unformulated conidia. This contradictory outcome was attributed to the physical properties of the formulation. Similarly, we demonstrated that spray dried formulation of blastospores resulted in lower insecticidal activity at higher dosages (LC_{80}) and retained lower persistence in relation to the air dried blastospores when bioassayed against whitefly nymphs. This difference in virulence and persistence between these blastospore formulations remain obscure and requires further elucidation.

To our knowledge, this is the first report of enhanced stability of sprayable formulations of *Beauveria* blastospores stored at high room temperature with persistence and excellent bioefficacy against whiteflies. Collectively, these results encourage the production of shelf stable, infective blastospores of *B. bassiana* as the active ingredient of mycopesticides.

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Table 7.1 - Production yield, desiccation tolerance, and moisture after spray drying for encapsulation of *Beauveria bassiana* blastospores in a skim milk matrix at inlet/outlet temperature regime of 95/48 ± 2 °C

Dehydration method	Formulation [a]	Production yield (%)	Moisture (%)	Water activity	Desiccation tolerance (% germination) [b]
Spray drying	20% SMP	88.4 (79.3 – 97.5)	4.48 (2.85 – 5.79)	0.1947 (0.0773–0.2817)	83.1 ± 1.2 [c]
	17.5% SMP + 2.5% Ascorbic Acid		4.47 (3.84 – 5.75)	0.2534 (0.1838–0.3243)	82.6 ± 0.7
Air drying	Diatomaceous earth	-	0.84 (0.56 – 1.31)	0.246 (0.2142–0.2876)	80.9 ± 1.0

[a] Ingredients for the microencapsulation of blastospores during spray drying: SMP = skim milk powder; air-dried blastospores were mixed with diatomaceous earth (1×10^{10} blastospores g^{-1} DE)

[b] Desiccation tolerance was measured as percent blastospores germinated upon rehydration in PD broth after 7 h incubation at 28 °C and 300 rpm

[c] Effect of formulation on desiccation tolerance (mean ± SE) was not significant ($P < 0.05$)

Table 7.2 - Half-lives estimated by exponential decay model for air dried and spray dried blastospores of *Beauveria bassiana* stored under different conditions

Drying method	Formulation [a]	Packaging	Temperature (°C)	Model parameters [c] *a*	*b*	*c*	R^2	$t_{½}$ (weeks) [d]
Spray drying	SMP	Vacuum sealed [b]	28	108.8	0.35	-	0.93	2.0
			4	100.7	-0.75	-0.004	0.91	NE
Air drying	DE	Vacuum sealed	28	102.9	0.05	-	0.98	13.3
			4	-	-	-	-	NE
			Absorbers (active packaging) + 21% atmospheric oxygen					
Spray drying	SMP	Control	28	99.9	0.3	-	0.99	2.3
		RP-3A	28	95.9	1.13	-0.096	0.94	NE
		ZM-1	28	101.3	0.22	-	0.98	3.2
		ZPT-50	28	102.4	0.32	-	0.99	2.2
		Silica gel	28	103.6	0.29	-	0.99	2.4
	SMP + ASA	Control	28	101.2	0.29	-	0.99	2.4
		RP-3A	28	96.8	0.14	-0.043	0.94	NE
		ZM-1	28	105.6	0.34	-	0.98	2.0
		ZPT-50	28	102.9	0.43	-	0.99	1.6
		Silica gel	28	102.2	0.23	-	0.99	3.0
Spray drying	SMP	Control	28	99.7	0.34	-	0.99	2.0
		ZM-1 + Silica	28	99.2	0.14	-	0.97	5.0

		gel						
		ZPT-50 + Silica gel	28	101.0	0.22	-	0.97	3.2
		ZM-1	28	101.4	0.20	-	0.97	3.5
		ZPT-50	28	101.2	0.38	-	0.99	1.8
		Silica gel	28	98.2	0.29	-	0.99	2.4
Air drying	DE	Control	28	102.0	0.23	-	0.98	3.0
		RP-3A	28	98.45	0.6	-0.038	0.90	NE
		ZM-1	28	101.6	0.22	-	0.98	3.2
		ZPT-50	28	100.9	0.53	-	0.99	1.3
		Silica gel	28	104.8	0.04	-	0.85	16.1

