University of São Paulo
"Luiz de Queiroz" College of Agriculture

Development of drying methods and evaluation of blastospores efficacy of *Beauveria bassiana*, *Cordyceps fumosorosea,* and *Metarhizium rileyi* against *Euschistus heros* and *Spodoptera* spp.

Bianca Corrêa

Dissertation presented to obtain the degree of Master in Science. Area: Entomology

Piracicaba
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Bachelor in Biological Sciences

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1. Fermentação líquida 2. Controle microbiano 3. Pragas da soja 4. Hypocreales I. Título

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“The only way to achieve the impossible is to believe it is possible. ”

Lewis Carroll – “Alice in Wonderland”

SUMMARY

RESUMO

Desenvolvimento de um método de secagem e avaliação da eficácia de blastosporos de *Beauveria bassiana*, *Cordyceps fumosorosea* e *Metarhizium rileyi* contra *Euschistus heros* e *Spodoptera* spp.

Blastosporos são células produzidas naturalmente no interior do corpo dos insetos durante o processo de infecção por muitos fungos entomopatogênicos. Artificialmente, estas células podem ser produzidas em meios líquidos nutricionamente ricos em condições de agitação e aeração adequadas. No Brasil, ainda não há nenhum biopesticida a base de blastosporos sendo comercializado, sendo os produtos a base de conídios aéreos. *Beauveria bassiana* e *Cordyceps fumosorosea* são fungos connhecidos por apresentarem a capacidade de infectar uma ampla gama de hospedeiros enquanto que *Metarhizium rileyi* é bem conhecido por ser específico a lagartas. A cultura da soja é responsável por mais da metade dos pesticidas usados no país para o controle de pragas, seja por insetos sugadores ou lagartas desfolhadoras. O objetivo deste estudo foi selecionar isolados de *B. bassiana*, *C. fumosorosea* e *M. rileyi* com alta produtividade de blastosporos virulentos a uma ampla gama de hospedeiros e desenvolver métodos de secagem para estas células, etapas cruciais para o desenvolvimento de um bioproduto a base de blastosporos. A primeira etapa deste projeto consistiu na avaliação da produção de blastosporos de 12 isolados de *B. bassiana* (4), *C. fumosorosea* (4) e *M. riley* (4). Posteriormente, dois isolados de cada espécie foram selecionados para os ensaios de virulência com lagartas de segundo ínstar de *Spodoptera cosmioides*, *Spodoptera eridania*, *Spodoptera frugiperda* e adultos de *Euschistus heros*. A segunda etapa do projeto consistiu na realização de um screening de excipientes e formulações para serem utilizados em três métodos de secagem de blastosporos para *C. fumosorosea*, sendo *air drying* rápido, *air drying* lento e *spray drying*. Após a seleção de duas formulações, as mesmas foram utilizadas na secagem de blastosporos de *B. bassiana*, *C. fumosorosea* e *M. rileyi* de modo a avaliar o shelf life durante o armazenamento a 4 e 28°C. Os resultados mostraram que todos os isolados atingiram concentrações superiores a 10^8 blastosporos mL^{-1} e alguns produziram mais de 10^9 blastosporos mL^{-1}. Foi observada uma grande variabilidade na susceptibilidade das espécies de pragas aos blastosporos dos isolados das três espécies fúngicas. Os blastosporos se mostraram mais sensíveis ao processo de secagem via *spray drying*, para algumas formulações e especialmente para *M. rileyi*. Duas formulações F12 e F20 apresentaram viabilidade de blastosporos superiores a 80% nos três métodos de secagem. Após 120 dias de armazenamento a 4°C algumas combinações de formulações e métodos de secagem apresentaram viabilidade de blastosporos acima de 70% para *B. bassiana* e *C. fumosorosea*. Os resultados obtidos neste projeto revelam o potencial da utilização de blastosporos para o controle de pragas da soja, assim como apresenta avanços no desenvolvimento de métodos de secagem para estas estruturas fungicas.

Palavras-chave: Fermentação líquida, Controle microbiano, Pragas da soja, Hypocreales

ABSTRACT

Development of drying methods and evaluation of blastospores efficacy of *Beauveria bassiana*, *Cordyceps fumosorosea,* and *Metarhizium rileyi* against *Euschistus heros* and *Spodoptera* spp.

Blastospores are cells naturally produced inside the insect's body during the infection process by many entomopathogenic fungi. These cells can be produced in vitro in rich liquid media under proper agitation and aeration conditions. In Brazil, there is still no biopesticide based in blastospore being commercialized, all products based on aerial conidia. *Beauveria bassiana* and *Cordyceps fumosorosea* are known for their capacity to infect a broad range of hosts, while *Metarhizium rileyi* is well known for its specificity caterpillar. The soybean crop is responsible for more than half of the pesticides used in the country for pest control, whether by sucking insects or defoliating caterpillars. This study's objective was to select isolates of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* with high productivity of virulent blastospores to a wide range of hosts and to develop drying methods for these cells, crucial steps for the development of a blastospore-based bioproduct. The first part of this project consisted of evaluating the blastospore production of 12 isolates of the fungi, *B. bassiana* (4), *C. fumosorosea* (4), and *M. rileyi* (4). Later, two isolates of each species were selected for the virulence assays with second instar caterpillars of *Spodoptera cosmioides*, *Spodoptera eridania*, *Spodoptera frugiperda,* and *Euschistus heros* adults. The second part of the project consisted of performing screening of fillers and formulations with three blastospore drying methods for C. fumosorosea, referred to as fast air drying, slow air, and spray drying. After selecting two formulations, the dried blastospores' shelf life was determined for *B. bassiana*, *C. fumosorosea,* and *M. rileyi* stored at 4 and 28°C. The results showed that all the isolates attained concentrations upper 10^8 blastospores mL^{-1} and some reached higher than 10^9 blastospores mL^{-1}. Significant variability was observed in pest species' susceptibility to blastospores from isolates of the three fungal species. Blastospores were more sensitive to the drying process via spray drying, for some formulations and especially for *M. rileyi*. Two formulations F12 and F20 showed blastospores' viability greater than 80% in the three drying methods. After 120 days of storage at 4 °C, some combinations of formulations and drying methods showed blastospores viability above 70% for *B. bassiana* and *C. fumosorosea*. The results obtained in this project reveal the potential of using blastospores to control soybean pests and bring advances in the development of drying methods for these fungal structures.

Keywords: Liquid fermentation, Microbial control, Soybean pests, Hypocreales

1. INTRODUCTION

1.1. Soybean crop

Soybean [*Glycine max* (L.) Merril] is an economically important crop for Brazil, representing 14.1% of the Brazilian exportation and providing the country economy about R$40.9 billion in 2018 (Agrostat, 2019; Conab, 2019). The country is the second-largest producer of this crop, producing around 114.8 million tons in 2019 and representing approximately 28% of the world's planted area (Conab, 2019; USDA, 2019).

The Brazilian soybean has a very favorable international market, mainly to supply the soybean meal for animal consumption in the industrial farming of meat and products of animal origin (Ortega et al., 2005).

Among the soybean cultivate in Brazilian farming, there are two production systems: the conventional and the organic. The first is composed of the genetically modified (GM) and non-genetically modified soybeans varieties (non-GM), while the second is composed of soybeans varieties cultivate without the use of synthetic fertilizers and pesticides (Pashaei Kamali et al., 2017). About the transgenic modified soybean, the Roundup Ready (GM_{RR}) varieties are the most used, representing in 2006, 57% of the total área of genetically modified plants (Böhm and Rombaldi, 2010). The high production of a soybean crop can be influenced by the edapho-climatic conditions and the damage caused by diseases and pests during its cycle (Marques et al., 2017). The most common pests on soybean are defoliating caterpillars, whiteflies, and phytophagous bugs (Souza et al., 2013). Besides that, the use of multiple crops during the year and the overuse of conventional insecticides increased secondary pests, even as the extensive use of fungicides reduced the natural control of pests by entomopathogenic fungi (Panizzi, 2013). Among the different strategies to control soybean pests, integrated pest management is the best alternative to the prophylactic use of insecticides and biological control strategies. The interesting point is that the prophylactic use of insecticides does not result in higher productivity but only increases the natural imbalance caused by pesticides (Bueno et al., 2011).

1.2. *Spodoptera* spp. complex

The genus *Spodoptera* comprises a series of species that cause damages to several crops. In Brazil, *Spodoptera frugiperda* (J. E. Smith), *Spodoptera cosmioides* (Walker) and *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae) are the most important pests species of this genus, mainly in cotton and soybean crops (Santos et al., 2009). *Spodoptera cosmioides* and *S. eridania* are the most common in soybean crops, feeding on pods, grains, and leaves.

Currently, the control of *S. frugiperda*, *S. eridania,* and *S. cosmioides* can be performed by biological and chemical control, botanic extracts, and through the use of transgenic crops (Ayil-Gutiérrez et al., 2018). For soybean plants, a good alternative to perform the management of these species could be cultivars that express the Cry1Ac protein. However, there has been detected a reduced susceptibility to these genetically-modified soybeans (Bernardi et al., 2014).

Spodoptera frugiperda has been considered a global problem. It was introduced in 2016 into West and Central Africa (Goergen et al., 2016) and in 2018 into some Indian territories (Brévault et al., 2018; Shylesha et al., 2018). The crop losses in sub-Saharan Africa can attain about 13 billion dollars, becoming a severe problem for the millions of poor farmers (Harrison et al., 2019).

In maize and millet crops, *S. frugiperda* prefers leaves; in cotton crops to leaves, flower buds, and mainly of growing bolls; while in soybean crops, the caterpillars eat the leaves and young string bean (Barros et al., 2010). The *S. frugiperda* has a lot of importance, mainly in maize crops, feeding on all growth stages, consuming a large part of leaves, and may impair plant growth (Cruz· et al., 1999). Generally, *S. frugiperda* can be controlled by the use of insecticides and *Bt* crops. However, some features of this species can explain the high risk of the development of field-involved resistance to these control tactics, such as the polyphagous feeding habit, the high reproductive potential and dispersion capacity, favorable weather conditions, and the high selection pressure using insecticides and Bt crops (Bernardi et al., 2016). For the control of this species in maize of tropical regions, frequent insecticide applications are required to keep populations below economic thresholds (Storer et al., 2012).

Spodoptera cosmioides was used for a long time as a synonymy of *Spodoptera latifascia*; however, molecular, morphological, physiological, and behavioral differences between *S. latifascia* moths collected in North America and West Indies and moths coming from South America were detected. Thus, they were separated into two distinct species, considered the South American one called *S. cosmioides* (Silvain

and Lalanne-Cassou, 1997). This species has a broad range of hosts. However, its status as a pest occurs only in some crops, mainly when there is excessive use of broad-spectrum insecticides (Bavaresco et al., 2003; Cabezas et al., 2013). The use of Bt plants could be an alternative to insecticides; however, it has been verified that the development and reproduction are not affected when *S. cosmioides* larvae feed on Bt soybean expressing the Cry1Ac protein (Silva et al., 2016).

Spodoptera eridania can feed on different species with economic importance, such as soybean, apple, cotton, maize, and beans. Its capacity for defoliation, jointly with high population densities, can cause severe damage and critical economic losses (Favetti et al., 2015). For a long time, this species was not considered a cotton pest. However, the use of plants such as *Ipomoea grandfolia* after the soybean cycle and the cultivation of soybean and cotton side by side provided the increase of populations of this species in cotton crops (Santos et al., 2005). Studies performed with a diet containing purified Cry1Ac protein showed that the protein does not affect the mortality of *S. cosmioides* and *S. eridania* and larvae fed in Bt soybean plants, did not change their life table parameters (Bernardi et al., 2014).

1.3. *Euschistus heros*

The Neotropical brown stink bug *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) is a pest that has been considered a minor problem for a long time of soybean. However, this is currently the primary insect colonizing soybean on Neotropical fields (Panizzi and Oliveira, 1998; Sosa-Gómez and Silva, 2010). This species has a mean development time from egg to adult of 38 days, with a range of oviposition from 108.5 to 130.5 eggs per female (Costa et al., 1998).

This insect feeds directly on soybean grains, a habit that reduces the number and the quality of seeds, impacting the final production (Costa et al., 1998). This damage is caused due to the feeding mode of the pentatomid insects, where it injects salivary secretions with enzymes into the seeds to ingest the food slurry (Depieri and Panizzi, 2011). Moreover, in general, *Nezara viridula* and *Euschistus servus* prefer soybean plants compared to Bt and non-Bt cotton in the field, mainly in the reproductive stage of development (Bundy and McPherson, 2009; Olson et al., 2011).

Some factors can explain the increase of the importance of *E. heros* in Brazilian soybean crops, as the selection made by the main insecticides of resistant populations,

the high offer of insecticides with a variety of actions mechanisms, the questionable application technology, and the mistaken use of broad-spectrum insecticides causing an ecological imbalance (Bueno et al., 2015). The polyphagous eating habit of these insects is a point that should be considered when chose the crops that will be used in the offseason because only 30% of the lifetime of these insects occur in the soybean crop. It is crucial to control the populations in this period (Panizzi and Vivan, 1997). Besides that, in the overwintering season, *E. heros* remains under dead leaves to accumulate lipids (Panizzi and Grazia, 2001).

1.4. Utilization of *Beauveria bassiana*, *Cordyceps fumosorosea,* and *Metarhizium rileyi* as biological control agents

Fungal species of the genus *Beauveria*, *Cordyceps* (= *Isaria*), and *Metarhizium* belong to the order Hypocreales. These entomopathogenic fungi have a broad range of insect hosts, and they are capable of producing a larger quantity of infectious cells (Butt et al., 2016). Approximately 80% of the commercial products based on entomopathogenic fungi are formulated with fungi belonging to the genus *Beauveria* and *Metarhizium.* The most used species are *Beauveria bassiana*, *Metarhizium anisopliae*, *Cordyceps fumosorosea*, and *Beauveria brongniartii* (Faria and Wraight, 2007). In Brazil, there are 24 registered products for *M. anisopliae*, 13 for *B. bassiana,* and one for *C. fumosorosea*, all of them based on aerial conidia as the main active ingredient (Mascarin et al., 2019).

Beauveria bassiana (Bals). Vuill. is found in different ecological environments, as soil, plants, and insects (Imoulan et al., 2017). Among the other entomopathogenic fungi species, this is the most commonly found in the field occupying the habitat above ground (Meyling et al., 2011). Besides, it is a fungus that causes disease in a broad range of insects, ticks, and mites (Rehner et al., 2011), and it could be used for the control of arthropods with both conidia and blastospores (Mascarin and Jaronski, 2016). In Brazil, the registered products based in aerial conidia of *B. bassiana* are being widely used for whitefly management in soybean fields (1.5 million hectares treated annually). Besides, studies are being performed to verify these products' efficiency for the coffee berry borer in Brazilian coffee crops (Mascarin et al., 2019).

Cordyceps (= *Isaria*) *fumosorosea* (Wize) Kepler is isolated from different arthropods (main insects belonging to the order Lepidoptera), air, plants, other fungi, and more rarely in the ground (Zimmermann, 2008). In Brazil, only one product is registered for the citrus psyllid *Diaphorina citri* (Mascarin et al., 2019). *I*ts efficiency for this pest has already been demonstrated in the field (Conceshi et al., 2016; Ausique et al., 2017).

Metarhizium (*Nomuraea*) *rileyi* (Farlow) is an entomopathogenic fungus that infects and kills different insects (Wakil et al., 2013), mainly Lepidoptera from the superfamily Noctuidae (Suwannakut et al., 2005). Moreover, it is also capable of causing natural epizootics in insects of this superfamily in South American countries, as in *Anticarsia gemmatalis*, *Trichoplusia ni*, *Alabama argillacea,* and *Helicoverpa armigera* (Corrêa and Smith, 1975; Alves et al., 1978; Villani et al., 1984; Sujii et al., 2002; Costa et al., 2015).

1.5. Production of blastospores by liquid fermentation

The production of the fungi of the order Hypocreales through the submerged liquid fermentation consists of the utilization of an aerated culture medium, where the fungi grow as blastospores, submerged conidia, or microsclerotia (Jaronski and Mascarin, 2017). The utilization of liquid fermentation is the most economical way to produce microbial biocontrol agents. Other processes using the submerged fermentation, as the production of antibiotics, amino acids, ethanol, and organic acids, has been used as a basis for the optimization of the biopesticides production using the liquid culture (Jackson et al., 1997). Entomopathogenic fungi from the genus *Cordyceps*, *Beauveria*, *Lecanicillium,* and *Metarhizium* in submerged cultures produce yeast-like structures called blastospores (Jackson et al., 2010).

