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

Advances in *Metarhizium* blastospores production and formulation and transcriptome studies of the yeast and filamentous growth

Natasha Sant´Anna Iwanicki

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

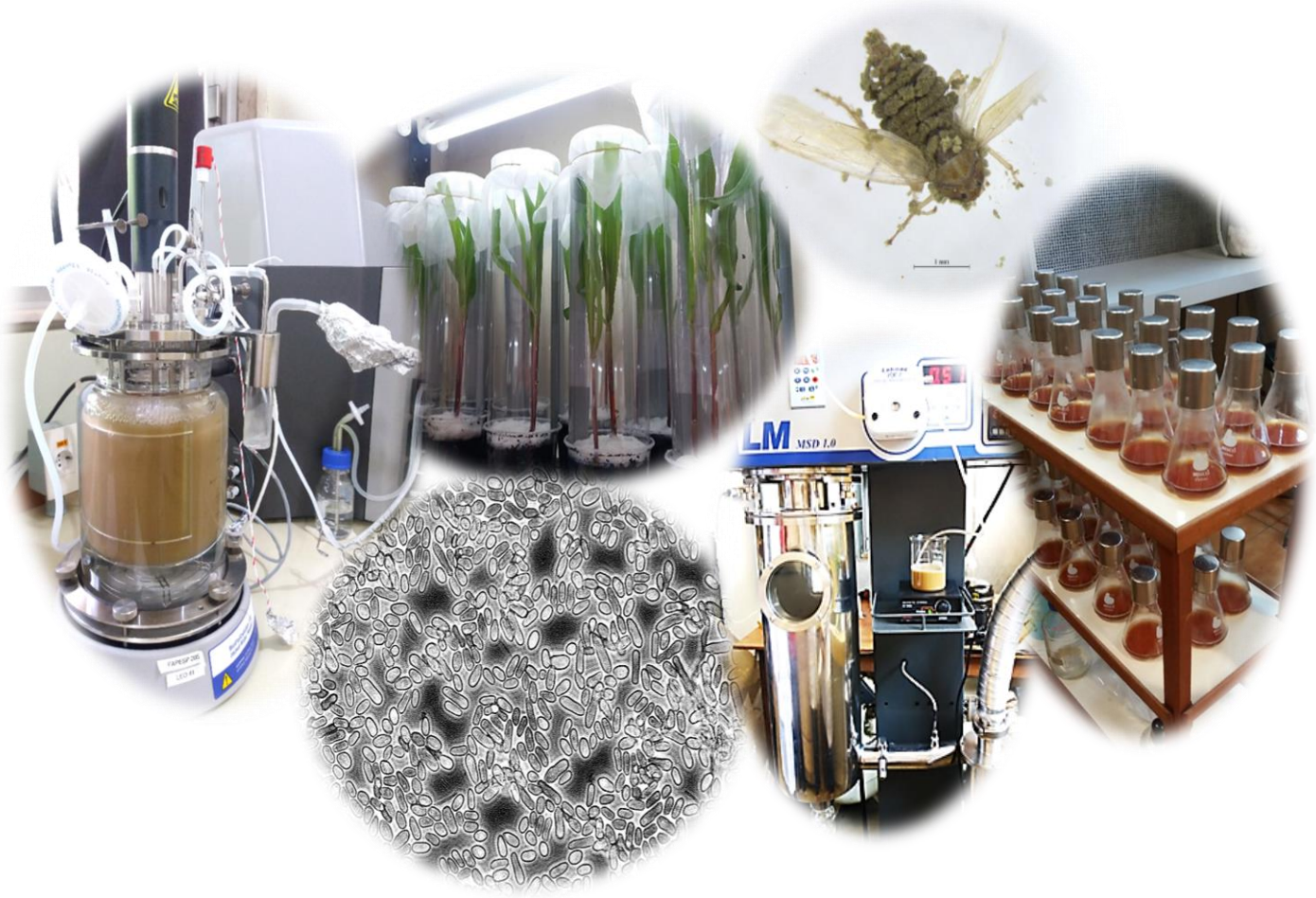
Piracicaba
2020

UNIVERSITY OF COPENHAGEN

FACULTY OF SCIENCE

Advances in *Metarhizium* blastospores production and formulation and transcriptome studies of the yeast and filamentous growth

PhD THESIS 2020 – Natasha Sant´Anna Iwanicki



Natasha Sant´Anna Iwanicki
Agronomic Engineer

Advances in *Metarhizium* blastospores production and formulation and transcriptome studies of the yeast and filamentous growth

Advisors:
Prof. Dr. **ITALO DELALIBERA JUNIOR**
Prof. PhD and Dr. agro **JØRGEN EILENBERG**

Thesis presented to obtain the double-degreee of Doctor in Science of the University of São Paulo and PhD at University of Copenhagen. Area: Entomology

Piracicaba
2020

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Iwanicki, Natasha Sant´Anna

Advances in *Metarhizium* blastospores production and formulation and transcriptome studies of the yeast and filamentous growth / Natasha Sant´Anna Iwanicki. - - Piracicaba, 2020.

248 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Blastosporos 2. Fermentação líquida 3. Dimorfismo fúngico 4. Fungos entomopatogênicos I. Título

USP
ESALQ

UNIVERSITY OF
COPENHAGEN

50 ANOS PPG ENTOMOLOGIA
Programa de Pós-Graduação
em Entomologia
USP/ESALQ

Laboratório de Patologia e
Controle Microbiano de Insetos

ACKNOWLEDGMENTS

First, I would like to thank my supervisors, Prof. Italo Delalibera Júnior and Prof. Jørgen Eilenberg for their confidence in my potential as a student, for the opportunities they gave me and the knowledge they shared, for their guidance and friendship over these years.

I also thank my co-advisors, Prof. Henrik H. De Fine Licht and Dr. Gabriel Moura Mascarin for their friendship, guidance, help in data analysis, in writing the articles, patience and motivation during my doctorate. I am grateful to have had the opportunity to work with them.

I would like to extend a special thanks to Dr. Gabriel Mascarin. Gabriel became a person very close to me during my doctoral journey. His enthusiasm, positivism, determination, professionalism, unconditional support and motivation helped me a lot in times of discouragement and changed the way I faced the challenges in academia. Thank you very much Gabriel.

To the endless support, patience understanding and collaboration of my parents, Ivania Sant´Anna and Jacek Lech Iwanicki, my wife Ana Luisa Martins Attie and my siblings Lara Sant´Anna Iwanicki and André Sant´Anna Iwanicki during these years.

To my aunt and uncle, Danuta and Prof. Edward Potworowski (Edek) for providing me with the opportunity to participate in the Workshop on Scientific Communication in Poland and to the family support during these years. I also thanks to my Polish family for hosting me during the Workshop in Poland.

A special thanks to my uncle Edek for teaching me scientific communication; for helping me to improve my writing of the scientific articles presented in this thesis and for the enormous support in the final moment of writing the thesis.

I thank to the team of the Laboratory of Pathology and Microbial Control of Insects - ESALQ / USP for all the support and moments shared.

A special thank for Bruna Oliveira and Sara Giro Moreno for all help, moments shared and friendship. You were my left arm during my research time in Brazil and I thank you very much.

I thank the technical assistance of Marcela Paduan for helping during RNA extraction protocols and sequencing, to the spray dryer team: Fernanda Ramos, always very helpful and Bianca Correa; to Anderson Silva for providing insects for bioassays; to Larissa Bueno for the friendship and support with oily formulation experiments;

To the technician Lenita Maria and Professor Marcos Yassuo Kamogawa for the help in the extraction and quantification of nitrogen in liquid culture media.

I thank the University of São Paulo (ESALQ) and University of Copenhagen (SOBI) for the academic structure and support offered so that I could carry out my research.

To all the professors from the department of Entomology and Acarology ESALQ / USP for the sharing their knowledge during all these years.

To the funding institutions: FAPESP and CAPES for the scholarship that sponsored my Ph.D research.

This work was supported by FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo)[Grant: 2017/048700 and 2016/206106], CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and by Projeto Universal CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Grant: 421629/2016-9

“Nie ten mocny, kto nie upada, ale kto ma siły sie podniesc”

Not the strong who does not fall, but who has the strength to rise

Não é o mais forte aquele que não cai, mas sim aquele que tem forças para se levantar

Sentence written in the 600 years history book of the Iwanicki´s family.

SUMMARY

RESUMO

Avanços na produção e formulação de blastosporos de *Metarhizium* e estudos do transcriptoma da fase de crescimento leveduriforme e filamentosa

O controle biológico de pragas é um mercado crescente no mundo. Espera-se que o uso de fungos entomopatogênicos no controle de pragas ocupe uma parcela importante desse mercado nos próximos anos. A maioria dos produtos à base de fungos no mundo é composta por conídios aéreos produzidos por fermentação sólida com grãos de cereais. Uma alternativa aos conídios aéreos é o uso de blastosporos, células hidrofílicas semelhantes a leveduras. Estas células podem ser produzidas em grandes quantidades por fermentação líquida em curto tempo (<4 dias), em um espaço pequeno e com pouco trabalho manual em comparação com o método de fermentação sólida. Portanto, os principais objetivos dos presente estudo foram: **primeiro:** otimizar um meio de cultura líquido, de baixo custo, para a produção de blastoporos de *Metarhizium*; **segundo**: avaliar a bioatividade dos blastosporos secos por secagem lenta contra o carrapato-do-boi *Rhipicephalus microplus*; **terceiro**: desenvolver uma formulação pó-molhável de blastosporos de *Metarhizium* com bioatividade contra a cigarrinha do milho *Dalbulus maidis*; **quarto**: buscar o incremento do tempo de prateleira das melhores formulações secadas pelos métodos de secagem lenta e spray-dryer, com adição de absorventes de oxigênio e umidade nas embalagens ou a vácuo e armazenadas em geladeira (± 4 °C) e em condições de ambiente (28 °C); **quinto**: determinar *in vitro* as alterações na expressão gênica entre as fases de crescimento filamentoso e leveduriforme (blastosporo) que possam estar associadas às principais alterações fisiológicas e metabolismos do dimorfismo de *M. anisopliae* e *M. rileyi*. Mostramos que a produção de blastosporos de *Metarhizium* varia entre isolados e espécies. As culturas enriquecidas com glicose e inoculadas com pré-culturas melhoraram a produção de *Metarhizium robertsii* ESALQ1426 ($5{,}9 \times 10^8$ blastospores / mL) em apenas 2 d. Os blastosporos de ESALQ1426 obtidos por secagem lenta mataram larvas de *R. microplus* com uma eficiência comparável a conídios aéreos. Tanto a pressão osmótica, induzida por altos teores de glicose, quanto a seleção de isolados se mostraram ser fatores críticos para produção de altos rendimentos de blastosporos de *Metarhizium*, que por sua vez, são infectivos a larvas de *R. microplus*. Os experimentos de cinética de crescimento de blastosporos e otimização da fonte de nitrogênio resultaram na definição de um meio de baixo custo usando milhocina (80 g/L) como fonte de nitrogênio atingindo rendimentos de $4{,}7 \times 10^8$ blastosporos/mL para ESALQ1426 em apenas 2 dias de cultivo a um custo de apenas US$ 0,30/L. Os blastosporos obtidos por secagem lenta foram tão virulentos quanto blastosporos frescos para adultos de *D. maidis* mantidos em plantas de milho. Co-formulantes foram selecionados para compor formulações que preservassem a viabilidade dos blastosporos de ESALQ1426 durante o processo de secagem. As formulações de blastosporos secos por secagem lenta e spray-dryer foram tão virulentas quanto os blastosporos frescos para a cigarrinha do milho, induzindo taxas de mortalidade variando de 60,3 a 78,2% após a pulverização com 5×10^7 blastosporos/mL. O LC_{50} foi significativamente maior para a formulação obtida por secagem lenta ($2{,}42 \times 10^7$) do que para a formulação obtida em spray-dryer ($4{,}65 \times 10^6$), sugerindo um possível efeito prejudicial da tecnologia anterior na virulência dos fungos. O transcriptoma de *M. anisopliae* revelou uma clara distinção na expressão gênica entre as fases leveduriforme e micelial. Os principais processos fisiológicos regulados nos

blastosporos durante a fermentação líquida foram estresse oxidativo, metabolismo de aminoácidos, processos respiratórios, transporte transmembranar e produção de metabólitos secundários. Por outro lado, os principais processos fisiológicos regulados nas hifas estavam associados ao crescimento e a reorganização da parede celular. Esses resultados destacam as principais funções metabólicas relacionadas a morfologia de crescimento dos blastosporos e hifas de *M. anisopliae*, respectivamente. Por outro lado, observou-se que células leveduriformes (blastosporos) de *M. rileyi* produzidas em meio líquido ativaram uma série de genes específicos relacionados à transdução de sinal e transportadores de membrana relacionados à aquisição de ferro, o que não foi observado nas hifas. O estresse oxidativo e a ativação de proteínas específicas “heat shock protein” foram fatores-chave envolvidos na formação de células leveduriformes. Por outro lado, a fase leveduriforme cultivada em meio sólido ativa um conjunto de genes únicos, não encontrados em outras espécies de *Metarhizium*, como proteínas específicas da membrana e vários fatores de virulência. Esses estudos demonstraram claras diferenças entre o metabolismo dos blastosporos e das hifas. Esses achados ilustram aspectos importantes da morfogênese em *M. anisopliae* e destacam as principais atividades metabólicas de cada propágulo sob condições de crescimento *in vitro*. Os estudos de transcriptoma estabeleceram as bases para a compreensão do metabolismo necessário para o crescimento de blastosporos em meio líquido e identificaram genes candidatos que servirão de base para futuras pesquisas sobre a otimização da produção de blastosporos de *M. rileyi*.

Palavras-chave: Blastosporos, Fermentação líquida, Dimorfismo fúngico, Fungos entomopatogênicos

ABSTRACT

Advances in *Metarhizium* blastospores production and formulation and transcriptome studies of the yeast and filamentous growth

Biological control of pests is a growing market in the world. It is expected that the use entomopathogenic fungi to control pests will take an important share of this market. Most fungal products in the world are based on aerial conidia produced by solid fermentation using cereal grains. An alternative for aerial conidia is the use of blastospores, yeast-like hydrophilic cells that can be produced in large amounts by liquid fermentation in a short time (<4 days), in a small space and with low hand labor compared to the solid fermentation method. Therefore, the main objectives of the present studies were **first** to optimize a liquid culture medium for low cost production of *Metarhizium* blastopores; **second**: to assess the bioactivity of air-dried blastospores against the cattle-tick *Rhipicephalus microplus*; **third**: to develop an air-dried and spray-dried *Metarhizium* blastospore formulation with bioactivity against the corn-leafhopper *Dalbulus maidis*; **fourth**: to improve the shelf-life of the best air-dried and spray-dried formulations stored in refrigerated (± 4°C) and in ambient conditions (28°C) using oxygen and moistures absorbrs or vacuum and **fifth**: to use comparative genome-wide transcriptome analyses to determine changes in gene expression between the filamentous and blastospore growth phases *in vitro* to characterize physiological changes and metabolic signatures associated with *M. anisopliae* and *M. rileyi* dimorphism. We showed that blastospore production of *Metarhizium* is isolate- and species-dependent. Glucose-enriched cultures inoculated with pre-cultures improved yields reaching optimal growth for *Metarhizium robertsii* ESALQ1426 (5.9×10^8 blastospores/mL) within 2 d. Resultant air-dried blastospores of ESALQ1426 were proved to quickly kill *R. micropulus* larvae with an efficiency comparable to that of conidia. We argue that both osmotic pressure, induced by high glucose titers, and isolate selection are critical to produce high yields of blastospores that hold promise to control *R. microplus* larvae. Fermentation experiments based on growth kinetics defined a low-cost medium using 80 g/L corn steep liquor as the most suitable nitrogen source for inducing blastospore growth in *M. robertsii* (4.7×10^8 cells/mL) in only 2 days of cultivation at a total cost of $0.30 USD per L. The dried blastospores were as high virulence as fresh cells to *D. maidis* adults fed on maize plants. Co-formulants were selected to compose formulations that allowed to keep *M. roberstii* blastospore viability after drying. The most promising resulting spray-dried and air-dried were as infective as fresh blastopores to the corn leafhopper inducing mortality rates ranging from 60.3 to 78.2% after spraying with 5×10^7 blastospores/mL. The LC_{50} was significantly higher for spray-dried formulation (2.42×10^7) than for the air-dried formulation (4.65×10^6), suggesting a possible detrimental effect of the former technology in fungal virulence. Comparative genome-wide transcriptome of *M. anisopliae* showed a clear molecular distinction between the blastospore and mycelial phases. The main physiological processes associated with up-regulated gene content in blastospores during liquid fermentation were oxidative stress, amino acid metabolism, respiration processes, transmembrane transport and production of secondary metabolites. In contrast, the up-regulated gene content in hyphae was associated with increased growth metabolism and cell wall re-organization, which underlines the specific functions and altered growth morphology of *M. anisopliae* blastospores and hyphae, respectively. Conversely, it was

observed that the *M. rileyi* yeast-like cells produced in liquid medium activated a series of specific genes related to signal transduction, and specific membrane transporters related to iron acquisition which was not observed in hyphae. Oxidative stress and activation of specific heat shock proteins were key factors involved in formation of yeast-like cells. On the other hand the yeast-like phase grown in solid medium activates a set of unique genes, not found in other *Metarhizium* spp., specific membrane proteins and several virulence factors. Significant transcriptomic differences between the metabolism of blastospores and of hyphae were demonstrated. These findings illustrate important aspects of fungal morphogenesis in *M. anisopliae* and highlight the main metabolic activities of each propagule under *in vitro* growth conditions. The genomic studies laid the foundation for understanding the main metabolism required for blastospores growth in liquid medium and identified candidate genes that will serve as a basis for future research on optimizing *M. rileyi* blastospore production.

Keywords: Blastospores, Liquid culture fermentation, Fungal dimorphism Entomopathogenic fungi

SAMMENDRAG

Biologisk bekæmpelse udgør et voksende marked og det kan forventes, at insektpatogene svampe til bekæmpelse af skadedyr vil udgøre en væsentlig del af dette marked. De fleste svampeprodukter i verden er baseret på luftbårne konidier produceret ved dyrkning på faste substrater (”solid fermentation”) såsom korn. Et alternativ til luftbårne konidier er blastosporer, som er gærcellelignende hydrofile celler, som kan produceres i store mængder på kort tid (> 4 dage) ved dyrkning i flydende substrater (”liquid fermentation”). Dette kan ske på mindre plads og med mindre manuel indsats sammenlignet med dyrkning på faste substrater.Derved var formålet for mine studier følgende: 1) Optimering af det flydende vækstmedium til at opnå omkostningseffektiv produktion af *Metarhizium* blastosporer, 2) Bestemmelse af den biologiske aktivitet af lufttørrede blastosporer overfor flåter på kvæg, *Rhipicephalus microplus;* 3) Udvikling af en formulering af lufttørrede og sprøjtetørrede *Metarhizium* blastosporer med aktivitet overfor cikader, *Dalbulus maidis,* på majs; 4) Forbedring af holdbarhed af udvalgte lufttørrede og sprøjtetørrede formuleringer, opbevaret køligt (± 4°C) eller ved skiftende betingelser (28°C), ved brug af ilt og absorption af fugtighed eller ved brug af vakuum; 5) Implementering af komparative transkriptomanalyser til sammenligning af total gen ekspression for to vækststadier *in vitro*: hyfer og blastosporer. Desuden karakterisering af de fysiologiske ændringer og metaboliske processer associeret med *M. anisopliae* og *M. riley* dimorfisme. Vi viste, at blastosporeproduktion af *Metarhizium* afhænger af svampeart og svampeisolat. Kulturer beriget med glukose og inokuleret med forkulturer forbedrede væksten som for *M. robertsii* ESALQ1426 i løbet af to dage nåede op på 5.9×10^8 blastosporer/mL. De opnåede lufttørrede ESALQ1426 blastosporer dræbte *R. micropulus* larver lige så effektivt som konidier. Vi mener derfor, at både osmotisk tryk, som induceres af øget glukose koncentration, samt udvælgelse af isolat, er essentielle elementer til at opnå høje udbytter af blastosporer med potentiale til at bekæmpe *R. micropulus* larver. Fermenteringsforsøg og målinger af vækstkinetik gav anledning til at definere et egnet vækstmedium (til lav pris på $0.30 USD pr liter) baseret på 80 g *corn steep liquor* pr liter. Denne kvælstofkilde inducerede blastosporedannelse af *M. robertsii* (4.7×10^8 celler/ml). Virulensen af tørrede blastosporer var lige så høj som friske over for *D. maidis* på majsplanter.Følgestoffer blev udvalgt med henblik på at sammensætte formuleringer, som øgede holdbarheden af *M. robertsii* efter tørring. De mest lovende resultater viste, at sprøjtetørrede eller lufttørrede blastosporer var lige så effektive til at inficere cikaderne som friske blastosporer, med mortaliteter mellem 60.3 og 78.2% efter sprøjtning med 5×10^7 blastosporer pr ml. LC_{50} var signifikant højere for sprøjtetørrede formuleringer, nemlig (2.42×10^7 sporer pr ml) end for lufttørrede formuleringer (4.65×10^6 sporer pr ml), hvilket kan skyldes en dæmpende effekt på virulensen af sprøjtetørring.

Komparative transkriptomanalyser af *M. anisopliae* viste en klar molekylær forskel mellem blastosporer og mycelium. De vigtigste fysiologiske processer relateret til opregulering af genekspression i blastosporer var iltstress, aminosyremetabolisme, respiration, transmembran transport samt produktion af sekundære metabolitter. For hyfer (mycelium) var opregulering af genekspression forbundet med øget vækstmetabolisme og reorganisering af cellevæggene. Dette understreger de specifikke funktioner og ændrede vækstmorfologi af *M. anisopliae* blastosporer og hyfer. Vi observerede, at gærcellelignende celler af *M. rileyi,* som var produceret i flydende medium, aktiverede en række specifikke gener relateret til signal transduktion samt specifikke membrantransportører relateret til jernakkumulering, hvilket ikke blev observeret i hyfer. Iltstress og aktivering af ”heat shock proteins” var nøglefaktorer involveret i dannelsen af gærcellelignende enheder. Samme fase, men dyrket på faste substrater, aktiverede en række unikke gener, som ikke er fundet i andre *Metarhizium* spp., nemlig specifikke membranproteiner og virulensfaktorer. De fundne transkriptomforskelle mellem metabolismen hos blastosporer og hyfer illustrerer væsentlige aspekter af morfogenesen af *M. anisopliae* og sætter fokus på de vigtigste metaboliske aktiviteter for hver enhed ved *in vitro* vækst. Disse transkriptomstudier har lagt grunden til en forståelse af den centrale metabolisme, som er koblet til blastosporer i flydende medium og vi har dermed identificeret kandidatgener, som kan danne baggrund for fremtidige undersøgelser til optimering af *M. rileyi* blastosporeproduktion.

.

Nøgleord: Blastospores, Flydende substrater, Svampedimorfisme, Insektpatogene svampe

1. INTRODUCTION

1.1. The biopesticide Market

The agricultural sector is an important component of the Gross Domestic Product (GDP) in many countries, especially those with large area. In Brazil, agribusiness represents 23% of Brazil's GDP (CNA, 2018). However, one of the main bottlenecks for increased productivity in the agricultural sector is the incidence of pests and diseases. The inefficiency in controlling pest is often related to the improper use of chemical pesticides, emergence of new pests and partial or complete lack of integrated pest control (IPM). Biological control in an obvious option (Van Lenteren., 2012; Steinberg, 2017).

Biological control is defined by Eilenberg et al. (2001) as: "*'The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be*". The growth of organic production as well as the search for control alternatives by producers adopting traditional pest control techniques have resulted in an increased use of biological control agents (macro and microorganism). Although the use of biological control currently represents only around 2% of the total pesticide market, its growth rate is estimated at between 20 and 25% in Brazil (ABCBio, 2019) and 16% worldwide (DunhamTrimmer's, 2019) in the next several years. Among factors that drive the advance of biological control is international pressure for pesticide-free products in exported foods and the inexorable problem of the evolution of chemical pesticide resistance in pest populations (Van Lenteren, 2012). In this context, microbial insect control (often termed "biopesticides") represents one efficient and safe alternative to chemical pesticide use. Entomopathogenic fungi are promising candidates, due to 1) the ease of relatively inexpensive production of infectious units on a commercial scale, 2) the existence of formulations that allow the application in the field through the use of the same equipment as is used for the application of chemical pesticides, 3) low environmental impact (Alves et al., 1998, Mascarin et al., 2018).

In Brazil, the biopesticide market has been growing significantly in recent years. Between 2011 and 2016, 50 biopesticide products were registered compared with 41 products over the previous 25 years (R.P. Dias, Ministry of Agriculture, Livestock and

Food Supply of Brazil, personal communication in Mascarin et al., 2018). Approximately 50% of the registered microbial biopesticides comprise fungi based on aerial conidia of the species *Metarhizium anisopliae* s.s and *Beauveria bassiana* (Mascarin et al., 2018).

However, it is known that there are many unregistered biopesticides in Brazil, most of them based on low tech production, many products are unformulated and/or of poor quality, some are commercialized while others are produced *on farm* for local use. This practice has raised concerns in society and in the scientific community as the efficiency of these products and their safety for the environment and to humans raises concerns. Furthermore, some of these products provide inconsistent and highly variable results in the field depending on the region and time in which they are applied.

Brazil is considered one of the largest reservoirs of natural biodiversity; therefore, the potential for rational exploitation of microorganisms for biological control purposes is invaluable, opening up major basic and applied research opportunities, which can create jobs in industry and in agriculture. The large amounts of fungal infective propagules needed require an economically viable production method to provide stable, infectious fungal propagules (Mascarin et al., 2018; Jackson et al., 2010).

1.2. The genus *Metarhizium*

The genus *Metarhizium* (Hypocreales: Clavicipitaceae) represents an important group of entomopathogenic fungi from Ascomycota (Zimmermann, 2007; Lenteren et al., 2017), with approximately 31 recognized species (Brunner-Mendoza et al., 2018; Luz et al., 2019). This genus has a worldwide distribution and the different species posess a versatile lifestyle allowing them to 1) inhabit the soil (Rocha et al., 2013; Kepler et al., 2015; Rezende et al., 2015; Iwanicki et al., 2019, Botelho et al., 2019), 2) infect insects and mites (Vega et al., 2009; Zimmermann, 2007). 3) live in association with roots or as plant endophytes (Wyrebek et al., 2011; Sasan and Bidochka, 2013; Liao et al., 2014; Steinwender et al., 2015; Canassa et al., 2019a, 2019b); 4) and act as antagonist of plant pathogens (Sasan and Bidochka, 2012).

The highest diversity of *Metarhizium* spp. is found in soil (Keyser et al., 2015; Iwanicki et al., 2018; Botelho et al., 2019) where it can persist for a long time even

when insects and crops are not present (Klingen and Haukeland, 2006). *M. robertsii* is the most abundant species found in Brazilian soils (Iwanicki et al., 2019 ; Rezende et al., 2015; Lopes et al., 2013a) while isolates from *M. anisopliae* Mani 2 subclade are the only ones found naturally causing disease in spittlebugs (Iwanicki et al., 2019; Rezende et al., 2015) ; specifically in Brazil, only that species is found in all available biological control products with *Metarhizium* (AGROFIT, 2019). It is estimated that two million hectares are treated annually with *M. anisopliae* to control spittlebugs (Parra, 2014). In addition to *M. robertsii* and *M. anisopliae,* several other species have been reported to occur naturally in Brazil: *M. pingshaense* (Botelho et al., 2019; Lopes et al., 2014)*, M. acridum, M. lepidiotae, M. majus* (Rezende et al., 2015; Lopes et al., 2014; 2013a; 2013b; Rocha et al., 2013; Driver et al., 2000) *M. brunneum* (Iwanicki et al., 2018; Lopes et al., 2014); *M. blattodeae* (Montalva et al., 2016), *M. pemphigi* (Rocha et al., 2013), *M. brasiliense*, *M. rileyi* (Kepler et al., 2014 ; Sujii et al., 2000) , *M. alvesii* (Lopes et al., 2017) and more recently, the species previously known as *Metarhizium* sp. indet. 1 (Iwanicki et al., 2018; Rocha et al., 2013; Rezende et al., 2015) now described as a new species, *M. humberii* . It is a sister lineage of *M anisopliae* (Luz et al., 2019).

The composition of the *Metarhizium* species can be quite diverse worldwide: in brazilian soils, *M. robertsii* is the most common species, *M. pingshaense* and *M. anisopliae* seem to be the most common species in Australian and Asian soils (Rehner and Kepler, 2017 ; Niu et al., 2019) while *M. brunneum* and *M. flavoviridae* are the most common in Danish soils (Steinwender et al., 2011; Steinwender et al., 2015; Kayser et al., 2015).

Metarhizium spp are dimorphic fungi, growing either in filamentous phase or in yeast-like phase during their infection cycle (Pedland and Boucias, 1997; Pedrini et al., 2017). This cycle starts with aerial conidia being attached to a susceptible host cuticle through hydrophobic and electrostatic mechanisms. Under conditions of high humidity, conidia germinate, form appressoria and by a combination of mechanical and enzymatic forces they overcome cuticular layers and reach the hemolymph, which is a liquid and nutrient rich environment inside the insects' body cavity. When present in the hemolymph *Metarhizium* spp. switches from invasive filamentous growth to yeast-like growth, called blastospores or hyphal bodies (Pedland and Boucias, 1997; Qu and Wang ,2018; Pedrini, 2017). These single cells can avoid

being detected by the host immune system, as they have fewer epitopes compared to conidia and hyphae (Wanchoo et al. 2009), and quickly multiply by septation or budding while competing for nutrients and invade the entire insects (Pedrini et al., 2017). Some isolates can produce toxic compounds, know as secondary metabolites, such as dextrusins that can facilitate the fungal spread inside of insect cavity as it modulate the host cellular immune responses (Pedland and Boucias, 1997; Hu et al., 2014) and act as an immunosuppressive conferring resistance against host defense (Trienens and Rohlfs, 2012) or are produced after insect death to avoid competition with other microorganisms (Skrobek and Butt 2005 ; Pedrini, 2017). After this process, the insects die and blastospores germinate and form an invasive mycelium growing saprophytically in the cadaver, later outgrowing from the dead insect body and into the environment (Wang et al., 2016).

M. rileyi (formely *Nomureae rileyi*), recently moved to *Metarhizium* genus (Kepler et al., 2014), is the only *Metarhizium* species commonly found causing field epizootics in the Lepidopteran species such as the economical important *Anticarsia gemmatalis* (Corrêa and Smith, 1975; Sosa-Gomez and Silva, 2003). The specificity of this fungus has been related to the cuticle composition of the host insects and the ability of *M. rileyi* to overcome the cuticle barrier, however the pathogenic mechanisms of *M. rileyi* are still poorly understood (Fronza et al., 2017). Nonetheless, *M. rileyi* has an different life-style compared to other *Metarhizium* species. The fungus requires a more complex medium such as SMAY (Sabouraud maltose agar with yeast) in order to germinate and sporulate when compared with other *Metarhizium* species (Alves et al., 1998, Edelstein et al., 2004, Boucias et al. 2016) that can easily grow in simple PDA (potato dextrose agar) medium. When grown in SMAY medium, *M. rileyi* conidial germ tubes switch to a yeast-like (blastospores) phase after 2-4 days of inoculation and grow in mucoid colonies for 1-7 days (depending on strain) (Song et al., 2018; Boucias and Pandland, 1984). After this period blastospores switch to mycelial growth followed by the formation of conidia. This transition conidia-blastospores-mycelial phase seems not to ocour in other *Metarhizium* species (*M. anisopliae*, *M. robertsii*, *M. brunneum*) as they switch directly from conidia to mycelial even in a nutritional rich medium as SMAY (personal information). The formation of blastospores in solid medium by *M. rileyi* seems to be related to nutritional factors of the culture medium. Pedland and Boucias, (1997)

reported that *M. rileyi* yeast-like phase can be by-passed by transferring germinating conidia from SMAY medium to Vogel´s medium (composed of salts, vitamins and inorganics nitrogen sources) instead of leaving them in SMAY. Thus, this unique growth pattern in *M. rileyi* makes it an very promising model for studying morphogenesis and compare cell phenotypes between *Metarhizium* groups.

Although *Metharizium* spp. are known mainly as biological control agents against insects, their entomopatogenicity characteristic arises later in the evolutionary history of *Metarhizium* in relation to its life-style associated with plants (Gao et al., 2011). Nonetheless, many investigations have focussed on the ability of these fungi to act as biostimulants of commercial plants (Sansan and Bidochka, 2012.2013; Golo et al., 2014; Jaber and Enkerli, 2017; Canassa et al., 2019a, 2019b) in addition to its effects in controlling insect and mites.

1.3. Genetics of *Metarhizium* spp.

In recent years, an increase in published studies related to comparative genomics between organisms has become possible due to the cheapness nowadays of sequencing techniques and also because of the advancement of tools for molecular data analysis (Shendure et al., 2017). Such studies allow researchers to access unique fitness-related characteristics of each species/isolate related to their lifestyles, adaptation to different niches, and interactions with other organisms and environment.

Many insect pathogenic fungi have been sequenced in recent years and their sequence data are deposited in globally recognized banks, such as NCBI (National Center for Biotechnology Information), Data Bank of Japan (DDBJ) and European Nucleotide Archive (ENA) for public use. Since November, 15th, 2019, eight species of *Metarhizium* had their genome sequenced (NCBI site, 2019): *M. anisopliae* (4 isolates), *M. acridum* (1 isolate), *M. rileyi* (2 isolates), *M. majus* (1 isolate), *M. brunneum* (1 isolate), *M. guizhouense* (1 isolate), *M. robertsii* (2 isolates) and *M. album* (1 isolate). *Metarhizium* spp. have a genome with approximately 31-42Mb in size, and predicted protein coding genes varying between 8,764 in *M. rileyi* isolate RCEF 4871 (Shang et al., 2016) to 12,036 in *M. robertsii*, isolate ARSEF 23(Gao et al., 2011) (NCBI, 2019) (Pattermore et al., 2014). These numbers are similar with

numbers found for other entomopathogenic fungi from Ascomycota: *Beauveria bassiana*, isolate ARSEF 2860 with 33Mb genome size and 10,364 predicted protein coding genes (Xiao et al., 2012) and *Cordyceps fumosorosea*, (formerly *Isaria fumosorosea*), isolate ARSEF 2679 with 33Mb genome size and 10,061 predicted protein coding genes (Shang et al., 2016) and also similar numbers compared to well known plant pathogens such as *Fusarium verticillioides*, isolate 7600, (genome size:41 Mb, predicted protein coding genes: 14,179) (Ma et al., 2010) and *Leptosphaeria maculans,* isolate JN3 (genome size: 45Mb, predicted protein coding genes:12,469) (Rouxel et al., 2011).

Metarhizium spp. contain species classified as generalists, with a broad host range (*M. robertsii*, *M. anisopliae* and *M. brunneum*) and specialists, with a more narrow host range (*M. rileyi*, *M. acridum*) (Boucias et al., 2000; Lacey and Kaya., 2007; Gao et al., 2011; Hu et al., 2014). The generalists such as *M. robertsii* can infect insects from several different orders like Orthoptera, Diptera, Hymenoptera, Hemiptera, Lepidoptera, and Coleoptera, whilst the specialist, *M. rileyi* and *M. acridum* are found infecting mainly Lepidopterans and Orthopterans, respectively (Driver et al., 2000; Boucias et al., 2000; Bing et al., 2008; Sosa-Gomez et al. 2010; Gao et al., 2011; Hu et al., 2014; Wang et al., 2016). The ability to infect different orders of insects can be genetically explained (Hu et al., 2014; Wang et al., 2016). Generalist species have expanded genes families with proteolytic capacities with more genes encoding for proteases and chitinases, more than two fold expansion of Pth11-like receptors (class of Fungal G protein-coupled receptors (GPCRs)) important for host recognition and virulence, fungal differentiation and appressoria formation, more gene clusters encoding for secondary metabolites, an expansion of genes related to detoxification, P450, and transports and more bacteria genes acquired by horizontal gene transfer. (Gao et al.,2011; Hu et al, 2014; Xu et al., 2016).All these expansions certainty played a pivotal role in generalists ability to colonize different insect hosts and their ability to live in association with plants (Behie et al., 2012; Shang et al., 2018). In generalist species extensive protein numbers were found through gene duplication events, while specialists were involved in the rapid evolution of existing protein sequences (Hu et l., 2014). These observations may reflect the coevolution process and consequently great efficiency in colonizing and evading the immune system of particular hosts as is the case with *M. rileyi*.

1.4. Study of the transcriptome and its applications

The first product of an organism's genome expression is the transcriptome, which represents the set of RNA molecules derived from protein-coding genes of which biological information is required by the cell at a specific time (Wang et al., 2009). Changes in the levels of gene expression are directly related to changes in physiology, metabolism and consequently to the process of cellular adaptation (Van Vliet, 2010). Researches of the transcriptome allows us to follow the gene expression in response to biotic and abiotic factors (Bhadauria et al., 2007), and to compare the expression of different genes simultaneously. Also, it allows us to study their interactions and to identify homologous transcripts of proteins and enzymes, by comparing these sequences with others already registered in recognized databases.

The use of transcriptomics to study interactions of entomopathogenic fungi and their hosts has been gaining increasing prominence in the scientific environment. In order to identify possible genes involved in the pathogenicity of these fungi, several authors examined the transcriptome obtained during different stages of the infective process in insects (Malagocka et al., 2015, Yek et al., 2013), fungal dimorphism (Tholander et al., 2005; Nigg et al., 2015 and 2017) and also under the development of the same fungus in different abiotic conditions, for example, under stress at high temperature (Wang et al., 2014) and growth in different culture media (Song et al., 2013). Many of these studies were able to identify not only the genes involved, but also the metabolic pathways that are activated under the analyzed conditions, thus clarifying the physiological responses of these fungi in different situations.

The understanding of the physiological mechanisms involved in the responses to the external environment allows understanding and manipulating the conditions under which these fungi develop in order to optimize the expression of genes and routes involved in pathogenic and growth processes under controlled conditions. The fungus *Saccharomyces cerevisiae*, widely used in the food industry, was the model organism for the development of the RNA-Seq technique (Nagalakshmi, 2008). Recently, several study groups have analyzed the *S. cerevisiae* fungus transcriptome under various production conditions (Zhao et al., 2015) and solid medium

(Aslankoohi et al., 2013). With the data they obtained, the authors were able to identify differences in gene expression in different stages of the fermentative process and stress situations, as well as to identify activated metabolic pathways and proteins produced.

1.5. Liquid culture fermentation and formulation of *Metarhizium*

Aerial conidia of. *Metarhizium* spp is the main fungal propagule commercialized by companies as a biocontrol agent. It is produced totally in solid substrate fermentation or in biphasic fermentation wherin liquid fermentation inoculum rather than a conidial suspension is used to inoculate solid substrate (Jaronski and Mascarin, 2017). Besides aerial conidia, *Metarhizium* spp. also produce other propagules, less explored, with the potential to be used in biological control programs such as submerged conidia (Kassa et al., 2004), microsclerotia (Mascarin et al., 2014) and blastospores (Iwanicki et al., 2018), this last, also commonly used as inoculum in the biphasic fermentation process. (Jaronski and Mascarin, 2017).