[a] Skim milk powder (SMP) at 20% (w/w); skim milk powder at 17.5% with ascorbic acid a 2.5% w/w (SMP + ASA); diatomaceous earth (DE)

[b] Vacuum packaging at 999 mbar pressure provided ≤ 0.021% atmospheric oxygen

[c] Exponential decay: $y = a*e^{(-bx)}$; 2nd order regression: $y = a + bx + cx^2$, where y = survival (%) and x = time (weeks)

[d] Half-life was calculated as $t_{½} = \ln(2)/b$. NE (not estimated) because the survival curve did not reach yet the median survival time, in other word, it indicates $t_{½} > 25$ weeks

Table 7.3 - Determination of water activity (a_w, mean ± SE) measured at 25 °C after 4 months of storage at 28 °C for different formulations and packaging systems for blastospores of *Beauveria bassiana* exposed to atmospheric air

Formulation	Packaging	Initial a_w	Final a_w
Spray dried	RP-3A	0.246 ± 0.021	< 0.023 ± 0.0
	Ageless ZM-1 + Silica gel		0.300 ± 0.008
	Ageless ZPT-50 + Silica gel		0.322 ± 0.005
	Ageless ZM-1		0.345 ± 0.005
	Ageless ZPT-50		0.440 ± 0.033
	Silica gel		0.270 ± 0.015
	No scavenger (control)		0.317 ± 0.013
Air dried	RP-3A	0.232 ± 0.006	0.171 ± 0.024
	Ageless ZM-1		0.470 ± 0.017
	Ageless ZPT-50		0.649 ± 0.014
	Silica gel		0.253 ± 0.015
	No scavenger (control)		0.415 ± 0.002

Table 7.4 - Virulence parameters based on lethal median concentration (LC_{50}) for comparison of air and spray-dried blastospores of *Beauveria bassiana* against 2nd instar whitefly nymphs (*Bemisia tabaci* biotype B)

Blastospore formulation [a]	n	Intercept	Slope [b]	χ^2 [c]	LC_{50} (CL95%) [spores cm^{-2}] [d]	RP_{50} (CL95%) [e]	LC_{80} (CL95%) [spores cm^{-2}]	RP_{80} (CL95%)
Air dried	2272	-4.02±0.33	1.19±0.09	173.09 (< 0.0001)	1,141 (818–1,502)	1.34 (0.98–1.83)	5,773 (4,537–7,514)	2.06 (1.55–2.74)
Spray dried	3529	-3.38±0.27	0.95±0.07	178.36 (< 0.0001)	1,530 (1,088–2,038)	-	11,903 (9,070–16,279)	-

[a] Air dried blastospores were formulated with diatomaceous earth, while the spray dried material was encapsulated with 20% skim milk powder

[b] Slope for mortality represents regression of proportion of nymph mortality versus log of spores cm^{-2}. Number of insects tested (n)

[c] χ^2 and *P* values represent the probability of slope $\neq 0$, rather than fit to Gompertz model

[d] Delivered median lethal concentration (LC_{50}) and respective confidence limits (CL95%) estimated by the Gompertz model (PROBIT procedure). Cumulative mortality censored up to day 6 post-application. Control (natural) mortality averaged 4.2 ± 0.9%

[e] Relative potency is the measure of relative efficacy of blastospores to aerial conidia within each fungal species: (LC_{50} air-dried / LC_{50} spray-dried). If the confidence limit for the LC ratio does not contain 1, hence the LC_{50} values are significantly different (WHEELER et al., 2007)