Blastospores are vegetative fungal cells produced in the hemolymph of the insects infected by conidia of entomopathogenic fungi and usually require a large number of nutrients and oxygen for their growth (Jackson et al., 2010). These cells can be produced in liquid media in a similar way that happens inside the infected insect's body (Jackson et al., 1997; Bernardo et al., 2018). Moreover, through the liquid fermentation process is possible to get high amounts of blastospores in a short time and at a low cost (Mascarin et al., 2015; Mascarin and Jaronski, 2016), better control

of the production conditions (pH, temperature, aeration rate) and the possibility of the production on a large scale with the use of bioreactors (Riaz et al., 2013).

Studies performed with *C. fumosorosea* demonstrated high amounts of blastospores produced in liquid media, with concentrations above 1×10^9 blastospores mL^{-1} in lab-scale (Mascarin et al., 2015). For *Beauveria bassiana* is possible to get concentrations above 1×10^9 blastospores mL^{-1} (Mascarin et al., 2015). Besides being possible to get high concentrations of these cells, the same is considered more virulent than conidia in susceptible hosts (Shapiro-Ilan et al., 2008), as well as observed in experiments performed with whitefly, aphids, beetles, ticks, and caterpillars (Jackson et al., 1997; Vandenberg et al., 1998; Behle et al., 2006; Mascarin et al., 2015; Iwanicki et al., 2018).

1.6. Drying methods for the development of blastospore formulations

To ensure the development of an economically viable biopesticide based on blastospores of entomopathogenic fungi, a production process that results in a large amount of drying tolerant blastospores should remain infective after the storage process (Sandoval-Coronado et al., 2001). A proper formulation can improve cells' viability after the drying process and ensure excellent shelf life and improve the virulence against insect pests (Banu, 2013). After the drying process and formulation, the final product should be safe and easy to be used. Moreover, the formulations can be composed of adjuvants, flours, proteins, oil, clays, and diatomaceous earth (Wraight et al., 2001).

Various processes could be used for dry blastospores, such as freeze-drying, air drying, and spray drying. The freeze-drying process is a method very used in the food industry. It consists of dehydration by sublimation of a frozen product, protecting the products' primary structure and shape with minimal reduction of volume. However, it is considered the most expensive drying method to be used on a large scale (Ratti, 2001). Blastospores of *Cordyceps fumosorosea* showed to be freeze-dried tolerant, presenting a survival rate of 67-85% after the freeze-drying process (Cliquet and Jackson, 1999). Moreover, the freeze-dried blastospore formulations of *C. fumosorosea* stored at 20 ºC present excellent viability for 12 months (Jackson et al., 2006).

The air-drying method consists of a drying chamber where the samples containing blastospores stay in touch with a moving air with a specified relativity humidity (Jackson and Payne, 2007). Studies demonstrated that air-dried blastospores of *C. fumosorosea*, *B. bassiana,* and *M. brunneum* with diatomaceous earth showed satisfactory viability after the air drying process (Corrêa et al., 2020; Iwanicki et al., 2018; Mascarin et al., 2016, 2015).

The other way to dry blastospores is the spray drying method, which consists of transforming a fluid state into a dried particulate form through the use of high temperatures using a Spray Dryer. The parameters that can influence the final results, mainly when is working with microbial cells, are the inlet temperature of the drying air, the drying air flow rate, the supply rate of the liquid stream, and the pressure and amount of atomizing air (Cal and Sollohub, 2010). The spray drying process for blastospores and submerged conidia is possible when used specific protective agents and temperatures (Stephan and Zimmermann, 1998). Even this drying method ensuring poor cell survival for some microorganisms, storage stability, and scale-up issues, blastospores of *B. bassiana* showed viability rates above 80% after the spray drying process. However, these blastospores were less infective to whitefly nymphs when compared to air-dried blastospores (Mascarin et al., 2016).

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2. PRODUCTION OF BLASTOSPORES OF *Cordyceps fumosorosea*, *Beauveria bassiana,* AND *Metarhizium rileyi* ISOLATES AND THEIR PATHOGENICITY AGAINST *Spodoptera* spp. AND *Euschistus heros*

Abstract

Soybean crop suffers significant losses of productivity due to a large number of insect pests. Aerial conidia of the fungal genera *Beauveria*, *Cordyceps,* and *Metarhizium* are widely used in Brazilian agriculture to control insects. The use of blastospores is an alternative to be explored, mainly due to ease of production. In this study, we aimed the development of new bioproducts, first evaluating the potential production of blastospores of twelve isolates of *Beauveria bassiana* (ESALQ540, ESALQ650, ESALQ1309 and ESALQ1451), *Cordyceps fumosorosea* (ESALQ1296, ESALQ3422, ESALQ3430 and ESALQ4556), and *Metarhizium rileyi* (ESALQ4946, ESALQ4947, ESALQ4948 and ESALQ4997). Based on their conidial productivity and isolate origin (host/soil), two isolates of each fungal species were selected for virulence studies against *Spodoptera cosmioides*, *Spodoptera eridania*, *Spodoptera frugiperda,* and *Euschistus heros*. The isolates were grown in a low-cost liquid media, and the blastospore concentration was evaluated at days two, three, and four of cultivation. For bioassays, the blastospores were air-dried with diatomaceous earth on the third day of culture, and suspensions of 1×10^7 were sprayed against 2nd instar larvae of *Spodoptera* spp. and adults of *E. heros*. A control containing only diatomaceous earth was also used, and the mortality and sporulation rates were evaluated for ten days. All isolates of each fungal species attained concentrations > 1×10^8 blastospores mL^{-1}, and only the isolates ESALQ1296 and ESALQ3422 of *C. fumosorosea* reached concentrations greater than 1×10^9 blastospores mL^{-1}. The isolates ESALQ540 and ESALQ1309 of *B. bassiana*, ESALQ1296, and ESALQ3422 of *C. fumosorosea,* and ESALQ4946 and ESALQ4947 of *M. rileyi* were tested against the target pests, and all of them presented higher mortality rates compared to the controls for each insect pest. These results demonstrated the potential of blastospores of different fungal species to control these target insects, considering the high production of virulent blastospores to *Spodoptera* spp. larvae and adults of *E. heros*. The possibility of producing infective blastospores of *M. rileyi* urge as a new alternative for field application.

Keywords: Noctuidae; Hemiptera; Hypocreales; Liquid fermentation; Biological control

2.1.Introduction

Soybean (*Glycine max*) is of primary economic importance for the Brazilian agribusiness (Bueno et al., 2017). The same occupies more than 35 million hectares of arable land in Brazil and significantly impacts the Gross Domestic Product (GDP) of national agriculture (Conab, 2018). This crop is one of the most harmed by a great diversity of pests, resulting in productivity losses (Pelizza et al., 2018). The most important species of the *Spodoptera* spp. complex in Brazilian soybean fields are *Spodoptera frugiperda*, *Spodoptera cosmioides,* and *Spodoptera eridania* (Santos et al., 2005). *Spodoptera frugiperda* is an important pest worldwide, recently invading Africa and India (Brévault et al., 2018; Chormule et al., 2019; Goergen et al., 2016). Another difficult pest to control is the Neotropical brown stink bug *Euschistus heros*.

The use of chemical pesticides has not resulted in effective control, and the emergence of new insecticidal molecules is increasingly rare. The use of genetically modified plants containing genes of the bacteria *Bacillus thuringiensis* is an important pest management tool for caterpillars. However, cases of resistance to this technology have been documented in the field for pests, such as *Spodoptera frugiperda* and *Helicoverpa zea* in maize and cotton cultivars, which can also occur in the future in transgenic soybean cultivars (Tabashnik et al., 2009). As a result of this, the microbial biopesticides market has expanded over the years, most notably those formulated from entomopathogenic fungi, representing about 60% of the 82 products already registered in Brazil (Mascarin et al., 2019).

In Brazilian soybean crops, fungi for pest control are recent, being *Beauveria bassiana,* the primary fungus used aiming the whitefly control over one million hectares (Mascarin et al., 2019). A product based on *Cordyceps fumosorosea* was recently registered in the country to manage *H. armigera,* an important pest of soybean. The efficacy of these fungal species in different soybean insect pests such as stink bugs and caterpillars is unknown. The control of stinkbugs with fungi is a great challenge since high doses of conidia are necessary for the infection. (Sosa-Gómez and Moscardi, 1998). *Metarhizium rileyi* is highly virulent against caterpillars. This fungus causes natural epizootics on soybean caterpillars (Kepler et al., 2014). However, due to the difficulties in mass production and this fungus's stability, there are no products available on the market based on this fungus.

In the Brazilian pesticides market, there are only products based on aerial conidia produced by solid fermentation. However, a production system widely used due to the low cost presents some disadvantages as the demand for more workforce and physical space (Mascarin and Jaronski, 2016). But not all fungi show high productivity in this system, as is *M. rileyi* (Fronza et al., 2017). Entomopathogenic fungi are also capable of producing blastospores in nutrient-rich culture media. An important aspect is that this propagule is more infective than conidia in several hosts (Iwanicki et al., 2018; Jackson et al., 1997; Mascarin and Jaronski, 2016; Wassermann et al., 2016), being highly desirable for products development.

The potential use of fresh and dried blastospores of *Beauveria bassiana* and *Cordyceps fumosorosea* for controlling the soybean pests, *Bemisia tabaci* (biotype b), *Chrysodeixis includens,* and *S. frugiperda* has been revealed (Corrêa et al., 2020). It is possible to obtain high concentrations of blastospores of both species through submerged fermentation (Jackson et al., 2003; Mascarin et al., 2015a). However, the production and infectivity of blastospores by *Metarhizium rileyi* still need to be investigated. Firstly, in this study, we evaluated the production of blastospores of isolates of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* in a low-cost culture medium. After that, the dried blastospores' virulence of two isolates of each fungal species was assessed against *S. frugiperda*, *S. cosmioides*, *S. eridania,* and *E. heros*. This study's main objective is to reveal the ability of blastospores of these species of fungus to control the soybean pest complex.

2.2. Material and Methods

2.2.1. Preparation of fungal inoculum

Four isolates of each fungal species were selected for the study, ESALQ540, ESALQ650, ESALQ1309, and ESALQ1451 of *Beauveria bassiana*, ESALQ1296, ESALQ3422, ESALQ3430, and ESALQ4556 of *Cordyceps fumosorosea* and ESALQ4946, ESALQ4947, ESALQ4948, and ESALQ5397 of *Metarhizium rileyi* (Table 1). The isolates were obtained from the Entomopathogenic Fungal Collection “Professor Sérgio Batista Alves,” located in the Insect Pathology Laboratory at ESALQ, University of São Paulo, state of São Paulo, Brazil.

Monosporic cultures were prepared for each isolate, which was stored at –80ºC in cryovials containing glycerol 10% for the use in all the experiments. The fungi were cultivated in the complete media – CM (KH_2PO_4, 0.36 g; $Na_2HPO_4.7H_2O$, 1.05 g; $MgSO_4.7H_2O$, 0.6 g; KCl, 1.0 g; glucose, 10.0 g; $NaNO_3$, 1.58 g; yeast extract, 5.0 g; agar, 20.0 g) for *B. bassiana* isolates, the potato dextrose agar – PDA Difco® for *C. fumosorosea* isolates and Sabouraud's maltose agar + yeast extract – SMAY (neopeptone, 10.0 g; yeast extract, 10.0 g; maltose, 40.0 g; $NaNO_3$, 5.0 g; KH_2PO_4, 1.5 g; $MgSO_4$, 0.5 g; $FeSO_4$, 0.01 g; $ZnSO_4$, 0.01 g e agar, 15.0 g) for *M. rileyi* isolates. Fungi were grown for ten days at 25 ± 1 ºC with a 12:12 h (L:D) photoperiod, and aerial conidia of these plates were used as inoculum for the liquid fermentation. The conidial suspensions were obtained by washing the culture plate with 10 mL with a sterile aqueous solution prepared with 0,04% of polyoxyethylene sorbitan monooleate (Tween® 80, Synth®, Diadema, SP, Brazil).

Table 1. List of isolates of each species used in the study.

Species	Isolates	Origin (Host/ Soil)	Location
Beauveria bassiana	ESALQ540	*Solenopsis* spp.	Cuiabá - MT
	ESALQ650	*Solenopsis* spp.nest	Alambari - SP
	ESALQ1309	*Euschistus heros*	Iepê - SP
	ESALQ1451	*Hypothenemus hampei*	Piracicaba - SP
Cordyceps fumosorosea	ESALQ1296	*Bemisia tabaci*	Jaboticabal - SP
	ESALQ3422	Soil of organic citrus	Itirapina - SP
	ESALQ3430	Soil of organic citrus	Itirapina - SP
	ESALQ4556	Soil of native vegetation	Pampa Biome
Metarhizium rileyi	ESALQ4946	*Anticarsia gemmatalis*	Itaara - RS
	ESALQ4947	*Anticarsia gemmatalis*	Itaara - RS
	ESALQ4948	*Anticarsia gemmatalis*	Itaara - RS
	ESALQ5397	*Spodoptera frugiperda*	Piracicaba - SP

2.2.2. Screening of isolates for blastospore production

Blastospores production was performed in two steps, the pre-culture, and the culture, wherein each one used a culture medium with different proportions of carbon and nitrogen. Both media were composed of a basal solution of salts with metals and vitamins described in Iwanicki et al. (2020), which contained per liter of distilled water: KH_2PO_4, 2.5 g; $CaCl_2{\cdot}2H_2O$, 1.0 g; $MgSO_4{\cdot}7H_2O$, 0.83 g; $FeSO_4{\cdot}7H_2O$, 0.3 g;

$CoCl_2 \cdot 6H_2O$, 29.6 mg; $MnSO_4 \cdot H_2O$, 12.8 mg; $ZnSO_4 \cdot 7H_2O$, 11.2 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 0.2 mg each; and folic acid, biotin, and vitamin B_{12}, 0.02 mg each. The pre-culture medium used for produce the fungal inoculum contained the basal medium supplemented with 80 g L^{-1} of glucose (Synth®, Diadema, SP, Brazil) and 80 g L^{-1} of corn steep liquor (Ingredion®, Mogi Guaçu, SP, Brazil), while the culture medium was supplemented with 140 g L^{-1} of glucose and 80 g L^{-1} of corn steep liquor. The glucose (14% p/v) was autoclaved separately from the rest of the medium. After autoclaving, the pH of the complete media was adjusted to 6.8.

Five mL of fungal suspensions at a standard concentration of 5×10^6 conidia mL^{-1} were used for inoculation in 250 mL baffled flasks containing 45 mL of pre-culture medium. Pre-culture lasted for two days and provided the blastospores for the culture inoculation. Five mL of a blastospore suspension from the pre-culture in a standard concentration of 5×10^7 blastospores mL^{-1} was used for inoculation in 250 mL baffled flasks containing 45 mL culture medium, which had a duration of four days. The culture flasks were placed in a rotatory shaker incubator with a 10 mm orbit diameter (Solab®, Piracicaba, SP, Brazil) maintained at 28 ± 1 ºC, 12:12 h (L:D) photoperiod and 350 revolutions per minute (rpm).

For each experiment, three flasks for each isolate were used, being each one of these flasks considered a replicate. A sample of 1 mL was taken from each flask on the second, third, and fourth day of culture to count the blastospores concentration. The count was performed on a Neubauer hemocytometer under a microscope at 400× magnification. The experiment was repeated three times.

2.2.3. Virulence bioassays with air-dried blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* against *E. heros* and *Spodoptera* spp.

2.2.3.1 Drying blastospores and preparation of fungal inoculum

To verify the efficacy of air-dried blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* against the target insects, two isolates of each fungal species were selected based on production and host (ESALQ540 and ESALQ1309 of *B. bassiana*,

ESALQ1296 and ESALQ3422 of *C. fumosorosea*, ESALQ4946 and ESALQ4947 of *M. rileyi*).

Blastospores obtained on the third day of culture were air-dried using diatomaceous earth (DE) as an inert material. Thus, the culture of each isolate/ species was mixed in 5% of DE (p/w) and filtered with a vacuum pump coupled to a Buchner funnel lined with disks of 8 cm diameter of Whatman® filter paper. The mixtures containing blastospores + DE were crumbled using an electric blender and placed in a drying chamber for 15 to 18 hours at 50 - 60% relative humidity. After this period, the humidity was reduced to 20 - 25% until the samples reached a water activity ≤ 3%.

2.2.3.2. Blastospore viability protocol

To verify the viability of the air-dried blastospores for each isolate, samples containing 0.1g of blastospores + DE were obtained, and a suspension adjusted to 1 × 10^6 blastospores/ mL was prepared for each sample. A volume of 150 µl was placed on Rodac® plates containing potato dextrose agar – PDA Difco®, which ones were incubated for 6 hours at 25º C. The viable and non-viable blastospores were evaluated under a microscope at 400× magnification. Viable blastospores were considered the ones with germination tube longer than their diameters.

Therefore, after the evaluation of blastospores viability, suspensions of blastospores at 1 × 10^7 viable blastospores / mL were prepared in distilled water + Tween® at 0.02% (v/v) for each isolate. The control treatments consisted of distilled water + Tween® at 0.02% (v/v) and distilled water + Tween® (0.02%) + DE (0,075g for each 0,1g of blastospores + DE), totalizing 8 treatments per experiment.