Blastospores have proven to be promising candidates for use in biological control (Shapiro-ilan et al., 2008; Mascarin et al., 2015a; Wasserman et al., 2016; Alkhaibar et al., 2016; Iwanicki et al.,2018). They are hydrophilic yeast cells with a thin cell wall; some isolates are highly infectious to insects and mites. Additionally, in liquid fermentation it is possible to produce large quantities of blastospores, in a short time (<4 days), in small a physical space and with low labor dependence compared to aerial conidia production.

In nature, blastospores are produced inside the hosts, in the arthropod hemolymph during fungal infection (Pedrini 2017). Generally, these propagules are nutrient and oxygen demanding (Mascarin et al., 2015b) and are easily produced in submerged fermentation systems, with greater control of the environment and quality of the final product in a short time (Jackson, 1997; Vega et al., 1999; Mascarin et al., 2015a).

Regardless of the propagule type, some technical and biological parameters of this propagule must be established in order to turn microbial agent into a commercial bioinsecticide; these include yield, nutritional requirements, tolerance to

drying method, compatibility to formulation additives/inert, stability during storage (shelf-life) , insecticidal activity (bioefficacy) (Humphreys et al., 1989; Jackson et al., 1997; Téllez-Martínez et al., 2016; Mascarin et al., 2019). After the liquid fermentation process is defined, a method for stabilizing the fungal propagule is required for subsequent use. The most practical method of stabilization consists of collecting fungal biomass and drying to a certain moisture content to reduce fungal metabolic activity and also unwanted contamination by microorganisms (Jaronski and Mascarin, 2017; Mascarin et al., 2016). Various methods for drying fungi biomass are known, such as freeze-drying, spray-drying, fluidized-bed drying and air-drying (Horaczek and Viernstein, 2004 ; Jaronski and Mascarin, 2017). While all of these drying methods have different benefits and specific applications, air-drying and spray-drying are the two method most commonly used for drying blastopsores (Iwanicki et al., 2018; Mascarin et al., 2018; Mascarin et al., 2016, Jackson and Payne, 2007; Jackson et al., 1997). In the first system it is used a slow drying protocol lasting from 20 to 24 hours, under ambient temperature (24~26°C) and controlled humidity (starting with 50-60% of humidity for 15~18h and more 3 ~ 4 h under <10% of humidity) for drying blastospores formulations. Conversely, the second system consists of quick-drying through atomization of the formulation into a chamber with an air flow at 80°C in average. Although formulations could take one day for drying in air-drying system, the main advantage of this methodology is its low cost and reduction of stress applied to the blastosproes during slow drying (Jackson and Payne 2007). On the other hand, it is possible to dry very quickly, high amount of formulations in spray dryer equipment although blastospores are submitted to high osmotic and thermal stress during drying. Spray drying produces dry powders from a solution or suspension by atomizing the wet product at high velocity within a chamber at high temperatures (varying from 50-200°C). This is a predominant process in the food industry as dairy and instantaneous coffee industry and it can be used to produce large amounts of ingredients with a relatively low cost (Morgan et al., 2006).

The most common conidia-based formulations of *Metahrizium* are wettable powder, with the advantage of being a product ready to be added directly to water (Faria and Wraight, 2007; Agrofit, 2019) and granulated formulations (mainly sold as sporulated fungus in rice) (Agrofit 2019). Although there is no oil formulations of

either blastospores or conidia of *Metarhizium* in the market (Agrofit 2019), the latter has been shown to be quite promising due to its greater protection against conidia desiccation, UV rays, high temperatures, better adhesion to arthropod hydrophobic cuticle and better target pest controls (Barreto et al ., 2016; Camargo et al., 2014; Samish et al., 2014; Luz et al., 2016). There is no blastospore oil formulation in the world market and only one publication related an attempt to formulate *Cordyceps fumosoroseae* (formerly *Isaria fumosoroseae*) blastospores (Kim et al., 2013). In that research, the authors showed a high level of control of whitefly *Trialeurodes vaporariorum*, although they did not test the effect of the solely formulation against this insect (Kim et al., 2013)

There are currently in the world three commercial blastospore formulated bioproducts, two formulated with *C. fumosorosea* strain Apopka 97 one being commercialized in the United States and in Europe by two companies with commercial names: PreFeRal WG® (Biobest company), and PFR-97 20% WDG® (CERTIS company) labeled for use on vegetable, fruit, and food crops. A third product is formulated with the species *Akanthomyces dipterigenus* (*Lecanicillium longisporum*) and comercialized in Europe as Mycotal® (Koppert company). In Brazil there are no fungal blastospore-based bioinsecticides so far, and the biofactories that produce entompathogenic fungi as biocontrol agents produce and commercialize aerial conidia using the cereal-based solid fermentation method, especially rice.

1.6. Corn leafhopper *Dalbulus maidis*

Maize is one of the key crops for the Brazilian economy with many industrial applications in the production of food, fuel, cosmetics, pharmaceuticals and animal feed. During the 2017-2018 crop season, 85 million tons of corn grains were harvested from approximately 16.5 million hectares (CONAB, 2018). The corn leafhopper, *Dalbulus maidis* (DeLong and Wolcott, 1923) (Hemiptera: Cicadellidae) has recently emerged as the most important sucking pest in maize crops in Brazil. This insect has a phloem feeding behavior and vectors three pathogens associated with the corn stunt disease complex: the maize bushy stunt phytoplasma, the Spiroplasma kunkelii and the virus Maize rayado fino virus (MRFV) (Nault and Delong 1980; Nault 1990). Symptoms in infected plants are chlorotic stipple-striping

in leaves, mainly caused by MRFV (Edwards et al., 2015), In the case of phytoplasma, red leaves, stunt and ear proliferation and consequently grain yield, kernel quality and the height of plants were reduced as the infective population increased leading to yield reductions of up to 91.3% (Toffanelli and Bedendo, 2002).

Since 2016, high levels of maize bushy stunt disease have been recorded in many Brazilian states leading to yield reduction of 90% in some cases (Canale et al., 2018).

D. maidis has a wide distribution throughout the Americas from Argentina to southeastern and southwestern U.S.A. (Triplehorn and Nault, 1985). This species developed to the adult stage within the temperature range of 17.5–35 °C and the complete biological cycle (egg-egg) is completed between 30 to 52 days (van Nieuwenhove et al., 2016). For controlling *D. maidis* seed treatment neonicotinoid and methyl carbamate are used, while the only biological alternative to control this insect is based on the foliar sprayable aerial conidia of Beauveria bassiana (AGROFIT, 2019). To the best of our knowledge, there is no report regarding the bioefficacy of entomopathogenic blastospores against this leafhopper. Earlier studies have indicated that blastospores of *B. bassiana* appeared to be less virulent than aerial conidia towards the green leafhopper *Nephotettix virescens* (Trinci and Gillispie, 1991). However, many attributes inherent to the fungal species and strain plus the quality of the infectious propagule can significantly influence the effectiveness of blastospores toward an insect target, and these aspects must be considered case-by-case.

1.7. Cattle-tick, *Rhipicephalus (Boophilus) microplus*

Ticks are worldwide ectoparasites of great importance for human and veterinary health as they transmit infectious agents during the feeding of their hosts' blood (Barros-Battesti et al., 2006). The *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) popularly known as cattle-tick, is a monoxenous (complete its life-cylce in only one host) species that has a short parasitic cycle, from 20 to 35 days (Hitchcock, 1995). After mating, the female feeds on blood until they reach a specific size that represents 200-600 her unfed weight (Sonenshine, 1991) and then it falls to the ground to oviposit, laying between 2000 to 4500 eggs (Wall and Shearer., 2001)

After this, the female dies. Larvae, which can survive for up to 8 months in ground (Hitchcock, 1955), climbs on leaf tips and waits for a host to grab. According to Cordovés (1997), it is estimated that only 5% of ticks are in their bovine hosts while the remaining 95% are in the field, in the non-parasitic phase that is mainly represented by unfed larvae. Larvae, nymphs and adults feeds on blood and live as hematophagous in cattle.

The tick *R. microplus* transmits two important disease-pathogens to cattle, the ricketsia *Anaplasma* and the protozoan *Babesia*, responsible for the complex called “bovine parasitic sadness” that cause great damage to the milk and cattle production system. By ingesting blood, the tick causes irritation in animals, stress and blood loss. Large infestations are responsible for reduced milk production, increased mortality in calves, reduced birth rates, weight loss and poor leather quality. What is even more serious, cases of acaricide resistance to chemical molecules like pyrethroids and organophosphates have been growing in the world, thus aggravating the losses caused by diseases and stress due to inefficiency of these products (Guerrero et al., 2012).

In this context, we have seen a growing interest for ecological alternatives to control these ticks, specially due to various economic damages caused by *R. microplus*, which reach the order of two billion dollars in Brazil per year (Grisi et al. 2002), increased resistance to acaricides and the constant concern about chemical residues in milk and meat.

Several authors have reported the efficiency of *Metarhizium* conidia in tick control (Bernardo et al., 2018; Samish et al., 2014; Rodríguez-Alcocer et al., 2014; Camargo et al., 2016; Nana, Pauli et al., 2016) however, few of them demonstrate virulence and efficiency of *Metarhizium* blastospores in tick control. In a recent report, Wasserman et al. (2016) indirectly applying *M. anisopliae* blastospores to *Ixodes ricinus* tick larvae and nymphs under semi-field conditions, obtained control of up to 81.5% of larvae and 93% of nymphs, while Bernardo et al., (2018) showed, in laboratory conditions, promising results for use of *Metarhizium robertsii* and *Beauveria bassiana* for cattle tick control.

Considering that most of the life cycle of *R. microplus* takes place in the field, in larvae stage and that they occur in an extremely high number, an alternative to the control of these ticks would be the application of products directly in the field, aiming

at controlling mainly the larval stage. In Europe, the United States and Canada field application of the fungus *Metarhizium brunneum* for tick control is a common practice; there is registered product Met52®/BIO1020® from the company Novozymes. In Brazil, there are no registered products based on entomopathogenic fungi for tick control. However, in light of the critical scenario, that has been gaining the tick control, the known pathogenicity of *Metarhizium* to ticks, and the use of this biological control agent in other regions of the world, we are seeing an increased interest from companies to develop bioproducts for controlling tick.

1.8. Objectives and hypotheses

Considering the worldwide trend of growing more sustainable practices in agriculture, such as biological control of pest and the huge but poorly explored potential of entomopathogenic fungal blastopores as biological control agents, the experiments developed in this thesis were divided into five work packages aimed to advance in the knowledge that will enable the use of *Metarhizium* blastospores as biological control agents and to clarify the metabolic characteristics during the yeast-like growth of two *Metarhizium* species. Therefore, the thesis addresses 1) the selection of blastospores-productive *Metarhizium* isolates and determine bioeficacy of blastospores against the veterinary pest: cattle-tick (*Rhipicephalus (Boophilus) microplus*) 2) the adaptation of culture media aiming at reducing medium cost and increasing blastospore production; establish the growth kinetics in liquid medium; optimize nitrogen; determine bioeficacy of blastospores against the agricultural pest, corn-leafhopper (*Dalbulus maidis*) 3) development of formulations using two drying methods; determine the wettability and physical stability of spray dried blastospore formulations; determine the shelf-life of blastospore formulation packaged at modified atmosphere; determine bioeficacy of formulated blastospores agains *D. maidis* 4) transcriptome studies of the vegetative growth phases (yeast and filamentous) of the fungi *Metarhizium anisopliae* and 5) *Metarhizium rileyi*.

Thus, the first work aimed first: to select two blastospores-productive isolates from 11 tested isolates of five *Metarhizium* species grown in Adamek medium, second: to increase blastospore production of the selected isolates in submerged liquid cultures and third: to determine the virulence of air-dried blastospores of one

strain compared with conidia toward larvae of tick , *Rhipicephalus microplus.* The hypotheses of this study were therefore: i) *Metarhizium* blastospore production is dependent on species and isolates; ii) Blastospore production is higher in modified Adamek´s medium supplied with 140g/L of glucose compared to production in the same medium supplied with 40g/L; iii) Dried-blastospores are more virulent to the cattle-tick larvae than conidia applied at the same concentration.

In the second study, the objective was first: to optimize the nutritional composition by lowering the cost of our modified Adamek's medium described in first study, second: to stablish the growth kinetics of *M. robertsii* blastospores in liquid medium and third: to measure blatospores virulence toward *D. maidis*. We therefore hypothesized that i) the nitrogen source affects blastospore yield; ii) there is an optimum nitrogen concentration in the medium allowing high blastospore yield; iii) fresh and dried blastospores can be equally or even more virulent against the corn-leafhopper *D. maidis* than aerial conidia applied at the same concentration.

The third study aimed to first: develop air-dried and spray-dried formulations of *Metarhizium* blastospores; second: to measured the shelf-life of the best air-dried and spray-dried formulations stored in refrigerated (± 4°C) and ambient conditions (28°C) and packaged in modified atmosphere and vacuum and third: Accessing the virulence of the best spray-dried and the best air-dried formulation against the corn-leafhopper *D. maidis*. Specifically, we hypothesized that i) There is an optimal combination of ingredients used for the spray-dried and air-dried blastospore formulations that preserve the high viability of blastospores after drying; ii) spray dryer formulations differ in their physical parameters such as wettability and stability iii) Shelf-life is longer for treatments stored with oxygen and moisture-absorbing sachets than those stored under vacuum or without sachets; iv) Formulations stored in the refrigerator have a longer shelf-life than those stored at room temperature; v) Lethal time (LT50) and lethal concentration (LC50) for corn leafhopper are lower for formulated bastospores compared to fresh blastospores

The fourth and the fifth studies aimed at using comparative genome-wide transcriptome analyses to determine changes in gene expression between the filamentous and blastospore growth phases *in vitro* to characterize physiological changes and metabolic signatures associated with *M. anisopliae* and *M. rileyi* dimorphism. Specifically in the case of *M. anisopliae* we hypothesized that changes

in gene expression can be related to i) phenotypic differentiation and growing processes in hyphae and blastospores, ii) potential differences in fungal cell-wall metabolism, iii) responses in cellular respiration and oxidative stress to liquid and solid media, iv) genes involved in arthropod pathogenicity that are differentially expressed between hyphae and blastospores, and v) specific classes of biosynthesis genes involved in secondary metabolism produced by each fungal structure.

Conversely, in the case of *M. rileyi* we hypothesized that changes gene expression of yeast-like phases (blastospores) grown in liquid medium can be associated to i) specific biological processes and up-regulated genes involved in the induction and maintenance of blastospores ii) *M. rileyi*-specific genes not found in other *Metarhizium* spp up-regulated only during growth in solid medium iii) genes related to virulence factors such as specific enzymes and toxins

1.9. Obtained results and future perspectives

The aim of these studies was to elucidate a number of questions related to the production and formulation of *Metarhizium* blastospores in order to provide reliable information that could be used for the production of a *Metarhizium* blastospore-based biopesticide and encourage further developments in this area. Optimizing liquid cultures for blastospore production of *Metarhizium* spp. has been a major challenge to researchers. Whereas the entomopathogenic fungi such as *Beauveria bassiana* and *Cordyceps fumosoroseae* are highly productive in liquid cultures (Jaronski and Jackson., 2012; Mascarin et al., 2015a, 2015b) and can achieve yields superior to 10^9 blastospores/mL in 2 or 3 days, there are only a few studies showing productivities equal to or greater than 10^8 *Metarhizium* blastospores per mL within 3-4 d of cultivation (Issaly et al., 2005; Ypsilos and Magan, 2005). Additionally, studies evaluating methods for stabilizing blastospores, shelf-life of formulated blastospores and bioefficacy of dried blastospores are quite advanced for *Beauveria bassiana* and *C. fumosoroseae* fungi but almost non-existent for *Metarhizium* blastospores.

Therefore, in our first chapter, (Iwanicki et al., 2018) 11 *Metarhizium* spp. isolates from four species were chosen for screening their blastospore production in Adamek´s medium. This led to the selection of two isolates, *Metarhizium robertsii* (ESALQ1426) and *M. anisopliae* (ESALQ4676), for the next series of experiments in

which we aimed to increase the production of these isolates by supplementing a modified Adamek's medium with increased glucose concentrations and accelerating fermentation time using a blastospore pre-culture as inoculum. Our results showed that blastospore production of *Metarhizium* is isolate- and species-dependent, highlighting the importance evaluating different isolates in order to select the most productive one. Glucose-enriched cultures of *M. robertsii* (ESALQ1426) inoculated with pre-cultures improved and grown under optimal conditions yielded within two days 5.9×10^8 blastospores/mL. This confirms the hypothesis that increased osmolality of the culture medium may promote increased blastospore production in a shorter culture time, as was previously shown for isolates of the fungus *Beauveria bassiana* (Mascarin et al., 2015a). Then, the resulting air-dried blastospores of *M. robertsii* ESALQ1426 were shown to infect and quickly kill cattle tick (*Rhipicephalus microplus*) larvae with comparable efficiency to that of conidia. As far as we know, this was the first time that dried blastospores of any species of *Metarhizium* were shown to be infective to ticks.

Despite the high yields of blastospores (5.9×10^8 blastospores/mL) obtained from *M. robertsii* ESALQ1426 (chapter one), the modified Adamek's medium still needed to be optimized for low-cost inputs in media formulation to replace yeast extract, an expensive protein source. Therefore, in the study presented in chapter two, our goal was to optimize nitrogen source and titers for maximum production of *M. robertsii* (ESALQ1426) blastospores cultures in shake flasks at highly aerated conditions and determine their virulence against the corn leafhopper *Dalbulus maidis*, an important vector of serious pathogens of maize crops worldwide. Our fermentation studies including growth kinetics showed that a low-cost corn steep liquor (CSL) was the most suitable nitrogen source for inducing blastospore growth in *M. robertsii* (ESALQ1426) with an optimal titer of 80 g/L that rendered up to 4.7×10^8 blastospores/mL with only 2 days of cultivation at a total cost of $0.30 USD per L. This is a very relevant finding to support the development of a blastospore-based biopesticide since the cost of the culture medium is one of the main expenses in production. Moreover, the blastospore growth kinetic was strongly dependent on glucose and nitrogen consumptions accompanied by a slight drop in the culture pH. We noted that it was the first time that nitrogen and glucose consumption by

Metarhizium blastospores was reported in the literature as well as the infectivity of fresh and dried *M. robertsii* blastospores on the corn leafhopper *D. maidis*.

Once an inexpensive and effective culture medium was obtained for growing high amounts of *M. robertsii* (ESALQ1426) blastospores, the next step of our research was to develop blastospores formulations and establish the effect of co-formulants on blastospore viability after spray-drying and air-drying. Additionally, we investigated wettability and stability characteristics of spray-dried formulations and shelf-life and virulence of two formulations against corn leafhopper (*Dalbulus maidis*). The data (chapter three) showed that bentonite clay was the inert that provided the highest viability after mixed with blastospores, air-dried and stored in ambient and refrigerated conditions, and we selected bentonite clay for blastospore formulations. In general, air-dried formulations presented higher viability than spray-dried formulations. The addition of co-formulants, besides bentonite, contributed little to preserve the viability of air-dried blastospores but were particularly important for preserving viability of spray-dried blastospores. We found that some co-formulants were important to keep blastopore viability during air-drying; these included skim milk and sucrose while maltodextrin, skim milk and bentonite are important for keep blastospores viability during spray-drying. Wettability time of spray-dried formulations differed between formulations, being higher for those with the highest amount of Ca-Lignin (5%) combined with the highest amount of maltodextrin (4%). Additionally, we reported spray dried and air dried formulations are as virulent as fresh blastopores to the corn leafhopper. On the other hand, the estimated median lethal concentration were significantly lower for spray dried formulation (LC_{50} = 2.42 × 10^7) then to air dried formulation (LC_{50} = 4.65 × 10^6). The results of bioassays from chapter 1, 2 and 3 all confirm that a concentration of 5 ×10^7 infective propagules/mL is crucial for achieving high mortality in cattle tick and corn leafhopper, regardless if the sprayed propagule is conidia, fresh, spray dried or air dried blastospores. Additionally, the addition of oxygen-moisture absorbers into sealed packages during storage substantially increased in three- or four-fold the half-life times of air-dried and spray-dried formulations stored either in fridge or ambient temperature compared to controls. We noted that this was the first report that demonstrated the effect of co-formulates on the viability of air-dried and spray-dried *M. robertsii* blastospores as

well as the first work to report aspects such as wettability and stability of blastospore formulations.

In order to understand the initiation of the yeast phase (blastospores) in *Metarhizium* and to characterize its metabolism during *in vitro* cultivation, the expressed genes and factors potentially inducing yeast growth in liquid and solid agar medium were investigated in this research. One generalist species, *M. anisopliae* (ESALQ4676), and one specialist species, *Metarhizium rileyi* (ESALQ4948) were selected which differ in in their response to growth conditions. *M. rileyi* is more fastidious, and is not stable for continuous production by solid substrate fermentation. *M. anisopliae* can easily grow in simple medium like potato dextrose agar (PDA). Additionally, *M. rileyi* produces a yeast phase in solid culture media and this morphotype, as far as we know, was never recorded for *M. anisopliae* and other generalist *Metarhizium* species. Results from transcriptome studies of yeast-phase grown in liquid and solid medium (in case of *M. rileyi*) compared to filamentous phase are presented in separate chapters for each species.

In chapter four our results of *M. anisopliae* (ESALQ4676) transcriptome showed clear differences between the metabolism of blastospores and filamentous phases. In total 6.4% (n=696) out of 10,981 predicted genes in *M. anisopliae* were differentially expressed between the two phases with a fold-change > 4. The main physiological processes associated with up-regulated gene content in blastospores during liquid fermentation were oxidative stress, amino acid metabolism, respiration processes, transmembrane transport and production of secondary metabolites. In contrast, the up-regulated gene content in hyphae were associated with increased growth metabolism and cell wall re-organization. We focused on presenting the main differences between blastospores and hyphae, highlighting the main processes that differ in cell wall metabolism, cell respiration and oxidative stress, expression of virulence factors and production of secondary metabolites. We found a clear difference in the composition of the cell wall of blastospores and hyphae, evidenced by a lower amount and diversity of Glycoside hydrolases (GH) related to the synthesis and degradation of cell wall components being expressed in blastospores than in hyphae. The thinner blastospore cell wall of *M. anisopliae* (ESALQ4676) are probably associated with increasing flexibility and permeability of the cell wall (Wang et al., 2005) that in turn enhance nutrient flux and are almost certainly induced in

response to different nutritional and environmental conditions. We found that blastopores grown in liquid medium are under oxidative stress, as evidenced by enriched gene ontology term related to oxidative stress and a high expression of antioxidant enzymes such as catalases, and antioxidants such as glutathione. In our study, the high agitation speed (350rpm) of culture flasks and the high glucose concentration of the medium (140g/L), resulted in high levels of dissolved oxygen (Giese et al., 2014; Mascarin et al., 2015a). This could expose blastospores to an increased oxygen triggering formation and reactive oxygen species (ROS) thus finally leading the fungus to experience oxidative stress. Another possible explanation for this physiological response would be the constitutive expression of antioxidant enzymes as a defense mechanism against the stressful conditions posed in the hemolymph of insects, an environment in which blastospores are naturally produced (Dubovskii et al., 2010a, b). An increase in intracellular ROS levels has been shown to induce morphogenesis of other fungi (Song et al., 2013, Schröter et al., 2000) and we speculate that it might be involved in blastospore formation. In addition to oxidative stress, we found genes related to TCA-cycle and respiration process highly expressed in blastospore compared to hyphae metabolism and this fact could be related to ROS production, as mitochondrial respiratory chain constitutes the main intracellular source of ROS (Turrens, 2005)

We found differences in the transport of substances between hyphae and blastospores, where transmembrane transport was more active in blastospores whereas vesicle transport of substance was more pronounced in hyphae. More genes related to secondary metabolite production were found up-regulated in blastospores than in hyphae, especially from terpenoid families. Additionally, blastospores produced a collagen-like protein MCL1 when grown in liquid culture medium. The MCL1 protein provided an antiadhesive protective coat that masks beta-glucan components of the blastospore cell wall and thereby hindered hemocytes from killing the fungal cells during growth in insect hemolymph (Wang et al., 2006). Since this protein was also expressed in blastospores grown during in-vitro cultivation in liquid media with agitation, it supports the idea that blastospores of *M. anisopliae* produced during in-vivo and in-vitro growth are comparable and that MCL1 does not require insect hemolymph to induce expression.

Overall, that study showed significant transcriptomic differences between the metabolism of blastospores and of hyphae and these findings illustrate important aspects of fungal morphogenesis in *M. anisopliae* and highlight the main metabolic activities of each propagule under *in vitro* growth conditions.

In chapter 5 our results of *M. rileyi* (ESALQ4948) transcriptome clearly showed that blastospores produced in solid (BS) and liquid (BL) medium are distinct *M. rileyi* yeast-like forms that activate a set of different genes to their maintenances. Nonetheless, we showed that BL up-regulated almost twice as many genes BS (n = 340) or hyphae (n=186). We showed that blastospores produced in liquid medium activate a series of specific genes related to signal transduction, that might be associated with cell growth as well as activation of specific membrane transporters related to iron acquisition. We showed that oxidative stress and activation of specific heat shock proteins are key factors involved in BL formation. On the other hand blastospores grown in solid medium have an unique set of genes associated to enzymes such as an aminoacid permease that are active, and not found in other *Metarhizium* spp. Nonetheless, BS activate a unique nitroreductase, specific membrane proteins and several virulence factors. Therefore we have clarified the main metabolism required for blastospores growth in liquid medium and identified candidate genes that will serve as a basis for future research on optimizing *M. rileyi* blastospore production. The fact that BS produces highly virulent factors opens new opportunities to explore blastospores produced in solid medium as components of biopesticides.

Altogether, these studies present relevant advances for liquid culture fermentation and morphogenesis of *Metarhizium* blastospores and raised other questions and hypotheses that should be further investigated, such as:

1) Studies to determine the effect of different concentrations of initial inoculum and constant pH cultivation on blastospore yield;
2) Further studies to understand the effects of self-inhibiting substances our a quorum sensing molecules produced by *Metarhizium* in liquid culture medium that impairs the increase in blastospore production;

2) Investigate continuous batch cultivation of *Metarhizium* blastospores in order to remove possible growth inhibitory substances that would be present in the culture medium;

4) Studies involving genomics approach to explain short shelf life of *Metarhizium* blastospores, even stored under low concentrations of oxygen and moisture;

5) Studies to describe particle size of spray dried formulations and the UV-light tolerance of spray dried blastospores provided by co-formulants and ca-lignin and activated charcoal;

6) Bio-efficiency of formulated blastospores compared to unformulated blastospores and conidia at field conditions;

7) Studies to clarify the quantity and the function of secondary metabolites produced by blastospores of *M. anisopliae* in liquid medium;

8) Laboratory studies to determine the bioefficacy of *M. rileyi* blastospores produced in solid medium compared to those produced in liquid medium;

9) Studies to determine the involvement of specific up-regulated genes found in blastospores produced in solid media in their formation and virulence.

References

ABCBio, 2019: Associação Brasileira das Empresas de Controle Biológico https://www.abcbio.org.br/ (Accessed 02 September, 2019).

AGROFIT, 2019. Sistema de agrotóxicos fitossanitários - ministério da Agricultura, Pecúaria e Abastecimento. http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. (Accessed 10 October, 2019).

Alkhaibari AM, Carolino AT, Yavasoglu SI, Maffeis T, Mattoso TC, Bull JC, Samuels RI, Butt TM (2016) *Metarhizium brunneum* Blastospore Pathogenesis in Aedes aegypti Larvae: Attack on Several Fronts Accelerates Mortality. PLoS Pathog 12.

Alves, S.B., 1998. Controle microbiano de insetos, second ed. Fealq, Piracicaba.

Aslankoohi E, Zhu B, Rezaei MN, Voordeckers K, Maeyer D De, Marchal K, Dornez E, Courtin CM, Verstrepen KJ (2013) Dynamics of the *Saccharomyces cerevisiae* transcriptome during bread dough fermentation. Appl Environ Microbiol 79:7325–7333.

Barros-Battesti DM, Arzua M, Bechara GH (2006) Carrapatos de importância médico-veterinária da Região Neotropical: Um guia ilustrado para identificação de espécies. São Paulo: Vox/ International Consortium on Ticks and Tick-borne Diseases (ICTTD-3)/Butantan, 223 p.

Barreto LP, Luz C, Mascarin GM, Roberts DW, Arruda W, Fernandes ÉKK (2016) Effect of heat stress and oil formulation on conidial germination of *Metarhizium anisopliae* s.s. on tick cuticle and artificial medium. J Invertebr Pathol 138:94–103.

Behie SW, Zelisko PM, Bidochka MJ (2012) Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science 336:1576–7.

Bernardo CC, Barreto LP, Silva CDSRE, Luz C, Arruda W, Everton KK (2018) Conidia and blastospores of *Metarhizium* spp. and *Beauveria bassiana* s.l.: Their development during theinfection process and virulence against the tick Rhipicephalusmicroplus. Ticks Tick Borne Dis 9:1334–1342.

Bhadauria V, Popescu L, Zhao WS, Peng YL (2007) Fungal transcriptomics. Microbiol Res 162:285–298.

Botelho ABRZ, Alves-Pereira A, Colonhez Prado R, Zucchi MI, Delalibera I (2019) *Metarhizium* species in soil from Brazilian biomes: a study of diversity, distribution, and association with natural and agricultural environments. Fungal Ecol 41:289–300

Boucias D, Liu S, Meagher R, Baniszewski J (2016) Fungal dimorphism in the entomopathogenic fungus *Metarhizium rileyi*: Detection of an in vivo quorum-sensing system. J Invertebr Pathol 136:100–108.

Boucias DG, Pendland JC (1984) Nutritional Requirements for Conidial Germination of Several Host Range Pathotypes of the Entomopathogenic Fungus *Nomuraea rileyi*. J Invertebr Pathol 43:288–292.

Boucias DG, Tigano MS, Sosa-Gomez DR, Glare TR, Inglis PW (2000) Genotypic properties of the entomopathogenic fungus *Nomuraea rileyi*. Biol Control 19:124–138.

Brunner-Mendoza C, Reyes-Montes MDR, Moonjely S, Bidochka MJ, Toriello C (2019) A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Sci Technol 29:83–102.

Camargo MG, Marciano AF, Perinotto WMS, Quinelato S, Galo PS, Angelo IC, Prata MCA, Bittencourt VREP (2014) Commercial formulation of *Metarhizium anisopliae* for the control of *Rhipicephalus microplus* in a pen study. Vet Parasitol 205:271–276.

Canale MC, Lopes JRS, Nesi CN, Prado SDS (2018) Role of *Dalbulus maidis* (Hemiptera: Cicadellidae) gender on maize bushy stunt phytoplasma transmission . Phytopathogenic Mollicutes 8:32.

Canassa F, Esteca FCN, Moral R a., Meyling N V., Klingen I, Delalibera I (2019a) Root inoculation of strawberry with the entomopathogenic fungi *Metarhizium robertsii*

and *Beauveria bassiana* reduces incidence of the twospotted spider mite and selected insect pests and plant diseases in the field. J Pest Sci (2004).

Canassa F, Tall S, Moral R a., Lara IAR d., Delalibera I, Meyling N V. (2019b) Effects of bean seed treatment by the entomopathogenic fungi Metarhizium robertsii and *Beauveria bassiana* on plant growth, spider mite populations and behavior of predatory mites. Biol Control 132:199–208.

CNA, 2018: Confederação da Agricultura e Pecuária do Brasil https://www.cnabrasil.org.br/cna/ (Accessed 12 November, 2019)

CONAB, 2018: Companhia Nacional de Abastecimento (Accessed 12 November, 2019)

Cordovés CO (1997) Carrapato: controle ou erradicação (2 ed.), Agropecuária, Guaíba 130 pp.

Corrêa BS, Smith JG (1975) *Nomuraea rileyi* attacking the velvetbean caterpillar, *Anticarsia gemmatalis*, in Paraná, Brazil. The Florida Entomologist, 58(4), 280.

Driver F, Milner RJ, Trueman WH (2000) A Taxonomic revision of *Metarhizium* based on sequence analysis of ribosomal DNA. Mycol Res 104:135–15.

Dubovskii I, Grizanova E, Chertkova E (2010a) Generation of reactive oxygen species and activity of antioxidants in hemolymph of the moth larvae *Galleria mellonella* (L.) (Lepidoptera: piralidae) at development of the process of encapsulation. J Evol Biochem Physiol. 46:35–43.

Dubovskiy I, Kryukov VY, Benkovskaya G (2010b) Activity of the detoxificative enzyme system and encapsulation rate in the Colorado potato beetle *Leptinotarsa decemlineata* (Say) larvae under organophosphorus insecticide treatment and entomopathogenic fungus *Metharizium anisopliae* (Metsch.) infection. Euroasian Entomol J. 9:577–582.

DunhamTrimmer's, 2019: The market research company. Data presented in BioControl Latan 2019 conference http://dunhamtrimmer.com/

Duran R, Cary JW, Calvo AM (2010) Role of the osmotic stress regulatory pathway in morphogenesis and secondary metabolism in filamentous fungi. Toxins (Basel) 2:367–381.

Edwards MC, Weiland JJ, Todd J, Stewart LR (2015) Infectious Maize rayado fino virus from cloned cDNA. Phytopathology 105:833–839.

Edelstein J, Lecuona R, Trumper E, (2004) Selection of culture media and in vitro assessment of temperature-dependent development of *Nomuraea rileyi*. Neotropical Entomology, Londrina, 33(6):737-742.

Faria MR, Wraight SP (2007) Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol Control 43

Fronza E, Specht A, Heinzen H, de Barros NM (2017) *Metarhizium* (*Nomuraea*) *rileyi* as biological control agent. Biocontrol Sci Technol 27:1243–1264.

Gao Q, Jin K, Ying SH, Zhang Y, Xiao G, Shang Y, Duan Z, Hu X, Xie XQ, Zhou G, Peng G, Luo Z, Huang W, Wang B, Fang W, Wang S, Zhong Y, Ma LJ, St Leger RJ, Zhao GP, Pei Y, Feng MG, Xia Y, Wang C (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7

Giese H, Azizan A, Kümmel A, Liao A, Peter CP, Fonseca JA, Hermann R, Duarte TM, Büches J (2013) Liquid films on shake flask walls explain increasing maximum oxygen transfer capacities with elevating viscosity. Biotechnol Bioeng 111:295–308.

Grisi L, Massard CL, Gonzalo MB, Pereira JB (2002) Impacto econômico das principais ectoparasitoses em bovinos no Brasil. A Hora Veterinária, v. 21, n. 125, p. 8-10, 2002.

Golo PS, Santos H a., Perinotto WMS, Quinelato S, Angelo IC, Camargo MG, Sá F a., Massard CL, Fernandes ÉKK, Roberts DW, Bittencourt VREP (2015) The influence of conidial Pr1 protease on pathogenicity potential of *Metarhizium anisopliae* senso latu to ticks. Parasitol Res 114:2309–2315.

Guerrero FD, Lovis L, Martins JR (2012) Acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus*. Rev Bras Parasitol Veterinária 21:1–6.

Hitchcock, LF (1955). Studies of the non-parasitic stages on the cattle tick, *Boophilus microplus* (Canestrini) (Acarina: Ixodidae).—Aust. J. Zool. 3 pp. 295–311.

Hu X, Xiao G, Zheng P, Shang Y, Su Y, Zhang X, Liu X, Zhan S, St Leger RJ, Wang C (2014) Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc Natl Acad Sci 111:

Humphreys AM, Matewele P, Trinci APJ, Gillespie AT (1989) Effects of water activity on morphology, growth and blastospore production of *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces farinosus* in batch and fed-batch culture. Mycol Res 92:257–264.

Issaly N, Chauveau H, Aglevor F, Fargues J, Durand A. (2005) Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. Process Biochem 40:1425–1431

Iwanicki NSA, Pereira AA, Botelho ABRZ, Rezende JM, Moral RDA, Zucchi MI, Delalibera Júnior I (2019) Monitoring of the field application of *Metarhizium anisopliae* in Brazil revealed high molecular diversity of *Metarhizium* spp in insects, soil and sugarcane roots. Sci Rep 9:4443.

Iwanicki NSA, Ferreira BDO, Mascarin GM, Júnior ÍD (2018) Modified Adamek's medium renders high yields of Metarhizium robertsii blastospores that are desiccation tolerant and infective to cattle-tick larvae. Fungal Biol 122:883–890.

Jaronski, ST, Jackson, MA (2012) Mass production of entomopathogenic hypocreales. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology, second ed. Academic Press, London, pp. 317-328.

Jaronski ST, Mascarin, GM (2017) Mass production of fungal entomopathogens. In L. A. Lacey (Ed.), Microbial control of insect and mite pests: *From theory to practice* (p. 141–155). Amsterdam: Academic Press.

Jaber LR, Enkerli J (2017) Fungal entomopathogens as endophytes: can they promote plant growth? Biocontrol Sci Technol 27:28–41.

Jackson MA, Dunlap CA, Jaronski ST (2010) Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55:129–145.

Jackson MA, Mcguire MR, Lacey LA, Wraight SP (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus.* Mycol Res 101:35–41.

Jackson M a., Payne AR (2007) Evaluation of the desiccation tolerance of blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) using a lab-scale, air-drying chamber with controlled relative humidity. Biocontrol Sci Technol 17:709–719.

Kassa A, Stephan D, Vidal S, Zimmermann G (2004) Production and processing of *Metarhizium anisopliae* var. *acridum* submerged conidia for locust and grasshopper control. Mycol Res 108:93–100.

Kepler RM, Humber RA, Bischoff JF, Rehner SA (2014) Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. Mycologia 106

Kepler RM, Luangsa-Ard JJ, Hywel-Jones NL, Quandt CA, Sung G-H, Rehner S a., Aime MC, Henkel TW, Sanjuan T, Zare R, Chen M, Li Z, Rossman AY, Spatafora JW, Shrestha B (2017) A phylogenetically-based nomenclature for Cordycipitaceae (Hypocreales) IMA Fungus 8:335–353.

Kepler RM, Ugine TA, Maul JE, Cavigelli MA, Rehner SA. (2015) Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ Microbiol 17(8), 2791–2804

Keyser CA, De Fine Licht HH, Steinwender BM, Meyling NV (2015) Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol 15:249.

Kim JS, Je YH, Skinner M, Parker BL (2013) An oil-based formulation of *Isaria fumosorosea* blastospores for management of greenhouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). Pest Manag Sci 69:576–581.

Klingen I, Haukeland S (2006) The soil as a reservoir for natural enemies of pest insects and mites with emphasis on fungi and nematodes. In: Eilenberg J, Hokkanen HMT (eds) An Ecological and Societal Approach to Biological Control. Springer Netherlands, Dordrecht, pp 145–211

Lane BS, Trinci APJ, Gillespie AT (1991) Endogenous reserves and survival of blastospores of *Beauveria bassiana* harvested from carbon- and nitrogen-limited batch cultures. Mycol Res 95:821–828.

Leles RN, Sousa NA, Rocha LFN, Santos AH, Silva HHG, Luz C (2010) Pathogenicity of some hypocrealean fungi to adult *Aedes aegypti* (Diptera: Culicidae). Parasitol Res 107:1271–1274.

Liao X, O'Brien TR, Fang W, St. Leger RJ (2014) The plant beneficial effects of *Metarhizium* species correlate with their association with roots. Appl Microbiol Biotechnol 98:7089–7096.

Lipa JJ, Lacey LA, Kaya HK. (Eds.) 2007 Field Manual of Techniques in Invertebrate Pathology. 2nd Edition. Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests. Springer, Dordrecht, 868 pp. ISBN 978-1-4020-5932-2.