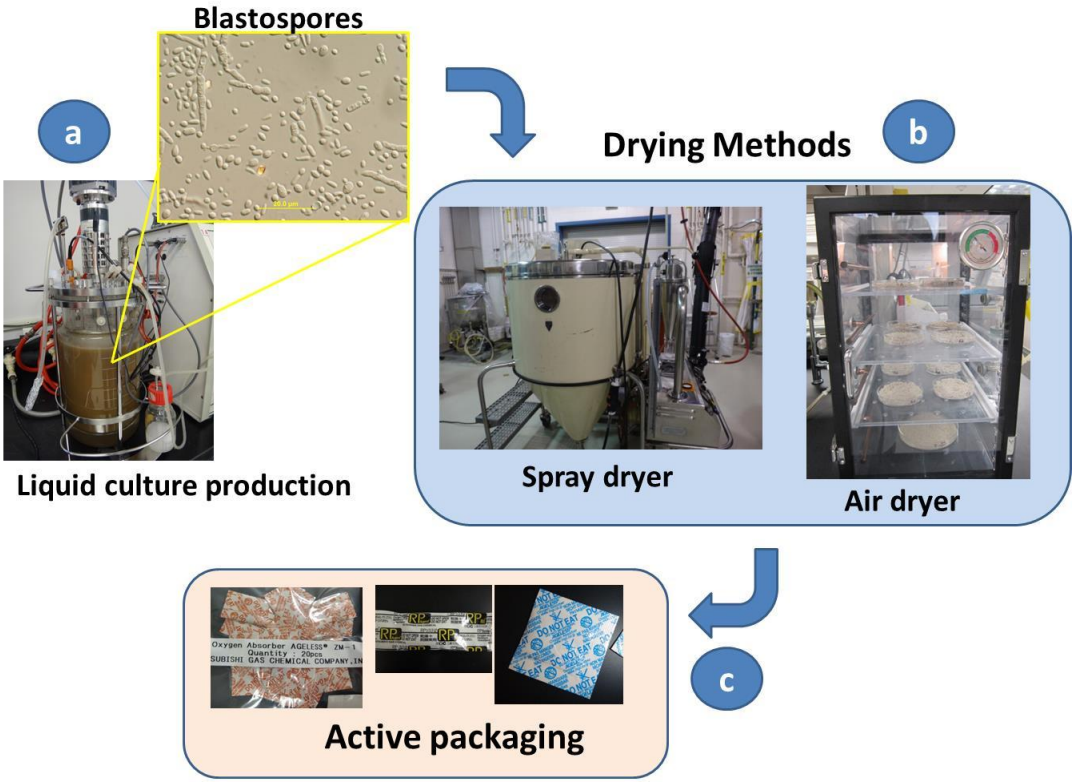

Figure 7.1 - Formulation, drying and packaging processes: a) Production of blastospores using 5-L fermentor for harvesting in 3 days; b) Convective spray and air drying systems to dehydrate blastospore cells; c) active packaging featuring different oxygen and moisture absorbers for extended shelf-life