2.2.3.3. Bioassays with *E. heros*

Adult *E. heros* from the Pathology and Microbial Control of Insects Laboratory, at the University of São Paulo, located in São Paulo, Brazil, were placed in Petri dishes (12.5 cm diameter) containing filter paper, string bean, peanut, and water-soaked in cotton. Each treatment was composed of 4 replicates containing 12 insects per replicate. The fungal suspensions were sprayed with a Potter Spray Tower (Burkard Manufacturing, Rickmansworth, UK) at 15 PSI, applying about 2 mL per replicate.

After spraying, the Petri dishes containing the insects were placed in an environmentally controlled chamber at 25 ± 1 ºC, 70-80% RH, and a 14:10 h (L:D) photoperiod. The mortality was evaluated daily for 10 days, and the cadavers were placed in a humidity chamber to provide the necessary condition for the fungal outgrowth. The mycosis level was then calculated as the percentage of cadavers showing fungal outgrowth compared to the total number of insects tested per replicate plate. The experiment was performed three times using new batches of fungi and insect cohorts.

2.2.3.4. Bioassays with *S. frugiperda, S. cosmioides,* and *S. eridania*

Eggs obtained from the rearing of Insect Biology Laboratory at the University of São Paulo, located in Piracicaba, SP, Brazil, were placed in plastic cups until hatching. *S. frugiperda* larvae were fed on pieces of maize leaves (Fórmula®, Syngenta, Brazil), while *S. cosmioides* and *S. eridania* larvae were fed on soybean leaves (BRS 264®, Embrapa, Brazil). Three or four days later, when larvae hit the second instar, the experiments were installed.

The larvae were placed in Petri dishes (12.5 cm diameter) containing filter paper and a piece of maize leaf for *S. frugiperda* and a soybean leaf for *S. cosmioides* and *S. eridania*. Each treatment (Petri dish) had five replicates with 12 larvae in each one. Two mL of fungal suspensions were sprayed per replicate with a Potter Spray Tower (Burkard Manufacturing, Rickmansworth, UK) at 15 PSI.

After the spraying, the Petri dishes containing the insects were placed in an environmentally controlled chamber at 25 ± 1 ºC, 70-80% RH, and a 14:10 h (L:D) photoperiod. For *S. frugiperda*, after two days, the larvae were individualized in polystyrene cups (41.70 × 33.50 × 52.00 mm), which ones were closed with acrylic covers. The mortality was evaluated daily for ten days, and new leaves of maize and soybean were offered to the larvae throughout the experiment. The cadavers were placed in a humidity chamber to provide the necessary condition for the fungal outgrowth. The mycosis level was calculated as the percentage of cadavers showing fungal outgrowth compared to the total number of insects tested per replicate. The experiment was repeated three times using new batches of fungi and insect cohorts.

2.2.4. Statistical analysis

Blastospore concentration data were transformed by log10(x) and then fitted to a linear mixed model (LMM) with fungal isolates and fermentation days as fixed factors, and a random effect attributed to replicate flasks accounted for different intercepts among treatments. Fixed effects attributed to fungal isolates and fermentation days in the model were assessed for significance with F-tests. The estimated means from treatments were statistically compared within each evaluation date by the Tukey HSD test. The P-values were adjusted with the false discovery rate (FDR), performed in the package “emmeans” (Lenth, 2018).

To assess the interaction effects of fungal species and isolates on the insect's mycosis rates, these parameters were fitted to GLMs with binomial distribution taking into account overdispersion and using a logit link function. The estimated means obtained from each fungal treatment were statistically compared with each other by the Tukey HSD test with FDR-adjusted P-values. The results were considered statistically significant at $P < 0.05$. All models chosen here to fit on these datasets were carefully selected based on their goodness-of-fit, using residual plots and half-normal plots based on the smallest Akaike’s Information Criterion (AIC) (Moral et al., 2017).

The survival analysis for censored data on insect mortality of these three soybean pests was undertaken with the “flexsurv” package in R (Jackson, 2016). Median survival times (ST_{50}) were estimated from the fitted model, and their respective 95% confidence limits (95% CL) were obtained from a parametric bootstrap procedure, using 10,000 samples (we set the seed as 1460 to allow for reproducibility).

2.3. Results

2.3.1. Screening of isolates from blastospore production

Among the *B. bassiana* isolates there was a distinct growth rate ($F = 14.21$, df = 11, 96, $P < 0.0001$) (Figure 1a). Except for isolate ESALQ540, there was no increase in blastospores' concentration after the second day of fermentation. The isolate ESALQ540 was the best blastospore producer, followed by ESALQ650 and ESALQ1309. The highest blastospore production was observed on day four by the

isolate ESALQ540, with 6.62 × 10^8 blastospores $mL^{-1,}$ while the isolate ESALQ1451 presented the worst yield at all days (< 3× 10^8 blastospores mL^{-1}). The isolates ESALQ540 and ESALQ1309 were selected for the virulence assays because the first presented the highest production. The second presented a great production and was isolated from a *Euschistus heros* cadaver, one of the pest insects used in this study.

For *C. fumosorosea*, the isolates ESALQ1296 and ESALQ3422 presented the higher growth rates at days 2 and 3 of culture, differing only at day 4 (F = 115.8, df = 11, 96, P < 0.0001) (Figure 1b). The two isolates attained concentrations above 1 × 10^9 blastospores mL^{-1} at days 3 and 4, ESALQ1296 reached a production of 2.21 × 10^9 blastospores mL^{-1} at four days of culture. In contrast, the isolates ESALQ 3430 and ESALQ4556 were the worst producers, attaining a maximum production of only 2.13 × 10^8 blastospores mL^{-1} and 8.89 × 10^7 blastospores mL^{-1} after two days. The isolates ESALQ 1296 and ESALQ322, the best blastospores producers were selected for the virulence study.

The isolates of *M. rileyi* presented different growth rates (F = 6.28, df = 11, 96, P < 0.0001) (Figure 1c). The isolates ESALQ4946 and ESALQ4947 presented the highest yields at days 2 and 3 of culture, attaining concentrations up to 3.11-5.46 × 10^8 blastospores mL^{-1}. On day 4, the isolates ESALQ4946 and ESALQ5397 obtained the highest blastospores production (>5 × 10^8 blastospores mL^{-1}). The blastospores concentration of the isolate ESALQ4948 did not increase along the evaluation period (3.24-3.57× 10^8 blastospores mL^{-1}). For virulence assays, the isolates ESALQ4946 and ESALQ4947 were selected.

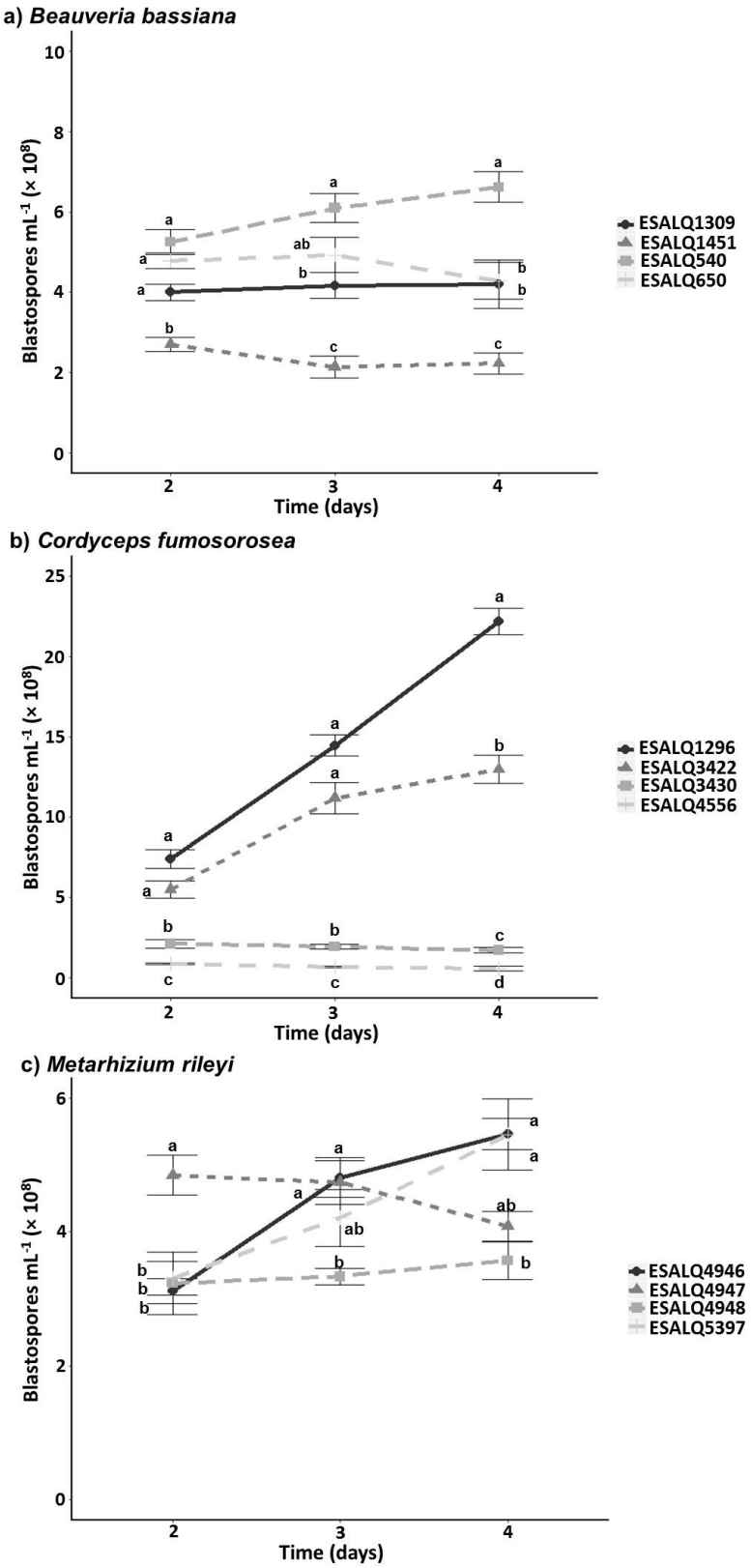

Figure 1. Blastospores yield of isolates of *Beauveria bassiana* (a), *Cordyceps fumosorosea* (b), and *Metarhizium rileyi* (c) grown during four days in a liquid culture medium supplemented with 140 g L^{-1} of glucose and 80 g L^{-1} of corn steep liquor at 28 ºC at 350 RPM. Means (± SE) followed by different letters within each day of incubation are statistically different according to the Tukey test ($P < 0.05$).

2.3.2. Virulence of air-dried blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* against *E. heros* and *Spodoptera* spp.

The survival time analysis of *E. heros* presented a significant difference among the treatments (χ^2 = 64.22; df = 7; P < 0.0001). The uninoculated treatments with and without diatomaceous earth presented insects' highest survival compared to the treatments with blastospores. Among the isolates tested, the isolate ESALQ540 of *B. bassiana* showed one of the lowest survival times of insects, with a ST_{50} of 9 days (Figure 2a). The ST_{50} for the other treatments were not calculated because they did not reach total mortality above 50%.

E. heros mycosis was the highest for the isolate ESALQ540 of *B. bassiana* (30.74%) and ESALQ3422 of *C. fumosorosea* (20.96%) (χ^2 = 22.42; df = 7; P = 0.0021). Cadavers from treatments with isolates ESALQ1309 of *B. bassiana* and ESALQ1296 of *C. fumosorosea* presented lower sporulation, with 11.11% and 12.63%, respectively (Figure 2b). No sporulation was confirmed for *E. heros* cadavers from treatments with the isolates ESALQ4946 and ESALQ4947 of *M. rileyi*.

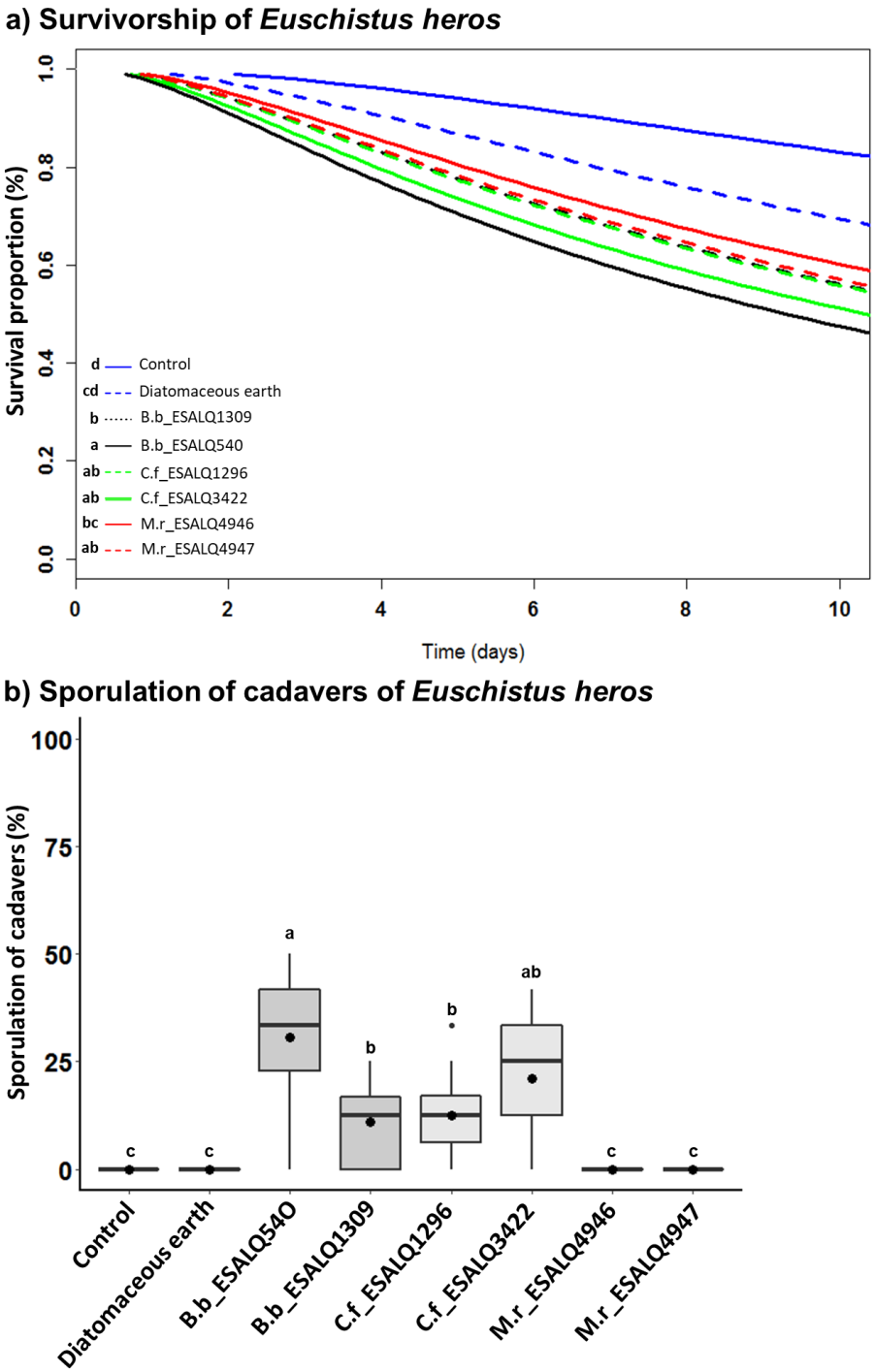

Figure 2. Survival proportion (a) of *Euschitus heros* after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. According to the log-likelihood ratio test, distinct letters correspond to significant differences between survival curves ($P < 0.05$). Sporulation of cadavers (b) of *Euschitus heros* after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. Boxes show the median, 25th, and 75th percentiles, while error bars show 10th and 90th percentiles. Dots outside of the box range represent outliers. A black dot (•) inside the box denotes the mean value of each treatment. According to the Tukey HSD test, means followed by different letters within each treatment indicate significant differences ($P < 0.05$).

The isolate ESALQ1296 of *C. fumosorosea* resulted in the highest reduction of S. frugiperda larvae's survivorship, followed by the isolate ESALQ3422 of the same fungal species (χ^2 = 133.20; df = 7; P < 0.0001). (Figure 3a). These treatments also presented the lowest values of ST_{50}, being three days for ESALQ1296 and five days for ESALQ3422. The isolates of *B. bassiana* (ESALQ540 and ESALQ1309) and *M. rileyi* (ESALQ4946 and ESALQ4947) presented a significant reduction of larval survival compared to the uninoculated treatments.

The mycosis rates of the *C. fumosorosea* isolates ESALQ3422 and ESALQ1296 was 32.80% and 29.01%, respectively (χ^2 = 14.19; df = 7; P = 0.0478). Only 15.55% and 17.82% of larvae treated with the isolates ESALQ540 and ESALQ1309 of *B. bassiana* presented fungal sporulation (Figure 3b). The larvae killed by the isolates of *M. rileyi* (ESALQ4946 and ESALQ4947) did not present a fungal outgrowth.