Lopes RB, Faria M, Souza DA, Bloch C, Silva LP, Humber RA (2014) MALDI-TOF mass spectrometry applied to identifying species of insect-pathogenic fungi from the *Metarhizium anisopliae* complex. Mycologia 106:865–78.

Lopes RB, Mesquita LM, Tigano MS, Souza DA, Martins I, Faria M (2013a) Diversity of indigenous *Beauveria* and *Metarhizium* spp. in a commercial banana field and their virulence toward cosmopolites sordidus (Coleoptera: Curculionidae). Fungal Ecol 6:356–364.

Lopes RB, Souza DA, Oliveira CM, Faria M (2013b) Genetic Diversity and Pathogenicity of *Metarhizium* spp. Associated with the White Grub *Phyllophaga capillata* (Blanchard) (Coleoptera: Melolonthidae) in a Soybean Field. Neotrop Entomol 42:436–438.

Luz C, D'Alessandro WB, Rodrigues J, Fernandes ÉKK (2016) Efficacy of water- and oil-in-water-formulated *Metarhizium anisopliae* in *Rhipicephalus sanguineus* eggs and eclosing larvae. Parasitol Res 115:143–149.

Luz C, Rocha LFN, Montalva C, Souza DA, Botelho ABRZ, Lopes RB, Faria M, Delalibera I (2019) *Metarhizium humberi* sp. nov. (Hypocreales: Clavicipitaceae), a new member of the PARB clade in the *Metarhizium anisopliae* complex from Latin America. J Invertebr Pathol 166:107216.

MA LJ, Van Der Does HC, Borkovich KA., Coleman JJ, Daboussi MJ, Di Pietro A, Dufresne M et al. (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464:367–373.

Malagocka J, Grell MN, Lange L, Eilenberg J, Jensen AB (2015) Transcriptome of an entomophthoralean fungus (*Pandora formicae*) shows molecular machinery adjusted for successful host exploitation and transmission. J Invertebr Pathol 128:47–56.

Mascarin GM, Jackson M a., Behle RW, Kobori NN, Júnior ÍD (2016) Improved shelf life of dried *Beauveria bassiana* blastospores using convective drying and active packaging processes. Appl Microbiol Biotechnol.

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Dunlap CA, Delalibera I (2015a) Glucose concentration alters dissolved oxygen levels in liquid cultures of *Beauveria bassiana* and affects formation and bioefficacy of blastospores. Appl Microbiol Biotechnol 99:6653–6665.

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Delalibera I (2015b) Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. J Invertebr Pathol 127:11–20.

Mascarin GM, Kobori NN, de Jesus Vital RC, Jackson MA, Quintela ED (2014) Production of microsclerotia by Brazilian strains of *Metarhizium* spp. using submerged liquid culture fermentation. World J Microbiol Biotechnol 30:1583–1590.

Mascarin GM, Lopes RB, Delalibera Í, Fernandes ÉKK, Luz C, Faria M (2018) Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. J Invertebr Pathol 165:46-53.

Montalva C, Collier K, Rocha LFN, Inglis PW, Lopes RB, Luz C, Humber R a. (2016) A natural fungal infection of a sylvatic cockroach with *Metarhizium blattodeae* sp. nov., a member of the *M. flavoviride* species complex. Fungal Biol 120:655–665.

Morgan CA, Herman N, White PA, Vesey G (2006) Preservation of micro-organisms by drying; A review. J Microbiol Methods 66:183–193.

Murigu MM, Nana P, Waruiru RM, Nganga CJ, Ekesi S, Maniania NK (2016) Laboratory and field evaluation of entomopathogenic fungi for the control of amitraz-resistant and susceptible strains of *Rhipicephalus decoloratus*. Vet Parasitol 225:12–18.

Nagalakshmi (2008) The Transcriptional Landscape of the Yeast Genome Defined by RNA Sequencing. Science 320:1344–1349.

Nana P, Ekesi S, Nchu F, Maniania NK (2016) Compatibility of *Metarhizium anisopliae* with *Calpurnia aurea* leaf extracts and virulence against *Rhipicephalus pulchellus*. J. Appl. Entomol.140: 590–597.

Nault LR, Delong DM (1980) Evidence for Co-evolution of Leafhoppers in the Genus *Dalbulus* (Cicadellidae: Homoptera) with Maize and Its Ancestors1. Ann Entomol Soc Am 73:349–353.

Nault, LR (1990). Evolution of insect pest: maize and leafhopper, a case study. Maydica 35: 165- 175

NCBI, 2019: National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/ (Accessed 15 November, 2019).

Nigg M, Bernier L (2017) Large-scale genomic analyses of in vitro yeast-mycelium dimorphism in human, insect and plant pathogenic fungi: From ESTs to RNAseq experiments. Fungal Biol Rev 31:131–142.

Nigg M, Laroche J, Landry CR, Bernier L (2015) RNAseq Analysis Highlights Specific Transcriptome Signatures of Yeast and Mycelial Growth Phases in the Dutch Elm Disease Fungus *Ophiostoma novo-ulmi*. Genes|Genomes|Genetics 5:2487–2495.

Niu X, Xie W, Zhang J, Hu Q (2019) Biodiversity of Entomopathogenic Fungi in the Soils of South China. Microorganisms 7:311.

Parra JRP (2014) Biological control in Brazil: An overview. Sci Agric 71:420–429

Pattemore J a, Hane JK, Williams AH, Wilson B Al, Stodart BJ, Ash GJ (2014) The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. BMC Genomics 15:660.

Pedrini N (2017) Molecular interactions between entomopathogenic fungi (Hypocreales) and their insect host: Perspectives from stressful cuticle and hemolymph battlefields and the potential of dual RNA sequencing for future studies. Fungal Biol 122:538–545.

Pendland JC, Boucias DG (1997) In vitro growth of the entomopathogenic hyphomycete *Nomuraea rileyi*. Mycologia 89:66–71.

Qu S, Wang S (2018) Interaction of entomopathogenic fungi with the host immune system. Dev Comp Immunol 83:96–103.

Rezende JM, Zanardo ABR, da Silva Lopes M, Delalibera I, Rehner SA. (2015) Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. BioControl. 60(4):495-505.

Rocha LFN, Inglis PW, Humber R a., Kipnis A, Luz C (2013) Occurrence of *Metarhizium* spp. in Central Brazilian soils. J Basic Microbiol 53:251–259.

Rouxel T, Grandaubert J, Hane JK, Hoede C, Van De Wouw AP, Couloux A, Dominguez V, Anthouard V, Bally P, Bourras S, Cozijnsen AJ, Ciuffetti LM, Degrave A, Dilmaghani A, Duret L, Fudal I, Goodwin SB, Gout L, Glaser N et al. (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. Nat Commun 2:202.

Samish M, Rot A, Ment D, Barel S, Glazer I, Gindin G (2014a) Efficacy of the entomopathogenic fungus *Metarhizium brunneum* in controlling the tick *Rhipicephalus annulatus* under field conditions. Vet Parasitol 206:258–266 .

Samish M, Rot A, Ment D, Barel S, Glazer I, Gindin G (2014b) Efficacy of the entomopathogenic fungus *Metarhizium brunneum* in controlling the tick Rhipicephalus annulatus under field conditions. Vet Parasitol 206:258–266.

Sasan RK, Bidochka MJ (2012) The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. Am J Bot 99:101-107

Sasan RK, Bidochka MJ (2013) Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. Can J Plant Pathol 35:288–293.

Schröter C, Hipler UC, Wilmer A, Künkel W, Wollina U (2000) Generation of reactive oxygen species by *Candida albicans* in relation to morphogenesis. Arch Dermatol Res 292:260–264.

Shang Y, Xiao G, Zheng P, Cen K, Zhan S, Wang C (2016) Divergent and convergent evolution of fungal pathogenicity. Genome Biol Evol 8(5):1374-1387.

Shapiro-Ilan DI, Cottrell TE, Jackson M a., Wood BW (2008) Virulence of Hypocreales fungi to pecan aphids (Hemiptera: Aphididae) in the laboratory. J Invertebr Pathol 99:312–317.

Shendure J, Balasubramanian S, Church GM, Gilbert W, Rogers J, Schloss JA, Waterston RH (2017) DNA sequencing at 40: Past, present and future. Nature 550:345-353.

Skrobek A, Butt TM (2005) Toxicity testing of destruxins and crude extracts from the insect-pathogenic fungus *Metarhizium anisopliae*. Fems Microbiol Lett 251:23-28.

Sonenshine, D (1991). The Biology of Ticks. Oxford University Press, New York. pp 331-369.

Song Z, Yin Y, Jiang S, Liu J, Chen H, Wang Z (2013) Comparative transcriptome analysis of microsclerotia development in *Nomuraea rileyi.* BMC Genomics 14:411.

Song Z, Yang J, Xin C, Xing X, Yuan Q, Yin Y, Wang Z (2018) A transcription factor, MrMsn2, in the dimorphic fungus *Metarhizium rileyi* is essential for dimorphism transition, aggravated pigmentation, conidiation and microsclerotia formation. Microb Biotechnol 11:1157–1169.

Sosa-Gómez DR, López Lastra CC, Humber R a. (2010) An overview of arthropod-associated fungi from Argentina and Brazil. Mycopathologia 170:61–76.

Sosa-gómez DR, Delpin KE ,Moscardi F, Nozaki M (2003) The Impact of fungicides on *Nomuraea rileyi* (Farlow) Samson epizootics and on populations of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), on soybean. Neotropical Entomology, Londrina, 32(2):287-291

Steinwender BM, Enkerli J, Widmer F, Eilenberg J (2011) Molecular diversity of the *Metarhizium anisopliae* lineage in an agricultural field. Journal of Applied Entomology, Washington, 66:113–115.

Steinwender BM, Enkerli J, Widmer F, Eilenberg J, Kristensen HL, Bidochka MJ, Meyling N V. (2015) Root isolations of *Metarhizium* spp. from crops reflect diversity in the soil and indicate no plant specificity. J Invertebr Pathol 132:142–148.

Sujii ER, Tigano, Myrian S, Sosa-Gomes D (2002). Simulação do impacto do fungo *Nomuraea rileyi* em populações da lagarta da soja, *Anticarsia gemmatalis*. Pesquisa Agropecuária Brasileira, 37(11), 1551-1558.

Sun B Da, Liu XZ (2008) Occurrence and diversity of insect-associated fungi in natural soils in China. Appl Soil Ecol 39:100–108.

Téllez-Martínez MG, Alcalá-Gómez G, Jiménez-Islas H, Navarrete-Bolaños JL (2016) Design of an efficient fermentation process for the production of *Metarhizium acridum* blastospores. Biocontrol Sci Technol 26:1652–1667.

Tholander M, Fekete C, Rajashekar B, Friman E, Johansson T, Tunlid A (2005) Comparison of gene expression in trap cells and vegetative hyphae of the nematophagous fungus *Monacrosporium haptotylum*. 789–803.

Toffanelli CM, Bedendo IP (2002) Efeito da população infetiva de *Dalbulus maidis* na produção de grãos e no desenvolvimento de sintomas do enfezamento vermelho do milho. Fitopatol Bras 27:82–86.

Trienens M, Rohlfs M (2012). Insect-fungus interference competition - the potential role of global secondary metabolite regulation, pathway-specific mycotoxin expression and formation of oxylipins. *Fungal Ecol.* 5 191–199.

Triplehorn BW, Nault LR, Phylogenetic Classification of the Genus *Dalbulus* (Homoptera: Cicadellidae), and Notes on the Phylogeny of the Macrostelini (1985) *Annals of the Entomological Society of America*, v.78, p.291–315.

Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344.

Van Lenteren JC (2012) The state of commercial augmentative biological control: Plenty of natural enemies, but a frustrating lack of uptake. BioControl 57:1–20.

Van Lenteren JC, Bolckmans K, Köhl J, Ravensberg WJ, Urbaneja A (2017) Biological control using invertebrates and microorganisms: plenty of new opportunities. BioControl 1–21.

Van Nieuwenhove GA, Frías EA, Virla EG (2016) Effects of temperature on the development, performance and fitness of the corn leafhopper *Dalbulus maidis* (DeLong) (Hemiptera: Cicadellidae): Implications on its distribution under climate change. Agric For Entomol 18:1–10.

Van Vliet AHM (2010) Next generation sequencing of microbial transcriptomes: Challenges and opportunities. FEMS Microbiol Lett 302:1–7.

Vega FE, Goettel MS, Blackwell M, Chandler D, Jackson M a., Keller S, Koike M, Maniania NK, Monzón A, Ownley BH, Pell JK, Rangel DEN, Roy HE (2009) Fungal entomopathogens: new insights on their ecology. Fungal Ecol 2:149–159.

Vega FE, Jackson MA, McGuire MR (1999) Germination of conidia and blastospores of *Paecilomyces fumosoroseus* on the cuticle of the silverleaf whitefly, *Bemisia argentifolii*. Mycopathologia 147:33–35.

Ypsilos IK, Magan N (2005) Characterisation of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. Biocontrol Sci Technol 15:683–699.

Wall R, Shearer D (2001). In: Wall R, Shearer D. (Eds.), “Ticks(Acari),” in Veterinary Ectoparasites: Biology. Pathology and Control, second ed. Blackwell Science, 55-82.

Wang C, Hu G, St Leger RJ (2005) Differential gene expression by *Metarhizium anisopliae* growing in root exudate and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fungal Genet Biol 42:704–18.

Wang C, St Leger RJ (2006) A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci U S A 103:6647–6652.

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63.

Wang Z-X, Zhou X-Z, Meng H-M, Liu Y-J, Zhou Q, Huang B (2014) Comparative transcriptomic analysis of the heat stress response in the filamentous fungus *Metarhizium anisopliae* using RNA-Seq. Appl Microbiol Biotechnol 98:5589–97.

Wang JB, Leger RJ St., Wang C (2016) Chapter Three - Advances in Genomics of Entomopathogenic Fungi. In: Lovett B, Leger RJ St. (eds) Genetics and Molecular Biology of Entomopathogenic Fungi. Academic Press, pp 67–105

Wassermann M, Selzer P, Steidle JLM, Mackenstedt U (2016) Biological control of *Ixodes ricinus* larvae and nymphs with *Metarhizium anisopliae* blastospores. Ticks Tick Borne Dis. 7(5):768-771.

Wyrebek M, Huber C, Sasan RK, Bidochka MJ (2011) Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157:2904–11.

Xavier-Santos S, Lopes RB, Faria M (2011) Emulsifiable oils protect *Metarhizium robertsii* and *Metarhizium pingshaense* conidia from imbibitional damage. Biol Control 59:261–267.

Xiao G, Ying S-H, Zheng P, Wang Z-L, Zhang S, Xie X-Q, Shang Y, St. Leger RJ, Zhao G-P, Wang C, Feng M-G (2012) Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci Rep 2:483.

Xu YJ, Luo F, Li B, Shang Y, Wang C (2016) Metabolic conservation and diversification of *Metarhizium* species correlate with fungal host-specificity. Front Microbiol 7:1–12.

Yek SH, Boomsma JJ, Schiott M (2013) Differential gene expression in *Acromyrmex* leaf-cutting ants after challenges with two fungal pathogens. Mol Ecol 22:2173–2187.

Zhao H, Chen J, Liu J, Han B (2015) Transcriptome analysis reveals the oxidative stress response in *Saccharomyces cerevisiae*. RSC Adv 5:22923–22934.

Zimmermann G (2007) Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Sci Technol 17:879–920.

2. MODIFIED ADAMEK'S MEDIUM RENDERS HIGH YIELDS OF *Metarhizium robertsii* BLASTOSPORES THAT ARE DESICCATION TOLERANT AND INFECTIVE TO CATTLE-TICK LARVAE

Natasha Sant'Anna Iwanicki [a,*], Bruna de Oliveira Ferreira[a], Gabriel Moura Mascarin[b], Italo Delalibera Júnior[a]

[a] Department of Entomology and Acarology, Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo (ESALQ-USP), Av. Pádua Dias, 11, C.P. 9, Piracicaba, SP CEP 13418-900, Brazil

[b] Laboratory of Environmental Microbiology, Brazilian Agricultural Research Corporation, Embrapa Environment, Rodovia SP 340, Km 127.5, 13820-000 Jaguariúna, Brazil.

*Corresponding author: Natasha S. A. Iwanicki
E-mail address: natasha.iwanicki@usp.br
Department of Entomology and Acarology, "Luiz de Queiroz" College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

Manuscript submitted to the Journal Fungal Biology on 4 April 2018, accepted on 17 May 2018, and published online on 25 May 2018 (doi: 10.1016/j.funbio.2018.05.004)

Abstract

Blastospores are yeast-like cells produced by entomopathogenic fungi that are infective to arthropods. The economical feasible production of blastospores of the insect killing fungus *Metarhizium* spp. must be optimized to increase yields. Moreover, stabilization process is imperative for blastospore formulation as a final product. In this sense, our goal was to increase blastospore production of two *Metarhizium* isolates (ESALQ1426 and ESALQ4676) in submerged liquid cultures. A modified Adamek's medium was supplemented with increased glucose concentrations and the fermentation time was accelerated by using a blastospore pre-culture as inoculum. Virulence (speed of kill) of air-dried stable blastospores was compared with conidia toward larvae of the cattle tick, *Rhipicephalus microplus*. Our results revealed that blastospore production of *Metarhizium* is isolate- and species-dependent. Glucose-enriched cultures (140 g glucose/L) inoculated with pre-cultures improved yields with optimal growth conditions attained for *M. robertsii* ESALQ1426 that rendered as high as 5.9×10^8 blastospores/mL within 2 days. Resultant air-dried blastospores of ESALQ1426 were firstly proved to infect and quickly kill cattle tick larvae with comparable efficiency to conidia. Altogether, we argue that both osmotic pressure, induced by high glucose titers, and the proper isolate selection are critical to produce high yields of blastospores that hold promise to control cattle-tick larvae.

Keywords: Hypocreales, Fungal entomopathogen, Liquid culture, Pathogenicity, Rhipicephalus microplus.

2.1 Introduction

Metarhizium spp. (Ascomycota: Hypocreales: Clavicipitaceae) are cosmopolitan fungi used as biological control agents against arthropod pests in agriculture, livestock and vectors of human diseases (Alkhaibari et al., 2016; Camargo et al., 2016; Lee et al., 2015; Van Lenteren et al. 2017). Interestingly, asexual spores termed conidia of *Metarhizium anisopliae* have been applied in Brazil on more than 3 million hectares of sugarcane annually for controlling spittlebugs (Hemiptera: Cercopidae) (Mascarin et al., 2018; Parra, 2014). This is indeed one of the most successful biological control programs ever practiced using a single fungal entomopathogen in the world.

Nonetheless, besides conidia, other fungal infective structures have been investigated for use in biological control programs. One such type of propagule is a yeast-like cell termed blastospore. Blastospores are formed in the arthropod hemolymph

during fungus infection. When applied against some hosts, earlier reports document that blastospores can be even more virulent than conidia (Alkhaibari et al., 2016, 2017; Jackson et al., 1997; Kim et al., 2013; Mascarin et al., 2015a, 2015b; Mascarin et al., 2016; Wassermann et al., 2016); moreover, the process for blastospore production has a number of advantages over that for conidia production (Jackson et al., 1997; Jaronski and Mascarin, 2016).

Large amounts of blastospores (>10^8 blastospores/mL) can be achieved by liquid culture fermentation in a shorter time (less than 4 days), in a smaller space and with lower labor requirement, than the solid-substrate fermentation method used for conidia production. Furthermore, the liquid fermentation method has a standardized quality similar to that found in commercial yeast production, which facilitates its scale-up production and downstream processing. In spite of clear advantages in the system production of blastospores relatively to that of conidia, the former is highly vulnerable to environmental stress conditions (Jaronski and Mascarin, 2016).

Therefore, to develop a blastospore biopesticide, it is necessary to devise formulations that afford good stability and protection against deleterious environmental factors. With this in mind, one of the key conditions for developing a blastospore biopesticide arises from the stabilization process to tolerate drying. Stabilization is necessary to decrease the blastospore metabolism while keeping it viable for long periods. Once stabilized, blastospores can be formulated aiming at shelf life extension and meeting the requirements for commercialization (Jackson et al., 1997; Kim et al., 2013; Mascarin et al., 2016).

There are several technologies used for cell stabilization but one of the most feasible and economically viable is air-drying, which consists of a slow drying process where the relative humidity of the air flow is controlled throughout the course of drying in order to maintain cell integrity (Jackson and Payne, 2007; Mascarin et al., 2016). Blastospores of some fungi can resist the air-drying process and remain highly viable for up to 12 months (Mascarin et al., 2015a, 2016). Some studies with the entomopathogenic fungi of the genera *Beauveria* and *Isaria* showed that stabilized blastospores can be significantly more virulent than conidia toward some hosts (Jackson et al., 1997; Mascarin et al., 2015a, 2015b, 2016; Vandemberg et al., 1998); However, this outcome is highly dependent on the fungal isolate and on the medium composition.

The standard Adamek´s medium has been successfully used to produce *Metarhizium* blastospores although the yields rarely reach high concentrations (>10^8 blastospores/mL). Recently Adamek´s medium has been shown to support the infectivity of fresh blastospores produced against the larvae of mosquitoes (Alkhaibari et al., 2016, 2017) and of *Ixodes ricinus* ticks (Wassermann et al., 2016).

The cattle-tick, *Rhipicephalus microplus* Canestrini (Acari: Ixodidae), is a one-host ectoparasite of livestock that inhabits mainly the tropical and subtropical regions of the world (Estrada-Peña et al., 2006). This ectoparasite is a vector of important pathogens: the bacterium *Anaplasma marginale*, and the protozoa *Babesia bigemina* and *B. bovis* Babes, responsible for causing the bovine diseases anaplasmosis and babesiosis, which results in an economic loss of US$3.2 billion per year (Grisi et al., 2014) by dramatically reducing weight gain of cows, milk production, the price of leather and the survival of calves.

Cattle-ticks have hitherto been prophylactically controlled with synthetic acaricides applied mainly as a *pour-on* directly on cows. However, concerns about the residues of acaricides in milk, the increasing threat posed by acaricide resistance in tick populations (Abbas et al., 2014; Mendes et al., 2013) and the lack of other alternatives to cattle-tick control have highlighted biological control as a promising alternative for controlling ticks in the field.

It is estimated that only 10-20% of cattle-ticks are actually in their hosts, whereas the remaining 80-90% are in the non-parasitic phase in the field, a phase mainly represented by larvae (Leal et al., 2017) that can survive long periods without feeding. Considering that each engorged female lays 2000 to 4500 eggs on the ground (Wall and Shearer, 2001) and that larvae can survive for long periods, spraying fungal infective structures on grasslands is a potential alternative for integrated management of cattle-tick.

The efficiency of *Metarhizium* spp. conidia against ticks under field conditions has been well reported in the literature (Camargo et al., 2016; Murigu et al., 2016; Ojeda-Chi et al., 2010; Samish et al., 2014). However, the effective control of cattle ticks requires high concentrations of conidia (up to 1×10^9 conidia/mL), sprayed over extensive grassland areas (Fernandes et al., 2011), which may discourage the use of fungal entomopathogens due to the high cost of spraying.

Taking into account that liquid culture fermentation is relatively quicker as well as more cost-effective than solid-state fermentation and that blastospores can potentially replace conidia for tick control, the aim of this work was to i) Select *Metarhizium* isolates that achieve a high yield of blastospores, ii) Improve Adamek's medium for the production of high concentrations of blastospores in short fermentation times; iii) Assess efficiency and efficacy against *R. microplus* larvae of air-dried blastospores of the *Metarhizium* isolate that showed the highest blastospore yields in the improved medium in comparison with that of conidia.

2.2. Materials and methods

2.2.1. Isolate selection

Eleven *Metarhizium* spp. isolates were chosen from the Entomopathogenic Fungal Collection from ESALQ-University of São Paulo (Piracicaba, Brazil). We selected isolates from *M. anisopliae, M. robertsii, M. brunneum,* and from two species not phylogenetically determined, recovered from different Brazilian states, referred to as *Metarhizium* sp. undet 1*, Metarhizium* sp. undet 4. The selected isolates for each species were: *M. anisopliae*: ESALQ2787, ESALQ4676 and ESALQ1184, *M. brunneum*: ESALQ4999 and ESALQ5181, *M. robertsii*: ESALQ4130 and ESALQ1426, *Metarhizium* sp. indet 1. : ESALQ4164 and ESALQ4207; *Metarhizium* sp. indet. 4: ESALQ1660 and ESALQ1687.

For each isolate, a stock culture of conidia was obtained from monosporic cultures obtained in potato dextrose agar (PDA, Difco®, Sparks, MD, USA), and preserved as sporulated agar chunks immersed in sterile 10% glycerol solution at -80 °C.

2.2.2. Screening isolates for blastospores production in Adamek´s medium

The Adamek's medium contains per liter: 30 g cornsteep liquor (Sigma®, St. Louis, USA), 40 g glucose (Synth®, SP. Brazil), 40 g yeast extract (Amresco® OH, USA) , and 4 mL Tween 80® (polyoxyethylene sorbitan monooleate). A glucose stock solution at a concentration of 20% (200 g/L) was autoclaved separately from other media components to prevent Maillard reaction between reducing sugars and amino

acids under high autoclaving temperature. Media preparation was carried out in two steps: for making 50 mL of liquid medium we added 10 mL of 20% glucose stock solution to deliver a final concentration of 4% glucose into 35 mL of basal medium containing protein sources and Tween 80®. To add it up, we inoculated 5 mL of fungal inoculum. The initial pH of all liquid media was adjusted to 6.8 after autoclaving (Adamek, 1965).

Conidia were used to inoculate the Adamek´s medium. For that, isolates from the stock culture were cultured in Petri dishes containing PDA (Difco®, Sparks, MD, USA) and incubated in a growth chamber at 25 °C for 10-15 days with 12 h photoperiod. Conidial suspensions were obtained by washing the Petri dishes containing fully sporulated fungus with 10 mL of a sterile aqueous solution made with 0.02% Tween® 80. The standard concentration of 5×10^6 conidia/mL was used for inoculation of the Adamek's medium, delivering a final concentration of 5×10^5 conidia/mL (i.e., 10% v/v inoculum). Each fungal isolate was grown in 50 mL of medium in four replicates in 250-mL baffled Erlenmeyer flasks (Bellco® Glass, Vineland, NJ, USA) during a period of 4 days at 28 °C and 350 rpm using a rotatory incubator shaker (Marconi®, SP, Brazil). Every day the flasks were shaken by hand to minimize the mycelial growth on the flask's wall. The screening experiment were repeated twice using new fungal culture preparations on different dates (total of n = 8 replicates per fungal isolate).

Blastospore yields were evaluated after two, three and four days of culture. Each day, aliquots of 1 mL were taken from each flask for evaluating the concentration of blastospores/mL using a Neubauer chamber under a phase contrast microscope at 400X magnification.

2.2.3. Blastospores production in Modified Adamek's medium

The nutritional composition of Modified Adamek's medium per liter was: 80 g yeast extract, 40 g cornsteep liquor, 20 µL of Tween 80® and minerals, trace metals and vitamins adapted from Jackson et al. (1997) at the following concentrations per liter: KH_2PO_4, 2.5 g; $CaCl_2.2H_2O$, 1.0 g; $MgSO_4.7H_2O$, 0.83g; $FeSO_4.7H_2O$, 0.3g ; $CoCl_2.6H_2O$, 29.6 mg; $MnSO_4.H_2O$, 12.8 mg; $ZnSO_4.7H_2O$, 11.2 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 0.2 mg each; and folic

acid, biotin, vitamin B12, 0.02 mg each. The media were supplied either with 40 g/L or 140 g/L of glucose. Two glucose stock solutions (200 g/L and 700 g/L) were autoclaved separately and sterile solutions of vitamins and metals, previously filtered in Millipore® membrane (0.22 µm pore size), were added to the autoclaved medium. Filling medium volume was set to 50 mL in a 250-mL baffled Erlenmeyer flask and fungal growth carried out for three days at 28 °C and 350 rpm. There were four culture flasks (replicates) per each fungal isolate. The entire fermentation experiment was conducted twice over time with a total of n= 8 replicates (culture flasks for each treatment). The procedure for blastospores yield was evaluated as stated before for the screening experiment (see section 2.2).

2.2.4. Glucose-enriched medium with and without preculture

The experiment included two fungal isolates (*M. robertsii* ESALQ1426 and *M. anisopliae* ESALQ 4676) cultured in Modified Adamek's medium with either 140 or 40 g glucose/L. In experiment without preculture, conidia were used as an inoculum delivering a final concentration of 5×10^5 conidia/mL. In experiment with preculture, two-day-old blastospores produced in a Modified Adamek's medium with 40 g/L of glucose (pre-culture) were inoculated in Modified Adamek's medium with 140 g/L (culture). The cultures were inoculated with blastospores from pre-culture to deliver a final concentration of 5×10^6 blastospores/mL.

2.2.5. Stabilization of blastospores in air-drying system

For blastospore stabilization, we followed the protocol developed by Mascarin et al. (2015b). Briefly, 7.5% of diatomaceous earth was added in two days culture of *M. robertsii* isolate ESALQ1426 in medium with 140 g/L of glucose from the experiment with pre-culture**.** The mixture containing the fungal propagules + diatomaceous earth was filtered with a vacuum pump coupled to a Buchner funnel lined with disks of 8 cm diameter of Whatman® filter paper. The mixture was placed in an electrical blender to be broken down into smaller particles. Next, the mixture was placed into aluminum trays for drying under aseptic conditions in a controlled humidity air-dry chamber for 15-18 h with 50-60% RH and then for 2 more h in 15%

RH, as described by Mascarin et al. (2015a). The water activity of the mixture was evaluated using a water activity meter (Novasina - Labmaster AW). Then, the mixture was dried until it reached a moisture content of less than 5% (w/w), and was subsequently vacuum-packed in polyethylene bags and stored at 4 °C until the bioassay experiment.

2.2.6. Bioassay with stabilized blastospores against cattle tick larvae

Suspensions containing 5 $\times 10^7$ and 1 $\times$ 10^7 conidia or air-dried blastospores/mL were prepared with 0.02% Tween® 80. The viability of stabilized blastospores was assessed by diluting 0.35g of fungus + diatomaceous earth in 50 mL of sterile distillated water in Erlenmeyer flasks and left in shaker with 350 rpm for 10 minutes. After that, the suspension was filtered using two layers of cheesecloth and the concentration was adjusted to 10^6 blastospores/mL. A volume of 150 µL of the suspension was placed on 2% malt + 1% yeast extract agar in Petri dishes and incubated in a growth chamber at 25 ± 1 °C for 6 h followed by assessment of viable blastospores. In the case of conidia, 150 µL of a 10^6 conidia/mL suspension was placed on PDA plates and the viability was assessed after 20 h incubation at the same temperature and photoperiod used for blastospores viability.

Cattle ticks engorged females were collected directly from cows during milking in the College of Agriculture “Luiz de Queiroz” (University of São Paulo) on a weekly basis. The cows did not receive any chemical treatment against ticks in the previous 30 days. In the laboratory, they were washed in sterile water, dried on filter paper and placed in small dishes (6 cm diameter) with wet cotton in a climatic chamber at 27 ± 1 °C with a 12:12 h (L:D) photoperiod to stimulate oviposition. After two weeks, the dead engorged females were discarded and the eggs incubated in the same conditions until larval hatching.

Tick bioassays was conducted following a randomized experimental design with five fungal treatments with four biological replicates per treatment. The experimental unit *per se* consisted of small glass plates (6 cm diameter) with a mixture of plaster and charcoal (9:1) at the bottom and moistened with distilled water (Abbatiello, 1965). When moistened, plaster-charcoal mixture keeps a high humidity inside the experimental unit. Treatments were established with four replicates each

(total n = 80 larvae). Twenty larvae with 18-24 days old were transferred to each plate and covered with a plastic film. With the aid of a Potter Tower equipment (Burkard Ltd., UK), larvae in plates were sprayed with 2 mL of each fungal suspension and subsequently incubated in a growth chamber at 27 ± 1 °C with a 12:12 h (L:D) photoperiod. Dead larvae were recorded every two days during 13 days after the application. To confirm that the mortality was due to the pathogen, dead larvae were transferred to a humid chamber and kept at 28 °C until mycosis (fungal outgrowth) could be seen. The whole virulence bioassay was repeated twice on different occasions using different batches of fungal preparations and tick larvae collected from the field.

2.2.7 Statistical analyses

Blastospore concentration datasets were $\log_{10}$-transformed prior to analysis of variance in order to meet normality assumptions, and then transformed data were fitted to a linear mixed model with a random effect for experiment date and fixed effects attributed to fungal treatments and evaluation days. When the interaction term between “day x fungal treatment” was significant, means were separated by post-hoc Tukey’s HSD test ($P < 0.05$) within each evaluation day. Larval mortality data were fitted to a generalized linear mixed model (GLMM) with binomial distribution for errors. Fixed effect was scored to be both fungal propagules tested at two concentrations, while the random effect was attributed to experiment date. Treatment means were separated by Tukey’s HSD test at $P < 0.05$. Furthermore, survival analysis was perform with censored data for dead larvae until day 30 using a parametric model with Weibull distribution that can be written as: $S(t) = \exp(-\lambda t^k)$, where $S(t)$ is the survival probability of tick larva, t is the time in days, λ is the scale parameter, and k is the shape parameter. In that way, median survival times (ST_{50}) were estimated for each fungal treatment and compared with each other by Tukey’s HSD test ($P < 0.05$). In addition, Weibull survival curves were compared based on the log-likelihood ratio test at $P < 0.05$. All analyses were performed in the statistical software R (R Core Development, 2015) and graphics were performed on SigmaPlot version 14.0 (Systat Software, Inc., USA) and R (R Core Development, 2015).

2.3. Results

2.3.1. Screening isolates for blastospore production in Adamek's medium

There was a remarkable variation in blastospore concentration of fungal isolates of different *Metarhizium* species at 2, 3 and 4 days of culture, indicating that these fungal isolates exhibited distinct growth rates (F = 4.05, df = 20, 155, P < 0.0001) (**Fig. 1**). We noted that two isolates of *M. robertsii* (ESALQ1426 and ESALQ4130) were the best producers attaining concentrations up to 3.6-3.8 × 10^8 blastospores/mL by day 4. These isolates also had the quickest growth rates as blastospore yields increased from day 2 to day 4 more rapidly than the other isolates. In contrast, the three fungal isolates belonging to *M. anisopliae* (ESALQ1184, ESALQ2787, ESALQ4676), *M. brunneum* (ESALQ4999, ESALQ5181) and two unknown *Metarhizium* sp. (ESALQ4164, ESALQ4207, ESALQ1660, ESALQ1687) had yields lower than 1.5 × 10^8 blastospores/mL by day 4. *M. robertsii* isolates renders the highest blastospore yield in the screening experiment. Blastospore yields of both *M. robertsii* isolates ESLAQ1426 and ESALQ4130 did not differ statistically across any culture day. Therefore, we selected the *M. robertsii* isolate ESALQ1426 for further experiments. Nonetheless, as we wanted to include in further trials at least one *M. anisopliae* isolate, we selected isolate ESALQ4676, which achieved a higher blastospore yield than the other two *M. anisopliae* isolates at day 4 of cultivation. Therefore, two isolates (ESALQ1426 and ESALQ4676) were selected for further assays.

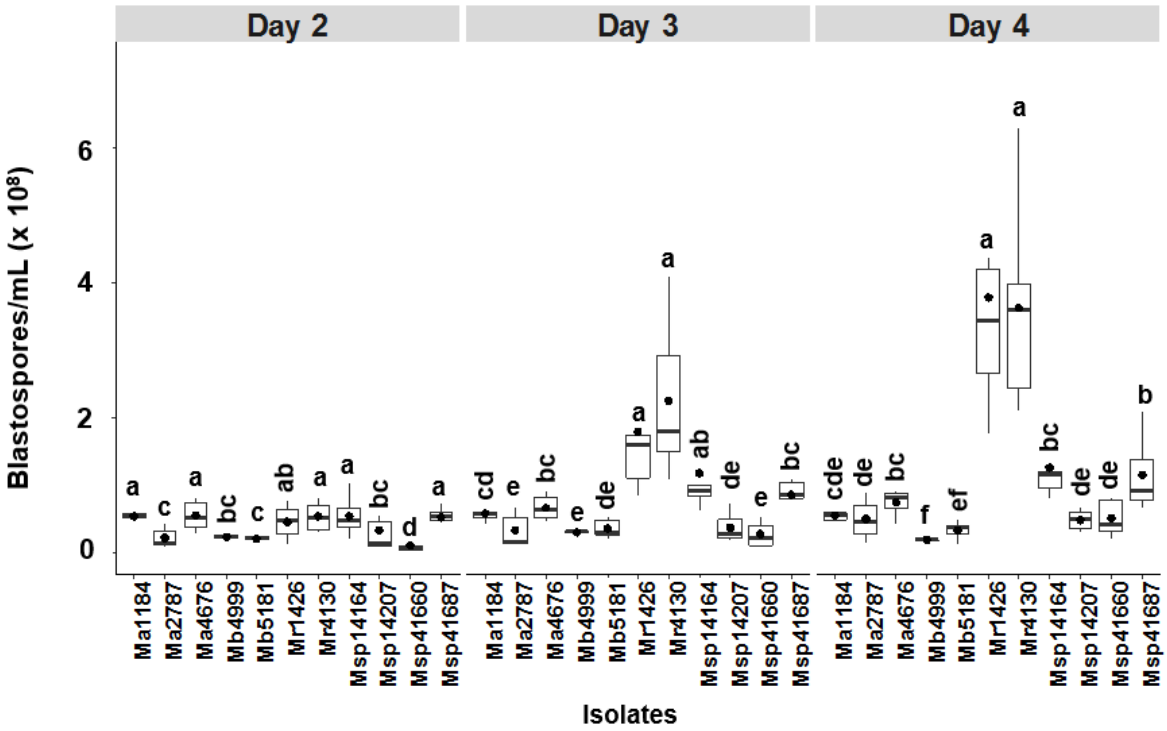


Figure 1. Blastospore yield from liquid cultures of 11 *Metarhizium* isolates grown in standard Adamek's medium and incubated at 28 °C and 350 rpm. Liquid cultures were inoculated with conidial suspensions to deliver a final concentration of 5×10^5 conidia /mL. Bars (mean±SE) followed by different letters, within each day, are significantly different from each other according to Tukey's HSD test ($P < 0.05$). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles. A black dot (.) denotes the mean value of the data.

2.3.2. Blastospore production on glucose-enriched medium

When grown in modified Adamek's medium we found that increasing glucose titers led to shorter fermentation times with a resultant higher concentration of blastospores ($F = 5.65$, df = 1, 56, $P = 0.0209$), irrespective of the fungal isolate ($F = 5.65$, df = 1, 56, $P < 0.0001$). In fact, *M. anisopliae* ESALQ4676 and *M. roberstii* ESALQ1426 at day 3 no longer responded to increased glucose titers as it did at day 2 (**Fig. 2**).

However, on the second day of culture in modified Adamek's medium with 40 g/L of glucose, blastospore yields were almost 2 times higher than in Standard Adamek's medium for ESALQ4676 and remained the same yield for ESALQ1426 (**Fig. 2**). On the other hand, on the same day of culture in modified Adamek's

medium amended with 140 g/L of glucose, blastospore yields were 3 times higher for ESALQ1426 and 3.2 times higher for ESALQ4676 than when cultured in Standard Adamek's medium.