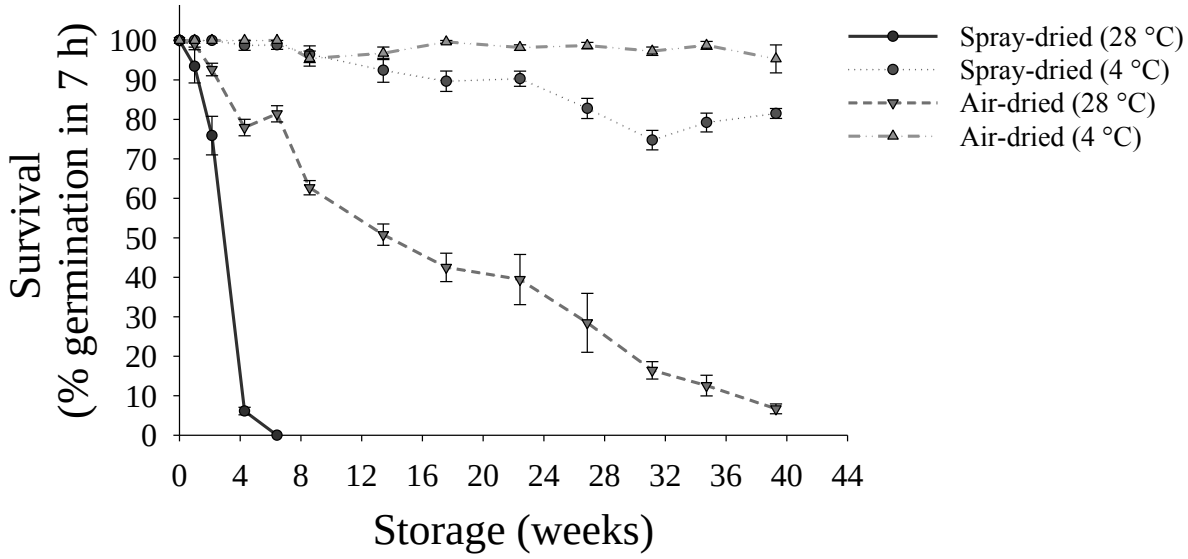

Figure 7.2 - Shelf life of dry preparations of *Beauveria bassiana* blastospores stored in vacuum-packed aluminum (mylar) bags at high room temperature (28 °C) or under refrigerated conditions (4 °C). Survival curves started at 100% viable spores at time 0

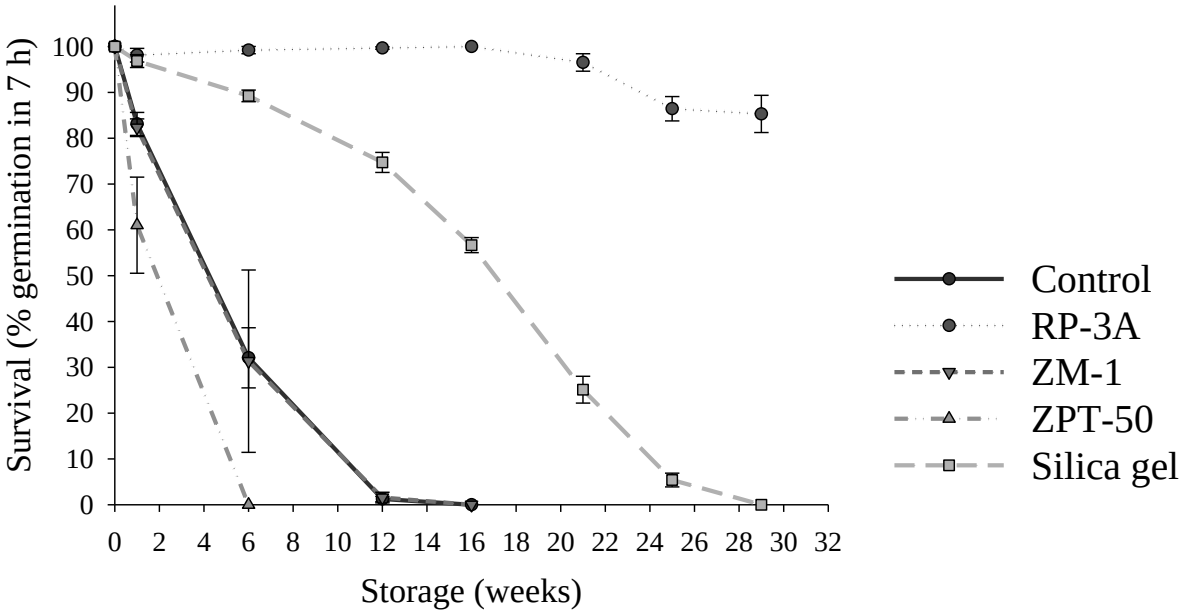

Figure 7.3 - Effect of active packing through oxygen and moisture scavengers on storage stability of air dried *Beauveria bassiana* blastospores stored at high room temperature (28 °C). Survival curves started at 100% viable spores at time 0