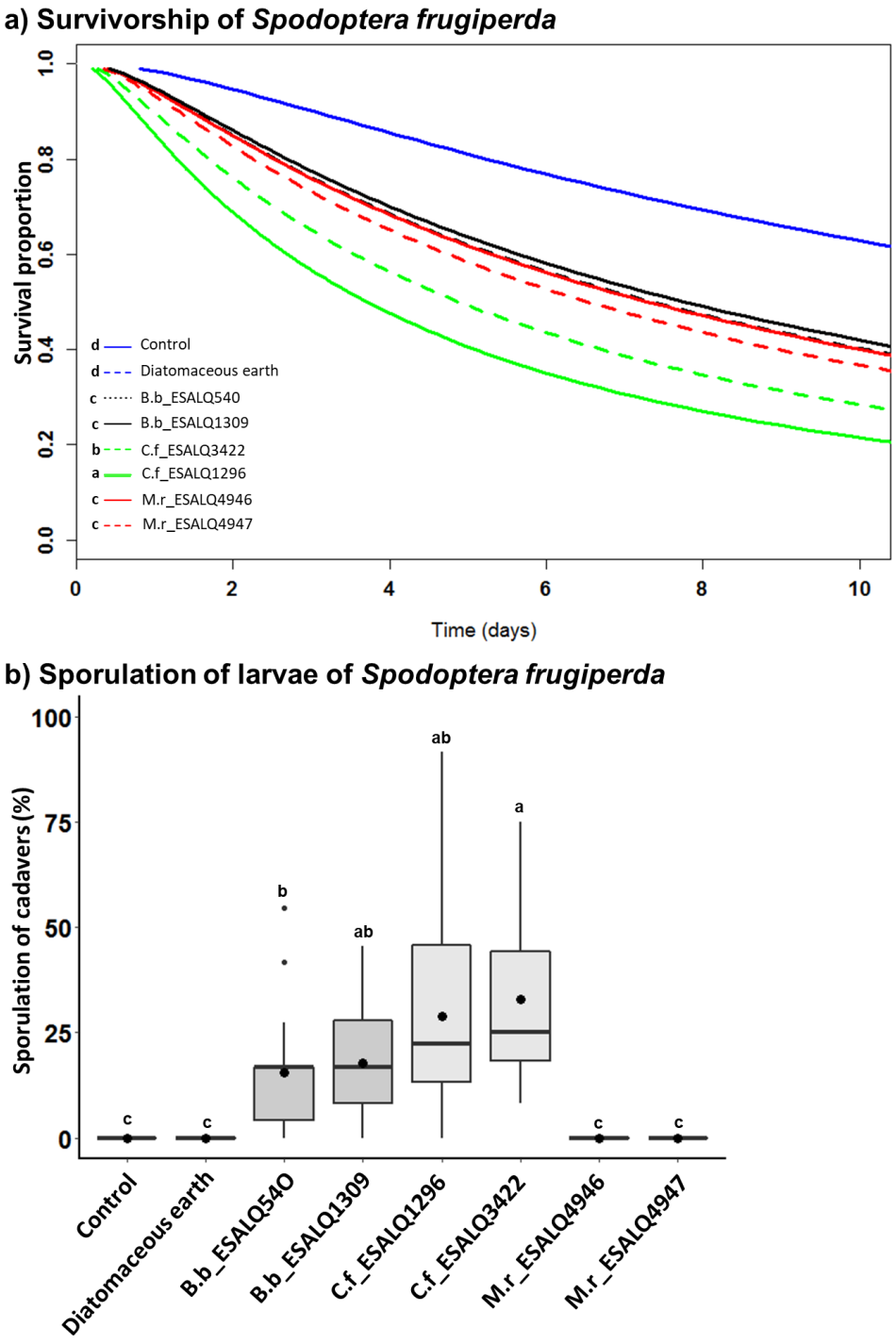

Figure 3. Survival proportion (a) of *Spodoptera frugiperda* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. According to the log-likelihood ratio test, distinct letters correspond to significant differences between survival curves ($P < 0.05$). Sporulation of cadavers (b) of *Spodoptera frugiperda* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. Boxes show the median, 25th, and 75th percentiles, while error bars show 10th and 90th percentiles. Dots outside of the box range represent outliers. A black dot (•) inside the box denotes the mean value of each treatment. According to the Tukey HSD test, means followed by different letters within each treatment indicate significant differences ($P < 0.05$).

For *S. cosmioides*, the uninoculated treatments with and without diatomaceous earth presented the highest survival of insects compared to the treatments with blastospores (χ^2 = 80.59; df = 7; *P* < 0.0001). These results indicate that the fungal isolates killed more and faster the larvae of *S. cosmioides* than the uninoculated treatments (Figure 4a). The ST_{50} for the treatments was not calculated because any of them reached total mortality above 50%.

The sporulation rates for *S. cosmioides* larvae were low for all the isolates and fungal species used in the study. The isolates ESALQ3422 of *C. fumosorosea* and ESALQ4946 and ESALQ4947 of *M. rileyi* resulted in 22.54%, 22.27% and 27.52% of larval with fungal outgrowing, respectively (χ^2 = 12.16; df = 7; *P* = 0.0325). The isolate ESALQ1296 of *C. fumosorosea* presented 19.14% of dead larvae with mycosis, while the isolates ESALQ540 and ESALQ1309 resulted in 13.89% and 7.78%, respectively (Figure 4b).

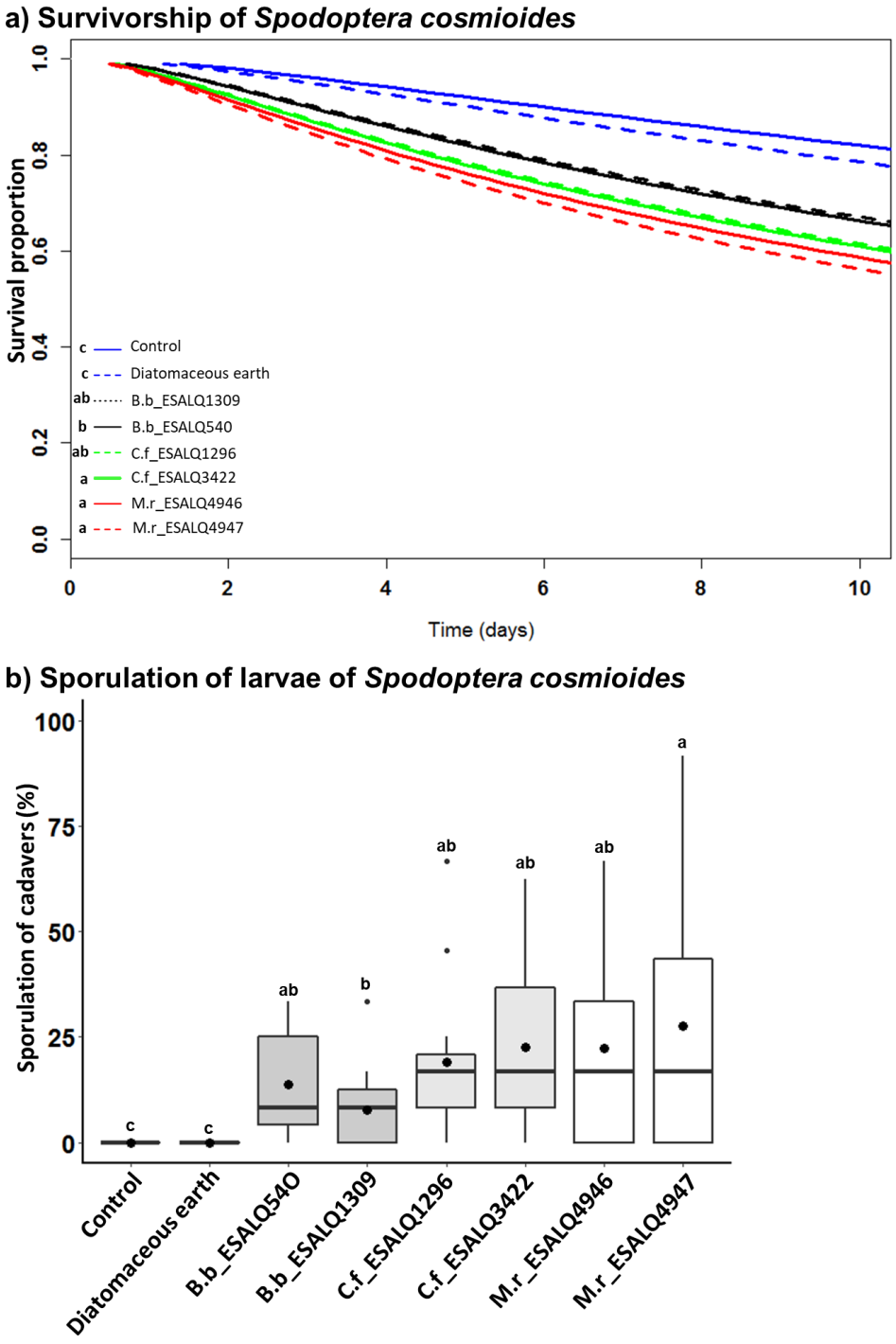

Figure 4. Survival proportion (a) of *Spodoptera cosmioides* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. According to the log-likelihood ratio test, distinct letters correspond to significant differences between survival curves ($P < 0.05$). Sporulation of cadavers (b) of *Spodoptera cosmioides* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. Boxes show the median, 25th, and 75th percentiles, while error bars show 10th and 90th percentiles. Dots outside of the box range represent outliers. A black dot (•) inside the box denotes the mean value of each treatment. According to the Tukey HSD test, means followed by different letters within each treatment indicate significant differences ($P < 0.05$).

The isolates of *B. bassiana* (ESALQ540 and ESALQ1309) and *M. riley* (ESALQ4946 and ESALQ4947) presented the lowest survival of *S. eridania* larvae (χ^2 = 50.08; df = 7; *P* < 0.0001). The uninoculated treatment with and without diatomaceous earth resulted in the highest survival of larvae (Figure 4a).

The number of sporulated larvae was very low for this species, and the mortality rates were not significantly different (χ^2 = 5.33; df = 7; *P* < 0.6195). The isolate ESALQ540 of *B. bassiana* presented 15% of sporulation, followed by the isolates ESALQ3422 and ESALQ1296 of *C. fumosorosea*, with 12.78% and 11.26%. The other isolate of *B. bassiana* (ESALQ1309) presented 10% of dead larvae with fungal outgrowth. The isolate ESALQ4947 showed a sporulation rate of only 6.11%, while any larvae killed by the isolate ESALQ4946 presented fungal outgrowth (Figure 4b).

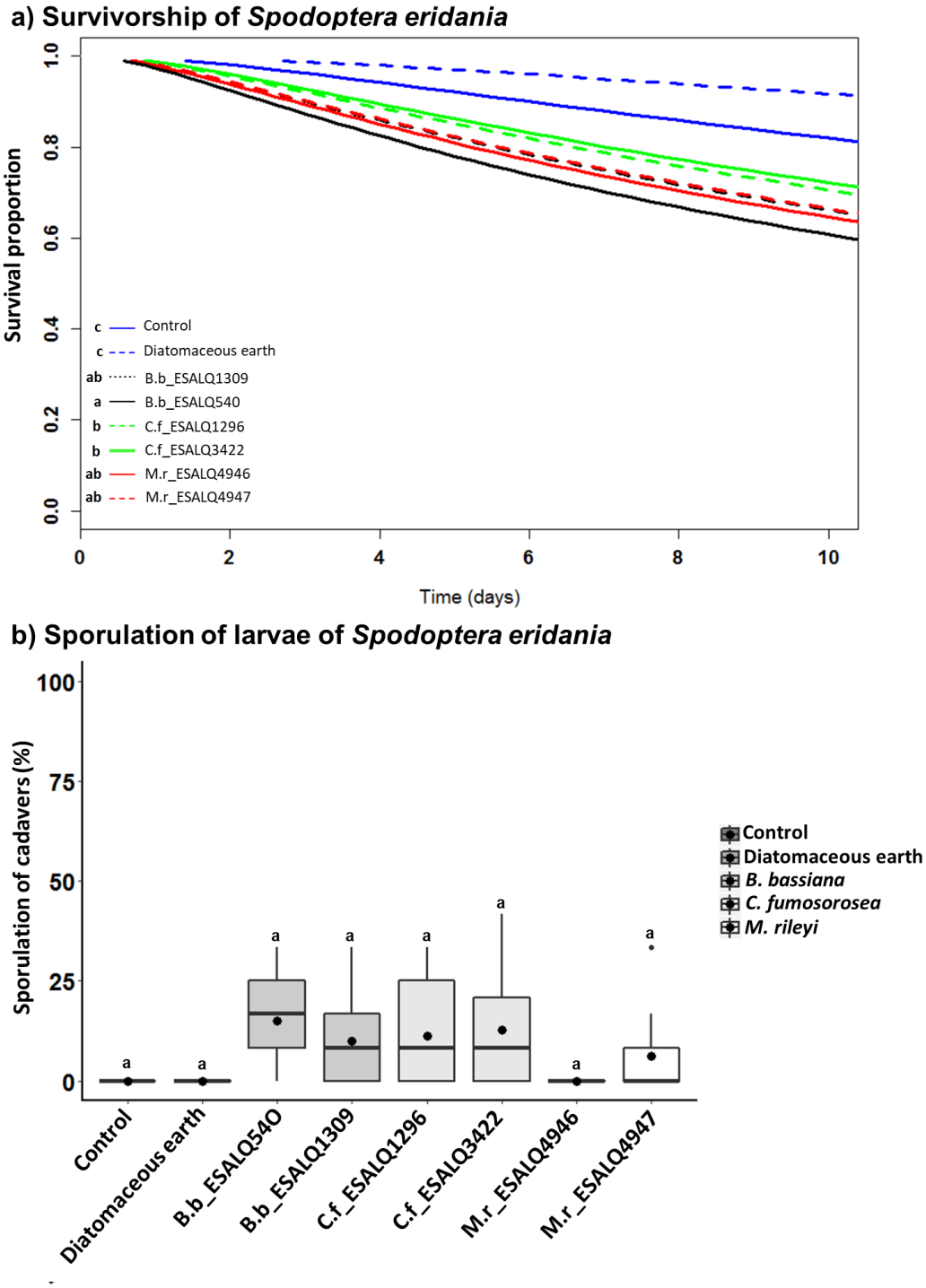

Figure 5. Survival proportion (a) of *Spodoptera eridania* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. According to the log-likelihood ratio test, distinct letters correspond to significant differences between survival curves ($P < 0.05$). Sporulation of cadavers (b) of *Spodoptera eridania* larvae after spraying with dried blastospores of *Beauveria bassiana* (B.b), *Cordyceps fumosorosea* (C.f), and *Metarhizium rileyi* (M.r) isolates (10^7 viable blastospores mL^{-1}) under laboratory conditions. Boxes show the median, 25th, and 75th percentiles, while error bars show 10th and 90th percentiles. Dots outside of the box range represent outliers. A black dot (•) inside the box denotes the mean value of each treatment. According to the Tukey HSD test, means followed by different letters within each treatment indicate significant differences ($P < 0.05$).

2.4. Discussion

A high concentration of blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* was possible to be obtained in a low-cost media using corn steep liquor as a nitrogen source. Besides that, dried blastospores of these three fungal species were infective to *E. heros* adults and *S. cosmioides*, *S. frugiperda,* and *S. eridania* second instar larvae. Therefore, this study reveals the potential of air-dried blastospores of these fungal species against *E. heros* and the three species of the *Spodoptera* complex. Furthermore, aerial conidia of *M. rileyi* is known to be infectious to lepidopteran pests; however, no studies reported M. rileyi blastospores' virulence to any insect pest. This is the first report of the fungal outgrowth in caterpillars sprayed with blastospores of *M. rileyi.*

When isolates of the same species were evaluated in this study, it was verified a significant variation in the blastospore production for the three fungal species, especially among *C. fumosorosea* isolates. Other studies also revealed a distinct production of blastospores among different isolates of the same species (Corrêa et al., 2020; Iwanicki et al., 2018; Mascarin et al., 2015a, 2015b), a fact that can be explained, in part, by the genetic variation between isolates. In this study, only the isolates ESALQ1296 and ESALQ3422 of *C. fumosorosea* obtained blastospore yields above 1×10^9 blastospore mL^{-1}. These isolates produce high concentrations of blastospore on the third day of culture ($>1\times10^9$ blastospore mL^{-1}) in a liquid medium supplemented with acid hydrolyzed casein (Corrêa et al., 2020). This study proved that these isolates showed similar growth patterns in a low-cost media supplemented with corn steep liquor. For the four isolates of *B. bassiana,* we obtained a production rate between 2 to 7×10^8 blastospore $mL^{-1,}$ and this could be a genetic feature of these isolates, once *B. bassiana* is already known to produce blastospores yields upper 1×10^9 blastospore mL^{-1} (Mascarin and Jaronski, 2016).