On the third day of culture in modified Adamek's medium with 40 g/L of glucose, blastospore yields were 1.8 times higher for ESALQ1426 and 3.2 times higher for ESALQ4676 than when cultured in Standard Adamek's medium. When grown in modified Adamek's medium with 140 g/L of glucose, blastospore yields were 2.5 times higher for ESALQ1426 and 4.2 times higher for ESALQ4676 than when grown in Standard Adamek's medium.

Taken together, these data show a significant improvement in blastospore yield for both selected isolates when cultured in modified Adamek's medium with both glucose concentrations compared to Standard Adamek's medium.

As a strategy to shorten up the fermentation time for our liquid cultures, we explored the possibility of using pre-cultures based on blastospores grown in only 2 days. The question therefore arose as to whether blastospore pre-culture in modified Adamek's medium amended with 40 g/L of glucose followed by culturing in modified Adamek's medium amended with 140 g/L of glucose was a viable approach to enhance blastospore concentration in shorter fermentation time.

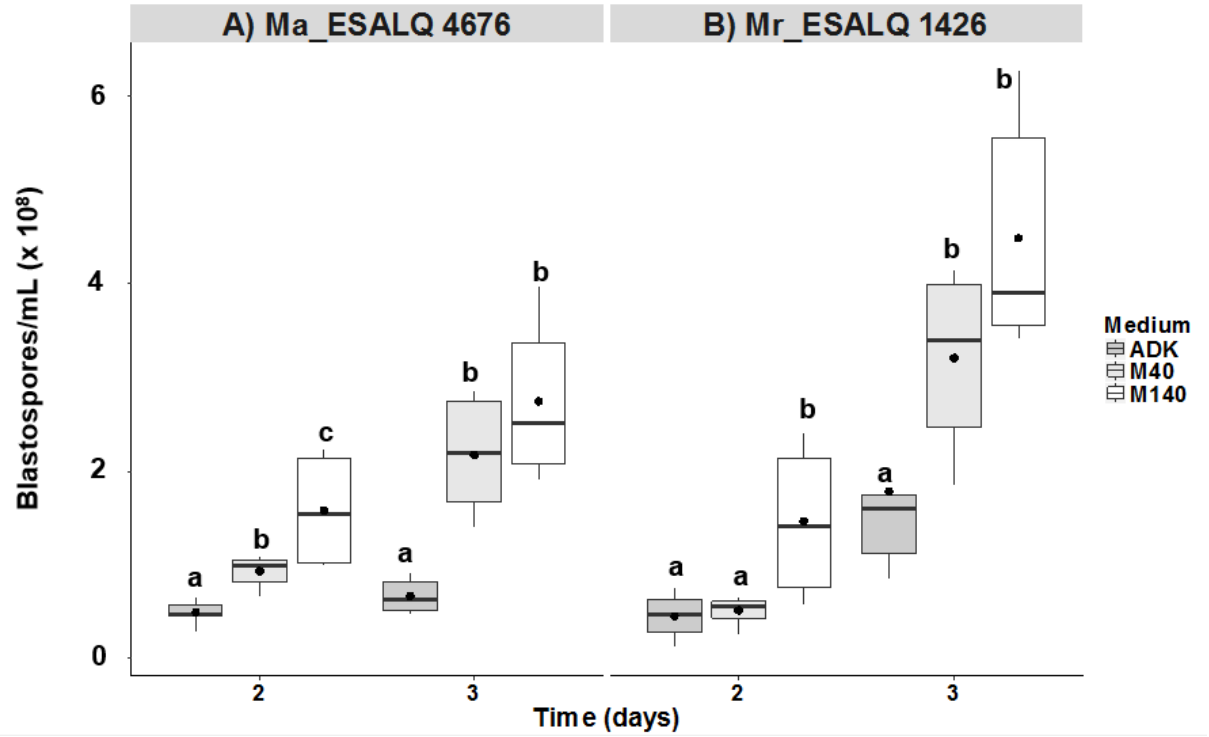


Figure 2. Blastospore yield from liquid cultures of *Metarhizium anisopliae* (Ma_ESALQ4676) (A) and *Metarhizium robertsii* (Mr_ESALQ1426) (B) grown in Adamek´s medium and Modified Adamek's medium with two glucose concentrations (140 g/L or 40 g/L). Incubation temperature, 28 °C and 350 rpm. Bars (mean±SE) followed by different letters, within each day, indicate significant differences according Tukey's HSD test ($P < 0.05$). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles. A black dot (.) denotes the mean value of the data.

2.3.3. Blastospore production by pre-culture followed by cultivation in glucose-enriched modified Adamek's medium

Here we tested, for the two selected isolates, whether blastopore pre-cultured for two days on modified Adamek's medium with 40 g/L of glucose and inoculated in the same medium with 140 g/L of glucose could provide a higher blastospore yield in a shorter period compared to the experiment without pre-culture.

Our data showed that ESALQ1426 achieved a yield of 5.9×10^8 blastospores/mL in only two days of culture in modified Adamek's medium with 140 g/L of glucose (**Fig.**

3). This was the highest yield obtained in this study within the shortest time. This was 4 times higher than in the same conditions but without preculture.

ESALQ4676 produced 4 × 10^8 blastospores/mL in modified Adamek's medium with 140 g/L of glucose at day 2 (**Fig. 3**). This means 2.5 times more blastospores than the experiment with 140 g/L of glucose but without pre-culture, on day 2. The most relevant observation in this experiment was that there was no increase in blastospore yield at day 3 for both isolates (**Fig. 3**).

On the basis of the above results, we selected isolate ESALQ1426, which showed the highest yields in both experiments with modified Adamek's medium, for assessing its infectivity and virulence against cattle-tick larvae.

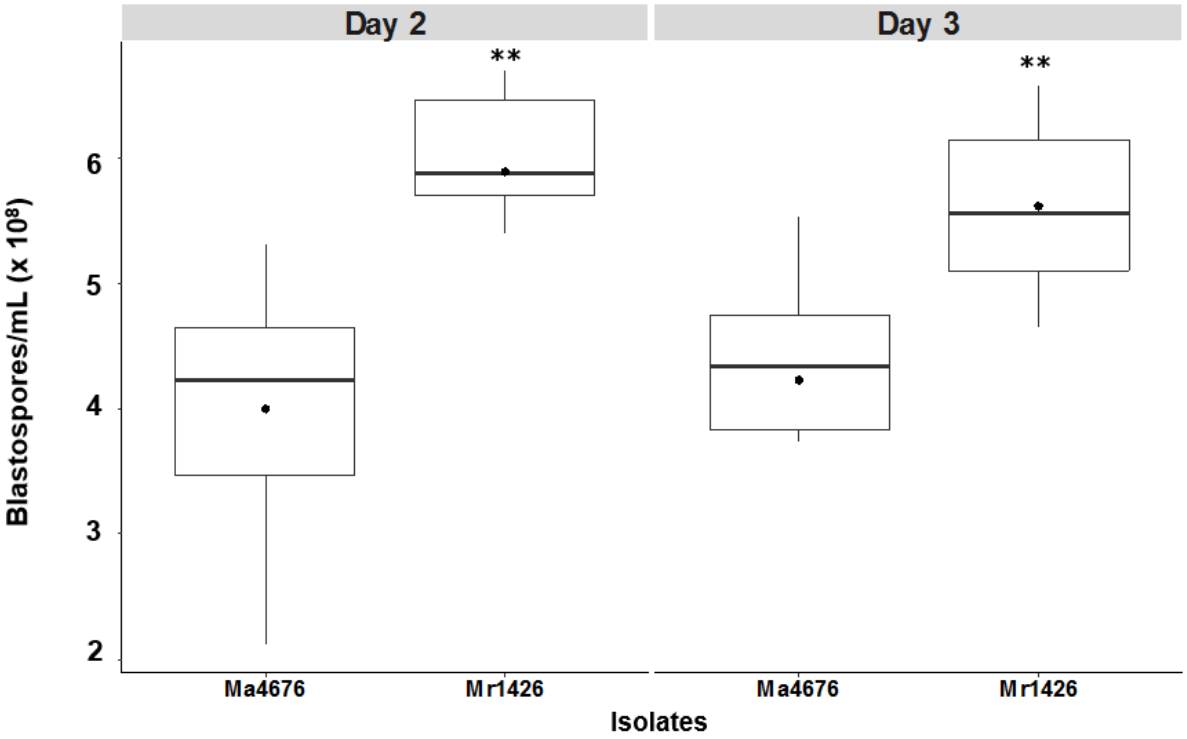


Figure. 3. Blastospore yield from liquid cultures of *Metarhizium robertsii* (Mr_ESALQ1426) and *Metarhizium anisopliae* (Ma_ESALQ 4676) grown in modified Adamek's medium with 140 g glucose/L and incubated at 28 °C and 350 rpm. Liquid cultures were inoculated with blastospore-based pre-cultures to provide a final concentration of 5 × 10^6 blastospores/mL. Bars (mean±SE) followed by **, within each day, indicate significant differences according to *t*-Student test ($P < 0.05$). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles. A black dot (.) denotes the mean value of the data

2.3.4. Bioassay with stabilized blastospores against cattle tick larvae

In this experiment, we evaluated the infectivity of stabilized blastospores of ESALQ1426 against cattle-tick larvae, the mycosis as a confirmation of mortality and the time the blastopores take to kill 50% of the cattle tick larvae. This was compared to results obtained with conidia.

Our data showed that ESALQ1426 blastopores successfully tolerated the air-dried stabilization process with viability ranging from 60 to 65% after 6 h incubation on malt extract agar. At that moment, germ tubes were longer than length of blastospore and were at an appropriate moment to be counted. The viability for conidia was higher than 92% in all cases, but it took 20 h incubation.

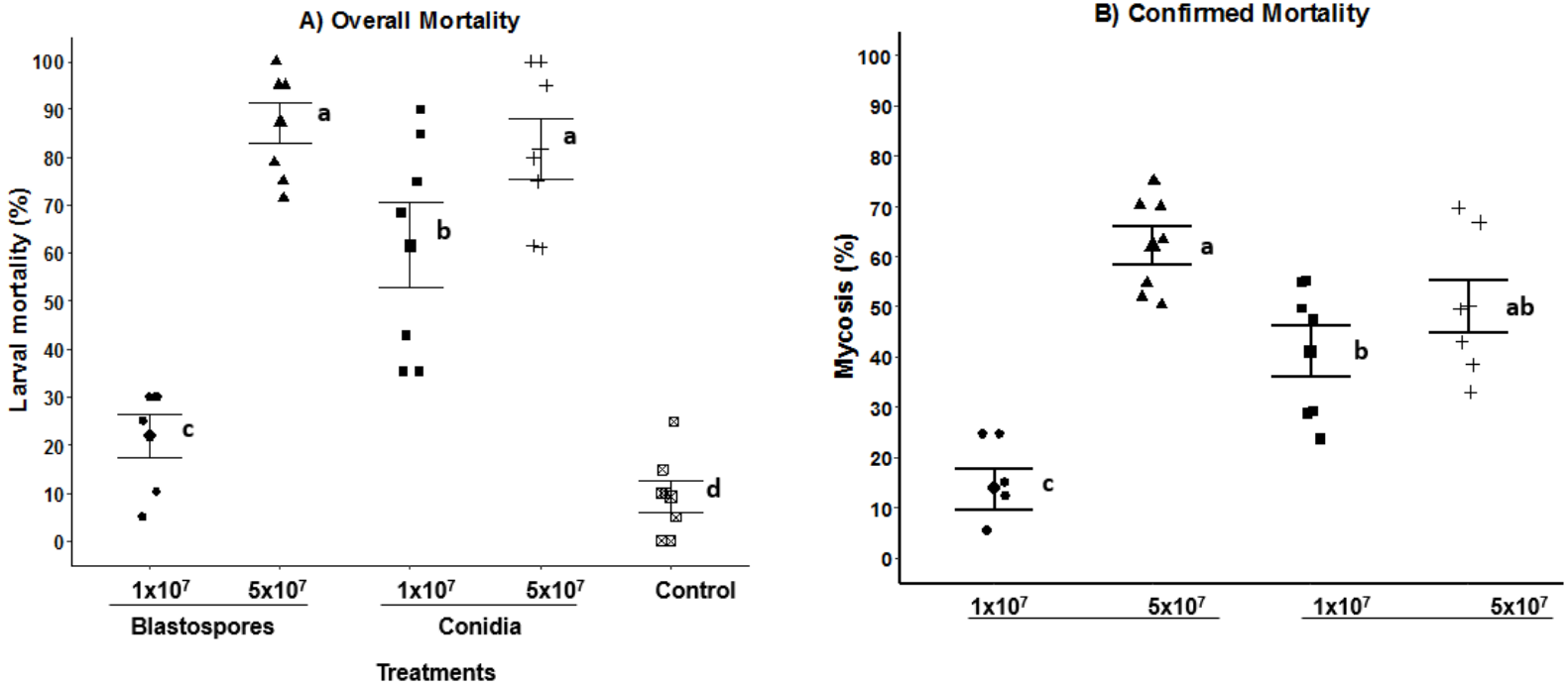
On the 13th day of the bioassay, stabilized blastospores and fresh conidia, both at 5×10^7/mL, killed respectively 87.2% (ST_{50} of 8.18 days) and 81.8% (ST_{50} of 8.84 days) of the cattle-tick larvae (**Fig. 4A**). On the other hand, at the 10^7/mL concentration, blastospores and conidia killed respectively 21.8% (ST_{50} of 19.53 days) and 61.8% (ST_{50} of 10.85 days) of the cattle-tick larvae, showing a strong positive relationship between tick mortality and fungal concentration, regardless of the propagule tested.

Tick larvae after death depicted typical symptoms and signs of mycosis by *M. robertsii* that produced conspicuous masses of light greenish spores emerging from cadavers upon incubation in humid environment. The 5×10^7/mL concentration of stabilized blastospores or conidia rendered 62.2% and 50.1% mycosis, respectively (**Fig. 4B**). At the 10^7/mL concentration, mycosis rates were 13.8% and 41.2%, respectively. In this regard, mycosis rate followed the same pattern of mortality-response dependency on fungal concentration (X^2= 70.68, df = 3, $P < 0.0001$). There was no statistical difference for confirmed mortality at the 5×10^7/mL between stabilized blastospores and conidia (**Fig. 4B**).

In the final study, we examined the time for achieving the mortality rates for the treatments described above. The survival time analysis showed that at 5×10^7/mL both fungal propagules equally reduced larval survival (X^2= 2.14, df = 1, P = 0.14). Median survival times (ST_{50}) were significantly lower (8-9 days) for those tick larvae treated with 5×10^7 fungal propagules/mL than when treated with 10^7/mL (10-

20 days) (**Fig. 4C)**. However, for the lower concentration treatments, tick larvae exposed to stabilized blastospores maintain the higher survival rate than when larvae were exposed to conidia (X^2= 48.77, df = 1, P < 0.0001).

Untreated cattle ticks larvae exhibited higher survival time than those larvae sprayed with either stabilized blastospores or conidia of ESALQ1426, regardless of the concentration tested, indicating that fungal treatments were effective in killing faster and more larvae than the untreated control (X^2 = 259.98, df = 4, P < 0.00001).



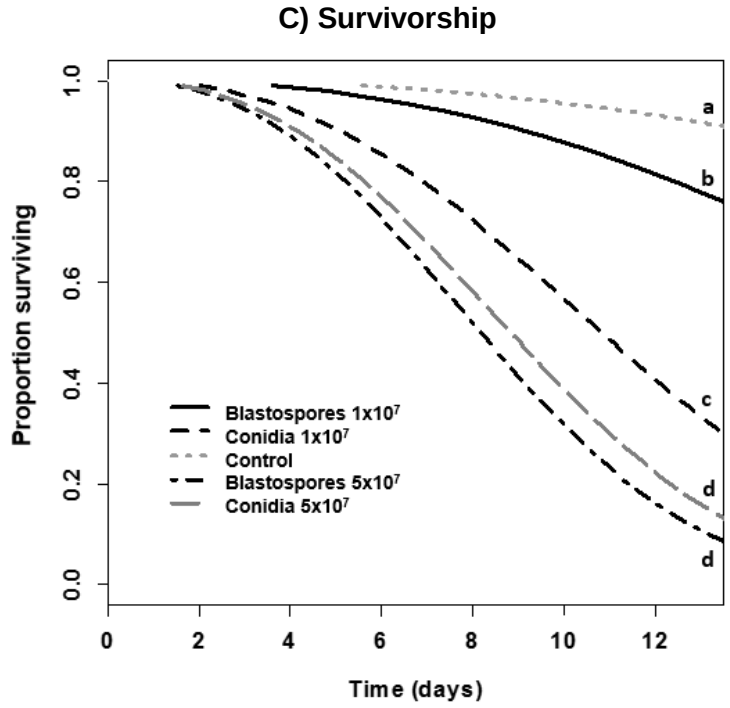


Fig. 4. Percent mortality (A) and confirmed mortality (B) in cadavers of cattle tick larvae exposed to air-dried blastospores or conidia of *Metarhizium robertsii* (ESALQ1426) at two different concentrations and 13 days post-incubation at 26 °C with >80% RH. Distinct letters correspond to significant differences between means ± standard error (SE), according to Tukey's HSD test ($P < 0.05$). Survival proportion of cattle-tick larvae after exposure to air-dried blastospores or conidia of *M. robertsii* (ESALQ1426) at different concentrations during an incubation period of 13 days at 26 °C with >80% RH. Distinct letters correspond to significant differences between survival curves, according to the log-likelihood ratio test ($P < 0.05$) (C).

2.4. Discussion

The present work highlights for the first time the infectivity of stabilized air-dried blastospores of *M. robertsii* (ESALQ1426) against cattle-tick larvae. Tolerance to stabilization processes is known to vary according to the species and/or isolate and the growing medium. Thus, we showed that blastospores produced by isolate ESALQ1426, grown in modified Adamek's medium with salts, vitamins, trace metals and high glucose concentrations (140 g/L) survive well to the air-drying stabilization process. Moreover, in modified Adamek's medium, the mentioned isolate reached unprecedented yields of 5.9×10^8 blastospores/mL in only 2 days of fermentation under the conditions stated in this study. This outcome represents an important progress in blastospore production of this fungus and shed light into the path for further improvements in this bioprocess.

Optimizing liquid cultures for blastospore production of *Metarhizium* spp. has posed a major challenge to researchers. Whereas the entomopathogenic fungi *Beauveria* and *Isaria* are highly profuse in liquid cultures (Jaronski and Jackson., 2012; Mascarin et al., 2015a, 2015b) and can achieve yields superior than 10^9 blastospores/mL in 2 or 3 days, there is only a few studies showing productivities equal to or greater than 10^8 *Metarhizium* blastospores per mL within 3-4 days of cultivation (Issaly et al., 2005; Ypsilons et al., 2005). In the present report, we show that *M. roberstii* (ESALQ1426) reached yields up to 5.9×10^8 blastospores/mL in only 2 days of liquid culturing. Among the factors that may be responsible for this successful blastospore production, we assumed that good oxygenation under high agitation speed (350 rpm and 50 mL of culture volume) coupled with a high glucose concentration to liquid cultures are crucial, as earlier suggested by others when investigating different entomopathogenic fungi (Jaronski and Jackson., 2012; Mascarin et al., 2015a, 2015b).

Additionally, the wide variation in blastospore production offers an opportunity to better understand the molecular and genetic mechanisms underlying this dimorphic growth among *Metarhizium* species and isolates.

In Brazil there is no commercial product based on *M. robertsii* up to date (Mascarin et al., 2018), the most common species found in Brazilian soils (Rocha et al., 2013; Castro et al., 2016), aside from the fact that this species has shown promise in different agricultural applications including its role as a bioestimulant for plants (Behie et al., 2012;

Sasan and Bidochka, 2012) and as a bioinsecticide for different pests (Ansari and Butt, 2012; Ment et al., 2012).

The isolate ESALQ1426 of *M. robertsii* not only showed to be the highest productive isolate but also was able to tolerate the desiccation from air-drying process. Obtaining stabilized blastospores is a crucial step for the further development of commercial fungal formulations. It should be noted that no attempts to dry and stabilize these fungal structures prior to effectiveness assays against different pests had been reported earlier (Alkhaibari et al., 2016, 2017; Dong et al., 2016; Fargues et al., 2002; Ramle and Kamarudin, 2014; Wassermann et al., 2016). As far as we know, our results are therefore the first evidence that air-dried blastospores of *M. robertsii* are as effective in killing cattle tick larvae at the same rate and concentration as that observed for conidia. This finding underlies the basis for establishing that, in the natural habitat of cattle tick larvae, blastospores could be as effective as aerial conidia for tick control. Thus, blastospores could be potentially used for the biocontrol of noxious ticks of medical and veterinary importance as well as in organic dairy farms, where chemical acaricides are precluded.

Taken together, the data presented here clearly demonstrate the viability of producing large amounts of *M. robertsii* blastospores in only 2 days of submerged cultivation, using a set of optimized environmental and nutritional conditions in modified Adamek´s medium. The cost of bulk ingredients of the modified Adamek's medium, especially regarding source and concentration of nitrogen, should be addressed in future research to make it more economically feasible for industrial mass production.

We further highlight that blastospores produced in that medium survived well the air-drying stabilization process and were as effective as aerial conidia for controlling cattle tick larvae. Further studies are warranted to shed light on the economic viability of producing *M. robertsii* blastospores in industrial deep-tank bioreactors. Lastly, our findings emphasize the importance of further media optimization for development of an effective and eco-friendly microbial acaricide based on blastospores of *M. robertsii* to fight ticks in Brazil.

Acknowledgments

This work was funded by Coordination for the Improvement of Higher Education Personnel (CAPES), The Brazilian National Council for Scientific and Technological

Development (Cnpq) [grant number 421629/2016-9] and São Paulo Research Foundation (FAPESP) [grant number 2016/20610-6]. We thank E. Potworowski for critically reviewing an earlier draft of this manuscript.

References

Abbas R.Z, Zaman M.A, Colwell D.D., Gilleard.J., Iqbal. Z., 2014. Acaricide resistance in cattle ticks and approaches to its management: the state of play. *Vet Parasitol* **203**: 6-20.

Abbatiello, M. J., 1965. A culture chamber for rearing soil mites. *Turtox News* **43**: 162–164.

Adamek, L., 1965. Submerse Cultivation of the Fungus *Metarrhizium anisopliae* (Metsch.). *Folia Microbiol* **10**: 255–257.

Alkhaibari, A. M., Carolino, A. T., Bull, J. C., Samuels, R.I., Butt, T.M., 2017. Differential Pathogenicity of *Metarhizium* Blastospores and Conidia Against Larvae of Three Mosquito Species. *J. Med. Entomol*, **54**: 696–704.

Alkhaibari, A.M., Carolino, A.T., Yavasoglu, S.I., Maffeis, T., Mattoso, T.C., Bull, J.C., Samuels, R.I., Butt, T.M., 2016. *Metarhizium brunneum* Blastospore Pathogenesis in *Aedes aegypti* Larvae: Attack on Several Fronts Accelerates Mortality. *PLoS Pathog* **12**:7.

Ansari, M. A., Butt, T. M., 2012. Susceptibility of different developmental stages of large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods. *J. Invertebr. Pathol* **111**: 33–40.

Behie, S. W., Zelisko, P. M., Bidochka, M. J., 2012. Endophytic Insect-Parasitic Fungi Translocate Nitrogen Directly from Insects to Plants. *Science* **336**: 1576–1577.

Camargo, M.G., Michel, R.S.N., Marciano, A.F., Perinotto, W.M.S., Coutinho-Rodrigues, C.J.B., Scott, F.B., C, Isabele., Prata, A.M.C.A., Bittencourt, V.R.E.P., 2014. Commercial formulation of *Metarhizium anisopliae* for the control of *Rhipicephalus microplus* in a pen study. *Vet. Parasitol* **205**: 271–276.

Camargo, M.G.; Nogueira, M.R.S., Marciano, A.F., Sá, F.A., Perinotto, W.M.S., Quinelato, S., Gôlo, P.S., Angelo, I.C., Prata, M.C.A., Bittencourt, V.R.E.P., 2016. *Metarhizium anisopliae* for controlling *Rhipicephalus microplus* ticks under field conditions. *Vet. Parasitol* **223**: 38–42.

Castro, T., Mayerhofer, J., Enkerli, J., Eilenberg, J., Meyling, N.V., Moral, R.A., Demétrio, C.G.B, Delalibera, Jr. I., 2016. Persistence of Brazilian isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *M. robertsii* in strawberry crop soil after soil drench application. *Agric. Ecosyst. Environ* **233**: 361–369.

Dong, T. Y., Zhang, B. W., Weng, Q. F.; Hu, Q. B., 2016. The production relationship of destruxins and blastospores of *Metarhizium anisopliae* with virulence against *Plutella xylostella*. *J. Integr. Agric* **15**: 1313–1320.

Estrada-Peña, A., Bouattour, A., Camicas, J. L., Guglielmone, A., Jongejan, F., Latif, A., Pegram, R., Walker, A.R., 2006. The known distribution and ecological preferences of the tick subgenus *Boophilus* (Acari: Ixodidae) in Africa and Latin America. *Exp. Appl. Acarol* **38**: 219–235.

Fargues, J., Smits, N., Vidal, C., Vey, A., Vega, F., Mercadier, G., Quimby, P., 2002. Effect of liquid culture media on morphology, growth, propagule production, and pathogenic activity of the Hyphomycete, *Metarhizium flavoviride*. Mycopathologia. **154**: 127–138.

Fernandes, E.K., Bittencourt, V.R., Roberts, D.W., 2011. Perspectives on the potential of entomopathogenic fungi in biological control of ticks. *Exp Parasitol* **130**: 300-5.

Grisi, L., Leite, R. C., Martins, J. R., Barros, A.T., Andreotti, R., Cancado, P.H.D., Angel Pérez, L.A., Pereira, J.B., Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev. Bras. *Parasitol. Veterinária* **23**: 150–156.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang X., Liu, X., Zhan, S., St Leger, R.J., Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. *Proc. Natl. Acad. Sci. U. S. A*. **111**: 16796-801.

Issaly, N., Chauveau, H., Aglevor, F., Fargues, J., Durand, A., 2005. Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. *Process Biochem* **40**: 1425–1431.

Jackson, M.A., Payne, A.R., 2007. Evaluation of the desiccation tolerance of blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) using a lab-scale, air-drying chamber with controlled relative humidity. *Biocontrol Science and Technology*. **17**: 709–719.

Jackson, M. A., Jaronski, S.T., 2009. Production of microsclerotia of the fungal entomopathogen *Metarhizium anisopliae* and their potential for use as a biocontrol agent for soil-inhabiting insects. *Mycol. Res* **113**: 842–850.

Jackson, M.A., McGuire, M.R., Lacey, L.A., Wraight, S.P., 1997. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Mycol Res* **101:** 35–41.

Jaronski, S. T, Mascarin G.M., 2013. Mass Production of Entomopathogenic Fungi: State of the Art. Mass Production of Beneficial Organisms: Invertebrates and Entomopathogens, pp. 357–413

Jaronski, S.T., Jackson, M.A., 2012. “Mass production of entomopathogenic Hypocreales,” in Manual of techniques in insect pathology. ed. Lacey L. (Ed). Academic Press, London. 2° ed, pp 317-328.

Kaaya, G.P., Samish, M., Hedimbi, M., Gindin, G., Glazer, I., 2011. Control of tick populations by spraying *Metarhizium anisopliae* conidia on cattle under field conditions. *Exp. Appl. Acarol* **55**, 273–81.

Kim, J. S., Je, Y. H., Skinner, M., Parker, B. L., 2013. An oil-based formulation of *Isaria fumosorosea* blastospores for management of greenhouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). *Pest Manag. Sci* **69**: 576–581.

Leal, B., Thomas, D.B., Dearth, R., 2017. Cattle fever tick, *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae): Potential control on pastures by the application of urea fertilizer. *Vet Parasitol* **241**: 39-42.

Lee, S. J., Kim, S.; Yu, J. S., Kim, J.C., Nai, Y.S., Kim, J.S., 2015. Biological control of Asian tiger mosquito, *Aedes albopictus* ,Diptera: Culicidae) using *Metarhizium anisopliae* JEF-003 millet grain. *J. Asia Pac. Entomol.* **18**: 217–221

Mascarin, G. M., Jackson, M. A., Behle, R. W., Kobori, N. N., Delalibera, Í. Jr., 2016. Improved shelf life of dried *Beauveria bassiana* blastospores using convective drying and active packaging processes. *Appl. Microbiol. Biotechnol* **100**: 8359-8370.

Mascarin, G. M., Jackson, M. A., Kobori, N. N., Behle, R.W., Dunlap, C.A. et al., 2015a. Glucose concentration alters dissolved oxygen levels in liquid cultures of *Beauveria bassiana* and affects formation and bioefficacy of blastospores. *Appl. Microbiol. Biotechnol* **99**: 6653–6665.

Mascarin, G.M., Jackson, M.A., Kobori, N.N., Behle, R.W., Delalibera, I. Jr., 2015b. Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. *J. Invertebr. Pathol* **127**: 11–20.

Mascarin, G.M., Lopes, R.B., Delalibera, I.Jr., Fernandes, E.K.K., Luz, C., Faria, M., 2018.Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. *J. Invertebr. Pathol*. "in press".

Mendes, E. C., Mendes, M. C., Sato, M. E., 2013. Diagnosis of amitraz resistance in Brazilian populations of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) with larval immersion test. *Exp. Appl. Acarol*. **61**: 357–369.

Ment, D., Churchill, A. C., Gindin, G., Belausov, E., Glazer, I., Rehner, S.A., Rot, A., Donzelli, B.G.G., Samish, M., 2012. Resistant ticks inhibit *Metarhizium* infection prior to haemocoel invasion by reducing fungal viability on the cuticle surface. *Env. Microbiol* **14**: 1570–83.

Murigu, M. M., Nana, P., Waruiru, R. M., Chege, J.N., Sunday, E., Nguya, K.M., 2016. Laboratory and field evaluation of entomopathogenic fungi for the control of amitraz-resistant and susceptible strains of *Rhipicephalus decoloratus*. *Vet. Parasitol* **225**: 12–18.

Ojeda-Chi, M. M., Rodriguez-Vivas, R. I., Galindo-Velasco, E., Lezama-Gutiérrez, R., 2010.Laboratory and field evaluation of *Metarhizium anisopliae* (Deuteromycotina:

Hyphomycetes) for the control of *Rhipicephalus microplus* (Acari: Ixodidae) in the Mexican tropics. *Vet. Parasitol* **170:** 348–354.

Parra, J. Biological control in Brazil., 2014. *Sci. Agric* **71**: 345–355.

R Core Team., 2015. R: A language and environment for statistical computing Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org.

Ramle, M., Norman, K., 2014. The use of palm kernel cake in the production of conidia and blastospores of *Metarhizium anisopliae* var. major for control of *Oryctes rhinoceros*. *J. Oil Palm Res* **26**: 133–139

Riaz, A., Shah, F.A., Butt, T.M., 2013. Intra-specific variability among *Metarhizium anisopliae* strains in their ability to produce blastospores in liquid culture media. *Pakistan Journal of Botany*. **45**: 1099–1103.

Roberts, D. W.; Leger, R. J. ST., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. *Adv Appl Microbiol*, v. 54, p. 1–70

Rocha, L. F., Inglis, P. W., Humber, R. A., Kipnis, A., Luz, C., 2013. Occurrence of *Metarhizium* spp. in Central Brazilian soils. *J. Basic Microbiol* **53**: 251–259.

Samish, M., Rot, A, Ment, D.,Barel, S.,Glazer, I.Gindin, G., 2014. Efficacy of the entomopathogenic fungus *Metarhizium brunneum* in controlling the tick *Rhipicephalus annulatus* under field conditions. *Vet. Parasitol* **206**: 258–266.

Sasan, R. K., Bidochka, M. J., 2012.The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Am. J. Bot* **99**: 101–107.

Vandenberg, J.D, Jackson, M.A, Lacey, L.A., 1998. Relative efficacy of blastospores and aerial conidia of *Paecilomyces fumosoroseus* against the Russian wheat aphid. *J. Invertebr. Pathol* **72**: 181–183.

Van Lenteren, J.C.; Bolckmans, K.; Köhl, J.; Ravensberg, W.J.; Urbaneja, A., 2017. Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl*, **63**: 39–59.

Wall, R., Shearer, D., 2001. “Ticks(Acari),” in Veterinary ectoparasites: Biology. Pathology and Control, 2nd ed. Wall, R., Shearer, D. (Blackwell Science), pp 55-82.

Wassermann, M., Selzer, P., Steidle, J. L. M., Mackenstedt, U., 2016. Biological control of *Ixodes ricinus* larvae and nymphs with *Metarhizium anisopliae* blastospores. *Ticks Tick. Borne. Dis* **5**: 768-771.

Ypsilos, I.K, Magan N., 2005. Characterisation of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. *Biocontrol Sci Technol* **15**: 683–699.

3. GROWTH KINETICS AND NITROGEN SOURCE OPTIMIZATION FOR LIQUID CULTURE FERMENTATION OF *Metarhizium robertsii* BLASTOSPORES AND BIOEFFICACY AGAINST THE CORN LEAFHOPPER *Dalbulus maidis*

Natasha Sant´Anna Iwanicki[a], Gabriel Moura Mascarin[b], Sara Giro Moreno[a], Jørgen Eilenberg[c], Italo Delalibera Júnior[a]

[a]Department of Entomology and Acarology, Escola Superior de Agricultura “Luiz de Queiroz”, University of São Paulo (ESALQ-USP), Av. Pádua Dias, 11, C.P. 9, Piracicaba, SP CEP 13418-900, Brazil

[b]Laboratory of Environmental Microbiology, Brazilian Agricultural Research Corporation, Embrapa Environment, Rodovia SP 340, Km 127.5, 13820-000 Jaguariúna, Brazil.

[c]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark.

*Corresponding author: Natasha S. A. Iwanicki
E-mail address: natasha.iwanicki@usp.br
Department of Entomology and Acarology, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

Abstract

The cosmopolitan entomopathogenic and root endophytic fungus *Metarhizium robertsii* exhibits a versatile lifestyle and undergoes during liquid fermentation intriguing dimorphic transformations from hyphae to conidia or microsclerotia, or from hyphae to blastospores. In all cases the processes are mediated by environmental and nutritional cues. Blastospores have potential for use in spray applications to control arthropod pests above ground and they may serve as an attractive alternative to the traditional solid-grown aerial conidial spores of *Metarhizium* spp. found in commercial products. Nitrogen is a vital nutrient in cell metabolism and growth, yet it is the expensive component in liquid cultures of entomopathogenic fungi. Our goal in this study was to optimize nitrogen sources and titers for maximum production of *M. robertsii* blastospores cultures in shake flasks at highly aerated conditions and determine virulence against the corn leafhopper *Dalbulus maidis*, an important vector of serious pathogens of maize crops worldwide. Our fermentation studies based on growth kinetics showed that a low-cost corn steep liquor (CSL) was the most suitable nitrogen source for inducing blastospore growth in *M. robertsii* with an optimal titer of 80 g L^{-1} that rendered up to 4.7×10^8 cells mL^{-1} in only 2 days of cultivation at a total cost of $0.30 USD per L. Moreover, the blastospore growth kinetic was strongly dependent on glucose and nitrogen consumptions accompanied by a slight drop in the culture pH. Insect bioassays evidenced a high virulence of these blastospores, either as dried or fresh cells, to *D. maidis* adults fed on maize plants. Taken together, our findings contribute to gaining insights on the nutritional requirements for optimal and cost-efficient production of *M. robertsii* blastospores and expand our knowledge on the potential of blastospores as a valuable ecofriendly biocontrol agent against the corn leafhopper.

Keywords: biocontrol, fungal entomopathogen, Hypocreales, virulence

3.1 Introduction

The cosmopolitan ascomycete arthropod pathogenic genus *Metarhizium* is widely explored as biopesticides against various insect pests (Faria and Wraight 2007) and more recently, it has been studied as plant biostimulants (Garcia et al. 2011; Jaber and Enkerli 2016; Jaber and Alananbeh 2018; Canassa et al. 2019). More intriguingly, some species act as plant endophytes contributing to enhancing plant health and development (Krell et al. 2018; Canassa et al. 2019). In Brazil, it is estimated that 2 million hectares are treated annually with aerial conidia of *Metarhizium anisopliae* to control spittlebugs in sugarcane and grasslands, which makes this approach the largest microbial control program with an entomopathogenic fungus worldwide (Mascarin et al. 2019; Parra 2014). Besides aerial conidia, *Metarhizium* spp. produce another infectious propagule, the so-called blastospores.

Blastospores are single, yeast-like vegetative cells produced in vivo inside of the insect body during infection (more specifically in the hemolymph), or they are produced in highly aerated and nutrient-rich liquid culture media, where source and amount of nitrogen greatly affect their productivity and ecological fitness (Jackson et al. 1997; Jaronski and Mascarin 2016; Mascarin et al. 2015, 2019).

Commercial interest in the use of blastopores as the active ingredient of biocontrol products has increased lately due to attributes comparable to or even superior than aerial conidia. In contrast to aerial conidia, blastospores need shorter fermentation time, reduced labor requirement, and feasible automation and scaling-up production process tied to versatile downstream processing strategies (Jackson et al. 1997; Jaronski and Mascarin 2016; Mascarin et al. 2019). Furthermore, blastospores possess the unique ability to germinate faster than aerial conidia, thus exhibiting in many cases higher virulence than the latter (Alkhaibari et al. 2016, 2017; Jackson et al. 1997; Kim et al. 2013; Mascarin et al. 2015a, 2015b, 2016; Wassermann et al. 2016; Shapiro-Ilan et al. 2008; Behle et al. 2006). Although the efficacy of *Metarhizium* spp. blastospores has already been demonstrated in several studies (Alkhaibari et al. 2016, 2017; Wassermann et al. 2016; Iwanicki et al. 2018; Bernardo et al. 2018), a formulated product containing such cells does not yet exist on the Brazilian market.

Several aspects of the liquid culture production system should be further optimized, such as screening for a virulent and productive isolate, development of a cost-efficient liquid medium, selection of the most amenable drying techniques to stabilize the selected isolate, and development of formulations that can prolong storage stability and improve fungal performance in the field. Particularly, there are very few studies addressing liquid culture medium optimization in *Metarhizium* species (Issaly et al. 2005; Ypsilos and Magan 2005), which remains a quite challenging task to be overcome. Recently, our group has shown the amenability of producing massive blastospore concentrations (5.9×10^8 blastospores mL^{-1}) of *Metarhizium robertsii* within only 2 days of cultivation in a modified Adamek´s medium with enriched glucose supply supplemented with yeast extract and cornsteep liquor (Iwanicki et al. 2018). Despite the high yields of blastospores, modified Adamek's medium still needs to be optimized for low-cost inputs in media

formulation by employing agro-industrial byproducts especially for substitution of yeast extract, considered an expensive protein source.

Cornsteep liquor (CSL) is an inexpensive byproduct derived from the wet-milling process commonly used as organic nitrogen source and vitamins in many microbial bioprocess industry, such as the large-scale production of penicillin with *Penicillium chrysogenum*. Remarkably, the complex composition of CSL appears to boost spore germination of filamentous fungi in the early stages of the cultivation process (Posch et al. 2012). The exact mechanisms of this phenomenon has not been studied in detail because of the complexity of CSL and the complex metabolic system of fungi, which metabolize not only the carbon substrates available in CSL but also amino acids and vitamins. Thus, even though CSL seems to be a suitable nitrogen and carbon source for blastospore production with *M. robertsii*, yet it remains to be tested and compared with other nitrogen sources.