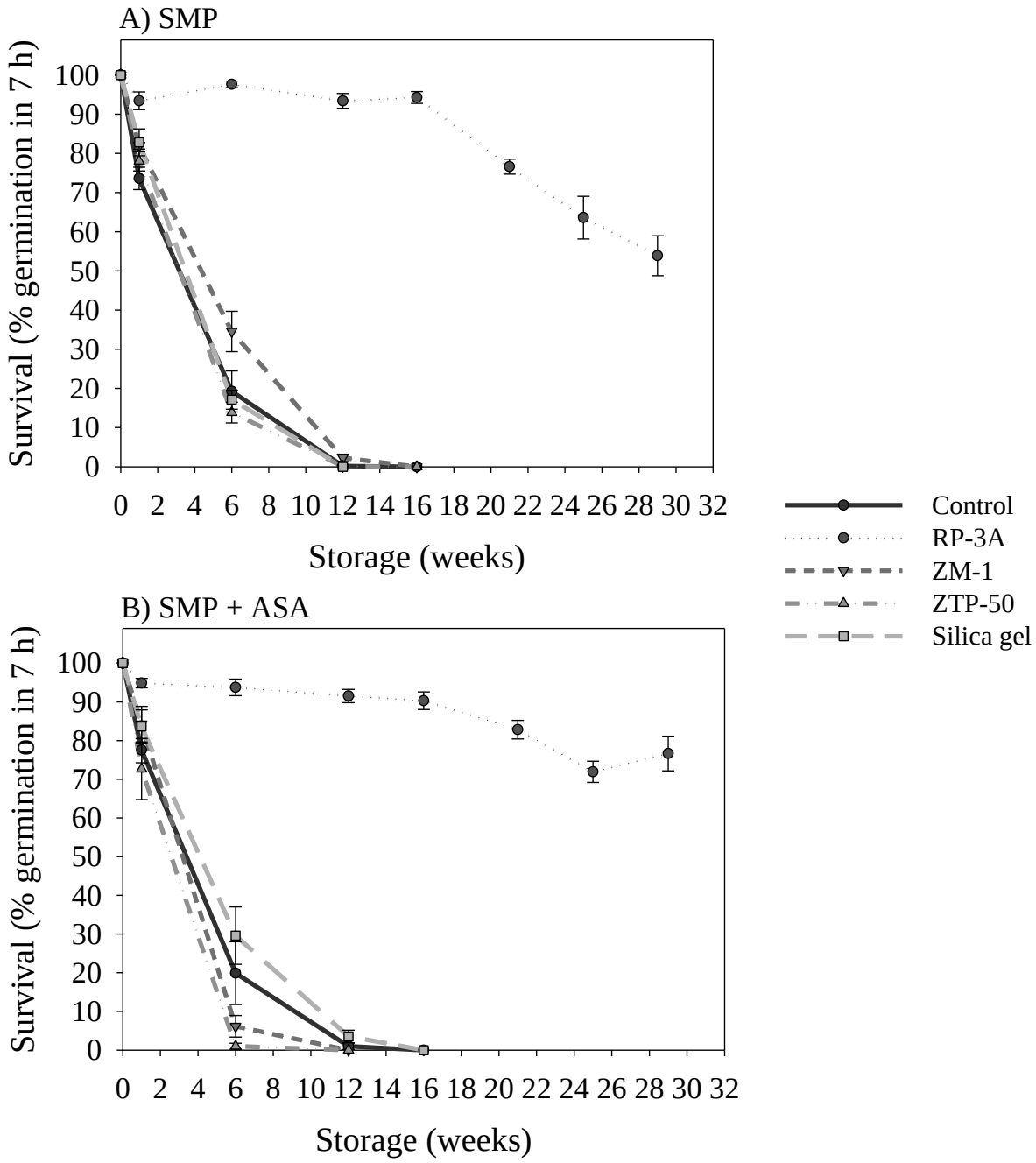

Figure 7.4 - Effect of active packing through oxygen and moisture scavengers on storage stability of spray-dried *Beauveria bassiana* blastospores encapsulated with either 20% skim milk powder (SMP) or 17.5% SMP + 2.5% ascorbic acid (ASA), and stored at high room temperature (28 °C). Survival curves started at 100% viable spores at time 0. After 16 weeks of storage, viability reached 0% for all treatments, except for RP-3A