M. rileyi is already known for the microsclerotia production in submerged fermentation (Song et al., 2017, 2016, 2014). Only one study reported the potential of this species to produce high concentrations of blastospores, up to 1×10^9 blastospores mL^{-1} using yeast extract as a nitrogen source (Abati, 2015). In this study, for the isolates of *M. rileyi,* we attained blastospore concentrations between 3 to 6×10^8 blastospore mL^{-1} using corn steep liquor as a nitrogen source. Some studies revealed that the nitrogen source and its concentration in the media could affect the blastospore

yield (Jackson et al., 1997, 2003; Mascarin et al., 2018; Iwanicki et al., 2020). In this way, using other nitrogen sources in the media can increase the blastospores yield for these isolates. Besides that, blastospores of *M. rileyi* up-regulate one iron permease, which can indicate, according to Iwanicki (2020), that this cell uses distinct mechanisms to acquire iron or that they need a large amount of this metal. In this way, a study involving different proportions of iron in the media can help us with this question. However, this not-so-high blastospores production can be a genetic feature of the isolates, once a similar blastospore growth pattern was observed for *Metarhizium robertsii* isolates (Iwanicki et al., 2018). Therefore, the capacity to obtain a high concentration of blastospores of this fungal species using a low-cost media is a great alternative instead of the use of aerial conidia, once actually the use of *M. rileyi* in the field is harmed by the difficulty of mass production of this fungi propagule (Fronza et al., 2017).

Some studies reported the virulence of conidia of *B. bassiana* against stink bugs, as *E. heros* (Oliveira et al., 2016; Sosa-Gómez and Moscardi, 1998). However, in this study, we show for the first time the virulence of blastospores of this fungal species to the brown stink bug, beyond also confirmed the possibility to use blastospores of *C. fumosorosea* and *M. rileyi* for *E. heros* control. For adults, the isolates of *B. bassiana* and *C. fumosorosea* were the promising species for controlling this pest, especially the isolate ESALQ540 of *B. bassiana*. This isolate was responsible for the highest mortality of adults (>50%) and the lowest value for the ST_{50}; moreover, most insects sprayed with the dried blastospores of this isolate presented fungal outgrowing after death. On the other hand, *M. rileyi,* a fungal species well known for infect lepidopteran species, gave a significant mortality rate compared to the control. However, the dead insects exposed to the dried blastospores of this fungal isolate did not sporulate. This fact could be explained by some fungi' ability to use toxins to cause host mortality instead of the extensive invasion of organs (BUTT, 2002). We already knew that *M. rileyi* could produce active metabolites against insects (Ignoffo et al., 1976), which showed toxic activity against *Heliothis zea* and *Heliothis virescens* (Mohamed and Nelson, 1984). Another hypothesis to explain the mortalities is that the diatomaceous earth sprayed with the dried blastospores removed or abraded the epicuticular wax, causing the dehydration of the insects (Butt et al., 2016). Because of this, the insects could be more susceptible to fungal infection. Therefore, to explain correctly the deaths caused by *M. rileyi* and the virulence of it to *E. heros,* it is

necessary a study involving scanning microscopy to confirm the blastospore penetration and colonization in the insect hemolymph.

When the dried blastospores of the selected isolates were used against the *Spodoptera* complex, it was verified a distinct mortality pattern among each insect species. *S. frugiperda* was the most susceptible species, presenting more than 50% of larvae mortality with the dried blastospores of the three fungal species. The isolates ESALQ1296 and ESALQ3422 of *C. fumosorosea* were the most virulent isolates for this species. They presented the lowest values for ST_{50} (< 5 days) when they were sprayed at a concentration of 1×10^7 blastospores mL^{-1}. This high susceptibility was also observed when a concentration of 5×10^7 blastospores mL^{-1} was used against *S. frugiperda* third instar larvae (Corrêa et al., 2020). The larvae died for the dried blastospores of *B. bassiana,* and *C. fumosorosea* presented fungal outgrowing, which was not observed for *M. rileyi*, similar to what happened with *E. heros*. However, unlike the latter, aerial conidia of *M. rileyi* is virulent to *S. frugiperda* larvae (Grijalba et al., 2018; Johnson et al., 1976; Maniania and Fargues, 1985), indicating the possible virulence of the blastospores of this fungal species too, even not presenting mycosis. For *S. cosmioides*, the highest mortality was caused by *M. rileyi* isolates, and for *S. eridania,* the highest rates were obtained by *B. bassiana* isolates. Different from what happened with individuals of *E. heros* and *S. frugiperda* died with *M. rileyi*, it was observed some larvae of *S. cosmioides* and *S. eridania* with fungal outgrowing. The nutrients presented in the larvae hemolymph of this species can provide the fungal colonization and extrusion, even the larger body size of these larvae compared to *S. frugiperda* larvae. Besides that, the use of blastospores of *M. rileyi* for pest control in the field can be possible due to hydrophobins' presence. These proteins can be responsible for the virulence and resistance to environmental stress that is up-regulated by this fungal propagule (Iwanicki, 2020).

The use of blastospores to pest control has some advantages compared to conidia, the most used fungal propagule. Firstly, the liquid fermentation process requires a short time to obtain many cells with better control of the growth conditions and with a low cost of production. However, these cells require a nutritionally rich media to be obtained, so there is a necessity to use a low-cost liquid media in the liquid fermentation process to keep reduced production costs (Mascarin et al., 2019). Besides that, several studies demonstrated the virulence of the blastospores to several insect pests, as *B. tabaci* (Corrêa et al., 2020; Mascarin et al., 2015a), *C. includens*,

S. frugiperda (Corrêa et al., 2020), *Diaphorina citri* (Morales-Reyes et al., 2018), *Rhipicephalus microplus* (Iwanicki et al., 2018) and *Dalbulus maidis* (Iwanicki et al., 2020). In this way, in this study, we showed that it is possible to produce blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* in a low-cost media, which ones are virulent to *E. heros*, *S. frugiperda*, *S. cosmioides,* and *S. eridania*. There is any biopesticide based in blastospores in the Brazilian market; thereby, this could be a new approach to insect pest control in soybean fields, being necessary studies involving the formulation of blastospores to ensure the shelf-life, protect the cells against UV radiation and improve the virulence of the fungi to the insects in the field. Besides that, the production of virulent blastospores of *M. rileyi* urges as a great alternative to the mass production of this fungal species to be used in the field for insect control, which is not possible yet with the use of conidia.

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3. EVALUATION OF DRYING METHODS FOR THE DEVELOPMENT OF BLASTOSPORES BASED FORMULATIONS OF *Cordyceps fumosorosea*, *Beauveria bassiana* AND *Metarhizium rileyi*

Abstract

Hypocrealean fungi are widely used as micopesticide. These fungi produce different propagules such as aerial and submerged conidia, blastospores, microsclerotia, and mycelium. Although it is possible to produce high concentrations of blastospores for developing a biopesticide based on this type of propagule, there is still a need to create technology for the formulation, drying to increase shelf life. In this study, we evaluated the influence of three drying methods and two storage temperatures on the blastospore viability of *Cordyceps fumosorosea* (isolate ESALQ1296), comparing eight fillers and several co-formulants comprising twenty formulations. The fillers were mixed with liquid media containing the blastospores in a proportion of 7.5% and 30% (w/v) and were air-dried. After that, eight formulations were evaluated according to three drying methods: fast air drying, slow air drying, and spray drying. Pareto chart was used for Plackett Burman design of 12 new formulations. The two best formulations were selected for the shelf life studies with *Beauveria bassiana* (isolate ESALQ540), *C. fumosorosea* (ESALQ1296), and *Metarhizium rileyi* (ESALQ4947) blastospores, which were dried by the fast air drying and spray drying method. All the formulations were packaged and stored for 180 days at 4°C and 28°C. When fillers were added at a proportion of 7.5%, Filler C and Filler E ensure high blastospore viabilities (87.7% and 82.9%, respectively). Still, it was only statistically different from Filler F, and Filler H. No difference in blastospore viability was observed when fillers were incorporated at 30%. No difference in blastospore viability was observed between the fast and slow air drying methods in 18 out of 20 formulations. The spray drying method affected drastically the blastospore viability in 12 out of the 20 formulations, compared to the air-drying methods. The formulations F12 and F20 presented >80% blastospore viabilities in the three drying methods and were selected for the shelf-life studies. After 120 days of storage at 4 °C, some combinations of formulations and drying methods showed blastospores viability above 70% for *B. bassiana* and *C. fumosorosea*. Blastopore viability decreased drastically for *C. fumosorosea* and *M. rylei* at 28°C for air drying and spray drying methods. Greater than 50% *B. bassiana* blastospores remained viable after 60 days at 28°C, and there were no differences between the air drying and spray drying methods for the two formulations. At 4°C, the shelf lives of *M. rileyi* blastospores formulations were lower than those of the two other fungi. This study revealed the potential of co-formulants and fillers to protect blastospores during spray drying, but studies to improve shelf-life are still needed.

Keywords: Entomopathogenic fungi; Liquid fermentation; Air-drying; Spray drying.

3.1. Introduction

The search for eco-friendly and economically viable alternatives for pest control has increased the use of microbial biopesticides every year (Damalas and Koutroubas, 2018). The advantage of using this control method is its high specificity to insect pests and the low risk to non-target organisms (Czaja et al., 2015). Products based on hypocrealean entomopathogenic fungi are essential in this market. The species belonging to this order can produce *in vitro* aerial conidia and submerged conidia, blastospores, microsclerotia, and mycelium (Jaronski, 2013). However, even though it is possible to produce them massively, there are several challenges for developing a biopesticide based on these fungal propagules,

Blastospores are propagules easily obtained in artificial media; on the other hand, they present short shelf life, and there still a need for more study to demonstrate the field efficacy (De La Cruz Quiroz et al., 2015). The stabilization of these propagules can be accomplished by drying, freeze-drying, spray-drying, and air-drying, being some methods used (Jackson and Payne, 2007). The freeze-drying and spray drying methods are the most recommended for the drying of microorganisms on an industrial scale, ensuring the microbial cells' viability for a longer time. However, they are expensive and require sophisticated equipment for their implantation (Hamoudi-Belarbi et al., 2016). Some studies using these drying methods for blastospores have already been tested, presenting excellent viability (Stephan and Zimmermann, 1998; Mascarin et al., 2016). Meanwhile, the air-drying process is cheaper and can be very efficient to ensure the viability of the cells (Nyanga et al., 2012), providing a high survival of the propagules after drying (Jackson and Paine, 2007; Mascarin et al., 2016).

A formulation is characterized as the mixture of an active ingredient with a diluent, dispersants, wetting agent, ultraviolet radiation protectors, synergists, penetrants, suspending agents, sticking agents virulence promoters (Latgé and Moletta, 1988; Jones and Burges, 1998). The evaluation of the compatibility of the components of a formulation with the microbial cell viability is one of the first steps when a biopesticide is developed. Formulations of entomopathogenic fungi can be classified as technical concentrates, wettable powders, and oil dispersion (Faria and Wraight, 2007), and the propagules used as an active ingredient can be hyphae (mycelium), aerial or submerged conidia, and blastospores (Leite et al., 2003). For the development of a biopesticide formulation based on these microorganisms is

necessary that the propagule remains stable for a long time. Thus, the successful use of an entomopathogenic fungus in pest management requires that the propagules be virulent, thermotolerant, and stable during the storage time (Moore et al., 1995; Fernandes et al., 2007).

Beauveria bassiana (Balsamo-Crivell) Vuillemin and *Cordyceps fumosorosea* (Wize) Kepler (Hypocreales: Cordycipitaceae) are entomopathogenic fungi capable of producing high concentrations of infective blastospores in artificial medium and can infect several pests, as *Bemisia tabaci*, *Diaphorina citri*, *Spodoptera frugiperda,* and *Chrisodeixis includens* (Corrêa et al., 2020; Mascarin et al., 2015; Morales-Reyes et al., 2018). Besides that, some studies had already revealed the drying tolerance of these propagules for these fungal species when different drying methods were used, as freeze-drying, spray drying, and air drying (Cliquet and Jackson, 1997; Jackson and Payne, 2007; Mascarin et al., 2016; Sandoval-Coronado et al., 2001), however, in biopesticides Brazilian market, there is no bioproduct formulated based in blastospores. On the other hand, *Metarhizium rileyi* (Farlow) Kepler (Hypocreales: Clavicipitaceae) is an epizootic entomopathogenic fungi. However, due to this fungus' instability, there no commercial product even with aerial conidia (Fronza et al., 2017). The possibility of developing the first bioproduct based on stable formulated blastospores of *M. rileyi* has excellent appeal. Previous studies were conducted in our research group, revealing that it is possible to obtain a large concentration of blastospores in liquid media (Abati, 2015; Corrêa, 2020, unpublished data); however, it is not yet known whether blastospores are drying tolerant.

The present study's central aim is to develop a drying method for blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi,* comparing the air and spray drying process using co-formulants. Thus, a set of formulations was developed to ensure the blastospore viability during the drying process and three different drying methods.

3.2. Material and Methods

3.2.1. Fungal inoculum

The isolates ESALQ540 of *Beauveria bassiana*, ESALQ1296 of *Cordyceps fumosorosea,* and ESALQ4947 of *Metarhizium rileyi* were obtained from the Entomopathogenic Fungal Collection “Professor Sérgio Batista Alves” located in the

Insect Pathology Laboratory at ESALQ, University of São Paulo, in the state of São Paulo, Brazil.

The fungi were grown for 10 days at 25 ± 1 ºC with a 12:12 h (L:D) photoperiod on complete media – CM (KH_2PO_4, 0.36 g; $Na_2HPO_4.7H_2O$, 1.05 g; $MgSO_4.7H_2O$, 0.6 g; KCl, 1.0 g; glucose, 10.0 g; $NaNO_3$, 1.58 g; yeast extract, 5.0 g; agar, 20.0 g) for *B. bassiana* isolates, the potato dextrose agar – PDA Difco® for *C. fumosorosea* isolates and SMAY medium - Sabouraud's maltose agar + yeast extract (neopeptone, 10.0 g; yeast extract, 10.0 g; maltose, 40.0 g; $NaNO_3$, 5.0 g; KH_2PO_4, 1.5 g; $MgSO_4$, 0.5 g; $FeSO_4$, 0.01 g; $ZnSO_4$, 0.01 g e agar, 15.0 g) for *M. rileyi* isolates. Monosporic cultures were prepared for each isolate, stored at – 80ºC in cryovials for use in all the experiments.

3.2.2. Blastospores production

Stock cultures of *Beauveria bassiana* (ESALQ540), *Cordyceps fumosorosea* (ESALQ1296), and *Metarhizium rileyi* (ESALQ4947) were grown for ten days at 25 ± 1 ºC with a 12:12 h (L:D) photoperiod in MC, PDA Difco®, and SMAY media, respectively.

A conidia suspension in a standard concentration of 5×10^6 conidia mL^{-1} was prepared by washing the plate with 10 mL with a sterile aqueous solution made with 0.04% of polyoxyethylene sorbitan monooleate (Tween® 80, Synth®, Diadema, SP, Brazil) and it was used for the pre-culture inoculation. The pre-culture period had two days for *B. bassiana* and *C. fumosorosea* and three days for *M. rileyi*. The basal medium contained per liter of distilled water: KH_2PO_4, 2.5 g; $CaCl_2{\cdot}2H_2O$, 1.0 g; $MgSO_4{\cdot}7H_2O$, 0.83 g; $FeSO_4{\cdot}7H_2O$, 0.3 g; $CoCl_2{\cdot}6H_2O$, 29.6 mg; $MnSO_4{\cdot}H2O$, 12.8 mg; $ZnSO_4{\cdot}7H_2O$, 11.2 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 0.2 mg each; and folic acid, biotin, and vitamin B_{12}, 0.02 mg each (Iwanicki et al., 2020). The pre-culture medium was supplemented with 80 g L^{-1} of glucose (Synth®, Diadema, SP, Brazil) and 80 g L^{-1} of corn steep liquor (Ingredion®, Mogi Guaçu, SP, Brazil). After this period, a blastospore suspension in a concentration of 5×10^7 blastospores mL^{-1} was prepared for the culture inoculation. The culture medium was composed of the basal medium supplemented with 140 g L^{-1} of glucose and 80 g

L^{-1} of corn steep liquor. The two media's initial pH was adjusted to 6.8, and it was not controlled throughout the fermentation.

Five mL of fungal inoculum was transferred to 250 mL baffled flasks (Bellco® Glass, Vineland, NJ, USA) containing 45 mL of medium, totalizing 50 mL. The culture flasks were placed in a rotatory shaker incubator with a 10 mm orbit diameter (Solab®, Piracicaba, SP, Brazil) maintained at 28 ± 1 ºC, 12:12 h (L:D) photoperiod and 350 revolutions per minute (rpm).