Maize is one of the key crops worldwide with many industrial applications in food production, fuel, cosmetics, pharmaceuticals and animal feed. The corn leafhopper, *Dalbulus maidis* (DeLong and Wolcott, 1923) (*Hemiptera*: Cicadellidae) has recently emerged as the most important sucking pest in maize crops in Brazil. This insect has a phloem feeding behavior and vectors three pathogens associated with corn stunt disease complex: the maize bushy stunt phytoplasma, the *Spiroplasma kunkelii* and the virus Maize rayado fino virus (MRFV) (Nault and Delong 1980; Nault 1990). Since 2016, high levels of maize bushy stunt disease have been recorded in many Brazilian states leading to yield reduction of 90% in some cases (Canale et al. 2018). For controlling *D. maidis* seed treatment neonicotinoid and methyl carbamate are used, while the only biological alternative to control this insect is based on the foliar sprays of aerial conidia of *Beauveria bassiana* (AGROFIT 2019). To the best of our knowledge, there is no report regarding the bioefficacy of entomopathogenic blastospores against this leafhopper. Earlier studies have indicated that blastospores of *B. bassiana* appeared to be less virulent than aerial conidia towards the green leafhopper *Nephotettix virescens* (Lane et al. 1991a). However, many attributes inherent to the fungal species and isolate plus the quality of the infectious propagule can significantly influence the effectiveness of blastospores toward an insect target, and these aspects must be considered case-by-case.

The present study aimed to optimize nutritional composition as well as reducing the cost of our modified Adamek's medium, while maintaining the insecticidal activity against *D. maidis*, which is firstly described here. More specifically, we hypothesized that i) the nitrogen source affects blastospore yield, ii) there is an optimum nitrogen concentration in the medium allowing high blastospore yield and iii) blastospores can be equally or even more virulent against *D. maidis* than aerial conidia.

3.2. Materials and methods

3.2.1. Fungal selection and inoculum preparation

The *M. robertsii* isolate ESALQ1426 was chosen for this study due to our previous results (Iwanicki et al. 2018), in which we showed that this isolate produced a fair amount of blastospores when grown in modified Adamek's medium (5.9×10^8 blastospores mL^{-1}), which retained pathogenicity to the cattle tick (*Rhipicephalus microplus*). The ESALQ1426 isolate is deposited in the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil).

A fungal stock culture was established by preserving sporulating agar chunks immersed in a sterile 10% glycerol solution at -80 °C. Weekly, conidial inocula were obtained by growing the fungus on potato dextrose agar (PDA, Difco, Sparks, MD, USA) during 10-14 days in a growth chamber at 26 °C with 12:12 h photoperiod.

3.2.2. Media and culture conditions

The isolate ESALQ1426 was grown in 50 mL liquid medium in two-three replicate 250-mL baffled Erlenmeyer flasks (Bellco® Glass, Vineland, NJ, USA) during a period of 3-4 days at 28±0.5 °C and 350 rpm using a rotating incubator shaker with an orbit diameter of 10 mm (Solab®, Piracicaba, SP, Brazil). The fungus was first grown during 3 days in a preculture medium composed by 40 g L^{-1} glucose monohydrate (Ingredion®, Mogi Guaçu, SP, Brazil), 40 g L^{-1} yeast extract (Sigma-Aldrich®, São Paulo, SP, Brazil), mineral salts, trace metals and vitamins adapted from Jackson's medium (Jackson et al. 1997) at the following concentrations per liter:

KH_2PO_4, 2.5 g; $CaCl_2.2H_2O$, 1.0 g; $MgSO_4.7H_2O$, 0.83g; $FeSO_4.7H_2O$, 0.3g ; $CoCl_2.6H_2O$, 29.6 mg; $MnSO_4.H_2O$, 12.8 mg; $ZnSO_4.7H_2O$, 11.2 mg; 0.2 mg each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid; and 0.02 mg each of folic acid, biotin, and vitamin B12 with a carbon-to-nitrogen (C:N) ratio of 12:1. Preculture medium was inoculated with 5 mL of a suspension containing 5×10^6 conidia mL^{-1}.

Blastospores produced in preculture medium were used to inoculate culture medium composed by 80 g L^{-1} cornsteep liquor (Ingredion®, Mogi Guaçu, SP, Brazil) (see composition of cornsteep liquor in Appendix C) 140 g L^{-1} glucose monohydrate (Ingredion®, Mogi Guaçu, SP, Brazil) and mineral salts, trace metals and vitamins at the same concentration as indicated for the preculture medium with a carbon-to-nitrogen (C:N) ratio of 30:1. The culture medium was inoculated with 5 mL of a suspension containing 5×10^7 blastospores mL^{-1} and left for growing during a period of 3-4 days. The initial pH of all liquid media was adjusted to 6.8 after autoclaving (Adamek 1965) and was not further monitored during the entire cultivation period. Glucose solutions were autoclaved separately from the basal media amended with the nitrogen source in order to avoid the Maillard reaction between sugars amino acids.

3.2.3. Screening nitrogen sources

Cornsteep liquor and yeast extract were chosen based on results of preliminary testing (blastospore yield) of nine protein sources (autolysed yeast (Lyscell®, ICC Industrial Comércio, Exportação e Importação Ltd, São Paulo, SP, Brazil), hydrolyzed yeast (Hilysis®, ICC Industrial Comércio, Exportação e Importação Ltd, São Paulo, SP, Brazil), cotton seed flour (Pharmamedia®, Traders Protein, Memphis, TN, USA), urea (Dinâmica®, Indaiatuba, SP, Brazil), glutamic acid (Dinâmica®, Indaiatuba, SP, Brazil), malt extract (Microgen®, New Delhi, India) and hydrolyzed casein (Sigma®), cornsteep liquor CSL (Ingredion®, Mogi Guaçu, SP, Brazil) and yeast extract (Microgen®, New Delhi, India)) (Appendix A)

The fungus *M. robertsii* (ESALQ1426) was first cultured in a preculture medium, detailed previously in media and culture conditions section, and then inoculated in culture media with either 80 g L^{-1} yeast extract or 80 g L^{-1} cornsteep liquor, as the

nitrogen source, to grow during additional 4 days. Blastospore yields were evaluated daily until 96 h of culture (fourth day). Each flask was vigorously hand-shaken daily to minimize the mycelial growth on the sides of the flasks. Aliquots of 1 mL were taken from each flask for evaluating the concentration of blastospores per mL using a Neubauer chamber under a phase contrast microscope at 400x magnification.

In a second experiment, we evaluated blastospore yield using different concentrations of cornsteep liquor as the nitrogen source (20, 40, 60, 80 and 100 g L^{-1}), supplemented with 140 g L^{-1} of glucose during 96 h of cultivation at the same conditions as the first experiment described previously. For both experiments, each treatment had two replicates and the experiment was independently repeated three times at different occasions.

To measure fungal efficiency in producing blastospores, two growth parameters were measured: a) maximum specific growth rate (SGR) given by $ln(N_2/N_1)=k(t_2-t_1)$, *where* N_1 *is the initial concentration of blastospores (*5×10^6 blastospores mL^{-1}) *at time zero (*$t_1 = 0$ *h) and* N_2 *is the maximum concentration of blastospores at the log-phase; while* b) doubling time (DT) or generation time is defined by the time required for the number of cells to increase by a factor of 2 and can be expressed as $Td = \ln 2/\mu_{max}$.

3.2.4. Growth kinetics experiment

The growth kinetics of *M. robertsii* (ESALQ1426) blastospores were investigated by measuring blastospore yield, pH variation in medium, and glucose and nitrogen consumptions at every 12 h during a period of 84 h (4 days) of culturing. The fungus was previously grown in a preculture medium, detailed previously in media and culture conditions section, followed another 4 days in culture medium, as described in Media and culture conditions section. The initial pH was adjusted to 6.8 and monitored further during the growth course. Glucose and nitrogen concentrations in the medium were measured before fungus inoculation. Through a destructive sampling method, every 12 h two culture flasks were taken from rotatory incubator shaker for determining the concentration of blastospores mL^{-1}, pH and glucose consumption by the fungus. Glucose consumption was measured by a blood glucose meter (OneTouch®, Ultramini, LifeScan, Pennsylvania, Switzerland). Next, each flask

was poured in a 50 mL Falcon® tube, centrifuged at 5,000 rpm for 5 min at 10 °C and the supernatant was transferred to 1.5-mL Eppendorf® tube for another centrifugation under 12,000 rpm for more 5 min. Through this procedure, any blastospores and hyphae from liquid medium has precipitated. Then the supernatant (i.e., spent medium) was checked for presence of blastospores and hyphal fragments and then transferred to a sterile 15 mL Falcon® tube. The supernatant was used to measure the nitrogen content by Kjeldahl method (Galvani and Gaertner 2006). Nitrogen and glucose consumptions, and pH variations were recorded indirectly by the difference between the initial pH, nitrogen and glucose titers in medium before fungus inoculation and in spent medium where blastospores were grown during a course of 84 h. Both nitrogen and glucose concentrations of the medium started off with 2.47 ± 0.04 g L^{-1} and 155 ± 4.2 g L^{-1}, respectively. Such high level of glucose in the culture medium was augmented by the CSL that contains free sugars. The entire experiment was repeated three times using different fungal batches (total of n=6 replicates per treatment for each 12 h of culture).

3.2.5. Bioassay against the corn leafhopper *Dalbulus maidis*

The virulence of fresh and air-dried *M. robertsii* (ESALQ1426) blastospores was assessed and compared to the virulence of conidia against adults of the corn leafhopper *D. maidis.* Blastospores were grown as described in medium and culture conditions section above. Blastospores were harvested from liquid medium after 3 days of culture and mixed with 7.5% (w/v) diatomaceous earth (Sigma-Aldrich®, St. Louis, USA). This mixture was filtered through a vacuum pump coupled to a Buchner funnel lined with a filter paper disc of 8 cm diameter (Whatman® No.1, Piscataway, NJ, USA) and the retained material was crumbled with an electrical blender. The resultant crumbled fungal preparation was placed on 10 cm glass Petri-dishes for drying inside an air drying chamber during 15-18 h with 50-60 % RH and then for additional 2 h at 15% RH, as described by Mascarin et al. (2015a), or until water activity was equal or less than 5% (w/w), measured with a water activity analyzer (Novasina - Labmaster Aw, Pfäffikon, Switzerland). Thereafter, the dried blastospore-DE mixture was vacuum-packed in polyethylene bags and stored at 4 °C until use in subsequent bioassays (between 7 to 10 days).

Fresh blastospores were obtained by setting up a culture experiment to coincide the third day of culture with the date of bioassay against corn leafhopper. Blastospores were cultured as described before and washed twice with a potassium buffer saline solution with the following composition per liter: NaCl 8.0 g, KCl 0.2 g; Na_2HPO_4 1.44 g; KH_2PO_4 0.24 g, and pH adjusted to 6.0, in order to remove spent medium prior to adjusting concentrations for spraying.

The viability of dried blastospores was assessed by rehydrating 0.3 g of fungus-DE mixture in an air-drying chamber with 85-90% RH during 30 min. Then, the mixture was diluted in 50 mL sterile distilled water in Erlenmeyer flasks and left on a rotating shaker with 350 rpm for 10 min at 28 °C. After that, the suspension was filtered using two layers of cheesecloth and the concentration adjusted to 10^6 blastospores mL^{-1}. A volume of 150 mL of that suspension was placed on potato dextrose agar in Petri dishes (Rodac™ type, Dickinson and Co, Franklin Lakes, NJ, USA) incubated in a growth chamber at 25 ± 1 °C for 6 h prior to assessment of viable and non-viable blastospores. In the case of conidia and fresh blastospores, 150 mL of 10^6 PBS-washed blastospores or conidia per mL suspension were placed on potato dextrose agar medium poured on Rodac™ type plate and incubated for 6 h in case of blastospores, and 20 h in case of conidia at the same temperature and photoperiod used for access the dried blastospores viability. The viability was determined in duplicates per replicate between 200-250 blastospores or conidia were counted and categorized as germinated or not germinated. Germ tubes equal to or longer than half the length of the blastospores or conidia were counted as germinated.

Unsexed adults of corn leafhoppers with 5-8 days old after emergence were obtained from a colony kept by the Laboratory of Insect Vectors of Plant Pathogens, Department of Entomology and Acarology from University of São Paulo in Piracicaba, SP, Brazil. The bioassays were conducted following a randomized experimental design with six fungal treatments and six biological replicates (experimental unit) per treatment. Experimental unit consisted of a cage containing a maize (*Zea mays* L.) (simple hybrid, Fórmula®, Syngenta, Brazil) plant in a plastic pot with 10.5 cm diameter and 10 cm height (volume: 865 cm^3) filled with a mixture of clay and sand (50%/50% v/v) sown with three to four non-transgenic maize plants with 30-40 cm high each one, a tube with 40-45 cm high made with acetate to isolate

corn plants and a screen cover made by nylon voile fabric fastened with a rubber band (Appendix B). To avoid dead insects getting into contact with soil or becoming infected by fungi on the soil, pieces of moisten cotton were placed on top of the soil to isolate it from insect contact. Between 10 and 12 adults were transferred to each experimental unit one day before of spraying fungal suspension.

Adults were sprayed with suspensions of fresh or dried blastospores and conidia at two concentrations, 1×10^7 or 5×10^7 blastospores or conidia mL^{-1}, prepared with 0.02% Silwet® L-77 surfactant (OSi Specialties, Inc., Danbury, CT, USA). A small airbrush was employed to apply through direct spray contact 2 mL of each treatment. The application was performed from the bottom up by slightly lifting the cage from the plastic pot to introduce the tip of the airbrush toward the base of the plant and then spraying the leaves. After spraying cages were kept in a room with 26-28 °C, 12 h of photoperiod and uncontrolled humidity. Dead adults were recorded every 2 days during 10 days after the application. To confirm that the mortality was due to the fungal infection (i.e., mycosis rate), dead adults were transferred to a humid chamber and kept at 28 °C until mycosis (fungal outgrowth) could be seen. The whole bioassay was repeated three times using different insect cohorts and fungal preparations, totalizing 18 biological repetitions (3 experiments x 6 replicates per treatment).

3.2.5. Statistical analysis

All experiments were conducted in a completely randomized design and repeated three times on different dates to ensure data reproducibility. Blastospore concentration was $\log_{10}$-transformed in order to meet normality assumptions and then fitted in a linear model (GLM). In fitted model, fixed effects were attributed to protein source, protein concentration and evaluation days according to experimental design. When the interaction between "day and protein source" or “day and protein source concentration” were significant, Student’s *t* test ($P < 0.05$) was used to compare two groups of means or post-hoc Tukey´s HSD test ($P < 0.05$) for multiple comparisons between treatments within each culture day. In order to explain the data obtained for growth kinetics of *M. robertsii* blastospores, we used regression commonality analysis with

correlated predictors (nitrogen and glucose consumption and pH variation) to decompose the variance in terms of R^2 (Ray-Mukherjee et al. 2014). Therefore, the growth kinetics data were fitted to a linear model with $\log_{10}$-transformed blastospore yields as response variable and nitrogen and glucose consumption and pH variation as fixed effects. Additionally, the optimal blastospore yield was determined based on the glucose and nitrogen uptake by fitting raw data to a logistic growth model (sigmoidal with three parameters) according to the equation: $y= a/(1+\exp(-(x- x_0)/b))$, where y is the blastospore yield mL^{-1}, x is nitrogen (mg) or glucose consumption (g) and a, b and x_0 are constants estimated by the interactive analysis performed with the software SigmaPlot 14.0 (Systat Software Inc., San Jose, CA,USA). To explain the blastospore yield as a function of time, empirical data were fitted to a quadratic polynomial model according to the equation: $y = -119520202 + 18553177*x -150609.6x^2$, $R^2= 0.52$, where y is blastospore yield mL^{-1} and x is time (h). Adult mortality and mycosis datasets were fitted to a generalized linear mixed model with binomial-logit-normal distribution for errors in which a random effect is included in the linear predictor accounting for extra-binomial variation among experimental repetitions. Fixed effects were attributed to propagule types at the two concentrations tested. Treatment means were statistically compared with each other by the multiple pairwise Tukey's HSD test at $P < 0.05$. Additionally, survival analysis was performed with censored data for dead adult until day 12 and fitted to a parametric Weibull survival model using the package "flexsurv" from R (Jackson, 2016). Estimated 50% (median) and 90% lethal times (LT) and their respective confidence intervals (CI 95%) were computed from the Weibull survival functions. All analyses were performed in the statistical software R (R Core Develpment, 2015) and plots were either built on SigmaPlot version 14.0 (Systat Software, Inc., USA) or with "ggplot2" package in R (Wickham 2016).

3.3. Results

3.3.1. Blastospore production with corn steep liquor or yeast extract as nitrogen source

Blastospore concentrations differed significantly between medium amended with CSL and YE ($F_{1,56}$ = 326.61, P < 0.001) and across culture days ($F_{3,56}$ = 57.72, P < 0.001), with higher production falling within 48 to 72 h for both protein sources. *M. robertsii* cultured in medium supplemented with CSL produced almost twice the amount of blastospores than in medium amended with YE (Fig. 1a). The peak of blastospore production was reached after 48 h of culture in liquid medium for both protein source: CSL (4.74 ± 0.28 × 10^8 blastospores mL^{-1}) and YE (2.35 ± 0.27 × 10^8 blastospores mL^{-1}) with no statistical difference when compared to blastospores yield after 72h of culture in medium with CSL ($F_{1,14}$ = 3.057, P = 0.102) and YE ($F_{1,14}$ = 3.42, P = 0.085)

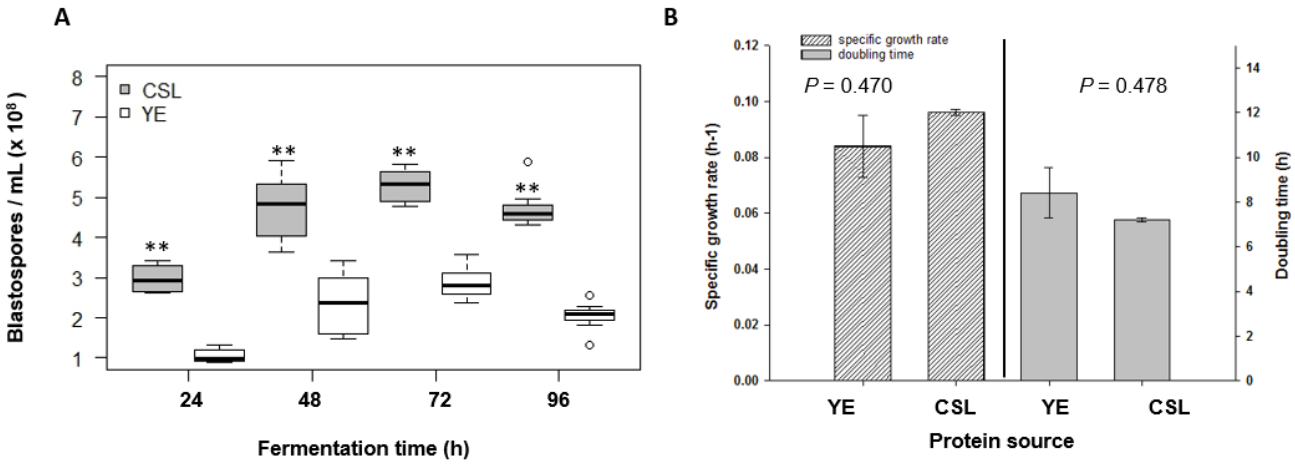


Figure 1. Blastospore yield from liquid cultures of *Metarhizium robertsii* (ESALQ1426) grown in medium with two different nitrogen source: cornsteep liquor (CSL) and yeast extract (YE) and incubated at 28°C and 350 rpm. Fitting curves of blastospore production when grown in CSL protein source: y = 0.0668+0.145*x - 0.001*x^2, R^2= 0.7449 and YE: y = -1.433+0.124x 0.0009*x^2, R^2 =0.6756. Means (±SE) followed by **, within each day, indicate significant differences according to *t*-Student test (P < 0.05). Boxes show the median, 25th and 75th percentiles, error bars show10th and 90th percentiles (A). Specific growth rate and doubling time of *M. robertsii* blastospores. Means (±SE) were compared according to Welch´s t-test (P <

0.05)(B). Liquid cultures were inoculated with blastospore-based precultures to provide a final concentration of $5x10^6$ blastospores mL^{-1}.

Although the blastopores production achieved higher values in CSL medium, we did not find statistical differences in the Specific growth rate (SGR) of blastospore grown with CSL (0.096 h^{-1}) or YE (0.084 h^{-1}). Furthermore, there was no statistical difference between doubling time (DT) of blastospores when cultured with CSL or YE, corresponding to 7.2 h and 8.4 h, respectively (Fig. 1b).

3.3.2. Impact of CSL concentration on blastospore productivity

We observed a significant effect of CSL concentration on blastospore yield ($F_{3,79}$ = 7.97, P < 0.001) and a positive correlation between these two variables (CSL concentration and blastospore yield) (Pearson correlation: r = 0.6, P < 0.001). The highest production of blastospores was obtained after 48 h for the medium with 80 g L^{-1} CSL (Fig. 2a). On the other hand, small agglomerates of hyphae instead of blastopores were formed in culture flasks when *M. robertsii* was grown in medium with the lowest concentration of CSL (20 g L^{-1}). After 48 h of cultivation, there was no significant increase in blastospore concentration irrespective with the CSL concentration. The optimal CSL concentration for this blastospore production within short time was around 80 g L^{-1} and the minimum required for blastospore development were between 20 to 40 g L^{-1}.

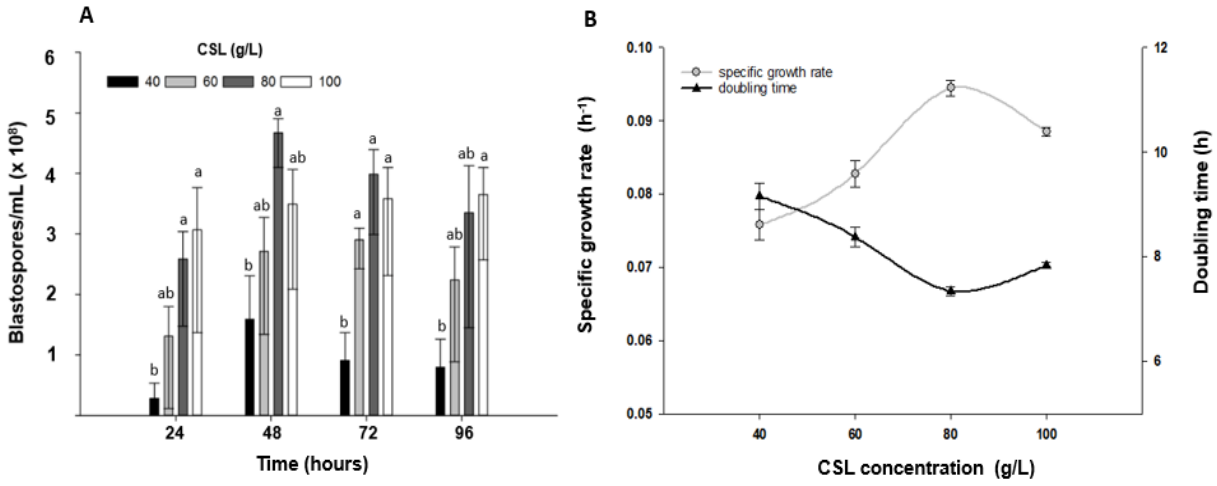


Figure. 2. Blastospore yield from liquid cultures of *Metarhizium robertsii* (ESALQ1426) grown in medium supplemented with 40, 60, 80 or 100 g L^{-1} of cornsteep liquor (CSL) as nitrogen source and incubated at 28 °C and 350 rpm. Means (±SE) followed by different letters, within each day, indicate significant differences according Tukey's HSD test ($P < 0.05$) (**a**). Specific growth rate and doubling time of *Metarhizium robertsii* blastospores (ESALQ1426) grown in media with five different concentrations of CSL (**b**). Liquid cultures were inoculated with blastospore precultures to provide a final concentration of 5×10^6 blastospores mL^{-1} and incubated at 28 °C and 350 rpm.

Next, the specific growth rate and doubling time for blastospore cultures were determined for each concentration of CSL in liquid medium. The highest SGR and the lowest value for DT were achieved in cultures amended with 80 g L^{-1} (SGR = 0.094 h^{-1}; DT = 7.33 h) and 100 g L^{-1} (SGR = 0.088 h^{-1}; DT = 7.83 h) of CSL (Fig. 2b) , although they were not statistically different, indicating that the former titer provided the maximum specific growth rate accompanied by the fastest doubling time. Conversely, the lowest SGR (0.075 h^{-1}) and the highest DT (9.15 h) were found when blastospores were cultured in the medium with 40 g CSL L^{-1} (Fig. 2b). It is important to note that at 20 g CSL L^{-1} *M. robertsii* cultures did not grow in yeast-like form and thus blastospore yield was precluded.

3.3.3. Growth kinetics

Blastospores in medium with 80g L^{-1} CSL throughout the time course of 84 h revealed significant increase in yield. This was linked with an increased consumption of both nitrogen and glucose, and followed by a slight decrease in pH. At the end of the fermentation course, 54.25% of the total nitrogen (1.34 ± 0.1g) and 42.00% (65.25 ± 4.54 g) of the total glucose amount in the liquid broth were utilized by the fungus (Table 1 and Fig. 3), while the pH showed a small drop from 6.8 to 6.08 ± 0.05.

Table 1. Cumulative average consumption of glucose and nitrogen (Means ±SE and percentage) of *M. robertsii* (ESALQ1426) blastospores measured every 12 h during

84 h of fermentation batch filled with 50 mL in 250-mL baffled shake flasks of modified Adamek medium supplemented with 80 g L^{-1} of corn steep liquor, 140 g L^{-1} of glucose and incubated at 28 °C and 350 rpm.

Average cumulative consumption				
	Glucose		Nitrogen	
Interval (h)	**(g L^{-1})**	**(%)**	**(g L^{-1})**	**(%)**
0 to 12	18.51 ± 4.20	11.60	0.32 ± 0.01	12.95
12 to 24	31.62 ± 4.98	20.42	0.79 ± 0.01	31.98
24 to 36	50.00 ± 1.73	32.25	1.15 ± 0.04	46.55
36 to 48	57.52 ± 1.81	37.09	1.27 ± 0.08	51.41
48 to 60	59.53 ± 3.05	38.38	1.23 ± 0.03	49.79
60 to 72	67.70 ± 4.59	43.67	1.28 ± 0.06	51.82
72 to 84	65.25 ± 4.54	42.09	1.34 ± 0.01	54.25

The peak of blastospore yield occurred between 12 to 24 h of cultivation, as illustrated by the highest SGR of 0.32 ± 0.08 h^{-1} and the lowest DT of 2.15 ± 0.05 h (Fig. 3 and Appendix D). Furthermore, this time interval corresponded to the maximum nitrogen and glucose consumptions, more specifically when blastospore yield achieved 2.03×10^{8} blastospores mL^{-1} and 2.29×10^{8} blastospores mL^{-1} respectively, according to the logistic model fitted to the raw data, and coupled with the greatest dropout in pH in the fermented culture broth. After 48 h of fermentation, nitrogen and glucose consumptions almost ceased along with a small drop in pH (Fig. 3). These results indicate that the lag-phase in the growth kinetic curve of *M. robertsii* (ESALQ1426) most likely fell within 0 to 12 h of culturing with formation of only hyphae but not blastospores, then followed by the log-phase around 24 to 48 h with profuse development of blastospores; the stationary phase from 48 to 60 h and, finally, the decline phase coinciding with 72 to 84 h, as illustrated by the photos in

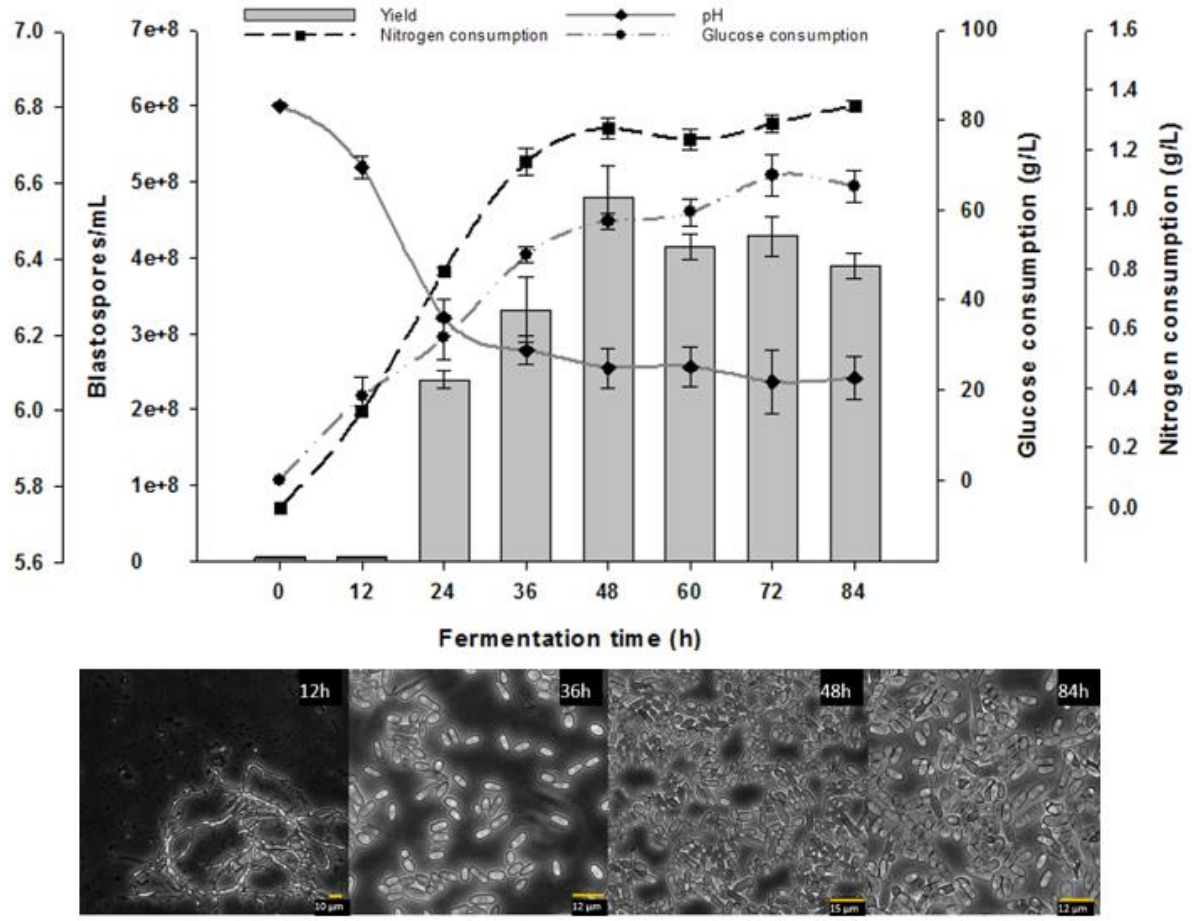


Figure. 3. Growth kinetics of *Metarhizium robertsii* (ESALQ1426) blastospores grown in medium with 80 g L^{-1} cornsteep liquor (CSL), 140 g L^{-1} glucose and incubated at 28°C and 350 rpm. Glucose consumption (g L^{-1}), nitrogen consumption (g L^{-1}) and pH variation are represented by solid and dashed lines, while bars represent blastospore yield. Means (±SE). Below the graphic, phase-contrast microphotographs of blastospores produced in liquid culture (400x) after 12, 36, 48 and 84 h of culturing.

Additionally, we found a positive correlation between glucose and nitrogen consumptions (Pearson correlation: r = 0.8, P < 0.001), whilst a negative correlation was observed between glucose consumption and pH (Pearson correlation: r = -0.6, P < 0.001) and for the relationship between nitrogen consumption and pH (Pearson correlation: r = -0.59, P < 0.001)

The predictor variables in the regression explained 54.6% (R^2 = 0.5458, $R^2_{adj.}$ = 0.4988, F = 11.62 df = 3, 29, P = 3.578e-05) of the total variance in blastospore

concentration (Table 2). The standardized regression equation from the beta coefficients (Table 2) yields the prediction equation:

$$\hat{Y}(log_{10}blastopore) = +0.00165 * Glucose + 0.3943 * Nitrogen + 0.10226 * pH$$

The commonality regression analysis showed that only the nitrogen predictor had a strong influence on blastospore production of *M. robertsii* (*t* = 2.973, *P* = 0059) and was indeed the best unique predictor of blastospore concentration (Table 2, Fig. 3), explaining 13.84% of variation in Y. A substantial amount of the regression effect (93.75%) involved positive commonality coefficient stemmed from the high correlation between associated nitrogen and glucose consumptions. Although pH decrease accompanied the increase in blastospore production by *M. roberstii* cultures, its contribution was only 12.25% of the whole R^2 regression. On the other hand, nitrogen and glucose consumptions accounted each for more than 70% of total regression variance.

Table 2. Blastospore growth kinetics of *Metarhizium robertsii* (ESALQ1426) influenced by glucose and nitrogen consumption rates and pH, tested using commonality analysis. Regression results includes multiple R^2 = 0.5458, adjusted R^2 = 0.4988, beta coefficients (β), beta coefficients standard error (SE), structure coefficients (r_s), squared structure coefficient (r_s^2), each predictor's total unique (U), total common (C) and total variance (r^2) in the regression equation.

Predictors	**β**[a]	**SE**	**Commonality coefficients**			**Contribution**
						(% of R^2)[b]
			U	*C*	*Total*	
Glucose	0.001658 †	0.00194	0.0114	0.3948	0.4062	74.42
Nitrogen	0.393434 **	0.13234	0.1384	0.3929	0.5313	97.34
pH	0.102257 †	0.14901	0.0058	0.1151	0.1225	22.44

[a] Significance codes: 0 <*** 0·001 <** 0·01<* 0·05 <† 0·1. [b] Contribution was given by % of R^2 = Total/R^2 and is also known as the squared structure coefficient (r_s^2).

3.3.4. Bioassay with *Dalbulus maidis*

Differences between treatments (blastospores *versus* conidia) were detected (χ^2 = 52.84, df = 6, *P* < 0.0001) with blastospores (both fresh and dry) and conidia

strikingly greater mortality levels of *D. maidis* adults through direct spray application than that observed for the control group. Aplication of 5×10^7 conidia mL^{-1} resulted in higher mortality level (88.2% ± 5.3%) than treatment with 1×10^7 dry blastospores mL^{-1} (63.7% ± 2.8%), all other comparisons were not statistically different (Fig. 4a). Mycosis in cadavers has little variation among fungal propagules, but the only statistical difference was the higher mycosis level attained by treatment with 5×10^7 conidia mL^{-1} in relation to 1×10^7 fresh blastospores mL^{-1}, while the rest of the treatments presented similar outcomes ($\chi^2 = 12.32$, df = 5, $P = 0.031$) (Fig. 4b, 4d).
The survival analysis revealed blastospores (both fresh and dry) and conidia sharply declined survival of *D. maidis* adults through direct spray application in relation to control (untreated) group ($\chi^2 = 144.64$, df = 6, $P < 0.0001$). Statistical differences were found between dry blastospores applied at concentration of 1×10^7 mL^{-1} and conidia at concentration of 5×10^7 mL^{-1} (Fig. 4c). *M. robertsii* blastospores and conidia killed 50% of the leafhoppers (LT_{50}) within 6 to 8 days. Conidia at concentration of 5 ×1 0^7 mL^{-1} attained the lowest LT_{50} value (6.25 days) while the highest LT_{50} value was attributed to dry blastospores at concentration of 1×10^7 mL^{-1} (7.84 days) (Table 3).

Table 3. Estimated median lethal time (LT_{50}), 90% lethal time (LT_{90}), lower confidence interval (lci) and upper confidence interval (uci) of the corn leafhopper adults (*D. maidis*) after being sprayed with dried blastospores, fresh blastospores or aerial conidia in two concentrations: 1 or 5×10^7 fungal propagules mL^{-1}. Time is measured in days.

		Median Lethal time (LT_{50})			**90% Lethal time (LT_{90})**		
Fungal propagule	**Concentration (propagules mL^{-1})**	**Estimated**	**lci***	**uci***	**Estimated**	**lci**	**uci**
Dried blastospores	1×10^7	7.84	6.99	8.79	14.30	12.71	16.11
	5×10^7	6.92	6.22	7.69	12.66	11.37	14.1
Fresh blastospores	1×10^7	7.39	6.63	8.24	13.52	12.1	15.13
	5×10^7	6.82	6.22	7.48	12.48	11.34	13.72
Aerial conidia	1×10^7	6.96	6.29	7.74	12.72	11.47	14.18
	5×10^7	6.25	5.72	6.84	11.43	10.44	12.53

*lci = lower confidence interval 95% and uci = upper confidence interval 95%.

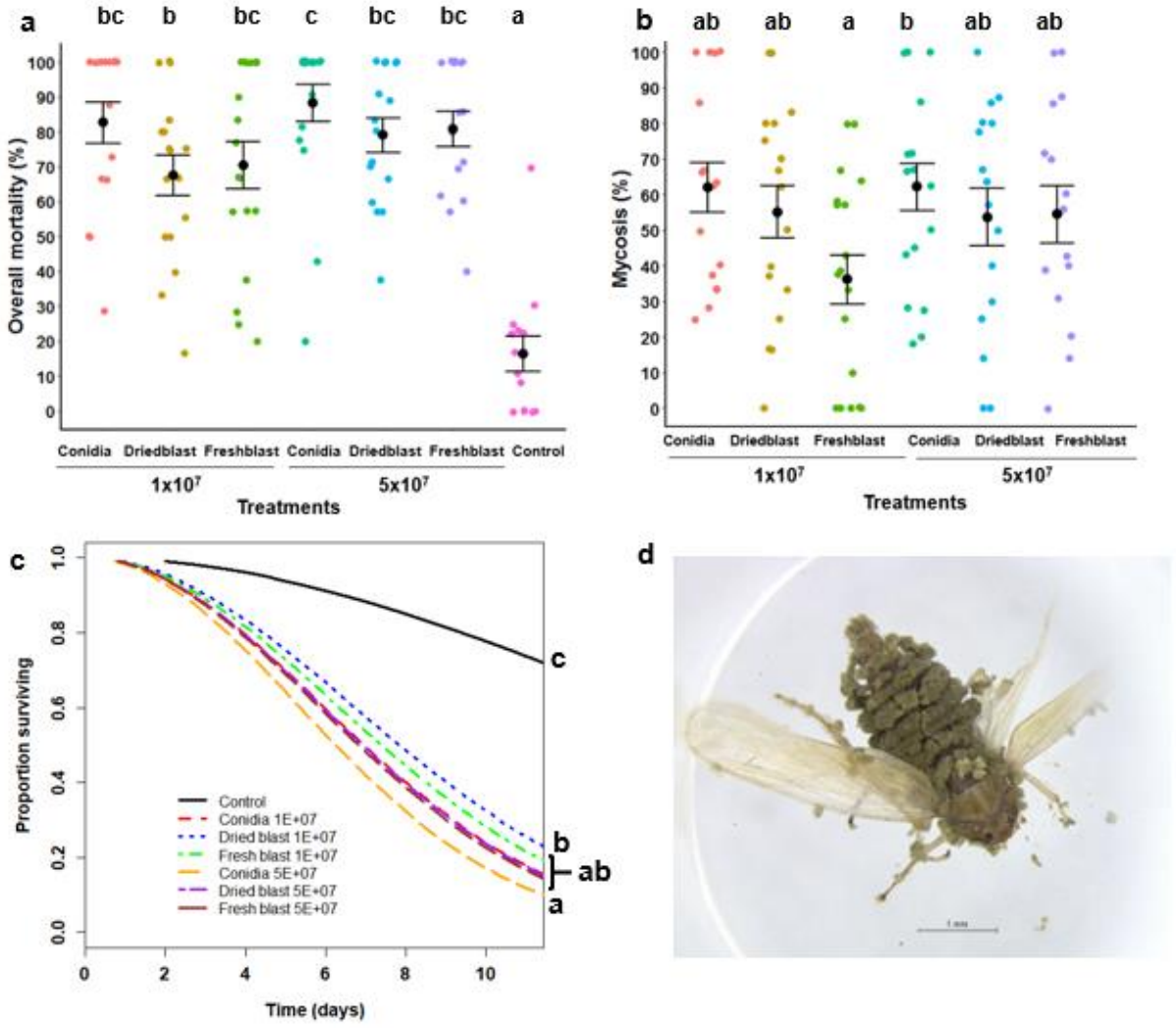


Fig. 4. a) and **b**): Overall mortality (**a**) and mycosis (**b**) in adults of leafhoppers *D. maidis* treated with fresh or dried blastospores, or fresh conidia of *M. robertsii* (ESALQ 1426) at two concentrations (1 x 10^7 or 5 × 10^7 conidia or blastospores mL^{-1}). Mean ± standard error (n = 18). Distinct letters indicate significant differences in overall mortality and mycosis between treatments according to Tukey's HSD test ($P < 0.05$). **c**): Proportion surviving of leafhoppers *D. maidis* after exposure to fresh or dried blastospores or fresh conidia of *M. robertsii* (ESALQ 1426) at two concentrations. Distinct letters indicate significant differences between survival curves according to the log-likelihood ratio test ($P < 0.05$) D): Mycosis caused by *M.*

robertsii in adult leafhopper *D. maidis* post-spraying with dry blastospores six days after death (**d**)

3.4. Discussion

We demonstrated for the first time the importance of the selection of a suitable and low-cost protein source to produce *M. robertsii* blastospores. Cornsteep liquor CSL is an inexpensive byproduct (a bulk product) from the corn oil industry and appeared to be the preferred nitrogen source for blastospore production of this fungus with a production peak being achieved using 80 g L^{-1} and within 48 h of cultivation, resulting in high and fast yield. Resultant blastospores from this culture medium showed desiccation tolerance after slow air-drying and the dry preparation of blastospores showed a virulence similar to aerial conidia and fresh blastospores, to the corn leafhopper *D. maidis*.