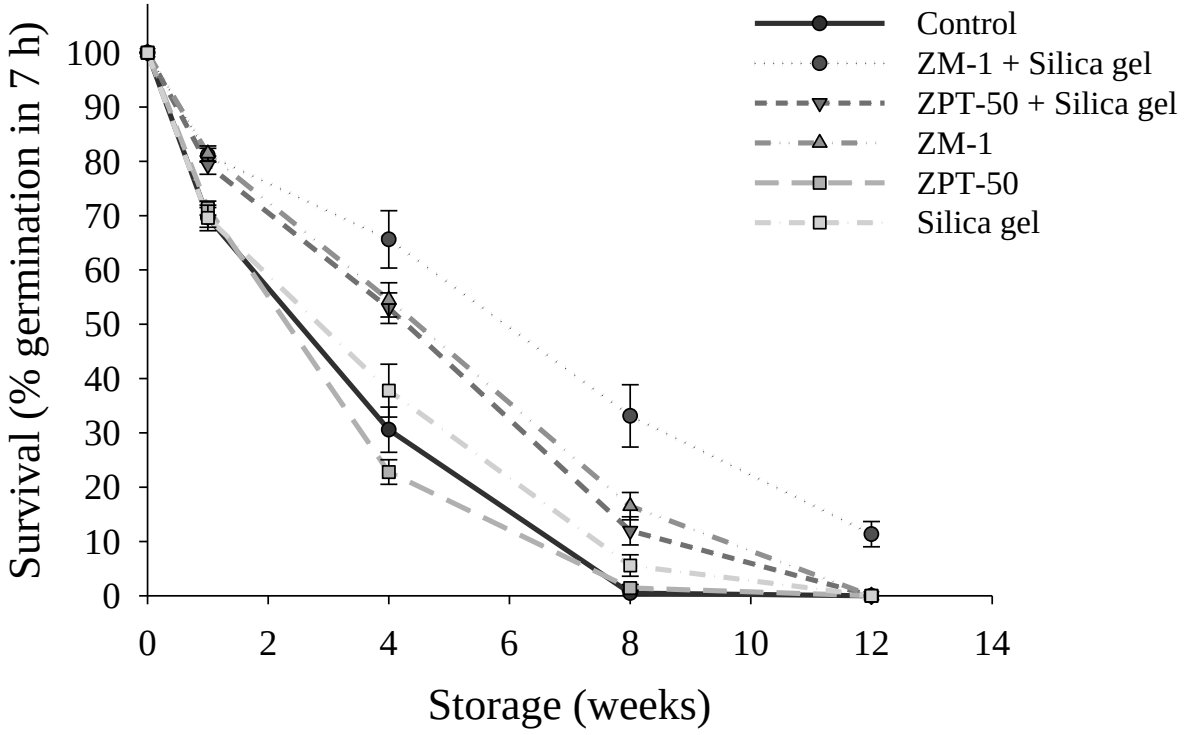

Figure 7.5 - Effect of active packing using single or combined oxygen and moisture scavengers on the storage stability of spray-dried *Beauveria bassiana* blastospores encapsulated with 20% Skim milk powder and maintained at high room temperature (28 °C). Survival curves started at 100% viable spores at time 0