3.2.3. Compatibility of fillers (inert) with blastospores in the air-drying process

Filters from the ceramic industry selected for this study were Filler C, Filler E, Filler I, Filler H, Filler G, Filler A (All from Mão na Massa®, São Paulo, Brazil)], Filler F (Apti®, Araras, SP, Brazil) and Diatomaceous Earth (Sigma-Aldrich®, Bazil). The blastospore cultures were obtained from the isolate ESALQ1296 of *C. fumosorosea*.

On the third day of culture, each filler was added to the fermented broth to get 7.5 g of inert material for each 100 mL of liquid medium + fungi. The mixture containing the fungal propagules + inert material was filtered with a vacuum pump coupled to a Buchner funnel lined with Whatman® filter disks (12.5 cm diameter and 25µm pore size). After that, the filtrate was removed from the filter paper and crumbled in an electric blender to get a homogeneous material. The content was placed in Petri dishes, which were placed in a drying chamber. The material remained for 15-18 hours in a relative humidity of 50-60%, and after that, the same was reduced to 20-25% until the samples reached a water activity ≤ 3% (Novasina - Labmaster AW, Switzerland). The samples were vacuum-sealed in polypropylene plastic packages, and they were stored at 4 ºC. The blastospore viability was verified one day after drying. Independent experiments were performed three times using different fermentation batches, and each treatment was performed in duplicate.

3.2.4. Increment of fillers (w/v) in the air-drying process

This experiment was proposed to develop a formulated product based on blastospores using only one filler jointly with the fungal propagules. For this, it was selected the fillers which provided the best results for blastospore viability: Filler C,

Filler E, Filler I, Filler G, and Filler A. Filler D WP® was also added as a treatment, whereas the same is composed of 95% of Filler I and 5% of adjuvants.

The isolate selected for this study was the ESALQ1296 of *C. fumosorosea*. On the third day of culture, each filler was added to the fermented broth to get 30 g of inert material for each 100 mL of liquid medium + fungi. The filtering and drying process followed the protocol described in 3.2.2. The blastospore viability was evaluated after the drying process. Independent experiments were performed three times using different fermentation batches, and each treatment was performed in duplicate.

3.2.5. Blastospore viability protocol

For the evaluation of the blastospore viability after the air air-drying process, 0.5 g of each moisture of inert material + fungi were added in Erlenmeyer flasks containing 30 mL of distilled water, and the flasks were maintained in a rotatory shaker incubator at 350 revolutions per minute (rpm) for about 20 minutes. Suspensions for each treatment were adjusted to 1×10^6 blastospores mL^{-1} and 150 µl were placed in Rodac® plates containing potato dextrose agar – PDA Difco® and the same were incubated at 25 ± 1 ºC for 6 hours. The viable and non-viable blastospores were evaluated after counting 100-150 propagules under a 400× magnification microscope; viable blastospores were considered those with germ tubes longer than their diameters.

3.2.6. Screening of formulations for spray and air drying

3.2.6.1. Fungus preparation

For the screening of formulations, the isolate ESALQ1296 of *C. fumosorosea* was used. The batches of fermentation obtained on the third day of culture were centrifuged for 10 minutes at 6000 rpm at 10 ºC to remove the spent medium. The resultant blastospore pellets were re-suspended in sterile deionized water to achieve a suspension used for the drying. For each experiment, new batches of blastospores were prepared, with about 1-2 $\times 10^9$ blastospores mL^{-1}. For every formulation, a

suspension of 100 mL was prepared, and they were dried at three different times for each drying method.

3.2.6.2. Formulations composition

Two sets of formulations were used, with 8 and 12 different combination of ingredients, which were proposed by the Plackett Burmann experimental design.

Table 1. Selected compounds and their respective percentages in eight tested formulations.

	Formulation (%)							
Co-formulants	F1	F2	F3	F4	F5	F6	F7	F8
UV Protector A	5,0	5,0	5,0	2,5	5,0	2,5	2,5	2,5
UV Protector B	0,0	2,5	2,5	2,5	0,0	2,5	0,0	0,0
Filler B	2,0	2,0	4,0	4,0	4,0	2,0	4,0	2,0
Filler A	5,0	2,5	2,5	5,0	5,0	5,0	2,5	2,5
Dispersant A	2,5	5,0	2,5	2,5	5,0	5,0	5,0	2,5
Osmoprotector A	4,0	0,0	4,0	0,0	0,0	4,0	4,0	0,0
Osmoprotector B	4,0	4,0	0,0	4,0	0,0	0,0	4,0	0,0

Table 2. Selected compounds and their respective percentages in 12 tested formulations.

	Formulation (%)											
Co-formulants	9	10	11	12	13	14	15	16	17	18	19	20
UV Protector A	4,0	4,0	4,0	0,0	4,0	0,0	0,0	0,0	4,0	4,0	0,0	4,0
UV Protector B	0,0	4,0	4,0	4,0	0,0	4,0	0,0	0,0	4,0	4,0	4,0	0,0
Filler B	0,0	5,0	5,0	5,0	0,0	5,0	0,0	0,0	5,0	5,0	5,0	0,0
Osmoprotector B	2,0	2,0	4,0	4,0	4,0	2,0	4,0	2,0	2,0	4,0	4,0	4,0
Osmoprotector C	4,0	0,0	0,0	4,0	4,0	4,0	0,0	0,0	0,0	0,0	4,0	4,0
Dispersant A	2,5	5,0	2,5	2,5	5,0	5,0	5,0	2,5	2,5	2,5	2,5	5,0
Filler A	4,0	1,0	4,0	1,0	1,0	4,0	4,0	1,0	4,0	1,0	1,0	1,0
Filler C	4,0	4,0	1,0	4,0	1,0	1,0	4,0	1,0	1,0	4,0	1,0	1,0
Filler D	4,0	1,0	1,0	1,0	4,0	4,0	4,0	1,0	4,0	1,0	1,0	4,0
Antioxidant A	2,0	2,0	0,0	0,0	0,0	2,0	2,0	2,0	0,0	2,0	0,0	0,0
Antioxidant B	2,0	0,0	2,0	0,0	0,0	0,0	2,0	2,0	2,0	0,0	2,0	0,0

3.2.6.3. Air drying (“slow drying”)

The co-formulants were added to the blastospores suspensions in distilled water and homogenized with a magnetic agitator. Each formulation was submitted to a filtering process using a vacuum pump coupled to a Buchner funnel lined with Whatman® filter disks (12.5 cm diameter and 25 µm pore size). After that, the moisture was removed from the filter paper, crumbled in an electric blender to get a homogeneous material, and placed in Petri dishes. The samples were prepared in a drying chamber where they remained for 15-18 hours in a relative humidity of 50-60% and reduced to 20-25% until the samples reached a water activity ≤ 3% (w/w) (Novasina - Labmaster AW, Switzerland).

3.2.6.4. Air drying (“fast drying”)

The preparation of the filter-cakes of formulations for this drying method was similar to that described for the “slow drying.” The samples were placed in a humidity chamber, and they were submitted to dry air. The initial and final relative humidity (RH) was about 20-25%. The samples were submitted to this process for 8-9 hours until they reached a water activity ≤ 3% (w/w) (Novasina - Labmaster AW, Switzerland).

3.2.6.5. Spray drying

The formulation components were added to the blastospores suspensions in distilled water and homogenized with a magnetic agitator's aid. The formulated blastospore suspensions were spray-dried using a LabMaq® MSD 1.0 Spray Dryer (Ribeirão Preto, SP, Brazil) with a spinning disc atomizer. The drying conditions used were 80 ± 2 ºC of inlet temperature, 50 ± 2 ºC of outlet temperature, 0.5L h^{-1} feed rate, 40L min^{-1} of compressed air flow, and 4 bar of compressed air pressure. All the formulations attained a water activity ≤ 3% after the drying.

3.2.6.6. Blastospore viability protocol

For each formulation, three samples of 0.1 g were obtained, and suspensions were adjusted to 1 × 10^6 blastospores mL^{-1}. 150 µl these suspensions were placed in Rodac® plates containing potato dextrose agar – PDA Difco® and incubated at 25 ± 1 ºC for 6 hours. The viable and non-viable blastospores were evaluated after counting 100-150 propagules under a microscope at 400× magnification; viable were considered those with germ tubes longer than their diameters.

3.2.7. Shelf-life of dried blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi*

Based on the results obtained for the desiccation tolerance in screening different formulations at the three drying methods, formulations 12 and 20 were selected. The fungi selected were ESALQ540 for *B. bassiana*, the ESALQ1296 for *C. fumosorosea,* and ESALQ4947 for *M. rileyi*. The formulated blastospores were dried using the "fast air drying" and spray drying method following all the parameters described above. The drying was performed at three different times with new batches of blastospores.

About 0.1 g of each dried fungus formulation were vacuum-sealed in plastic packages (Equapack®, São Paulo-SP, Brazil) protect from light. Three packages were prepared per treatment for each evaluation time (0, 30, 60, 90, 120, and 180 days) and storage temperature (4 and 28 ºC). For each sample, suspensions were adjusted to 1 × 10^6 blastospores mL^{-1}, and 150 µl were placed in Rodac® plates containing potato dextrose agar – PDA Difco® and the plates were incubated at 25 ± 1 ºC for 6 hours. The viable and non-viable blastospores were evaluated after counting 100-150 propagules under a microscope at 400× magnification; viable were considered those with germ tubes longer than their diameters.

3.2.8. Statistical analysis

To assess the interaction effects of fillers or formulations and drying methods on the blastospore viability, the data were fitted to GLMs with binomial distribution taking into account overdispersion and using a logit link function. The estimated means obtained from each fungal treatment were statistically compared with each other by

the Tukey HSD test with FDR-adjusted P-values. The text and figures' values were presented as mean ± standard error, and the results were considered statistically significant at $P < 0.05$. All models chosen here to fit on these datasets were carefully selected based on their goodness-of-fit, using residual plots and half-normal plots based on the smallest Akaike's Information Criterion (AIC) (Moral et al., 2017). The effect of co-formulates on the blastospore viability was analyzed using a Plackett-Burman design for eight and twelve runs and seven and eleven factors, respectively. Data frames were fitted to a linear model with proportion data on blastospore viability as a dependent variable and co-formulants as a fixed effect. The magnitude of the impact of each co-formulatant on the blastospore viability was determined and illustrated in a Paretto-Chart with the respective *p-value*. Survival data over shelf life estimation was fitted separately for *B. bassiana*, *C. fumosorosea,* and *M. rileyi*. For each fungal species, the data were fitted independently for formulations stored at 4 and 28°C to generalized linear models with binomial distribution for errors and logit link function assuming formulations, storage temperatures, and storage time as fixed factors.

3.3. Results

3.3.1. Compatibility of fillers with blastospores in the air-drying process

When a variety of fillers were used in the air-drying process of blastospores of *C. fumosorosea* ESALQ1296, little differences were observed in the viability rates among treatments ($F = 4.57$, df = 7, $P = 0.0008$) (Figure 1). The blastospores dried with Filler C, Filler E, and Filler I attained rates of viability above 80% (87.7%, 82.9%, and 82.2%), while Filler A, Filler G, and Diatomaceous Earth ensure 79.9%, 77.8%, and 77.1% of viable blastospores, respectively, however, the viability rates of these fillers were not statistically distinct. Filler F and Filler H presented the lowest viability rates, close to 70% (68.8% and 68.9%).

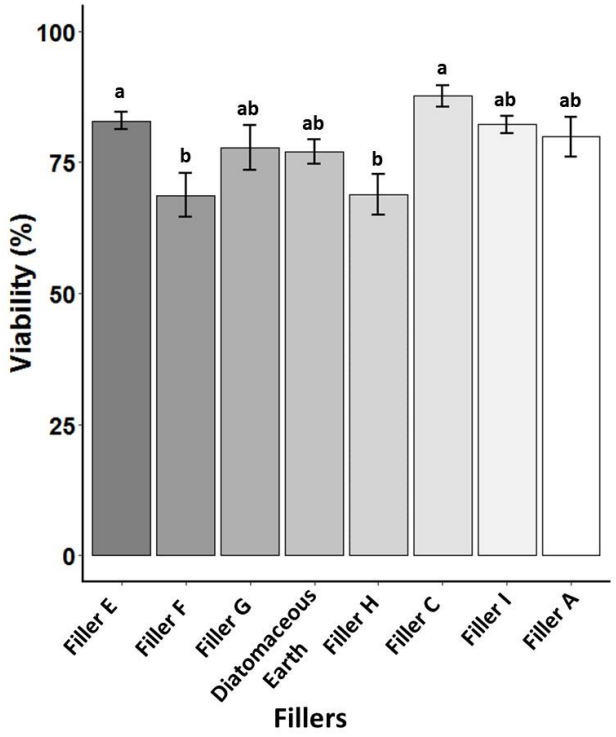

Figure 1. Blastospore viability (%) after the air drying process with different fillers (7.5 g of filler for each 100 mL of liquid medium + fungi). Means (± SE) followed by different letters denote statistical differences according to the Tukey test (P < 0.05).

3.3.2. Increment of fillers (w/v) in the air-drying process

After the first screening with eight fillers to be used with blastospores in the air-drying process, six were selected based on the viability rates, with the addition of another one (filler D) to evaluate the effect of a higher concentration of the filler. At 30% (w/v) of each filler mixed with the batch of blastospores before drying, the viability rates were similar among treatments (F = 1.61, df = 5, P = 0.1862) (Figure 2). The viability rates varied from 76.40% to 88.13%. The increase of filler concentration from 7% to 30% did not affect the viability rate.

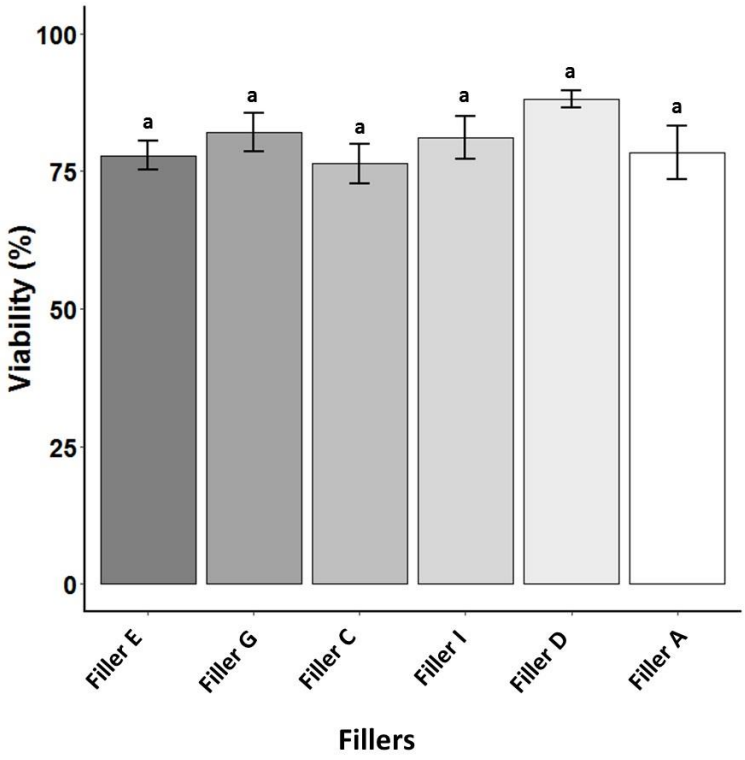

Figure 2. Blastospore viability (%) after the air drying process with the increment (w/v) of different fillers (30 g of filler for each 100 mL of liquid medium + fungi). Means (± SE) followed by different letters denote statistical differences according to the Tukey test (P < 0.05).

3.3.3. Screening of blastospores formulations for spray drying and fast and slow air drying

3.3.3.1. Set of 8 formulations

The formulation composition and the drying method used had a significant influence on the blastospores viability of the *C. fumosorosea* ESALQ1296 (F = 7.70, df = 14, 192, P < 0.0001) (Figure 3). All formulation resulted in viability rates above 80%, using the fast air-drying method, and only the formulations F7 and F3 showed viability rates below 85% (83% and 82%, respectively). For the slow air-drying method, the formulations F2 and F8 presented the lowest viability rates (78.2% and 67%, respectively). F7, F4 and F1, resulted in 87.4%, 87.2% and 87.1% viable blastospores, respectively. The spray drying method also resulted in high viability rates; blastospores dried with F2, F7, F3, and F4 presented viability rates above 80% (85.8%, 82.9%,

81.3%, 81.3%, respectively). The lowest viability rate for this drying method was obtained by F (61.1%).

According to the drying method, the composition of formulations F1, F5, and F8 influenced blastospore protection. In these formulations, the spray drying resulted in the lowest viability rates. For the F1 formulation, the difference between the slow drying and the spray drying was 11%; for F2 the difference between the fast air drying and spray drying was 23.38%, and for F3 there was a 25.67% difference between the viability rates obtained by the fast air drying and spray drying method. Except for the formulation F8, all the formulations presented similar viability rates comparing the two air-drying methods.