Optimization of nutrient concentration of an inexpensive protein source that supports high productivity of blastospores is of utmost importance to enable a production to take place at a large scale by the industry. In the current work, we optimized the modified Adamek's medium for the production of *M. robertsii* blastospores, previously published by our group (Iwanicki et al. 2018). The great advance was the significant decrease in the cost of the medium from approximately U$ 13.55 L^{-1} (modified Adamek's medium) to U$ 0.36 / liter (optimized medium), while the productivity was only slightly affected, resulting in 4.79 x 10^8 blastospores mL^{-1} (optimized medium) when compared to 5.9 × 10^8 blastospores $mL^{-1}$of the modified Adamek's medium, after 48 h of fermentation. This represents a breakthrough in the medium cost and was achieved with the selection of bulk ingredients of glucose and only one source of protein (the byproduct of the wet-milling industry, CSL), while in our previous modified Adamek's medium we had supplied it with two protein sources (i.e., yeast extract + CSL), plus monohydrate glucose, all with high purity and high cost (Iwanicki et al. 2018). Nonetheless, our optimized medium has shown very promising to produce blastospores of other Hypocrealean species: *Metarhizium rileyi*, *Metarhizium anisopliae*, *B. bassiana* and *Cordyceps fumosorosea* (N.S. Iwanicki, unpublished data).

We demonstrated that the production of *M. robertsii* blastospores is boosted when organic and complex sources of nitrogen are used, such as yeast extract and CSL, instead of inorganic sources such as glutamic acid and urea, in agreement with Mascarin et al. (2018a) that reported similar results for *B. bassiana* blastospores cultured with different organic and inorganic nitrogen sources. We contend that our results are associated with CLS's complex and rich composition of minerals and vitamins that act as growth factors (Liggett and Koffler 1948).

We demonstrated the main growth phases of *M. robertsii* in liquid medium by describing its kinetics for blastospore production. The phase of greater cell multiplication and biosynthesis cell material (log-phase) took place between 12 to 48 h of growth. Additionally, it was during this interval that we noticed the highest glucose and nitrogen consumption accompanied by a rapid drop in the culture's pH while corresponding to the highest specific growth rate. The final products of the aerobic fermentation process of *M. robertsii* cultures were partly composed of organic acids and carbonic acid due to the respiration process in liquid medium and this can explain why the medium turned more acidic. We speculate that the slight decline in pH coincides with the higher increase in blastospore yield while matching as well with the peak in glucose and nitrogen consumption due to the intense cell proliferation and metabolism during the log-phase. Initially, we hypothesized that the lack of nitrogen or glucose would be limiting factor for high blastospore density in liquid culture, however we showed that, after 48 h of cultivation, the fungus had consumed only 37% (± 57.3 g L^{-1}) and 51% (1.25 g L^{-1}) of glucose and nitrogen, respectively, supplied in the culture medium.

Besides being used as carbon source CLS can be used to increase the osmolarity of the culture medium, a previously proven condition favouring the production of blastospores (Iwanicki et al. 2018; Mascarin et al. 2015b), hence a significant reduction of this ingredient should be avoided to preserve high osmolarity. Additionally, we have for the first time documented the relationship between blastospore growth and nitrogen consumption in liquid culture of *M. robertsii*, and this can be considered a strong predictor to drive the growth of blastospores as shown by our commonality regression analysis.

The amount of oxygen dissolved in the culture medium is extremely important for the maintenance of blastospore growth (Jackson 2012; Mascarin et al. 2015b).

However, under culture conditions in shake flasks, the dissolved oxygen level is not controlled and usually falls under the critical dissolved oxygen concentration required for high cell density. In order to provide adequate oxygen supply in shake flask cultures of *B. bassiana*, for instance, it is possible to increase the osmotic pressure of the medium to enhance the dissolved oxygen level resulting in better blastospore yields (Mascarin et al. 2015b). After 48 h of cultivation, we noticed that the medium began to become more viscous due to the transformation of blastospores into hyphae. Therefore, we believe that even in the presence of nutrients, these *M. robertsii* cultures may have lacked oxygen supply for optimal blastospore growth promotion and consequently the fungus to some extent entered the stationary phase, producing hyphae and secondary metabolites instead of blastospores. Another hypothesis is that growth of the blastospores ceased due to the presence of high amount of secondary metabolites or other compounds excreted by the blastospores in the medium that impaired cell growth. Although *auto-inhibitory* metabolites have not been described as a limiting growth factor in *Metarhizium* spp. liquid cultures, this hypothesis remains to be elucidated and cannot be discarded.

For the first time we reported the infectivity of fresh and dried *M. robertsii* blastospores on the corn leafhopper *D. maidis* with mortality values varying from 65 to 77%. Additionally, our work with the isolate ESALQ1426 showed that the blastospore efficiency in controlling the corn leafhopper is similar compared to aerial conidia at both concentrations (1×10^7 and 5×10^7 propagules mL^{-1}) and the same for controlling larvae of the cattle-tick, at higher concentration (5×10^7 propagules mL^{-1}) (Iwanicki et al. 2018). Equal efficiency of aerial conidia and blastospores was reported by Ramle and Norman (2014) after applying another species, *Metarhizium major,* for controlling third instar larvae of the Asiatic rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabeidae) and by Morales-Reyes et al. (2018), who compared the virulence of *C. fumosorosea* and *B. bassiana* aerial conidia and blastospores against the Asian citrus psyllid, *Diaphotina citri* (Hemiptera: Liviidae). Some studies have even shown a greater efficiency of *M. brunneum* (Alkhaibari et al. 2016) and *B. bassiana* (Mascarin et al. 2015a) blastospores in relation to aerial conidia.

The virulence of blastospores can be enhanced by manipulation of the composition of liquid culture medium (Mascarin et al. 2015b; Lane et al. 1991b). The

medium lethal time (LT_{50}) for the adult of green rice leafhopper, *Nephotettix virescens* (Hemiptera: Cicadellidae) of conidia and blastospore from nitrogen-limited cultures batches were significantly lower than that for blastospores produced in carbon-limited culture (Lane et al.1991a). One reason for this outcome is attributed to the greater adhesion to the insect cuticle of blastospores produced in media with lack of nitrogen due they appear with thicker cell wall than those produced in carbon-limited culture (Lane et al. 1991b). Additionally, as shown by Mascarin et al. (2015b), *B. bassiana* blastospores grown in culture with high amount of glucose (140 g L^{-1}) are smaller and more virulent to whitefly nymphs, *Bemisia tabaci* (Hemiptera: Aleyrodidae) when compared to those grown in medium with lower glucose level (40 g L^{-1}). In our study, we did not explore the effect of the culture medium on the virulence of blastospores, yet we indeed demonstrate that after drying, the blastospores derived from our cost-effective liquid medium remained as virulent as fresh blastospores, which prompts blastospores for development of dry formulations for spray applications. Nevertheless, future studies are warranted to confirm that the virulence of these blastopores are maintained unaltered after being stored for long storage periods.

Considering the great importance of the corn crop for the Brazilian economy, the damage caused by leafhoppers and the inefficient chemical control options, our work places a great advance by reducing the cost of liquid culture media for producing blastospores, which represents one of the biggest limitations expenses for industry. These blastospores, either freshly harvested or air dried for cell stabilization, may offer an excellent biological tool to control this noxious maize pest. Our results therefore pave the way for the industrial large-scale production of *M. robertsii* blastospores in deep-tank bioreactors, and with the resultant air-dried blastospores as a potential bioinsecticide for rapidly controlling *D. maidis* on maize plants. The blastospore production seems indeed to be a realistic and attractive alternative to synthetic insecticides. Additional studies should investigate the presence of *M. robertsii* auto inhibitory compounds during liquid cultivation, which may hamper blastospore's growth; to study the effect of dissolved oxygen in liquid culture medium on blastospore production and finally and as continuation of the present study, *Metarhizium* blastospores formulations should be developed for enabling the widespread commercial use of this fungal propagule

Acknowledgments

The authors are grateful to the technician Lenita Maria and Professor Marcos Yassuo Kamogawa from Exact Sciences Department of University of São Paulo/ESALQ for the help in the extraction and quantification of nitrogen in liquid culture media and Anderson Ramos for granting the insects for the bioassays.

Also, we are thankful to The Brazilian National Council for Scientific and Technological Development (CNPq) [grant number 421629/2016-9] for financial support. The first author thanks the São Paulo Research Foundation (FAPESP) [grant number 2016/20610-6] for the scholarship during the PhD project and GMM thanks to the Embrapa [grant # 02.13.12.003.00.00].

Conflict of interest

Authors declare not having any competing interest.

Ethical statement

This article does not contain studies with human participants or vertebrates performed by any of the authors.

Authors´ contribution

NSI, GMM and ID planned the research and designed the experiments. NSI and SGM performed the experiments. GMM and NSI analyzed data. NSI, ID, JE and GMM wrote the manuscript. All authors discussed results and commented on the manuscript.

References

Adamek L (1965) Submerse Cultivation of the Fungus *Metarhizium anisopliae* (Metsch.). Folia Microbiol (Praha) 10:255-257.

AGROFIT, 2019. Sistema de agrotóxicos fitossanitários - ministério da Agricultura, Pecúaria e Abastecimento. http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. (Accessed 10 October, 2019).

Alkhaibari AM, Carolino AT, Bull JC, Samuels RI, Butt TM, Andreadis T (2017) Differential pathogenicity of *Metarhizium* blastospores and conidia against larvae of three mosquito species. J Med Entomol 54:696–704. https://doi.org/10.1093/jme/tjw223

Alkhaibari AM, Carolino AT, Yavasoglu SI, Maffeis T, Mattoso TC, Bull JC, Samuels RI, Butt TM (2016) *Metarhizium brunneum* blastospore pathogenesis in *Aedes aegypti* larvae: Attack on Several Fronts Accelerates Mortality. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1005715

Behle RW, Garcia-Gutierrez C, Tamez-Guerra P, McGuire, MR, Jackson, MA (2006). Pathogenicity of blastospores and conidia of *Paecilomyces fumosoroseus* against larvae of the mexican bean beetle (Coleoptera: Coccinellidae). Southwest. Entomol. 31:289–295.

Bernardo CC, Barreto LP, Silva CDSRE, Luz C, Arruda W, Everton KK (2018) Conidia and blastospores of *Metarhizium* spp. and *Beauveria bassiana* s.l.: Their development during the infection process and virulence against the tick *Rhipicephalus microplus*. Tick Borne Dis 9:1334–1342 . https://doi.org/10.1016/j.ttbdis.2018.06.001

Canale MC, Lopes JRS, Nesi CN, Prado SDS (2018) Role of *Dalbulus maidis* (Hemiptera: Cicadellidae) gender on maize bushy stunt phytoplasma transmission. Phytopathogenic Mollicutes. https://doi.org/10.5958/2249-4677.2018.00005.1

Canassa F, Esteca FCN, Moral RA., Meyling NV, Klingen I, Delalibera I (2019) Root inoculation of strawberry with the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* reduces incidence of the two spotted spider mite and selected insect pests and plant diseases in the field. J Pest Sci. https://doi.org/10.1007/s10340-019-01147-z

Elena GJ, Beatriz PJ, Alejandro P, Lecuona RE (2011) *Metarhizium anisopliae* (Metschnikoff) Sorokin Promotes Growth and Has Endophytic Activity in Tomato Plants. Adv Biol Res 5:22–27

Faria MRD, Wraight SP (2007) Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of

formulation types. Biol Control 43:237–256. https://doi.org/10.1016/j.biocontrol.2007.08.001

Galvani F, Gaertner E (2006) Adequação da metodologia Kjeldahl para determinação de nitrogênio total e proteína bruta (Circular Técnica 63). Corumbá: Embrapa Pantanal, 9p. (In Portuguese)

Wickham H, (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Issaly N, Chauveau H, Aglevor F, Fargues J, Durand A (2005) Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. Process Biochem 40:1425–1431. https://doi.org/10.1016/j.procbio.2004.06.029

Iwanicki NSA, Ferreira BDO, Mascarin GM, Júnior ID (2018) Modified Adamek's medium renders high yields of *Metarhizium robertsii* blastospores that are desiccation tolerant and infective to cattle-tick larvae. Fungal Biol. https://doi.org/10.1016/j.funbio.2018.05.004

Jaber LR, Alananbeh KM (2018) Fungal entomopathogens as endophytes reduce several species of *Fusarium* causing crown and root rot in sweet pepper (*Capsicum annuum* L.). Biol Control 126:117–126. https://doi.org/10.1016/j.biocontrol.2018.08.007

Jaber LR, Enkerli J (2017) Fungal entomopathogens as endophytes: can they promote plant growth? Biocontrol Sci Technol 27:28–41. https://doi.org/10.1080/09583157.2016.1243227

Jackson MA (2012) Dissolved oxygen levels affect dimorphic growth by the entomopathogenic fungus Isaria fumosorosea. Biocontrol Sci Technol 22:67–79. https://doi.org/10.1080/09583157.2011.642339

Jackson MA, Mcguire MR, Lacey LA, Wraight SP (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycol Res 101:35–41. https://doi.org/10.1017/S0953756296002067

Jaronski ST (2013) Mass Production of Entomopathogenic Fungi: State of the Art. Mass Prod Benef Org Invertebr Entomopathog 357–413 . https://doi.org/10.1016/B978-0-12-391453-8.00011-X

Kim JS, Je YH, Skinner M, Parker BL (2013) An oil-based formulation of Isaria fumosorosea blastospores for management of greenhouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). Pest Manag Sci 69:576–581. https://doi.org/10.1002/ps.3497

Krell V, Unger S, Jakobs-Schoenwandt D, Patel AV. (2018) Endophytic *Metarhizium brunneum* mitigates nutrient deficits in potato and improves plant productivity and vitality. Fungal Ecol 34:43–49 . https://doi.org/10.1016/j.funeco.2018.04.002

Lane BS, Trinci APJ, Gillespie AT (1991a) Influence of cultural conditions on the virulence of conidia and blastospores of *Beauveria bassiana* to the green leafhopper, *Nephotettix virescens*. Mycol Res 95:829–833. https://doi.org/10.1016/S0953-7562(09)80046-4

Lane BS, Trinci APJ, Gillespie AT (1991b) Endogenous reserves and survival of blastospores of *Beauveria bassiana* harvested from carbon- and nitrogen-limited batch cultures. Mycol Res 95:821–828. https://doi.org/10.1016/S0953-7562(09)80045-2

Liggett RW, Koffler H (1948) Corn steep liquor in microbiology. Bacteriol Rev 12:297–311

Mascarin GM, Jackson MA, Behle RW, Kobori NN, Júnior ÍD (2016) Improved shelf life of dried *Beauveria bassiana* blastospores using convective drying and active packaging processes. Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-016-7597-2

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Dunlap CA, Júnior ID (2015a) Glucose concentration alters dissolved oxygen levels in liquid cultures of *Beauveria bassiana* and affects formation and bioefficacy of blastospores. Appl Microbiol Biotechnol 99:6653–6665. https://doi.org/10.1007/s00253-015-6620-3

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Júnior ID (2015b) Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria*

bassiana and *Isaria fumosorosea* strains. J Invertebr Pathol 127:11–20. https://doi.org/10.1016/j.jip.2014.12.001

Mascarin GM, Kobori NN, Jackson MA, Dunlap CA, Júnior ID (2018) Nitrogen sources affect productivity, desiccation tolerance and storage stability of *Beauveria bassiana* blastospores. J Appl Microbiol 124:810–820. https://doi.org/10.1111/jam.13694

Mascarin GM, Biaggioni RL, Júnior ID, Fernandes EKK, Luz C, Faria M (2019) Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. J Invertebr Pathol. 165: 46-53, https://doi.org/10.1016/j.jip.2018.01.001

Morales-Reyes C, Mascarin GM, Jackson MA, Hall D, Sánchez-Peña SR, Arthurs SP (2018) Comparison of aerial conidia and blastospores from two entomopathogenic fungi against *Diaphorina citri* (Hemiptera: Liviidae) under laboratory and greenhouse conditions. Biocontrol Sci Technol 28:737–749. https://doi.org/10.1080/09583157.2018.1487028

Nault, L.R. (1990) Evolution of an insect pest: Maize and the corn leafhopper, a case study. Maydica, 35, 165–175.

Parra J (2014) Biological control in Brazil. Sci Agric 71:345–355. https://doi.org/10.1590/0103-9016-2014-0167

Posch AE, Spadiut O, Herwig C (2012) Switching industrial production processes from complex to defined media: method development and case study using the example of *Penicillium chrysogenum*. Microb Cell Fact https://doi.org/10.1186/1475-2859-11-88

Ramle M, Norman K (2014) The use of palm kernel cake in the production of conidia and blastospores of *Metarhizium anisopliae* var. *major* for control of Oryctes rhinoceros. J Oil Palm Res 26:133–139

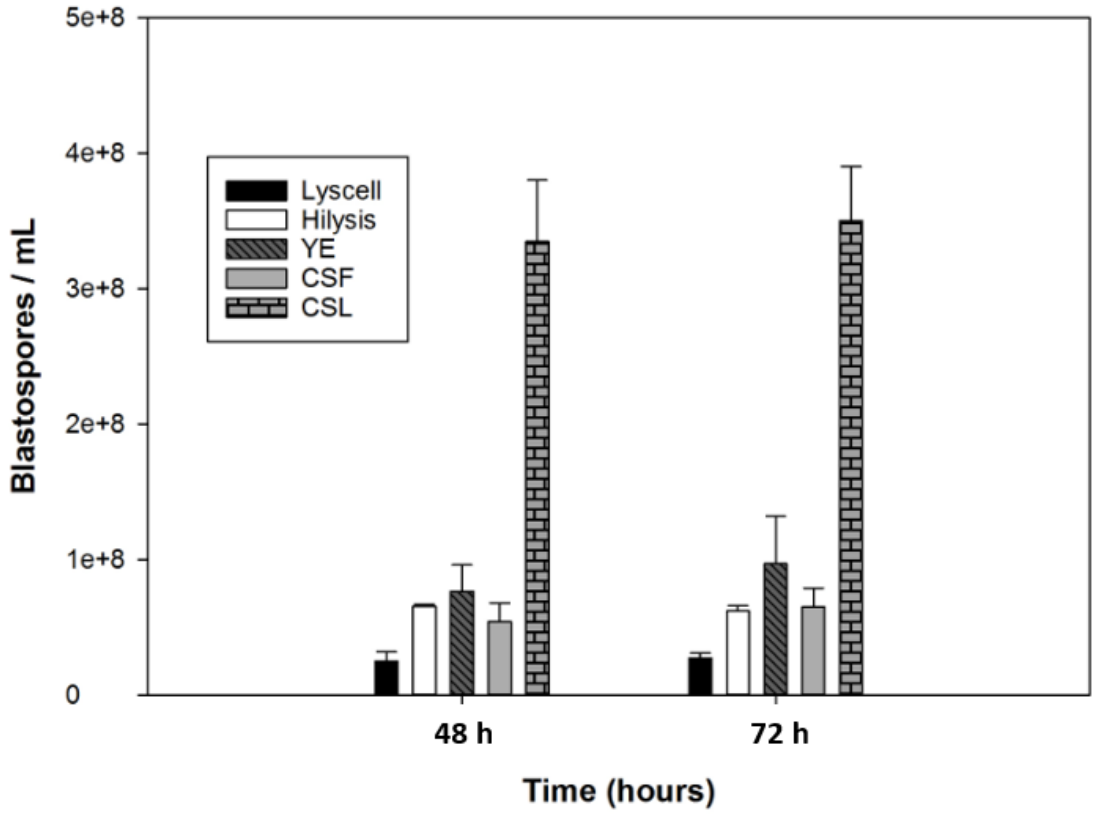
Ray-Mukherjee J, Nimon K, Mukherjee S, Morris DW, Slotow R, Hamer M (2014) Using commonality analysis in multiple regressions: A tool to decompose regression effects in the face of multicollinearity. Methods Ecol Evol 5:320–328. https://doi.org/10.1111/2041-210X.12166

Shapiro-Ilan DI, Cottrell TE, Jackson MA, Wood BW (2008) Virulence of Hypocreales fungi to pecan aphids (Hemiptera: Aphididae) in the laboratory. J Invertebr Pathol 99:312–317. https://doi.org/10.1016/j.jip.2008.07.001

Wassermann M, Selzer P, Steidle JLM, Mackenstedt U (2016) Biological control of Ixodes ricinus larvae and nymphs with *Metarhizium anisopliae* blastospores. Ticks Tick Borne Dis. https://doi.org/10.1016/j.ttbdis.2016.03.010

Ypsilos IK, Magan N (2005) Characterisation of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. Biocontrol Sci Technol 15:683–699. https://doi.org/10.1080/09583150500136774

Appendix



Appendix A - Blastospore yield of *M. robertsii* (ESALQ1426) after 48hr and 72hr when grown on modified Adamek´s medium (Iwanicki et al., 2018) amended with 14% of glucose and 4% of one of the five protein source: Lyscell (autolyzed yeast), Hilysis (hydrolyzed yeast), YE: yeast extract, CSF:Cotton seed flour, CSL:Corn steep liquor. Blastospore yield presented values lower than 1×107 for treatments with: urea (Dinâmica®, Indaiatuba, SP, Brazil), glutamic acid (Dinâmica®, Indaiatuba, SP, Brazil), malt extract (Microgen®, New Delhi, India) hydrolyzed casein (Sigma®).

Appendix B - Experimental unit used in bioassays. Cage containing a maize (*Zea mays* L.) plant in a plastic pot filled with a mixture of clay and sand (50%/50% v/v) sown with three to four non-transgenic maize plants with 30-40 cm high each one, a tube with 40-45 cm high made with acetate to isolate corn plants and a screen cover made by nylon voile fabric fastened with a rubber band.

Appendix C - Cornsteep liquor composition from Company: Ingredion Brasil Industrial Ingredients

Composition	Amount
Density	1.15
Calcium	2.00%
Potassium	2.90%
Electric conductivity	1.52mS/cm
Magnesium	0.95%
Total phosphorus	1.12%
Sulfur	0.25%
Saline Index	14.16%
Nitrogen	3.41%
Iron	647.5mg/kg
NPK (Sum)	7.43%
Total Organic Carbon	16.72%
C/N	4.9
Copper	22.5mg / kg
Zinc	152.5mg / kg
Boron	0.08%
Sodium	0.08%
Manganese	57.5mg/kg
pH	4

Appendix D - Specific growth rate and doubling time of Metarhizium robertsii blastospores (ESALQ1426) grown in medium with 80 g L-1 of corn steep liquor, 140 g L-1 of glucose and incubated at 28 °C and 350 rpm. SGR and DT were determined each 12 hours of culture until 84 hours.

Interval (h)	Specific growth rate (h-1)	Doubling time (h)
0 to 12	0	0
12 to 24	0.32 ± 0.08	2.15 ± 0.05
24 to 36	0.04 ± 0.02	21.04 ± 10.4
36 to 48	0.03 ± 0.02	33.48 ± 25.9
48 to 60	-0.01 ± 0.03	-10.97 ± 43.4
60 to 72	0.01 ±0.004	258.35 ± 251.2
72 to 84	0.00 ± 0.005	3360.49 ± 2938

4. SHELF LIFE OF SPRAY-DRYING AND AIR-DRYING FORMULATIONS OF *Metarhizium robertsii* BLASTOSPORES AND THEIR BIOEFFICACY AGAINST THE CORN LEAFHOPPER *Dalbulus maidis*

Natasha Sant´Anna Iwanicki[a], Gabriel Moura Mascarin[b], Sara Giro Moreno[a], Jørgen Eilenberg[c], Italo Delalibera Júnior[a]

[a]Department of Entomology and Acarology, Escola Superior de Agricultura “Luiz de Queiroz”, University of São Paulo (ESALQ-USP), Av. Pádua Dias, 11, C.P. 9, Piracicaba, SP CEP 13418-900, Brazil

[b]Laboratory of Environmental Microbiology, Brazilian Agricultural Research Corporation, Embrapa Environment, Rodovia SP 340, Km 127.5, 13820-000 Jaguariúna, Brazil.

[c]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark.

*Corresponding author: Natasha S. A. Iwanicki
E-mail address: natasha.iwanicki@usp.br
Department of Entomology and Acarology, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

Abstract

Formulation technology is key for the commercial development of robust, shelf stable and effective fungal biopesticides. In the current research, spray-drying and air-drying processes employed for *Metarhizium robertsii* blastospores were developed to obtain a wettable powder (WP) formulation. To achieve this, we investigated the effect of co-formulants on the viability of blastospores after drying along with the assessment of the wettability and stability properties in water of the resultant formulations. The effect of oxygen-moisture absorbers was assessed aiming to increase shelf life of these formulations stored under room temperature (28 °C) and cold storage at 4 °C for up to 90 days. Additionally, we determined the insecticidal activity of the best spray-dried and air-dried formulations toward the corn leafhopper *Dalbulus maidis*, an important corn pest in Brazil. The importance of the proper selection of co-formulants allowing increased cell viability after drying was clearly demonstrated. While sucrose and skim milk played an important role as drying protectants in preserving air-dried blastospores, maltodextrin, skim milk and bentonite were crucial to attain high cell survival for spray-drying. In general, the lowest wettability time was achieved with spray dried formulations containing less amounts of Ca^{2+}-lignin, while the most stable formulations after 60 minutes were those containing increased amounts of charcoal powder. The addition of oxygen-moisture absorbers into sealed packages increased from three to four-fold the half-life times of air-dried and spray-dried formulations stored either refrigerated (4 °C) or ambient temperature (28 °C) compared to the respective controls. However, the values obtained for half-life using modified atmosphere were less than 3 months. We demonstrated that spray-dried and air-dried formulations were as infective as fresh blastopores to the corn leafhopper sprayed at 5×10^7 blastospores mL^{-1} inducing mortality rates from 60.3 to 78.2% and with similar median lethal times, 8.9 and 8.2 days respectively. Conversely, the median lethal concentration was significantly greater for air-dried formulation (2.42×10^7) than that obtained with the spray-dried formulation (4.65×10^6), suggesting a possible detrimental effect of the former technology in fungal virulence. Our findings bring innovative advancement for blastospore formulation of *M. robertsii* through spray-drying technology highlighting the importance of protective matrices and the use of oxygen-moisture absorbers to keep cell viability during storage.

Keywords: Microencapsulation, Microbial Control, Formulation, Mycoinsecticide, Storage stability, Virulence.

4.1. Introduction

In recent years, there has been a significant expansion of the microbial biopesticides market. Entomopathogenic fungi of the phylum Ascomycota are among the main explored beneficial microbes for biological pest control in the world (Van

Lenteren et al., 2017). Aerial conidia produced by these fungi make up almost all of the fungus-based products marketed worldwide for biological control of agricultural pests, such as insects and mites (Faria and Wraight, 2007). Besides aerial conidia, many ascomycetous dimorphic entomopathogenic fungi produce a less explored but potentially infectious propagule termed blastospores. Blastospores are yeast-like single cells naturally formed during the infection process undergone in the insect hemolymph (Boucias and Pedland, 1998; Pedrini et al., 2017). Its function is associated with the spread of the fungal pathogen inside the insect body, as these cells are unrecognized by the host immune system. Nutrient consumption combined with toxin production by the blastospores lead to the death of the host and this outcome allows the fungus to completely colonize the host internally and externally (Pedrini et al., 2018). Although naturally produced in the insect hemolymph, blastospores can be produced artificially in vitro in a balanced liquid culture medium, within a short period of time and under specific growth conditions already described in many earlier studies (Iwanicki et al., 2018; Mascarin et al., 2015a; Jackson, 1997; Vega et al., 1999). Blastospores of the fungus *Metarhizium* have already been shown to be as infectious as aerial conidia to different arthropod hosts (Ramle and Norman, 2014; Iwanicki et al., 2018; Alkhaibari et al. 2017) or even more virulent (Alkhaibari et al., 2016; 2017; Dong et al., 2016). However, compared to aerial conidia, blastospores are more sensitive to osmotic and oxidative stresses induced by dehydration or other environmental stresses; thus, they require cautious manipulation during drying and formulation steps of the microbial biopesticide development process (Jaronski and Mascarin, 2016). In this context, the art of formulating blastospores with the appropriate ingredients (co-formulants: inerts, adjuvants, protective matrices etc.) represents an essential component for their protection from stressful factors during the drying and rehydration processes, as well as maintaining long storage viability without jeopardizing their efficacy in the field.

For many decades, the long term storage of microbial cultures has been preferably pursued by different drying methodologies documented in an extensive literature on preserving microorganisms for various purposes, but results are often specific to one particular isolate, which indeed poses a great challenge to microbial industry when dealing with different species and isolates of microbes of interest. Many methods for drying fungal biomass are known and hence can be explored for

industrial-scale formulation and stabilization purposes, such as freeze drying, spray-drying, fluidized bed drying and air-drying (Horaczek and Viernstein, 2004; Mascarin and Jaronski, 2016). While all of these drying methods have different benefits and specific applications, air-drying and spray-drying are the two most promising techniques for drying blastospores (Iwanicki et al., 2018; Mascarin et al., 2016; Mascarin et al., 2016, Jackson and Payne, 2007; Jackson et al., 1997). Although formulations could take long hours for air-drying, the main advantage of this methodology lies in its low cost and reduced stress upon the blastospores due to the slow drying characteristic (Jackson et al., 2007). On the other hand, it is possible to dry quickly high volumes of formulations properly designed for sensitive microbial cells using the spray dryer system at relatively high temperatures with minimal harmful impact on cell viability (Stephan and Zimmermann, 1998; Mascarin et al., 2016), including fungal blastospores that seems to be more prone to suffer from high osmotic and thermal stresses during this process.

Some previous efforts in stabilizing *Metarhizium* blastopores grown in liquid media through assessment of their desiccation tolerance after spray-drying resulted in cell viability rates up to 80% for one out of the three *M. anisopliae* isolates tested after storage of blastospores in 10% hydroxyethyl starch at 4 °C (Kleespies and Zimmermann (1994). Stephan and Zimmermann (1998) were pioneered in the use of the spray-drying technique for dehydrating submerged spores (blastospores and submerged conidia) of one strain of *M. anisopliae* and another strain of *Metarhizium flavoviride*. The authors reported high blastospores viability (> 80%) immediately after drying, although they neither evaluated the storage stability nor bioefficacy of such spray-dried cells. While studies focusing on blastospore stability of *Metarhizium* spp. are quite scarce, more advances have been made with the dissection tolerance of *Cordyceps fumosorosea* (formerly *Isaria fumosorosea*) and *Beauveria bassiana* blastospores resulting in high blastospores survival after freeze or air-drying (Cliquet and Jackson, 1997; Jackson et al. 1997, 2006; Mascarin et al., 2015a; Mascarin et al., 2016; Mascarin et al., 2018) and spray-drying (Stephan and Zimmerman., 1998; Mascarin et al., 2016). Additionally, some of these studies investigated the shelf life of dried blastopores and reported excellent cell viabilities of up to 90% when *B. bassiana* blastospores were packaged with modified atmosphere stored for nine months at 28 °C (Mascarin et al., 2016). However, to the best of our knowledge very

few studies deals with the shelf life of spray-dried or air-dried *Metarhizium* blastospores along with important technical physical parameters used to evaluate both stability and wettability of typical wettable (microgranule) powder formulations, and its bioactivity on a targeted pest. Such dry microgranule formulations in a dry state generally possess good shelf life, are easy to handle and to use with water for spray applications in agriculture, which are of considerable interest to the industry, as only few fungal-derived biopesticides containing blastospores haven been developed to date.

In our previous studies, we demonstrated the great potential of *M. robertsii* to produce high amounts of blastospores (4.7×10^8 mL^{-1}) in only two days by liquid fermentation under specific nutritional growth conditions (Iwanicki et al., 2018). We have optimized an inexpensive and productive culture medium and we demonstrated, for the first time, the efficiency of air-dried blastospores to control cattle-ticks (*Rhipicephalus microplus*) (Iwanicki et al., 2018) and corn leafhopper (*Dalbulus maidis*) (Iwanicki et al. unpublished results). Therefore, in this paper, we placed our focus on the following specific objectives: 1) selecting appropriate co-formulant ingredients for maintenance of good blastospore viability during spray-drying and air-drying by developing various wettable powder formulations; 2) determining the wettability and stability of these formulations; 3) assessing the shelf life of our two best formulations packaged with or without modified atmosphere technology and then stored under refrigerated (4 °C) and ambient temperature (28 °C); 4) determining the bioefficacy of these two best formulations (one obtained with spray-drying and another with air-drying) against corn leafhoppers (*D. maidis*) through spray applications of different blastospore concentrations on corn leaves. The insect host was chosen as it is an economically important pest due to its direct damage and the transmition of several pathogens to corn plants in Brazil.

4.2. Material and Methods

4.2.1. Fungal cultivation

The *M. robertsii* isolate ESALQ1426 was chosen for this study due to our previous results in which we showed that this fungus produced a fair amount of blastospores when grown in a low-cost composition of modified Adamek's medium

(4.7×10^8 blastospores mL^{-1}), which also exhibited good efficacy against the corn leafhopper *D. maidis* (Iwanicki et al. unpublished results). The ESALQ1426 isolate is officially preserved in the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) in 2-mL cryovials containing 10% sterile glycerol solution stored at –80 °C.

The isolate ESALQ1426 was cultured in 50 mL liquid medium in 250-mL baffled Erlenmeyer flasks (Bellco® Glass, Vineland, NJ, USA) over the course of 3 days at 28±0.5 °C and 350 rpm using a rotating incubator shaker with an orbit diameter of 10 mm (Solab®, Piracicaba, SP, Brazil). The fungus was first cultivated during 3 days in a preculture medium composed of 40 g L^{-1} glucose monohydrate (35% carbon, Ingredion®, Mogi Guaçu, SP, Brazil), 80 g L^{-1} cornsteep liquor (Ingredion®, Mogi Guaçu, SP, Brazil), mineral salts, trace metals and vitamins as described in Iwanicki et al. (2018) at the following concentrations per liter: KH_2PO_4, 2.5 g; $CaCl_2.2H_2O$, 1.0 g; $MgSO_4.7H_2O$, 0.83g; $FeSO_4.7H_2O$, 0.3g ; $CoCl_2.6H_2O$, 29.6 mg; $MnSO_4.H_2O$, 12.8 mg; $ZnSO_4.7H_2O$, 11.2 mg; 0.2 mg each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid; and 0.02 mg each of folic acid, biotin, and vitamin B12. Preculture medium was inoculated with 5 mL (i.e., 10% of total medium volume) of a suspension containing 5×10^6 conidia mL^{-1}.

Blastospores cultivated in precultures were used to inoculate new liquid cultures composed of 80 g L^{-1} cornsteep liquor (16.7% total organic carbon and 3.41% nitrogen on a dry basis, Ingredion®, Mogi Guaçu, SP, Brazil), 140 g L^{-1} glucose monohydrate (35% carbon on a dry basis, Ingredion®, Mogi Guaçu, SP, Brazil) and mineral salts, trace metals and vitamins at the same concentration as indicated above in the preculture medium with a carbon-to-nitrogen (C:N) ratio adjusted to 30:1 and with a total carbon of 70 g L-1. The culture medium was inoculated with a 150 mL of a suspension (i.e., 10% of total medium volume) containing 5×10^7 blastospores mL^{-1} and left to grow during two days in a 2 L bioreactor (Bioflo III Batch Continuous Fermentor Bioreactor, New Brunswick®, New Jersey, NJ, USA) containing 1.5 L of liquid medium plus the inoculum volume. The glucose solution was autoclaved separately from the basal media and added with the sterile vitamin solution to the medium once autoclaved and cooled off. The pH was initially adjusted to 6.8 before autoclaving. Gas flow was purged with filtered atmospheric air at a rate of 1 L min^{-1}, agitation speed set to 400 rpm and constant

temperature kept at 28 °C. These conditions were maintained during the cultivation period and the pH was left uncontrolled.

4.2.2. Screening co-formulants for blastospore formulations

After two days of cultivation, *M. robertsii* blastospores were harvested and filtered through Whatman® filter paper (pore size: 25µm) for removing the mycelium. 7.5% of each inert was added to the culture filtrate containing mostly blastospores: Albite (Mão na Massa®, São Paulo, Brazil), Grey-clay (Mão na Massa®, São Paulo, Brazil), Bentonite (Mão na Massa®, São Paulo, Brazil), Kaolin (Mão na Massa®, São Paulo, Brazil), Cream Phyllite (Mão na Massa®, São Paulo, Brazil), Dolomite (Mão na Massa, São Paulo, Brazil), Diatomaceous earth (Sigma-Aldrich®, Brazil). Then, the mixture of blastospores with inerts was filtered again through Whatman® filter paper (pore size: 6-8 µm) with the aid of a vacuum pump, for removing the spent medium. Next, a semi-solid paste composed of a mixture of blastospores and co-formulants were crumbled using an electric blender and then placed into Petri dish plates for overnight drying inside a controlled humidity air-drying chamber for 15-18 h with 50-60% RH in the air flow and an additional 2 h under 15% RH, as described by Mascarin et al. (2015a). The water activity of the mixture was evaluated using a water activity meter (Novasina - Labmaster AW, Pfäffikon, Switzerland). Then, when water activity of the mixture dropped to < 0.3, the fungal preparations (or mixtures) were vacuum-packed in polyethylene bags and stored in the refrigerator at 4 °C and another same group of formulations were stored at room temperature of 26 ± 2 °C inside a desiccator containing silica gel to maintain external RH constant and low. The blastospores viability was determined seven days after drying upon rehydration and after 30 days of storage, following procedures described in the blastospore viability section. For each evaluation date, the viability was determined from two independent bags each representing a destructive sample.