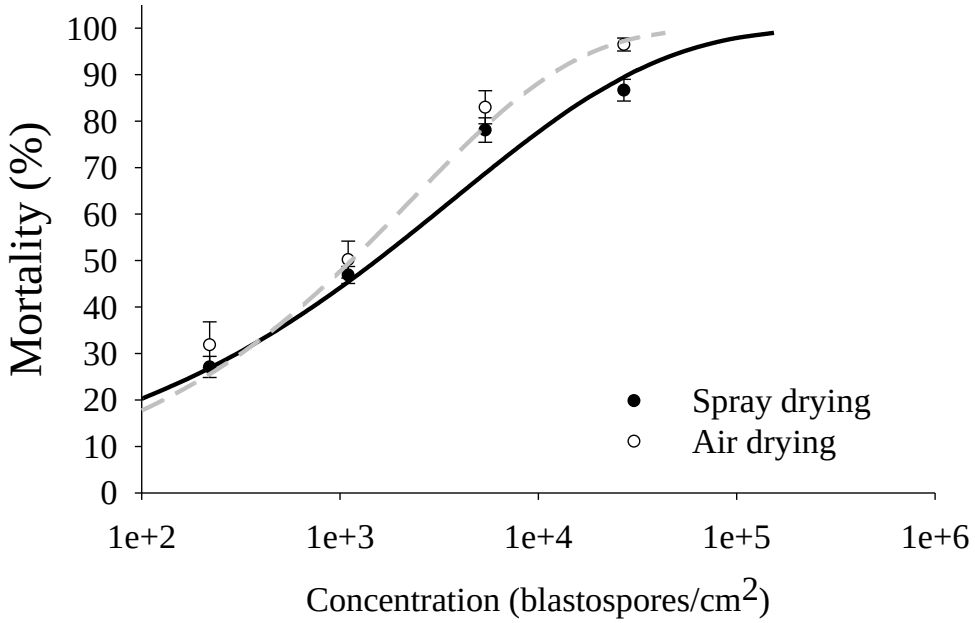

Figure 7.6 - Comparison of the convective drying methods (spray and air drying) for virulence of *Beauveria bassiana* blastospores against whitefly nymphs (2nd instar) of *Bemisia tabaci* biotype B after 6 days of incubation at 27 °C, 65% RH and 14 h photophase. Lines represent the fitted by Gompertz model, while filled circles are observed means (± SE)

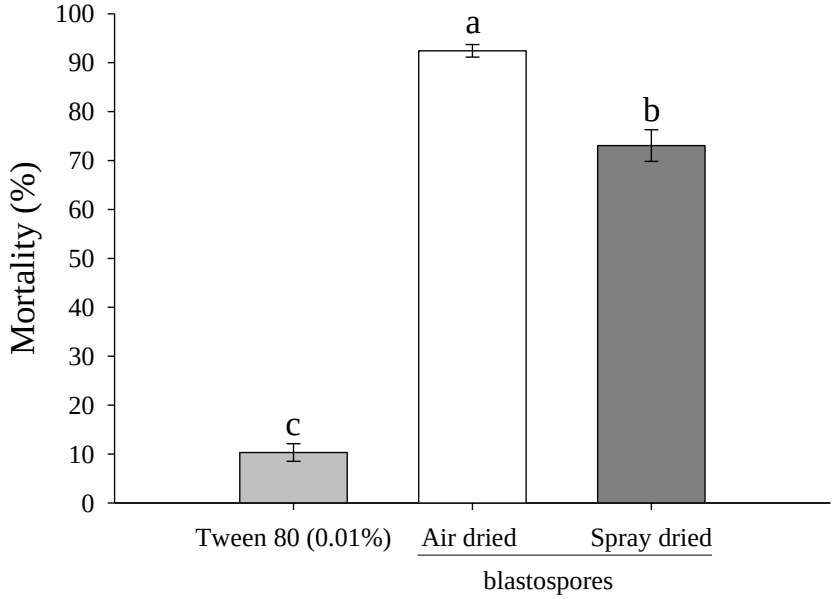

Figure 7.7 - Persistence of air and spray dried blastospores of *Beauveria bassiana* after 4 months of storage at 28 °C against second-instar whitefly nymphs using single-dosage assay based on LC_{80} estimates. Bars (mean ± SE) consist of a pool of three independent experiments

8 FINAL CONSIDERATIONS

The use of fungal entomopathogens against whiteflies has an important role in alleviating the pressure of synthetic insecticides, thus delaying pest resistance, and they are also compatible with other natural enemies. The present research comprises the importance of screening, producing and formulating promising candidates of fungal entomopathogens for use as biocontrol agents of whiteflies and possibly other insect pests. The selection of high virulent fungal strains against whiteflies coupled with their easiness of mass production either by solid or liquid fermentation and the design of appropriate formulations or mixtures with compatible additives will contribute to the progress of whitefly microbial control within integrated pest management programs. Particularly, the liquid fermentation process developed here provides a rapid method to produce high concentrations of desiccation tolerant and shelf stable blastospores of *B. bassiana*, and this technology is considered a groundbreaking advance for the mass production of this fungus and can also be adapted to other dimorphic filamentous entomopathogenic fungi. All in all, the results reported here will enhance the widespread adoption of liquid fermentation processes to produce effective, shelf-stable fungal propagules, such as blastospores, using low-cost media components, thus facilitating development and commercialization of these biocontrol agents by the biopesticide industry toward numerous insect pests in the mainstream agriculture.