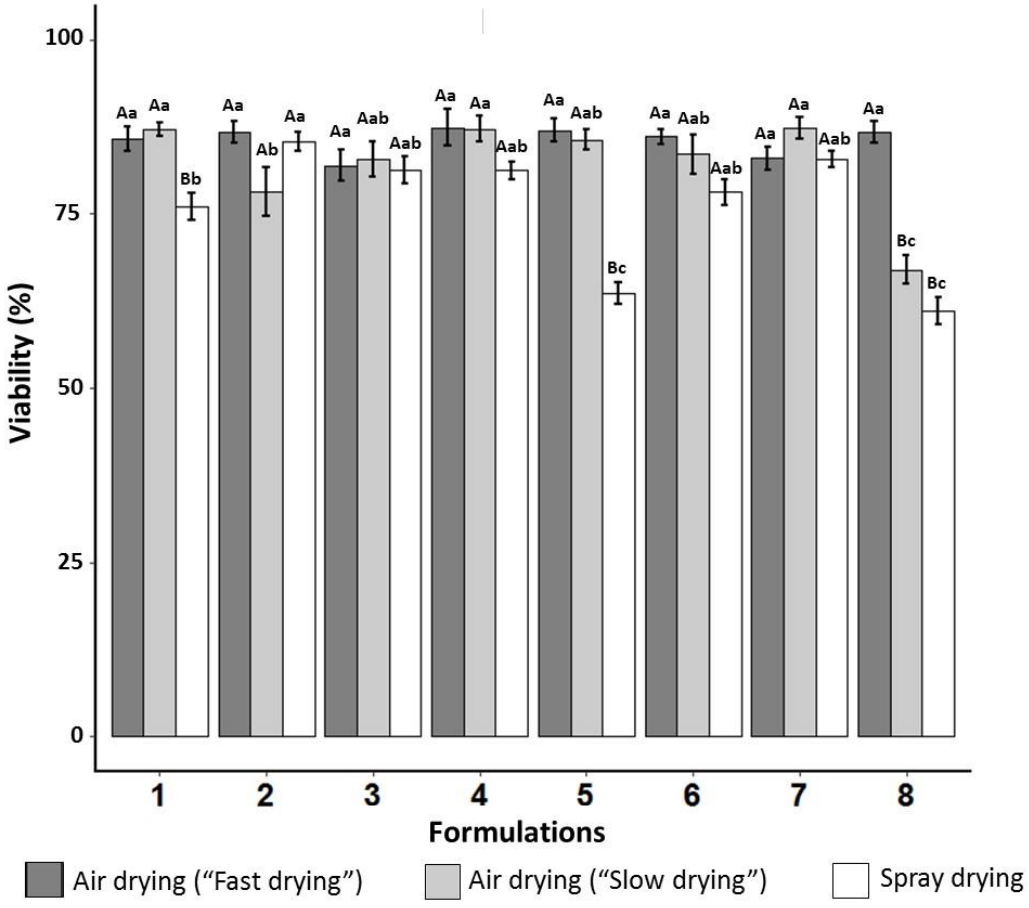

Figure 3. Viability (%) of 8 formulations with blastospores of the isolate ESALQ1296 of *C. fumosorosea* after the air dring (“fast” and “slow”) and the spray drying process. Means (± SE) followed by different uppercase letters denote the statistical difference between the formulations within each drying method, and means (± SE) followed by different lowercase letters denote the statistical difference between the drying methods within each formulation according to Tukey test ($P < 0.05$).

The co-formulants presented negative or positive effects on the blastospore viability for the fast air-drying method (F = 1.20, df = 7, 64, P = 0.3152; Multivariate equation to predict viability: Viability = - 0.22*UV Protector A – 0.04*UV Protector B – 0.74*Filler B + 0.98*Filler A + 0.11*Dispersant A – 1.38*Osmoprotector A + 0.15*Osmoprotector B). Among the seven factors studied, Osmoprotector A, Filler B, UV Protector A, and UV Protector B harmed the blastospore viability. In contrast, Filler A, Osmoprotector B, and Dispersant A positively affected (Figure 4a). Osmoprotector A (negative), Filler A (positive), and Filler B (negative) presented a higher magnitude of effect when compared with the other factors.

All co-formulants presented positive effects on the blastospore viability for the the slow air-drying method (F = 9.58, df = 7, 64, P < 0.0001; Multivariate equation to predict viability: Viability = 1.07*UV Protector A + 0.58*UV Protector B + 3.40*Filler B + 3.52*Filler A + 1.33*Dispersant A + 2.84*Osmoprotector A + 2.61*Osmoprotector B). Filler A, Filler B, Osmoprotector A and Osmoprotector B (Figure 4b) observed the highest magnitude of effects.

For the spray drying method, only the Filler A resulted in negative effect on the blastospore viability (F = 29.69, df = 7, 64, P < 0.0001; Multivariate equation to predict viability: Viability = 0.38*UV Protector A + 5.30*UV Protector B + 1.05*Filler B – 1.44*Filler A + 1.29*Dispersant A – 3.40*Osmoprotector A + 5.19*Osmoprotector B). Among the positive factors, UV Protector B, Osmoprotector B, and Osmoprotector A presented the highest magnitude of effect on the blastospore viability (Figure 4c).

Based on these results of the effect of each formulation composition (co-formulants) on viability rates, the UV Protector B, Filler B, Osmoprotector B, UV Protector A, and Filler A were selected for a new set of ingredients combinations to be tested with other compounds.

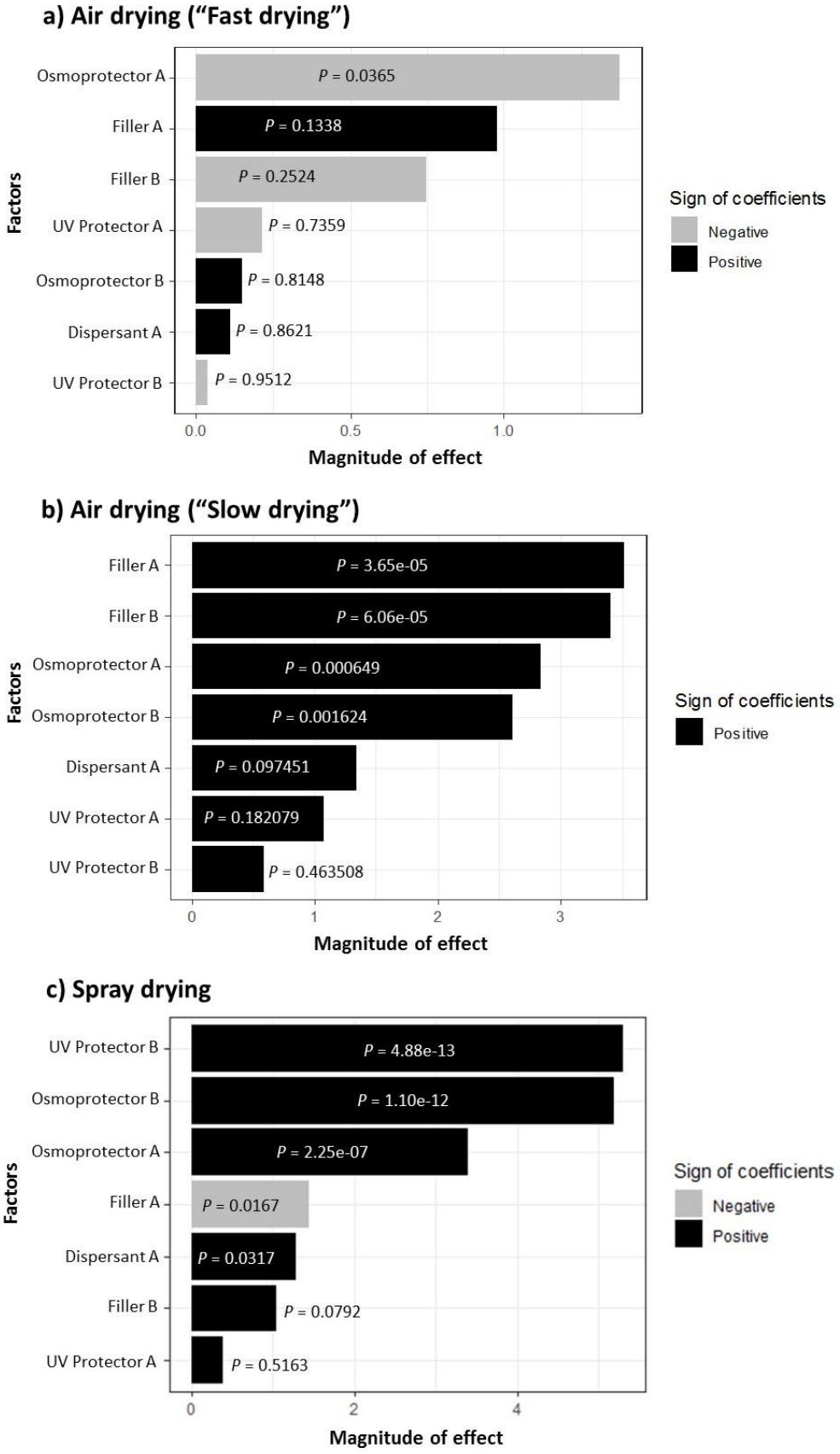

Figure 3. Pareto chart for Plackett Burman design for seven factors on blastospore viability in three different drying methods: a) fast Air drying, b) slow Air drying, and c) Spray drying.

3.3.3.2. Set of 12 formulations

When the 12 blastospore formulations of *C. fumosorosea* ESALQ1296 composed of 11 different co-formulants were tested, higher significant variations in the viability rates were obtained among the formulations and drying methods (F = 42.47, df = 22, 288, P < 0.0001) (Figure 4). Some formulations resulted in very low blastospore viabilities after spray-dried, e.g., F18, F16, and F9 resulted in 10.7%, 10.1%, and 10% viability, respectively. This shows that the spray drying method is more drastic to the cells, and the composition of the formulation influences the protection of the blastospores during the drying process. Using the fast air-drying a large number of formulations attained viability rates above 90%, among those are F20 (94.24%), F14 (94.10%), F19 (94.09%), F17 (93.51%), F12 (92.70%) and F13 (90.32%). Using this drying method, the worst viability rates were obtained by the formulations F15 and F9, with 68.9% and 14.2%, respectively. The slow air-drying method was also very efficient, although only the F19 formulation presented a viability rate above 90% (91%). Only three formulations attained viability rates below 70%, the F16 (76.2%), F15 (71%), and the worst of them, the F9 with 37.1%. The other formulations for this drying method resulted in viability rates around 87-89%. The great viability rates obtained with the spray drying method were F12, F20, F14, and F19 (86.6%, 86.5%, 86.1%, 83.5%, and 81.1%, respectively).

Only the formulations F12, F13, F14, F19, and F20 presents great viability rates in both drying methods (> 80%). On the other hand, the formulations F10, F11, F15, F16, F17, and F18 attained high blastopore viability after dried by air drying (“fast” and “slow”) and low values when they were dried by spray drying. Of all the formulations, only the F9 presented low viability rates in the three drying methods, not indicated for use in the drying process for blastospores.

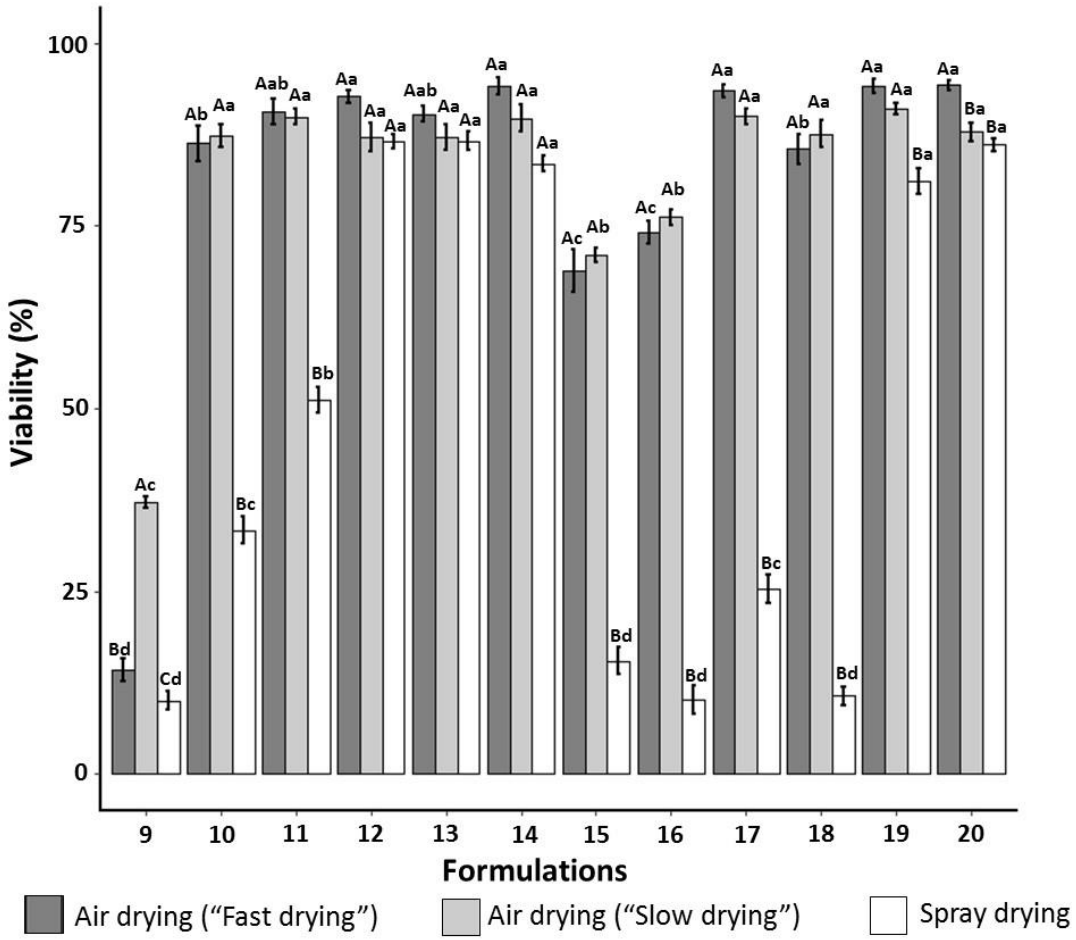

Figure 4. Viability (%) of 12 formulations with blastospores after the air drying ("fast" and "slow") and the spray drying process. Means (± SE) followed by different uppercase letters denote the statistical difference between the formulations within each drying method, and means (± SE) followed by different lowercase letters denote the statistical difference between the drying methods within each formulation according to Tukey test ($P < 0.05$).

According to the Pareto chart some co-formulants presented positive effect and others negative effect on blastospore viability for the fast air-drying method (F = 189.30, df = 11, 96, $P < 0.0001$; Multivariate equation to predict viability: Viability = -8.48*UV Protector A + 9.91*UV Protector B + 2.96*Filler B - 10.62*Osmoprotector B + 3.33*Osmoprotector C – 5.85*Dispersant A - 9.45*Filler A – 1.96*Filler C + 4.42*Filler D – 7.69*Antioxidant A – 8.76*Antioxidant B). The UV Protector B, Filler D, Osmoprotector C, and Filler B were the only positive effect factors. The UV Protector B was the one presenting the highest magnitude of effect (Figure 5a). Among all the factors with a negative effect, Filler C presented the lowest magnitude of effect, while the Osmoprotector B presented the highest value.

The co-formulants presenting positive effect by the slow air-drying method were the same as the fast air drying (UV Protector B, Filler D, Osmoprotector C, and Filler B) (F = 115.40, df = 11, 96, P < 0.0001; Multivariate equation to predict viability: Viability = - 3.92*UV Protector A + 8.15*UV Protector B + 2.79*Filler B – 7.36*Osmoprotector B + 2.49*Osmoprotector C – 4.20*Dispersant A – 6.64*Filler A – 0.29*Filler C + 2.58*Filler D – 3.74*Antioxidant A – 5.20*Antioxidant B). The UV Protector B presented the highest magnitude of positive effect, while the Osmoprotector B and Filler A showed the highest magnitude of negative effect (Figure 5b).

Beyond the UV Protector B, Osmoprotector C, and Filler B that also presented a positive effect in the air drying methods (“fast” and “slow”), the Osmoprotector B, Dispersant A, and the Filler C also presented positive effect in the spray drying method on the blastospore viability (Figure 5c) (F = 497.60, df = 11, 96, P < 0.0001; Multivariate equation to predict viability: Viability = -2.60*UV Protector A + 3.25*UV Protector B + 3.76*Filler B + 11.92*Osmoprotector B + 15.08*Osmoprotector C + 6.36*Dispersant A – 6.56*Filler A + 6.80*Filler C – 9.16*Filler D – 16.57*Antioxidant A – 9.25*Antioxidant B). The Osmoprotector C and the Osmoprotector B presented the highest magnitude of positive effect, while the Antioxidant A showed the highest magnitude of adverse effect.