4.2.3. Air-dried formulations

We developed eight formulations composed of different combinations of ingredients containing varying concentrations of silicon dioxide (Exôdo científica, São Paulo, Brazil) and bentonite clay (Mão na Massa, São Paulo, Brazil) used as

dispersant, activated charcoal powder (Exôdo científica, São Paulo, Brazil) and Ca-lignin (Borregaard LignoTech, Norway), used as UV light protectors and dispersant and the drying protectants: sucrose, (Exôdo científica, São Paulo, Brazil), skim milk (Piracanjuba®, Goiás, Brazil) and monosodium glutamate (Exôdo Científica, São Paulo, Brazil). The formulation strategy adopted in this part of our study followed a statistical designed based on Plackett-Burman (Table 1). For the sake of comparison, we used as a positive control a formulation composed of blastospores mixed only with 5% bentonite.

Blastospores were cultured during two days as described in the fungal cultivation section, harvested and filtered on Whatman® filter paper (pore size: 25 µm) for removing mycelium. Then, ingredients were added to 200mL of stirring filtered blastospores broth at amounts described in Table 1 and following the order: Bentonite, Ca-lignin, activated charcoal, SiO_2, Sodium glutamate, sucrose and skim milk. Formulated blastospores were filtered on Whatman® filter paper (pore size: 6-8 µm) using a vacuum pump, which removed the medium, and for drying the same above mentioned procedure was employed. Dried formulations were vacuum-packed in polyethylene bags for storage in refrigerator at 4 °C for one week. The entire experiment was repeated three times using different fungal batches in order to ensure reproducibility.

Table 1. *Metarhizium robertsii* (ESALQ 1426) air-dried blastospores formulations according to Plackett-Burman experimental design. The formulations are numbered in each line and the percentage of each ingredient per volume of medium is described in the respective column.

Formulations	Ca-Lignin (%)	Activated charcoal (%)	Sodium glutamate (%)	Bentonite (%)	SiO_2 (%)	Sucrose (%)	Skim milk (%)	Total solids (%)
1	5.0	0.0	2.0	5.0	2.0	4.0	4.0	22
2	5.0	2.5	2.0	2.5	4.0	0.0	4.0	20
3	5.0	2.5	4.0	2.5	2.0	4.0	0.0	20
4	2.5	2.5	4.0	5.0	2.0	0.0	4.0	20
5	5.0	0.0	4.0	5.0	4.0	0.0	0.0	18
6	2.5	2.5	2.0	5.0	4.0	4.0	0.0	20
7	2.5	0.0	4.0	2.5	4.0	4.0	4.0	21
8	2.5	0.0	2.0	2.5	2.0	0.0	0.0	9
9*	0.0	0.0	0.0	5.0	0.0	0.0	0.0	5

***** Formulation 9 represent the control, not planned based on the Plackett-Burman experimental design.

4.2.4. Spray-dried formulations

To determine the effect of formulation ingredients on blastospore viability, we examined eight formulations composed of the same ingredients used in the air-drying experiment, except that sucrose was replaced by maltodextrin, as the latter is a polysaccharide that provides thermos- and osmoprotection to cells during heating and drying. A positive control was formulated likewise mentioned in the previous experiment, composed of blastospores with 5% bentonite.

For each experiment, a volume of 200 mL of filtered blastospore suspensions were mixed with co-formulants following the amounts shown in Table 2 in the same order as found for the air-dried formulation section. Formulated blastospore suspensions were spray dried using a LabMaq®, MSD 1.0 Spray Dryer (Ribeirão Preto, SP, Brazil). Drying conditions were set to: 80 ± 2 °C of inlet temperature, 45-50 °C of outlet temperature, 0.5 L h^{-1} feed rate, 40 L min^{-1} of compressed air flow and 4 bar of compressed air pressure. All formulations reached water activity < 0.3 after spray-drying. Dried formulations were vacuum-packed in polyethylene bags and stored in the refrigerator at 4 °C for one week prior to assessing cell viability. The whole experiment was repeated three times on different occasions using distinct batches of fungal blastospores.

Table 2. *Metarhizium robertsii* (ESALQ 1426) spray dried blastospore formulations following a Plackett-Burman experimental design (formulation 1 to 8).The formulations are indicated in each line and the percentage of ingredients per volume of medium is described in the respective column.

Formulations	Ca-Lignin (%)	Activated charcoal (%)	Sodium glutamate (%)	Bentonite (%)	SiO_2 (%)	Maltodextrin (%)	Skim milk (%)	Total solids (%)
1	5.0	0.0	2.0	5.0	2.0	4.0	4.0	22
2	5.0	2.5	2.0	2.5	4.0	0.0	4.0	20
3	5.0	2.5	4.0	2.5	2.0	4.0	0.0	20
4	2.5	2.5	4.0	5.0	2.0	0.0	4.0	20
5	5.0	0.0	4.0	5.0	4.0	0.0	0.0	18
6	2.5	2.5	2.0	5.0	4.0	4.0	0.0	20
7	2.5	0.0	4.0	2.5	4.0	4.0	4.0	21
8	2.5	0.0	2.0	2.5	2.0	0.0	0.0	9
9[#]	0.0	0.0	0.0	5.0	0.0	0.0	0.0	5
10[*]	2.5	0.0	4.0	2.5	6.0	6.0	0.0	21

[#] Control formulation [*] cost-reduced treatment based on formulation 7. Both formulations not planned based on the Plackett-Burman experimental design.

4.2.5. Packaging and shelf life

We selected one spray-dried and one air-dried formulation to determine the shelf life under modified atmosphere packaging during storage conditions at ambient temperature (28 °C) and cold temperature (4-6 °C). Spray-dried and air-dried formulated blastospores were distributed in amounts of 0.45 g and packaged in 15 × 15cm Nylon-poly bags (Equapack®, São Paulo-SP, Brasil) 18 microns thick and made by 5 layers which ensure moisture and oxygen proof. Bags were either vacuum packed (to remove totally the atmospheric oxygen from inside), only sealed, or sealed in modified atmosphere in the active packing experiment using combinations of oxygen and moisture scavengers. One oxygen absorber (Ageless ZPT-100MBC, Mitsubish Gas Chemical America, New York, NY, USA), and two dual moisture/oxygen absorbers (RP-5AN and Agstend AS-100, Mitsubish Gas Chemical America, New York, NY, USA) were used in active packaging experiments. Each bag contained 0.45 g of the dried blastospore formulation, vacuum-packed, sealed or sealed with a single sachet in it were stored in refrigerator at 4 - 6 °C or at 28 °C. Sampling for viability of formulated blastospores was conducted after seven days and then followed by one, two, three and four months or until the blastospore viability had dropped to zero. For each assessment date, destructive samples represented by two bags per formulation were used to determine viability. The entire experiment was repeated three times using different fungal batches.

4.2.6. Blastospore viability protocol

For each evaluation date, the viability was determined from two independent bags, each representing a destructive sample of inerts or spay/air dried formulations. Per bag, an amount of 0.3 g was mixed with water at 25 °C and adjusted to a concentration of 10^6 blastospores mL^{-1}. A volume of 150 µL of this suspension was spread in potato dextrose agar (PDA) plates (Rodac™ type, Dickinson and Co, Franklin Lakes, NJ, USA) and left for 6 h of incubation at 26 °C.

In case of the inert screening experiment, air-dried blastospores were first rehydrated for 30 minutes in humid chamber (>85% relative humidity) before mixed with water. For fresh blastospores, spray dried and air dried formulations, the viability

was determined as described previously without rehydration prior to plating because in these cases, we observed no difference in the germination of previously hydrated and dehydrated formulated blastospores (data not shown).

The viability was determined by counting between 100-150 blastospores, which were categorized as germinated or non-germinated. Germ tubes equal to or longer than half the length of the blastospore cell were assigned as germinated (viable). Thus, the proportion of blastospore viability was given by: viability = number of germinated cells / total number of cells counted per sample.

4.2.7. Wettability

Wettability of spray-dried formulations was determined by transferring 50 mL of hard water (prepared according to BRASIL, ABNT NBR 13074) into 200 mL beaker. A sample of 0.1 g of each formulation was added individually at once to a beaker at a height of 5 cm from the top. The timer was started and the time taken to become completely wet was recorded. The counting was repeated twice for each formulation in each of the three repetitions of the experiment.

4.2.8. Physical stability

The physical stability of spray-dried formulations can be measured by their dispersion capability and was evaluated using optical analyzers (Turbiscan LAB ™, Formulaction Co., France). The Turbiscan LAB ™ allows the analysis of the physical properties of soft materials, such as particle size variation, concentration variation, and physical stability. Various systems such as colloidal dispersions and emulsions have been analyzed by this technique to detect destabilization phenomena such as sedimentation, particle migration and flocculation (Terayama et al., 2003; Yan Sun et al., 2019; Dwari and Mishra, 2019).

For each formulation, six samples were used taken from three spray-drying experiments repeated over time. Each sample had 1 g of dried formulation added to a borosilicate glass cup, 25 mm in diameter and 55 mm high, filled with 20 mL of distilled water and homogenized for 10 seconds. The sample was then scanned by a light beam composed of an electro-luminescent diode in the near infrared region (850 nm) and maintained at a constant temperature of 25 °C. Two optical sensors detected the light transmitted through the sample (180° from the incident light) and

the backscattered light from the sample (45° from the incident radiation). The optical reading scanned the entire length of the sample along the cell (up to 55 mm), obtaining transmission and backscatter data every 40 µm as a function of sample height in mm. Data acquisition was performed just after preparing the sample, time "0", after 30 and 60 minutes.

The profile obtained during the analysis allowed the detection of particle migration and tendency to destabilization of the system from the variation in signals obtained from transmitted or backscattered light. The stability results of the formulations were presented according to the Turbiscan Stability Index (TSI). According to the manufacturer: "*The TSI is a computation directly based on the raw data that you get with the instrument: Backscattering and Transmission signals. It sums up all the variations in the sample, to give as a result a unique number reflecting the stabilization of a given sample. The higher is the TSI, the stronger is the destabilization in the sample*". This index is a statistical factor and its value is calculated as following:

$$\mathrm{TSI} = \sum_{j=0}^{j-h} \left| scanref\left(h_j\right) - scan_i\ (h_j) \right|$$

Where $scanref$ and $scan_i$ are the initial backscattering value and the backscattering value at a given time, respectively, *hj* is a given height in the measuring glass and TSI is the sum of all the scan differences from the bottom to the top of the glass (Lesaint et al., 2009). The TSI of formulations as a function of time were obtained using the Turbiscan LAb computer software. The observed data set composed by TSI recorded for time intervals (0, 30, 60 min) for each formulation were fitted to a quadratic polynomial equation described as $y = y0 + ax + bx^2$ being y the TSI of each time and x the reading time (0, 30 or 60 min). Polynomial curves were adjusted in SigmaPlot® 14.0 (Systat Software Inc., San Jose, CA, USA).

4.2.9. Bioefficacy of blastospore formulations against the corn-leafhopper

The virulence of the spray-dried optimized formulation of *M. robertsii* (ESALQ1426) blastospores was assessed and compared to the virulence of fresh blastospores against adults of the corn leafhopper *D. maidis.* Blastospores were grown as described in the fungal cultivation section above. Blastospores were

harvested from liquid medium after 3 days of culture, formulated, spray-dried, vacuum-packaged in Nylon-poly bags and stored at 4 °C until used in subsequent bioassays (between 6-8 days). Fresh blastospores were obtained by setting up a culture experiment to make the third day of culture matching with the date of bioassay against corn leafhopper. The viability of fresh and spray-dried blastospores was determined as described in the blastospore viability section.

Unsexed adults of corn leafhoppers 8-10 days old after emergence were obtained from a colony kept by the Laboratory of Insect Vectors of Plant Pathogens, Department of Entomology and Acarology from University of São Paulo in Piracicaba, SP, Brazil. The bioassays were conducted using a randomized experimental design with six fungal treatments and six biological replicates (experimental unit) per treatment. The experimental unit consisted of a cage containing a maize (*Zea mays* L.) (Simple hybrid, Fórmula®, Syngenta, Brazil) plant in a plastic pot 10.5 cm diameter and 10 cm height (volume: 865 cm^3) filled with a mixture of clay and sand (50%:50% v/v) sown with two to three non-transgenic maize plants with 30-40 cm high each a tube 40-45 cm high surrounded by an acetate sheet to confine plants and topped with a screen cover made of nylon voile fabric fastened with a rubber band. To avoid dead insects getting into contact with soil or becoming infected by opportunistic fungi in the soil, pieces of moisten cotton were placed on top of the soil to avoid insect contact. Between 10 and 12 adults were transferred to each experimental unit one day before spraying fungal concentrations.

Initially, we estimated the amount of blastospores present in 0.1 g of each formulation. We then adjusted this value according to the viability, and weighed the amount needed to adjust the concentrations to: 1×10^6 , 5×10^6 , 1×10^7 or 5×10^7 formulated blastospores mL^{-1}. Fresh blastospores were adjusted at 1×10^6 and 5×10^7 mL^{-1} prepared with 0.02% Silwet® L-77 surfactant (OSi Specialties, Inc., Danbury, CT, USA). To evaluate the effect of co-formulants on the leafhopper mortality, we determined two positive controls that consisted of air dried or spray dried formulations without blastospores. For each formulation, we weighed the same amount needed to obtain 5×10^7 blastospores mL-1 and mixed with water previously to the spraying.

A small airbrush was used to apply through direct spray contact 2 mL of each treatment against insects. The application was performed from the bottom up by

slightly lifting the cage from the plastic pot to introduce the tip of the airbrush toward the base of the plant and then spraying the leaves. After spraying, cages were kept in a room at 26-28 °C, 12 h of photoperiod and uncontrolled humidity. Dead adults were recorded every 2 days during 10 days after the application. To confirm that the mortality was due to the fungal infection (i.e., mycosis rate), dead adults were transferred to a humid chamber and kept at 28 °C until mycosis (fungal outgrowth) could be seen.

4.2.10. Statistical analysis

Experiments were carried out with a completely randomized design and repeated three times except the bioassay experiment that was not reapeated. Proportion data on blastospore viability from inert, spray dried and air dried formulation assays were fitted to generalized linear models (GLM) with a quasi-binomial distribution for errors in which inerts or formulations comprised de fixed effects. Treatment means were separate by pos-hoc pair-wise multiple comparisons using Tukey´s HSD. The effect of co-formulates of spray and air dried formulations, on blastospore viability were analyzed using a Plackett-Burman design for eight runs and seven factors. Data frame were fitted to a linear model with proportion data on blastospore viability as dependent variable and co-formulants as fixed effect. Then, the magnitude of effect (positive or negative) of co-formulates on blastospore viability were determined and illustrated in a Pareto Chart with respective *p-value*. Data on wettability and stability (TSI: Turbiscan stability index) were fitted to a general linear model with Gaussian distribution for error. The dependent variable “wetting time” or “TSI” were root transformed prior to analysis to meet homoscedasticity assumptions. Spray dried formulations were implemented as fixed effects and treatment means were separate by the multiple pairwise Tukey´s HSD. Adult mortality and mycosis datasets were fitted to a generalized linear model with quasi-binomial distribution for errors. Fixed effects were attributed to fresh, spray dried or air dried blastospore formulation at the two concentrations tested. Treatment means were statistically compared with each other by the multiple pairwise Tukey’s HSD test at $P < 0.05$. Additionally, survival analysis was performed with censored data for dead adult until day 11 and fitted to a parametric Weibull survival model using the package “flexsurv” from R (Jackson, 2016). Estimated 50% (median) and 90% lethal times (LT) and their

respective confidence intervals (CI 95%) were computed from the Weibull survival curves. In addition, Weibull survival curves were compared based on the log-likelihood ratio test at $P < 0.05$. Median lethal concentration (LC_{50}) with its corresponding confidence limits (95% CL) and slope were calculated for spray and air dried formulations after proportion data on adult mortality were log transformed and fitted to a general linear model with binomial distribution for errors.

Survival data over time for estimation of shelf life were fitted separately for formulations stored in the refrigerator and at ambient temperature to generalized linear models with binomial distribution for errors and logit link function assuming formulations, storage temperatures and storage time as fixed factors with their triple and dual interaction terms, while a random effect was attributed to the observational level to account for data overdispersion. The half- and quarter-lives of blastospores in each formulation under these two storage conditions were estimated with the “drc” package in R, and values were not considered statistically significant when their 95% confidence intervals overlapped. All analyses were performed in the statistical software R (R Core Development, 2015) and plots were either built on SigmaPlot version 14.0 (Systat Software, Inc., USA) or with “ggplot2” package in R (Wickham. 2016).

4.3. Results

4.3.1. Screening inerts for development of blastospore formulations

One of the first steps for developing a formulation is to select an inert to be used as a vehicle and as osmoprotectant for drying blastospores with preserved viability. Therefore, here we screened seven inerts to select those that resulted in the best viability of blastospores after drying. We found that the viability of air-dried blastospores stored in the refrigerator for seven days was not significantly influenced by the inerts ($F_{6,72} = 0.965$, $P = 0.454$) and ranged from 55.7 ± 7.9 to 69.4 ± 4.6% for preparations of blastospores mixed with cream phyllite and diatomaceous earth, respectively (**Figure 1A**). During the same period, at ambient condition (25-26 °C) blastospore viability dropped to less than 20% for all treatments, except for those cells dried with bentonite (56.2 ± 4.8%) ($F_{6,77} = 12.1$, $P < 0.001$) (**Figure 1A).** Conversely, after 30 days of storage, the viability of air-dried blastospores stored at

ambient condition dropped to zero, whilst under refrigerator cells attained from 29.1 ± 4.4% to 57.7 ± 4.9% viability rates when formulated with grey clay and bentonite, respectively ($F_{6,76}$ = 3.5 , P < 0.001). The viability of blastospores air dried with bentonite comprised the only treatment that significantly differed from the others after seven days of storage at room temperature and after 30 days stored in the refrigerator. Therefore, bentonite was selected as the main inert diluent for the next phase of formulation development for air and spray-drying methods.

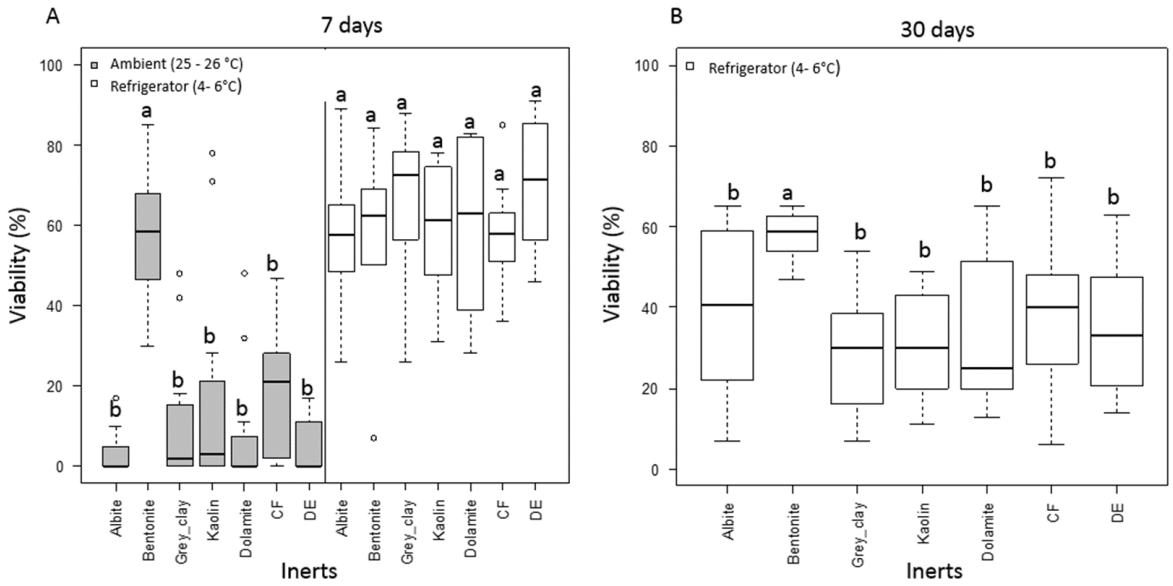


Figure 1. Viability of *Metarhizium robertsii* blastospores air-dried with 7.5% of one of the following inerts: Albite, Bentonite, Kaolin, Grey clay, Dolomite, Cream phyllite (CF), Diatomaceus earth (DE), stored in ambient conditions (25 - 26 °C) and in the refrigerator (4 - 6 °C) after seven (A) and thirty days (B). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles. Boxes followed by different letters are significantly different (Tukey's test at P < 0.05 and confidence interval for the data obtained after 30 days (B)).

4.3.2. Air- dried formulations

All formulations, including the control, blastospores dried with 5% bentonite (formulation 9), showed a viability higher than 67% after seven days of storage in refrigerator ($F_{8,42}$ = 3.994, P < 0.001). The formulation that provided the highest blastospore viability was the formulation 1 (90 ± 3.3%), which was statistically higher than formulations 8 and control (9) (Figure 2A). All ingredients that composed the formulations contributed positively to blastospore viability ($F_{7,16}$ = 1.805, P < 0.001)

(Figure 2B), although only the osmoprotectors skim milk and sucrose were statistically significant to maintain blastospore viability after air-drying ($P < 0.05$) (Figure 3B). Additionally, blastospore viability could be explained by a multivariate equation with all co-formulants and their respective estimated coefficients included in the linear model: Viability = 2.25 * lignin + 0.416 * charcoal + 0.583 * glutamate + 0.083 * SiO_2 + 4.083 * sucrose + 4.083 * skimmed milk + 0.583* bentonite ($F_{8,42}$= 3.99, $P < 0.001$, $R^{2 =} 0.44$)

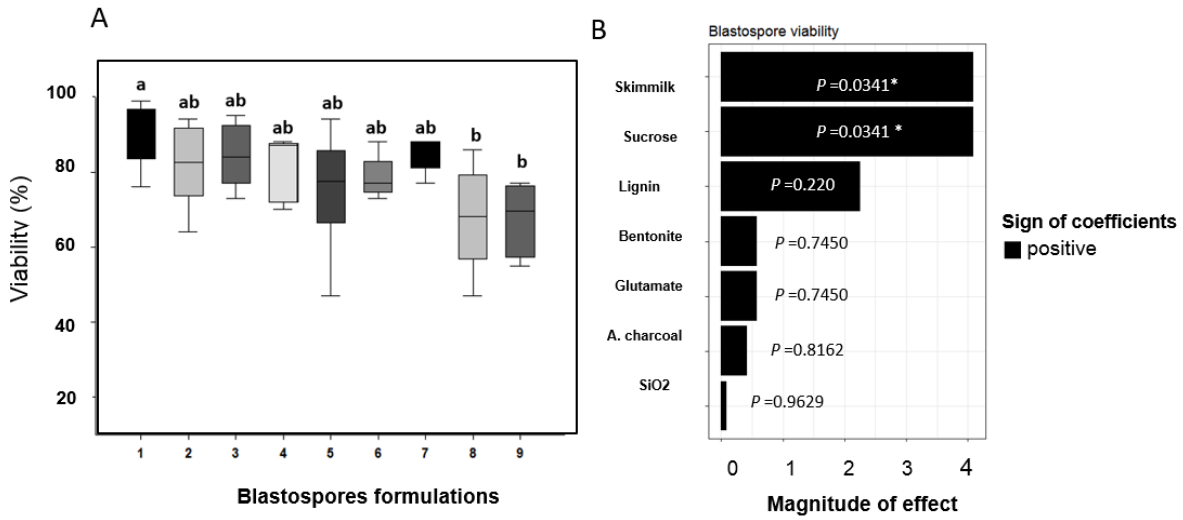


Figure 2. Viability of nine blastospore formulations after air-dried and stored for one week in the refrigerator (4 - 6 °C). The control formulation 9 consisted of blastospores on 5% bentonite. Means (±SE) followed by different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles (A). Pareto chart showing the magnitude of positive effect of seven ingredients that compose formulation, in blastospore viability after air-drying with respective p-values (* significance at $P < 0.05$) (B). The plotted data represent pooled data from three independent experimental repetitions carried out on different days using new fungal batches.

4.3.3. Spray-dried formulations

The results of the spray-drying showed higher differences than obtained with the air-drying method with a significant effect of formulation compositions on blastospore viability ($F_{8,45}$ = 15.58, $P < 0.001$) (Figure 3A). Formulations 1, 6 and 7 showed >

75% viability after spray-drying, with no statistical difference among them (Figure 3A). The control formulation 9 with 5% bentonite clay rendered the lowest viability (44.2 ± 3.1%) and was statistically different from formulations 1, 2, 4, 5, 6 and 7.

The ingredients used in the formulations affected positively or negatively the viability of blastospores ($F_{7,16}$ = 5.640, P < 0.001) (Figure 3B). However, only bentonite, maltodextrin and skim milk significantly contributed positively to improve blastospore viability (P < 0.05), whereas lignin had a negative impact on cell survival (P < 0.05). Blastospore viabilities could be explained by a multivariate equation with all co-formulants tested during the spray-drying according to their respective estimated coefficients (positive and negative) included in the linear model: Viability = -4.183*lignin -1.262*charcoal -1.896* glutamate + 3.178* SiO_2 + 5.275*maltodextrin + 3.952* skimmed milk + 3.310* bentonite ($F_{8,45}$= 15.58, P < 0.001, $R^{2\,=}$ 0.70).

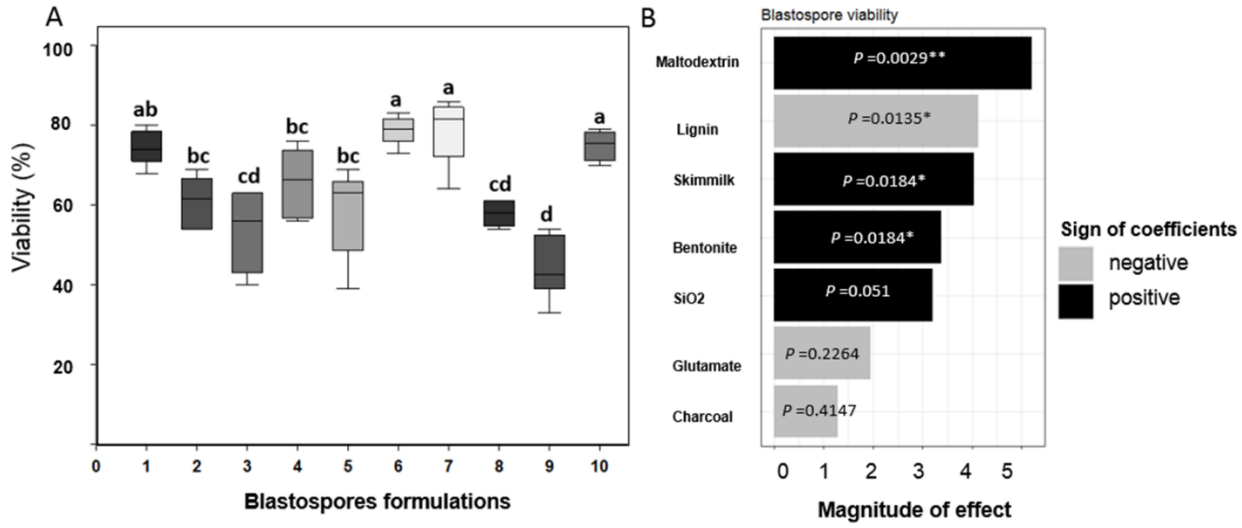


Figure 3. Viability of nine blastospore formulations after spray dried and storage for one week in the refrigerator (6 - 8 °C). Formulation 9 and 10 represents the control (blastospores and 5% of bentonite) and a low cost formulation based on formulation 7, respectively. Means (±SE) followed by different letters indicate significant differences according to Tukey's HSD test (P < 0.05). Boxes show the median, 25th and 75th percentiles, error bars show 10th and 90th percentiles (A). Pareto chart showing the magnitude of positive or negative effect of seven ingredients that compose formulation, in blastospores viability after spray-drying with respective p-values (significance at *P < 0.05 and at **P < 0.01) (B). The resulted charts are from

joined data of three repetitions of the experiment at different days with new fungal material each time.

4.3.4. Wettability and physical stability of spray-dried formulations

The wettability profile varied significantly with spray-dried formulations, indicating that the formulation composition significantly influenced wettability ($F_{9,40}$ = 13.05, P < 0.001). Wettability times ranged from 30.6 ± 2.3 seconds (formulation 1) to 85.1 ± 2.3 seconds (formulation 8) (Figure 4A). Formulations 5, 8 an 10 exhibited the lower wettability times than formulations 1, 2 and 3 and these formulations were not statistically different to the others. The ingredients used in the formulations affected the wettability time (Figure 3B), and only lignin, maltodextrin, and skim milk significantly contributed negatively on reduction of wettability time (P < 0.05), Wettability time could be explained by a multivariate equation with all co-formulants tested during the spray-drying according to their respective estimated coefficients (positive and negative) included in the linear model: Wettability time = 12.219*lignin + 3.094*charcoal -4.781* glutamate -5.052* SiO_2 + 10.677*maltodextrin +5.99* skimmed milk -1.719* bentonite ($F_{7,15}$= 7.892, P < 0.001, $R^{2\,=}$ 0.78).

In addition to wettability, dispersion stability of aqueous spray-dried formulations was investigated by comparing the TSI (turbiscan stability index) between formulations after 30 and 60 min of homogenization. The TSI curves of dispersions containing between 9 to 22% of solids showed an increased trend from time 0 to 60 min (Figure 5A). The TSI is influenced by the particle size, the density, and the quantity of the co-formulants in each formulation. Therefore, we found significant differences among formulations between TSI values after 30 ($F_{8,43}$ = 8.03, P < 0.001) and 60 min ($F_{8,37}$ =14.168, P < 0.001) (Table 3). The higher TSI and consequently the more unstable formulations were attributed to formulations 1, 5, 7 and 9, regardless of the measuring time. On the other hand, the more stable formulations were attributed to formulations 2, 3, 4, 6 and 8 after 30 minutes and to 3, 4 and 6 after 60 minutes (Table 3).

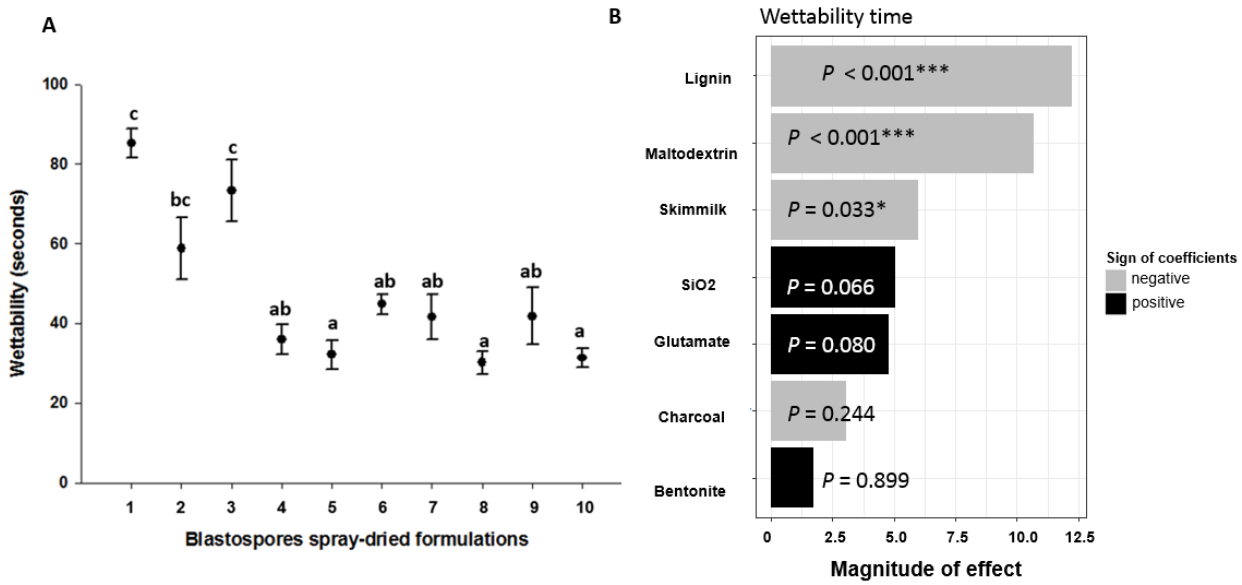


Figure 4. Wettability of nine blastospore formulations after spray dried and stored for one week in the refrigerator (6-8 °C). Formulation 9 and 10 represents the control (blastospores with 5% bentonite) and a low cost formulation based on formulation 7, respectively. Means (±SE) followed by different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$). The resulting charts are from joint data of three repetitions of the experiment on different days with different fungal batches each time (A). Pareto chart showing the magnitude of positive or negative effect of seven ingredients that compose formulations in wettability time with respective p-values (significance at *$P < 0.05$ and at **$P < 0.01$). Statistical significative negative sign values indicate ingredients that play a role in increasing wettability time. The resulted charts are from joined data of three repetitions of the experiment at different days with new fungal material each time (B).

The ingredients used in the formulations affected positively or negatively the turbiscan stability index (TSI) (Figure 5B). However, only charcoal significantly contributed positively to increase stability of formulations ($P < 0.001$), whereas lignin and SiO_2 had a negative impact in formulation stability ($P < 0.05$). TSI could be explained by a multivariate equation with all co-formulants tested during the spray-drying according to their respective estimated coefficients (positive and negative) included in the linear model: Viability = +1.546*lignin -2.966*charcoal +0.034* glutamate + 0.94* SiO_2 + 0.307*maltodextrin + 0.435* skimmed milk + 0.102* bentonite ($F_{7,16} = 25.08$, $P < 0.001$, $R^{2} = 0.91$).

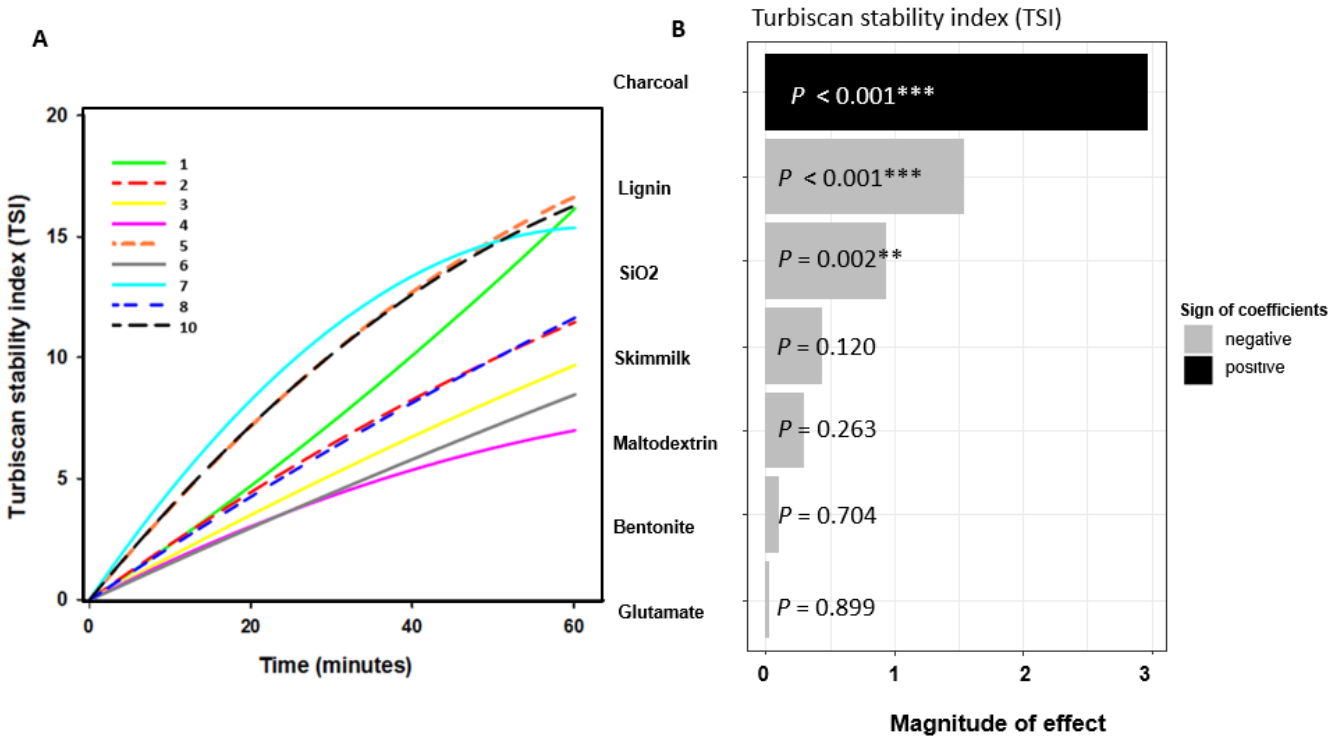


Figure 5. Second order polynomial fitted curves describing the physical stability of nine spray dried blastospore formulations according to Turbiscan Stability Index (TSI) at different time intervals. Formulation 10 represents a low cost formulation based on formulation 7. The resulting fitted curves were obtained by pooling together the data of three repetitions from independent experiments performed with different fungal preparations to ensure reproducibility (A). Pareto chart showing the magnitude of positive or negative effect of seven ingredients that compose spray dried formulations in Turbiscan stability index (TSI) measured after 60 minutes, with respective p-values (significance at $*P < 0.05$ and at $**P < 0.01$). Statistical significative positive sign values indicate ingredients that play a role in decreasing TSI thus, increasing dispersion stability. The resulted charts are from joined data of three repetitions of the experiment at different days with new fungal material each time (B).

Table 3. The Turbiscan stability index (TSI) of dispersed spray dried formulations after 30 min and 60 min suspended or mixed in water. Means (±SE) followed by

different letters in the same column indicate significant differences according to Tukey's HSD test ($P < 0.05$).

	Turbiscan stability index (TSI)	
Formulation	**0 to 30 min**	**0 to 60 min**
1	7.73 ± 0.69 **bcd**	17.03 ± 1.30 **cd**
2	6.80 ± 0.57 **abc**	12.08 ± 0.55 **bc**
3	5.46 ± 0.44 **ab**	10.22 ± 0.54 **ab**
4	4.54 ± 0.38 **a**	7.38 ± 0.37 **a**
5	9.43 ± 0.40 **cd**	17.54 ± 0.68 **d**
6	4.67 ± 0.56 **a**	8.94 ± 1.09 **ab**
7	11.8 1 ± 2.04 **d**	16.18 ± 1.96 **cd**
8	6.60 ± 0.37 **abc**	12.27 ± 0.58 **bcd**
10	9.32 ± 1.34 **bcd**	17.15 ± 2.16 **cd**

4.3.5. Optimizing cost of spray-dried formulation

Formulation 7 was chosen for optimization of the formulation cost as it showed one of the highest blastopores viability and lowest wettability time. The cost per kilo of the formulation n°7 (U$$: 2.28 based on values for the bulk ingredients marketed in Brazil) was reduced by removing the 4% of skim milk (one of the most expensive ingredients of the formulations) and increased from 4% to 6% the amount of maltodextrin and silicon dioxide. This low cost formulation was assigned to formulation 10 (U$$: 2.02).