Based on the results obtained for blastospores viability and the magnitude of each compound's effect in the screening of formulations (n=12), the formulations F12 and F20 were selected for the shelf life study. In general, the two formulations attained great viability rates in the three drying methods, and the majority of the compounds presented a positive effect on the blastospore viability.

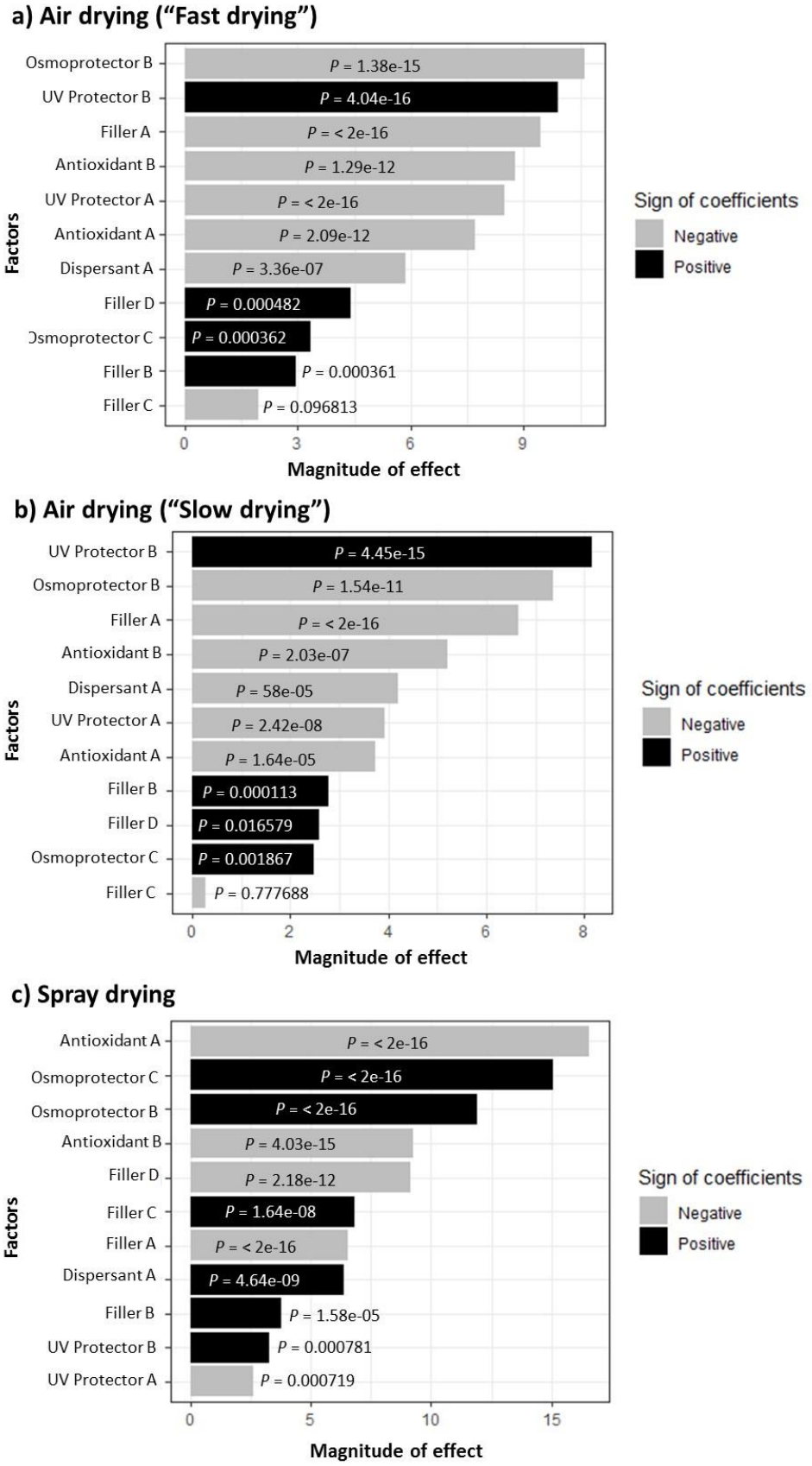

Figure 5. Pareto chart for Plackett Burman design for 11 factors on blastospore viability in three different drying methods: a) fast Air drying, b) slow Air drying, and c) Spray drying.

3.3.4. Shelf-life of formulated blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* dried by air drying and spray drying

B. bassiana ESALQ540 blastospores presented viability rates above 94% after drying by both formulations (F12 and F20) and drying methods (spray and air drying) (day 0) (Figure 6a). After 120 days of storage at 4°C, only the treatment F12 - Air drying and F20 - Spray drying presented viability rates above 70% (73.1% and 77.4%, respectively). After 180 days, all the treatments showed less than 15% of viable blastospores. At 28°C, after 30 days of storage, the treatment F12 – Spray drying and F12 - Air drying attained the highest viability rates, with 70.6% and 74.2%, respectively, and the treatment F20 – Spray drying showed a significant decrease in the viability rate (95.4% to 21%). After 60 days of storage, only the treatment F20 – Air drying presented more than 50% of viable blastospores (55.4%), and after 90 days, all the treatments showed viability rates below 30%.

The blastospores formulation F12 – Spray drying and F20 – Spray drying attained 87.8% and 90.1% viability for *C. fumosorosea* ESALQ1296 immediately after the drying process while the treatments F12 – Air drying and F20 – Air drying presented viability rates of 93.4% and 93.9%, respectively (Figure 6b). After 60 days of storage at 4°C, viability rates remained above 70 % for all treatments. Treatment F12 – Spray drying presented 70.6% viability after 120 days of storage. On the other hand, at the same time of storage, the other treatments showed a significant decrease in the viability rates. After 180 days, both the formulations presented less than 5% of viable blastospores. When the treatments were stored at 28 ºC, after 30 days, only the treatment F12 – Spray drying contained some viable blastospores (6.45%).

Blastospores of the isolate ESALQ4947 of *M. rileyi* were very sensitive to drying, especially to the spray drying method, resulting in low viability rates immediately after drying by the F12 – Spray drying and F20 – Spray drying treatments (43.2% and 38.5%, respectively (Figure 6c). The treatments F12 – Air drying and F20 – Air drying also showed low viability rates when compared to the results obtained from the other fungal species (74.9% and 67.2%, respectively). After 60 days of storage, only the treatments F12 and F20 – Air drying attained viability rates above 60%. After 120 days of storage, blastospores viability in these treatments were only 4.6% and 3.3%, respectively. At 28ºC, no blastospores remained viable after 30 days for any treatments.

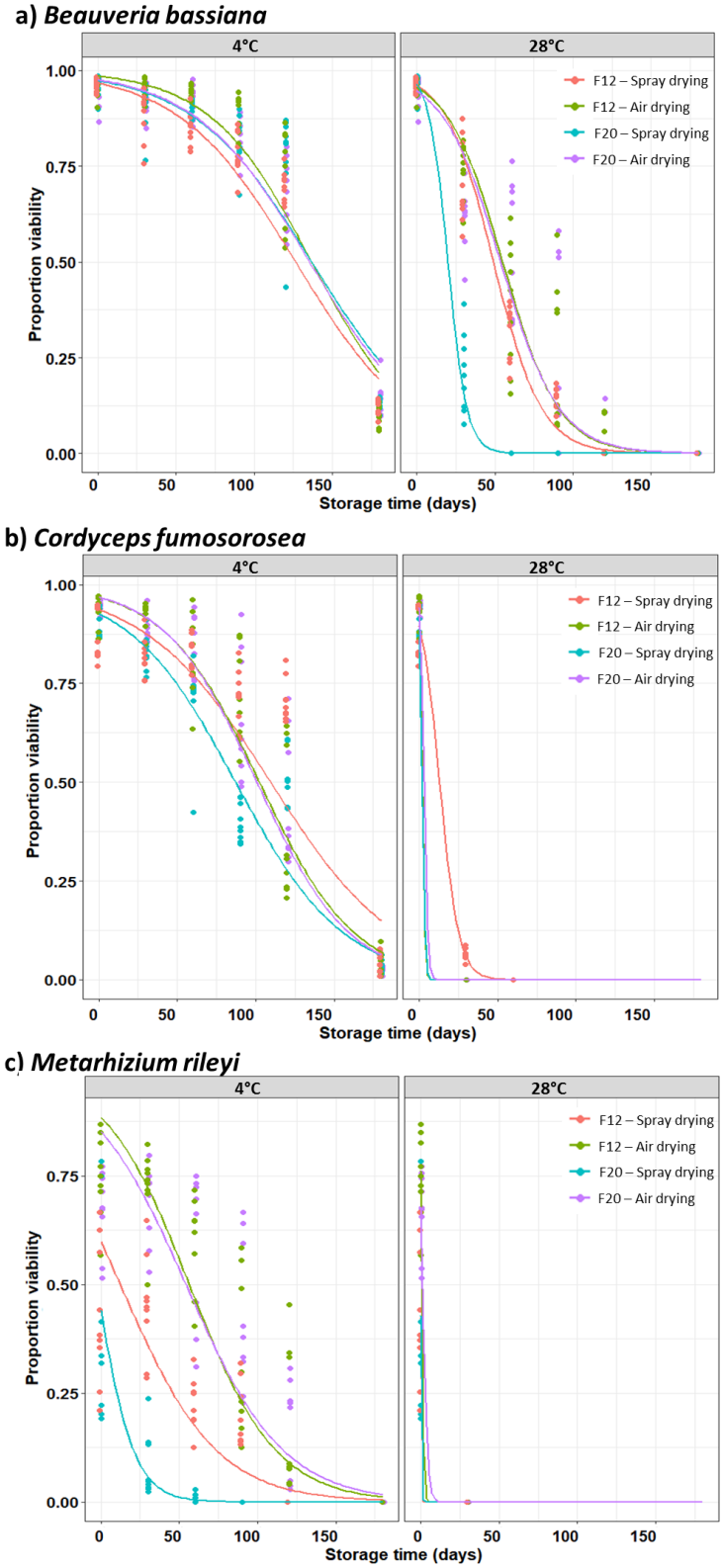

Figure 6. Proportion viability curves of blastospores of *Beauveria bassiana* (a), *Cordyceps fumosorosea* (b), and *Metarhizium rileyi* (c) formulated and dried by the *spray drying* e *air-drying* methods stored at 4°C and 28ºC for 180 days. F12 = UV Protector B, 4.0g; Filler B, 5.0g; Osmoprotector B, 4.0g; Osmoprotector C, 4.0g; Dispersant A, 2.5g; Filler A, 1.0g; Filler C, 4.0; Filler D, 1.0g; F20 = UV Protector A, 4.0g; Osmoprotector B, 4.0g; Osmoprotector C, 4.0g; Dispersant A, 5.0; Filler A, 1.0g; Filler C, 1.0g; Filler D, 4.0g.

3.4. Discussion

In this study, we aimed to develop formulations to protect blastospores during the drying process and ensure high viability during storage. First, we selected compatible fillers to be used with co-formulants. Besides increasing the volume of the formulation and avoid blastospores clustering, the filler also is an important agent to protect the microorganism against UV radiation, as it was observed with *Pantoea agglomerans* and *Trichoderma harzianum* immobilized in freeze-dried alginate beads containing Filler I as a filler (Zohar-Perez et al., 2003).

The searching for a filler with a low cost is necessary to make feasible the development of a biopesticide competitive in the market. The diatomaceous earth is the most used filler in studies involving the drying of blastospores; however, it has a high cost to be used on a large scale in Brazil. In this study, we also used some clays commonly used in the ceramic industry as potential fillers beyond diatomaceous earth. Of all of these, only the use of Filler F and Filler H resulted in lower blastopore viability, indicating the compatibility of the other inert materials at 7.5 or 30% w/v with the microorganism, presenting viability rates above 75%. This compatibility has already been observed for kaolin, talc, and corn starch mixed with blastospores of *C. fumosorosea* before the air drying process (Sandoval-Coronado et al., 2001). Among the fillers which ensure the highest blastospore viability rates, we have the Filler C, Filler E, and Filler D. The Filler C is a natural antioxidant. However, its use as a filler in formulations of entomopathogenic fungi has not been studied. When Filler E was used in the proportion of 30%, the time to dry the blastospores was longer than the other fillers; thereby, this filler was not selected for the composition of the formulations.

The majority of the studies involving the drying of blastospores used the air drying method last for at least 15 hours, referred to here as “slow” air drying and result in high values of relative humidity (Cliquet and Jackson, 1997; Jackson and Payne, 2007; Chong-Rodríguez et al., 2011). We demonstrated for the first time that it is possible to ensure high blastospore viability in an air-drying process with a duration of eight hours using a dry air current. Besides that, with the set of co-formulants used here, we also revealed the potential use of the spray drying for blastospores, ensuring high values of viable blastospores after the drying process. The use of this drying method for fungal cells such as conidia (Jin and Custis, 2011; Liu and Liu, 2009; Muñoz-Celaya et al., 2012) and blastospores (Mascarin et al., 2016) has been studied, showing a promising method, once it is a fast method compared to the air drying

method and allows the microencapsulation of the microorganisms. The advantage of cells' microencapsulation is the protection against mechanic and environmental stress (Rathore et al., 2013). In this way, we showed that it is possible to use different drying methods for the drying of blastospores of *C. fumosorosea*, once we attaine with viability rates above 80%.

This study used two sets of different complex blastospores formulations composed by fillers, osmoprotectants, dispersants, and UV protectors in the air and spray drying process. This diversification of components is critical because the blastospore stability, desiccation tolerance, and suspension stability can be enhanced by specific formulation components (Jackson et al., 2006). When we compared the different formulations within each drying method, we saw that the formulation composition is critical to ensure high blastospore viability in the spray drying process, which is less crucial for the air-drying process in the “slow” or “fast” manner. For the first set of formulations, the use of Osmoprotector B or Osmoprotector A was determinant for the viability of the blastospores dried by spray drying. In the absence of one of these two compounds, the viability rate of blastopores was lower. We hypothesized that the Osmoprotector B and Osmoprotector A are probably protecting the cels during the dehydration process by replacing the water, maintaining membrane integrity and protein conformation as demonstrated by other similar compounds.

On the other hand, from the results obtained for the second set of formulations, the absence of Osmoprotector C and the presence of Antioxidant A and Antioxidant B were harmful to the blastospore. The Osmoprotector C is used as a carrier material in several studies involving the use of spray drying due to its decreased tendency to bind and penetrate the cell membrane. This feature ensures the protection of the microbial cells during the dehydration process, maintaining the blastospores' viability. The use of Antioxidant A and Antioxidant B together presented some toxicity to the blastospores in this study.

Besides evaluating the blastospore viability right after the drying process, it is essential to verify the cells' stability in a formulation. In this study, we assessed the shelf life of the blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* using two formulations dried by “fast” air drying and spray drying stored at 4 and 28°C. At the storage temperature of 4°C, surprisingly, the stability of blastospores of *B. bassiana* was superior in the formulations dried by spray drying. These results indicate that storing *B. bassiana* blastospores in vacuum-packaged for up to 120 days, with 75%

viability, is possible. On the other hand, the blastospores of this fungal species did not survive as long at 28°C. In this way, the cold storage temperature is required for blastospore persistence, independently of the formulation or drying method. This temperature sensibility presented by submerged cells produced by *B. bassiana* was also observed in other studies, either with blastospores (Mascarin et al., 2016) or submerged conidia (Immediato et al., 2017). The effect of temperature was higher for blastospores of *C. fumosorosea* and *M. rileyi*; all blastospores of these fungi died after 30 days of storage at room temperature. Blastospores of *M. rileyi* did not present desiccation tolerance, mostly when a spray dryer dried it. This indicates that the formulations were not sufficient for this fungal species to protect the blastospores during drying. The fast-drying either by air or spray drying used and the high temperature in the spray dryer may be responsible for the reduced blastospore viability.

Low water content, low temperature, and reduced oxygen levels are required in the package system to increase the shelf life of a fungal biopesticide (Mascarin and Jaronski, 2016). Here, we developed promising drying methods (formulation + drying process) that can be used in a future biopesticide based in blastospores. However, there is still a need to increase the shelf life of blastospores of these fungi, especially in ambient temperatures. The use of modified atmosphere packaging systems or packaging less permeable to humidity can improve shelf life. This is the first attempt to use complex formulations for the drying of blastospores of *B. bassiana*, *C. fumosorosea,* and *M. rileyi* either by air drying or by spray drying, revealing the possibility to develop blastospores based biopesticide for these fungal species. Moreover, this is the first study reporting the low desiccation tolerance of *M. rileyi* blastospores, indicating that there is still a great challenge to use this species of fungus in Brazilian agriculture.

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