After spray-drying formulation 10, we did not see any difference in the viability of blastopores compared to the original formulation 7 (81 ± 8%) and its cheapest version with 6% of maltodextrin and silicon dioxide formulation 10 (79 ± 4%) (*t*-test: 2.11, $P = 0.079$) neither in physical stability (Table 3) and wettability time (Figure 4). This formulation was further use for shelf-life experiments and insect bioassay.

4.3.6. Shelf life of spray-dried and air-dried formulations

Here, the spray-dried formulation 10 and the air-dried formulation 1 were chosen to determine the shelf life after packaged at modified atmosphere under ambient condition and refrigerator during 90 days of storage. We found significative differences between spray-dried formulations stored in different modified atmosphere in refrigerator (LRT = 1905.00, df = 12, $P < 0.001$) or ambient condition (LRT = 991.00, df = 6, $P < 0.001$), and air-dried formulation stored in refrigerator (LRT = 4265.00, df = 12, $P < 0.001$) or ambient condition (LRT = 1990.8, df = 6, $P < 0.001$).

Formulations packaged with dual moisture/oxygen absorbers RP-5AN (represented by curve 3 in figure 6) and Agstend AS-100 (represented by curve 4 in figure 6) showed the higher half-life and quarter-life, regardless of storage and drying condition (Figure 6 and Table 4). The higher values for half-life were attributed to formulations stored in refrigerator (4 °C) and packed with the RP-5AN (spray dried: $t_{1/2}$ = 75.9 days ; air dried: $t_{1/2}$ = 75.7 days) and Agstend AS-100 (spray dried: $t_{1/2}$ = 50.8 days air dried: $t_{1/2}$ = 76.2 days), with no statistical difference between them, under the same drying condition (table 4). On the other hand, the lower half-life and quarter-life were attributed to formulations packaged with oxygen absorber ZPT (represented by curve 2 in figure 6), stored under refrigerator condition (spray dried: $t_{1/2}$ = 6.26 days; air dried: $t_{1/2}$ = 8.12 days) (Figure 6 and table 4). Under ambient condition, the control formulation, sealed without any absorber and no vacuum, (represented by curve 5 in figure 6) and formulation packed with oxygen absorber ZPT showed no blastospore viability after one week of storage, then, their respective curves are shown only in chart of refrigerator condition.

While both dual moisture/oxygen absorbers RP-5AN and Agstend AS-100 showed similar effect on blastospore viability stored in refrigerator, under ambient condition they showed statistical differences as the absorber RP-5AN (spray dried: $t_{1/2}$ = 43.4 days; air dried: $t_{1/2}$ = 53.1 days) improved blastospore survival in relation to Agstend AS-100 (spray dried: $t_{1/2}$ = 17.2 days; air dried: $t_{1/2}$ = 25.9 days), regardless of the drying condition (table 4). Vacuum sealed formulated blastospores showed lower half-life for both drying conditions stored in refrigerator (spray dried: $t_{1/2}$ = 35.1 days; air dried: $t_{1/2}$ = 34.3 days) and no blastospore viability at ambient conditions after seven day.

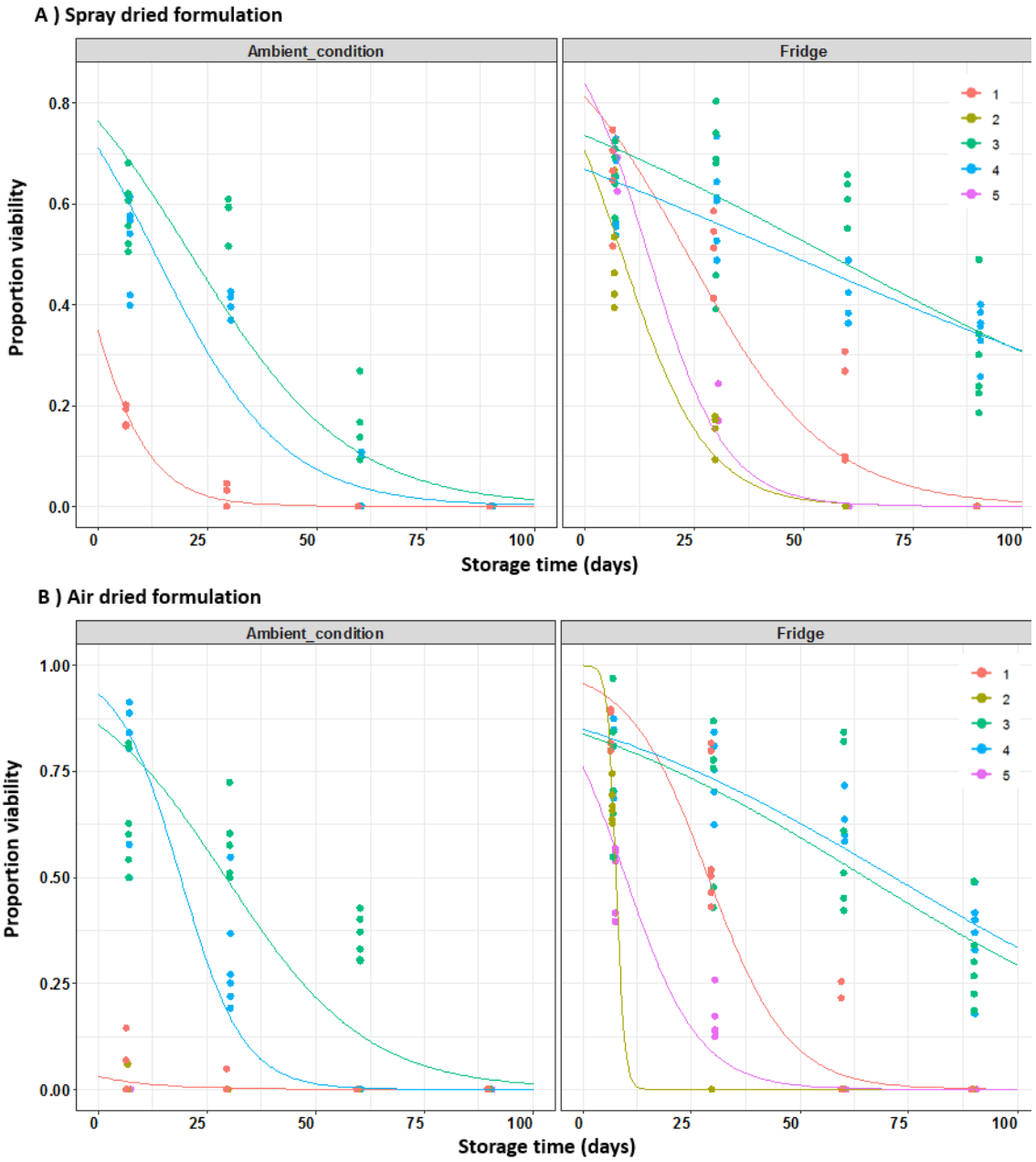


Figure 6. Proportion viability curves of one spray dried (A) and one air dried (B) *M. robertsii* blastospore formulation stored at ambient condition (28 °C) and refrigerator (4 °C) during 90 days. Curves represent formulation under one of the following treatments: 1: vacuum package; 2: sealed with ZPT oxygen absorber; 3: sealed with dual moisture/oxygen absorber RP-5AN; 4: sealed with dual moisture/oxygen absorber Agstend AS-100; 5: only sealed.

Table 4. Regression model parameters used to estimate half-life and quarter-life for air dried or spray dried *M. robertsii* blastospore formulations stored under refrigerator

(4 °C) and ambient condition (28° C). Control consisted of formulation sealed without any absorbers. ZPT: oxygen absorber; RP-5AN and AS–100: moisture/oxygen absorber. Means followed by different letters in the same column, at same drying method and storage temperature indicate significant differences between half-life and quarter-life with no overlap of confidence intervals.

Drying method	Absorbers	Storage temperature (°C)	Model * parameters			$t_{1/2}$ (days)	$t_{1/4}$ (days)
			a	*b*	*c*		
Vacuum sealed							
Spray-drying		28	2.03	0.2	57.99	-	-
		4	3.16	0.63	54.8	35.15	53.77
Air-drying		28	0.93	0.05	10.67	-	-
		4	2.75	0.85	42.87	34.29	46.28
Active packing without vacuum							
Spray-drying	control	28	-	-	-	-	-
	ZPT	28	-	-	-	-	-
	RP-5AN	28	6.54	0.58	57.99	43.41 **a**	56.5 **a**
	AS-100	28	3.22	0.52	45.94	17.16 **b**	41.76 **b**
	control	4	3.63	0.62	28.99	20.41 **b**	28.77 **b**
	ZPT	4	4.00	0.50	28.70	6.26 **c**	26.21 **b**
	RP-5AN	4	6.29	0.64	94.31	75.91 **a**	93.51 **a**
	AS-100	4	1.77	0.62	117.29	50.77 **a**	111.91 **a**
Air-drying	control	28	-	-	-	-	-
	ZPT-	28	-	-	-	-	-
	RP-5AN	28	7.08	0.63	65.22	53.1 **a**	64.52 **a**
	AS-100	28	3.97	0.74	32.66	25.9 **b**	33.38 **b**
	control	4	5.29	0.5	29.33	13.58 **b**	27.49 **b**
	ZPT-	4	5.17	0.85	9.16	8.12 **b**	9.53 **c**
	RP-5AN	4	4.44	0.75	92.61	75.67 **a**	94.63 **a**
	AS-100	4	3.03	0.79	98.16	76.24 **a**	102.99 **a**

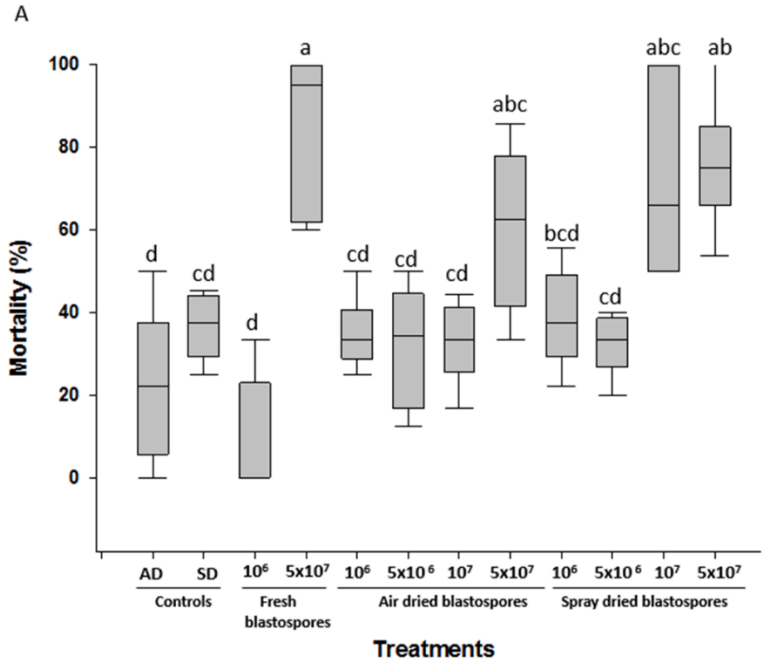
*Parameters estimated by the three-parameter Weibull model: $y = 0 + (b - 0)\exp(-\exp(a(\log(x) - c)))$, where y = proportional survival and x = time (days)

4.3.7. Bioassay

Here we evaluated the infectivity of one spray dried (9) and one air dried (1) blastospore formulation of ESALQ1426 against the leafhopper *D. maidis*, the mycosis, the time the blastospores take to kill 50% (LT_{50}) and 90% (LT_{90}) of leafhoppers and the median lethal concentration (LC_{50}) for each formulation. These results were compared to those obtained with fresh blastospores and controls consisting of formulations without blastospores.

Air dried formulation killed 60.3 % (LT_{50} = 8.95 d) and spray dried 75.6 % (LT_{50} = 8.18 d) of the leafhoppers compared with 78.2 % (LT_{50} = 7.69 d) of fresh blastospores when applied at the highest concentration, 5 × 10^7 blastospores mL^{-1}, with no statistically significant difference between the treatments after 10 days of fungal application (Figure 7A and Table 5). No statistical differences in leafhopper mortality were observed due to the concentrations of air-dried bastospores. Only the highest concentration differed from the control (AD). The same was observed with spray-dried formulations, where only the treatment with the highest concentration differed from the control SD (Figure 7A).

Mycosis was confirmed by symptoms of *M. robertsii* outgrowth and sporulation on leafhoppers represented by a masses of greenish conidia covering their bodies. We found, at the highest concentration (5 × 10^7 blastospores mL^{-1}) 61.1%, 42.2% and 74.3% mycosis for fresh, air dried and spray dried blastospores respectively, with no statistical difference between them. At lower concentrations (10^6 or 5 × 10^6) there was no statistical difference for mycosis between treatments (**Figure 7B**).



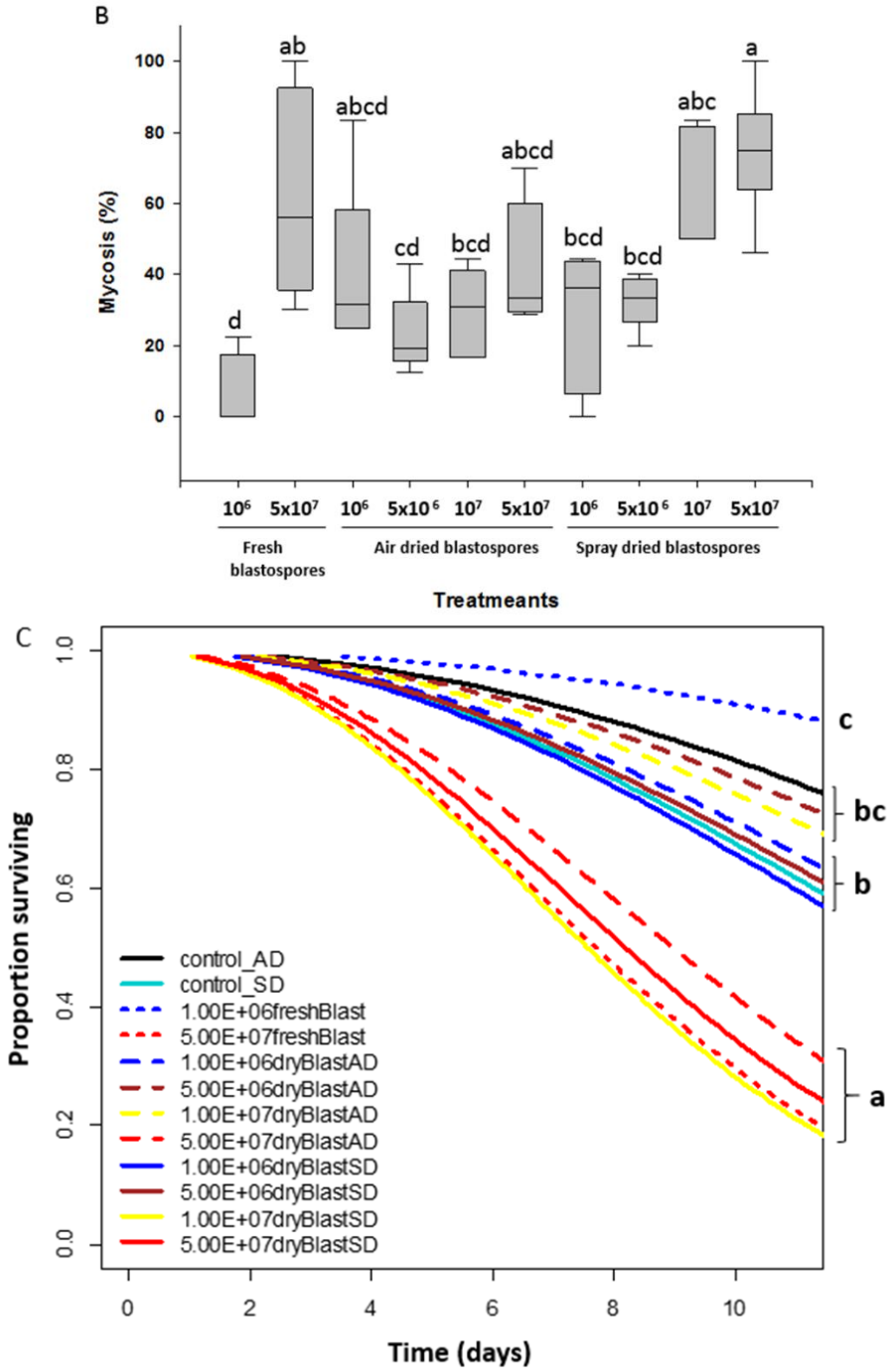


Figure 6. Mortality (A) and mycosis (B) in adults of leafhoppers *D. maidis* after spraying with fresh blastospores of *M. robertsii* (ESALQ 1426) at two concentrations 1 x 10^6 or 5 × 10^7, and spray dried (SD) and air dried blastospores (AD) at four concentrations (1 x 10^6 or 5 × 10^6 , 1 × 10^7 , 5 × 10^7 blastospores mL^{-1}). Controls: SD (spray dried), AD (air dried) formulations without fungus and weighed in the same amount as for the treatment with 5 ×10^7 blastospores mL^{-1}. Mean ± standard error (n = 6). Distinct letters indicate significant differences in mortality and mycosis between treatments according to Tukey's HSD test (P < 0.05). C): Proportion surviving of

leafhoppers *D. maidis* after exposure to fresh or spray/air dried blastospores of *M. robertsii* at two concentrations in case of fresh blastospores or four concentrations in case of air and spray dried blastospores. Distinct letters indicate significant differences between survival curves according to the log-likelihood ratio test ($P < 0.05$).

The survival curves of leafhoppers treated with fresh, dried blastospores or formulations without fungi (control AD and control SD) showed a clear difference in proportional survival and are splited in three groups with no statistical difference within each group. The first one, with the lowest median lethal time is represented by spray dried, air dried and fresh blastospores applied at 5×10^7 blastospores mL^{-1} and spray dried blastospores at 1×10^7 blastospores mL^{-1} with LT_{50} varying from 7.5 to 8.9 days (Table 5). The second groups, with an intermediate medial lethal time, is represented by air dried and spray dried blastopores applied at 1×10^6 blastospores mL^{-1}, spray dried blastospores applied at 5×10^6 blastospores mL^{-1} and control SD, varying from 12.6 to 13.9 days (Table 5). The third group, with only one representative is the fresh blastospores applied at 1×10^6 blastospores mL^{-1} with the highest LT_{50} of 25 days (Table 5).

Table 5. Estimated median lethal time (LT_{50}), 90% lethal time (LT_{90}), lower confidence interval (lci) and upper confidence interval (uci) of the corn leafhopper adults (*D. maidis*) after being sprayed with dried blastospores or fresh blastospores in four concentrations: 1 or 5 × 10^6 and 1 or 5 × 10^7 blastospore mL^{-1}. Time is measured in days.

		50% Lethal time (LT_{50})			**90% Lethal time (LT_{90})**		
Treatment	**Concentration (blastospores mL^{-1})**	**Estimated**	**lci***	**uci***	**Estimated**	**lci**	**uci**
Fresh blastospores	1 × 10^6	25.3	15.8	40.4	50.1	30.6	81.7
	5 ×10^7	7.6	6.6	8.9	15.2	12.9	17.8
Air dried blastospores	1 × 10^6	13.9	11.2	17.2	27.4	21.6	35.1
	5 ×10^6	16.3	12.6	21.0	32.3	24.4	42.8
	1 × 10^7	15.3	12.0	19.3	30.2	23.3	39.3
	5 × 10^7	8.9	7.4	10.7	17.7	14.6	21.6
Spray dried blastospores	1 × 10^6	12.6	10.0	15.6	24.9	19.6	31.7
	5 ×10^6	13.3	9.9	17.7	26.4	19.3	35.8
	1 × 10^7	7.5	6.2	9.1	14.9	12.1	18.3
	5 × 10^7	8.1	6.9	9.5	16.1	13.6	19.1

*lci = lower confidence interval 95% and uci = upper confidence interval 95%. Statistical differences between ST_{50} or ST_{90} are indicated when their 95% confidence intervals did not overlap.

Consistent with mortality results, the estimated median lethal concentration LC_{50} showed that spray dried blastopores exhibited 5.2 times improved insecticidal activity (virulence) compared with air-dried blastospores with LC_{50} represented by 4.65 × 10^6 and 2.42 × 10^7 blastospores mL^{-1} for spray dried and air dried formulation respectively (Table 6).

Table 6. Estimated median lethal concentration (LC_{50}), lower confidence interval (lci) and upper confidence interval (uci) of the corn leafhopper adults (*D. maidis*) after being sprayed with spray or air dried blastospores formulations in four concentrations: 1 or 5 × 10^6 and 1 or 5 × 10^7 blastospore mL^{-1}. Concentration is expressed as blastospores mL^{-1} .

	n**	Slope ± SE***	χ^2 (*p*-value)****	Median Lethal concentration (LC_{50})		
Blastospore formulation				**Estimated**	**lci***	**uci***
Air dried	184	0.26 ± 0.11	15.71 (0.014)	2.42×10^7	1.15×10^5	4.85×10^8
Spray dried	154	0.38 ± 0.11	19.41 (<0.001)	4.65×10^6	5.79×10^5	3.31×10^7

*lci = lower confidence interval 95% and uci = upper confidence interval 95%
** = Total number of leafhoppers in the experiment (6 replicates per blastospore-concentration)
*** = Slope for mortality represents regression of proportion of leafhoppers versus log (concentration) of blastospores
**** = χ^2 and *p*-values represent the probability of slope ≠ 0, rather than fit to logistic model.

4.4. Discussion

In the present work, we demonstrated that it is possible to develop cost-effective *M. robertsii* (ESALQ1426) blastospores formulations using a combination of co-formulates with well-defined functions that provides high blastospore viability rates (> 70%) after spray or air-drying. Carriers are inert substances such as clay, which aid in the process of cell drying and avoid caking by promoting dispersion of fungal material (Burges, 2012). In our study, bentonite clay was selected, among seven clays tested. Bentonite is composed mainly by montmorillonite clay (~ 60 to 80%) commonly used in microbial inoculants (Rani and Kumar, 2019). Clays have different physicochemical and mineralogical characteristics, such as density, porosity, electrical charges, composition of silicate and other mineral layers. However, more studies are needed to be able to explain the higher efficacy of bentonite in preserving the blastospore viability over other clays.

In addition to bentonite, we found that osmoprotectors such as skim milk and sucrose and maltodextrin and skim milk play a pivotal role in preserving the viability of air and spray dried blastospores respectively. Sugar solutions are known to improve survival of cells during drying as they act specially by reducing disruption of cellular membrane during dehydration (Burges et al. 2004) while skim milk mixed with different sugars were showed to be good protector agents during spray-drying of submerged spores of *M. anisopliae* (Stephan and Zimmermann. 1997), submerged conidia of *M. acridum* (Kassa et al., 2004) and, solely skim milk, during spray-drying of *Beauveria bassiana* blastospores (Mascarin et al. 2016). In our assay, we found

that formulation 1 protected blastospore during air-drying maintaining 90.0 ± 3.3% viability. This formulation is composed of higher amount of skim milk (4%), sucrose (4%), bentonite (5%) and Ca-lignin (5%) and it was statistically better than formulation 8 (71.2 ± 3.7%) with no skim milk and sucrose and from the control (67.5 ± 3.8%), with only 5% of bentonite. However, formulation 7 also has in its composition skim milk and sucrose but resulted in blastospore viability similar to formulation 8 and the control (9). Therefore, we believe that it is the specific combination of the type and the amount of co-formulants, not only the specific osmoprotectants that led to higher preservation of the blastospore viability. We highlighted that none of the co-formulates showed negative effect in the blastospore viability after air-drying but this was not the case for spray-drying formulations.

Comparing with air-drying, spray-drying is more convenient, saving time and results in higher yield, however, heat and osmotic stresses to the cells has to be minimized by optimizing spray-drying conditions and adjusting co-formulates in liquid suspension before drying (Fu and Chen, 2011). In general, spray dried formulations showed lower blastospore viability then air dried formulations. This finding can be attributed to heat stresses in addition to osmotic stresses suffered by blastospores during spray-drying and quickly dehydration process. Although skim milk and sucrose has been reported in literature as good protectors agent, in the present work we obtained high positive effect (P = 0.0029) of maltodextrin in preserving blastospore viability after spray-drying, being this effect more significant than those obtained for skim milk (P = 0.0184). Maltodextrin, as sucrose and skim milk are known to provide osmoprotection during bacterial cells drying procedures (Strasser et al. 2009; Fu and Chen, 2011), furthermore, maltodextrin can provides thermal protection to spray dried microbial cells (Muhammad et al. 2017; Luna-Solano et al. 2007). Therefore, our findings reinforce the dual-protection function of maltodextrin to spray dried *M. robertsii* blastospores. In addition to maltodextrin and skim milk, bentonite showed a positive effect (P = 0.0184) in preserving blastospore viability and it may have acted as dispersant, although blastospores mixed only with bentonite and spray dried showed lower viability (44.2 ± 3.1%) compared with the same air dried treatment (67 ± 3.7). After spray dried submerged spores (blastospores and submerged conidia) mixed with 5% of bentonite Stephan and Zimmermann (1997) showed a drastic reduction of spores' viability (5.5%). However, in that case, blastospores were

centrifuged and resuspended in deionized water, removing any effect the culture medium might have provided to preserve the viability of submerged spores. In our study, liquid culture medium was not removed and the remained glucose and other components from the medium may contributed to preserve blastospore viability after spray and air-drying.

Although, wettability and physical stability of *Metarhizium* blastospore formulations in water are very important parameters we don´t find in the literature these features characterized for other fungal formulations. Our spray dried formulations showed optimal wetting time (personal information), lower than 60 seconds, with the exception of the formulation 1 and 3. In fact, these last two formulations are composed of two co-formulates with varying degree of solubility greatly, and they were added at the highest amount tested in this experiment: ca-lignin (5%) and maltodextrin (4%). Maltodextrin is a product resulting from the hydrolysis of maize starch composed of glucose oligomers of different chain sizes. Although maltodextrin is more soluble than maize starch, the size of oligomer chains may influence its solubility in water, with bigger chains providing lower solubility (Chronakis et al. 2010). Conversely, lignin is poor soluble polymer, however, to separate it from plant biomass, lignin is processed in an aqueous solution using sulfite salts containing calcium, sodium and other components. This treated lignin is denoted as lignosulfonates (Meister. 2007) with solubility rates depending on the degree of sulfonation applied to lignin (Aro and Fatehi. 2017). Ca-Lignin is actually a lignin processed with Ca salt, that originate the Ca-lignosulfonate used. Therefore, it could be that the maltodextrin and Ca-lignin used in our experiment are partially soluble and added together in high amount, resulted in higher wetting time found for the formulation 1 and 3. Similar work has been done with spray dried formulations of the bacteria *Bacillus thuringiensis* to measured wettability (Teera-Arunsiri et al., 2003). By combining different co-formulates, the authors obtained different wetting times being 24 seconds the value attributed to the best formulation. In addition to measuring the viability and wettability of our spray dried formulations, we determined physical stability of water suspensions.

In practice, the stability of a bioproduct suspended in water is an important trait to ensure a homogeneous system over a given time without agitation. In our work, Turbiscan Stability Index (TSI) values were obtained from suspensions that simulated

a real concentration of product in a spray tank (equivalent to 500g of formulation in 100L of water).

Ca-lignin, charcoal and silicon dioxide are known by its good dispersion property in wettable formulations. In our experiment we found that the more stable formulations are those with charcoal (2.5%) (3, 4 and 6) with the exception of formulation 8, after 30 minutes. However, after 60 minutes, this formulation was not within the most stable ones while formulations 3, 4 and 6 still kept lowest stability index. Although formulation 7 and 10 showed excellent viability and wetting time, these formulations were the most unstable ones (table 3). These formulations, as well as formulations 5 and 1, do not contain carbon in their composition. Therefore, as demonstrated in pareto chart (figure 5), activated charcoal may assist in the stabilization process of these formulations. Physical stability is a measuring influenced by the composition of each formulation, like the quantity, the density and the particle size of ingredients added in the suspension (Turbiscan manufacturer guide). Our spray dried formulations is composed between 9 (formulation 8) to 22% (formulation 1) of solids however we did not find any relation between the amount of solids and Turbiscan Stability Index (TSI) since formulation 8 did not differ from formulation 1 (table 3). We found the TSI values ranged from 6 to 21 after 60 minutes, however, there is a trend of increasing the instability beyond 60 minutes demonstrated by the equation of the fitted curves for TSI along the time (supplementary material S1). This tendency indicate that the formulations did not reach stability after 60 minutes and physicochemical phenomena such as sedimentation, migration of particles, creaming and agglomeration may be acting in the suspension and should be further investigated to clarify the increasing instability and the exact effect of each co-formulate in physical stability of formulations.

In the present work, we determined the shelf life of the air dried formulation 1 and the spray dried formulation 10 packed under modified atmosphere by the use of oxygen and moisture absorbers. We highlighted that, in the light of our knowledge, this is the first report on shelf life of dried *Metarhizium* blastospores. We found that storing the formulations in packaging containing dual oxygen and moisture absorbers significantly increased the half-life of the formulations in 3.7 (spray dried) to 5.6 (air dried) times compared to control stored at 4 °C. Conversely, under ambient condition, blastospores showed no viability after stored for one week, but a half-life of

43 and 53 days after spray and air dried, respectively when stored with oxygen and moisture absorbers (RP-5AN). However, we found a great difference in half-life between the two oxygen and moisture absorbers tested. The composition of these absorbers is not disclosed and may responsible for the differential results. Similar findings are reported by Mascarin et al. (2016) with air and spray dried *Beauveria bassiana* blastospores. The authors attributed a longer shelf life of up to 13 months to blastospore formulation stored at 28 °C to the use of dual moisture and oxygen absorbers. The half-life obtained for *B. bassiana* was much longer than that obtained for *Metarhizium* blastospores. This fact can be attributed to higher sensitivity of the fungus *Metarhizium* compared to *Beauveria*, a fact already demonstrated for aerial conidia (Faria et al. 2009). Therefore, intrinsic factors of the *Metarhizium* genetics probably play a role on the ability of this fungus to remain viable during storage and should be addressed in future studies.

While we showed that oxygen and moisture absorbers significantly prolonged the shelf life, formulations stored with single oxygen absorbers rendered worse viability rates than those just vacuum-packed or packaged without any absorber. This fact can be explained due to the release of water inside the packaged resultant of the chemical reaction when oxygen molecules are absorbed by the compounds (iron oxides among others) present in the sachet. This residual water in contact with the powder provoked caking in the formulations probably mainly due to the reaction with the hydroscopic maltodextrin and milk powder, which in turn induced blastospore respiration and forced germination. Therefore, our results showed that this type of absorber is inappropriate for packing blastopore formulations.

In addition to accessing the shelf life of the air-dried formulation 1 and the spray-dried formulation 9, we determined the insecticidal activity of them by spraying blastopores suspension against an important maize pest in Brazil, the leafhopper *D. maidis*. In our previously study (Iwanicki et al. 2020), we had first reported the infectivity of fresh and air dried *M. robertsii* blastospores in controlling the corn leafhopper with similar results compared to aerial conidia at both concentrations tested (1×10^7 and 5×10^7 propagules mL^{-1}). Here, we took a step further and we investigated the insecticidal activity (virulence) of the two best dried formulations applied at four concentrations and determined the medium lethal concentration (LC_{50}). Our bioassay showed that air and spray dried blastospore formulations are as

virulent as fresh blastospores sprayed at higher concentration (5×10^7 blastospore mL^{-1}). Although the median lethal time of these treatments were similar, spray dried blastopores exhibited 5.2 times improved insecticidal activity (virulence based on Median Lethal concentrations) compared with air-dried blastospores. We highlight that this is the first time that spray dried *M. robertsii* blastospores are showed to be infective to a pest. *D. maidis* has recently emerged as the most important sucking pest in maize crops in Brazil. For controlling *D. maidis* seed treatment neonicotinoid and methyl carbamate are used, while the only biological alternative to control this insect is based on the foliar sprays of aerial conidia of *Beauveria bassiana* (AGROFIT, 2020). Here we demonstrated that *M. robertsii* blastospore is also an alternative against *D. maidis.*

There is no commercial biopesticide based on blastospores neither on *Metarhizium robertsii* in the Brazilian market to date, which is *per se* two key points that our results fill the gaps and then encourage the development of novel mycopesticides for pest control as well as for plant growth promotion, as it is well-known that this species of *Metarhizium* can endophytically colonizes the root systems of many dicot and monocot species benefiting plants with nutrients and alleviating abiotic stresses. Therefore, we have made some important advances in blastospore formulation for air-dried or spray-dried blastospore of *M. robertsii* that can be implemented in the production of mycopesticides. Additional studies are needed to validate our fermentation and formulation processes under large industrial scale as well as assessing the bioefficacy of these new blastospore formulations of *M. robertsii* under field conditions compared with traditional conidia-based formulations. Although significant advances were obtained in these studies, the shelf life for *M. robertsii* even under controlled atmosphere conditions was relatively short. Considering the cost of this technology, further studies are needed to enable the use of blastospores as an active ingredient of biopesticides.

References

AGROFIT, 2020. Sistema de agrotóxicos fitossanitários - ministério da Agricultura, Pecúaria e Abastecimento. http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. (Accessed 10 January, 2020).

Alkhaibari AM, Carolino AT, Bull JC, Samuels RI, Butt TM, Andreadis T (2017) Differential pathogenicity of *Metarhizium* blastospores and conidia against larvae of three mosquito species. J Med Entomol 54:696–704.

Alkhaibari AM, Carolino AT, Yavasoglu SI, Maffeis T, Mattoso TC, Bull JC, Samuels RI, Butt TM (2016) *Metarhizium brunneum* blastospore pathogenesis in *Aedes aegypti* Larvae: Attack on Several Fronts Accelerates Mortality. PLoS Pathog 12(7): e1005715.

Aro T, Fatehi P (2017) Production and Application of Lignosulfonates and Sulfonated Lignin. ChemSusChem 10:1861–1877.

Burges HD (1998) Formulation of mycoinsecticides. In: Burges HD (ed) Formulation of microbial biopesticides. Kluwer, Dordrecht, pp 131–185

Chronakis IS (1998) On the molecular characteristics, compositional properties, and structural-functional mechanisms of maltodextrins: A review. Crit Rev Food Sci Nutr 38:599–637.

Cliquet S, Jackson MA (1997) Comparison of air-drying methods for evaluating the desiccation tolerance of liquid culture-produced blastospores of *Paecilomyces fumosoroseus*. World J Microbiol Biotechnol 13:299–303

Dong TY, Zhang BW, Weng QF, Hu QB (2016) The production relationship of destruxins and blastospores of *Metarhizium anisopliae* with virulence against *Plutella xylostella*. J Integr Agric 15:1313–1320.

Dwari RK, Mishra BK (2019) Evaluation of flocculation characteristics of kaolinite dispersion system using guar gum: A green flocculant. Int J Min Sci Technol. 25 :745-755.

Faria MR, Wraight SP (2007) Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol Control 43:237–256.

Faria M, Hajek AE, Wraight SP (2009) Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium acridum*, and *Metarhizium anisopliae*. Biol Control 51:346–354.

Fu N, Chen XD (2011) Towards a maximal cell survival in convective thermal drying processes. Food Res Int 44:1127–1149.

Horaczek A, Viernstein H (2004) Comparison of three commonly used drying technologies with respect to activity and longevity of aerial conidia of *Beauveria brongniartii* and *Metarhizium anisopliae*. Biol Control 31:65–71.

Iwanicki NSA, Ferreira BDO, Mascarin GM, Júnior ÍD (2018) Modified Adamek's medium renders high yields of *Metarhizium robertsii* blastospores that are desiccation tolerant and infective to cattle-tick larvae. Fungal Biol 122:883–890.

Jackson MA, Payne AR (2007) Evaluation of the desiccation tolerance of blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) using a lab-scale, air-drying chamber with controlled relative humidity. Biocontrol Sci Technol 17:709–719.

Jackson MA, Mcguire MR, Lacey LA, Wraight SP (1997) Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycol Res 101:35–41

Jackson C (2016). flexsurv: A Platform for Parametric Survival Modeling in R. Journal of Statistical Software, 70(8) 1-33.

Jaronski ST, Mascarin, GM (2017) Mass production of fungal entomopathogens. In L. A. Lacey (Ed.), Microbial control of insect and mite pests: From theory to practice (p. 141–155). Amsterdam: Academic Press.

Kleespies RG, Zimmermann G (1994) Viability and virulence of blastospores of *Metarhizium anisopliae* (Metch.) Sorokin after storage in various liquids at different temperatures viability and virulence of blastospores of *Metarhizium anisopliae* (Metch .). Biocontrol Sci Technol 4:309–319.

Lesaint C, Glomm WR, Lundgaard LE, Sjöblom J (2009) Dehydration efficiency of AC electrical fields on water-in-model-oil emulsions. Colloids Surfaces A Physicochem Eng Asp 352:63–69.

Luna-Solano G, Salgado-Cervantes MA, Garcia-Alvarado MA, Rodriguez-Jimenes G (2000) Improved viability of spray dried brewer's yeast by using starch (grits) and maltodextrin as processing aids. J Food Process Eng 23:453–462.

Mascarin GM, Jackson MA, Behle RW, Kobori NN, Júnior ÍD (2016) Improved shelf life of dried *Beauveria bassiana* blastospores using convective drying and active packaging processes. Appl Microbiol Biotechnol 100: 8359–8370.

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Dunlap CA, Júnior ÍD (2015a) Glucose concentration alters dissolved oxygen levels in liquid cultures of *Beauveria bassiana* and affects formation and bioefficacy of blastospores. Appl Microbiol Biotechnol 99:6653–6665.

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Júnior ÍD (2015b) Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. J Invertebr Pathol 127:11–20.

Meister JJ (2002) Modification of lignin. Journal of Macromolecular Science - Polymer Reviews 42:235-289.

Muhammad Z, Ramzan R, Huo GC, Tian H, Bian X (2017) Integration of polysaccharide-thermoprotectant formulations for microencapsulation of *Lactobacillus plantarum*, appraisal of survivability and physico-biochemical properties during storage of spray dried powders. Food Hydrocoll 66:286–295.

Pedrini N (2017) Molecular interactions between entomopathogenic fungi (Hypocreales) and their insect host: Perspectives from stressful cuticle and hemolymph battlefields and the potential of dual RNA sequencing for future studies. Fungal Biol 122:538–545.

R Core Team, 2015. R: A language and environment for statistical computing Vienna, Austria. R Foundation for Statistical Computing. http://www.R-project. org.

Ramle M, Norman K (2014) The use of palm kernel cake in the production of conidia and blastospores of *Metarhizium anisopliae* var. major for control of *Oryctes rhinoceros.* J Oil Palm Res 26:133–139.

Stephan D, Zimmermann G (1998) Development of a spray-drying technique for submerged spores of entomopathogenic fungi. Biocontrol Sci Technol 8:3–11.

Strasser S, Neureiter M, Geppl M, Braun R, Danner H (2009) Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. J Appl Microbiol 107:167–177.

Sun Y, Deac A, Zhang GGZ (2019) Assessing Physical Stability of Colloidal Dispersions Using a Turbiscan Optical Analyzer. Mol Pharm 16:877–885.

Teera-arunsiri A, Suphantharika M (2003) Preparation of Spray-Dried Wettable Powder Formulations of *Bacillus thuringiensis*-Based Biopesticides. Sci Technol 96:292–299

Terayama H, Hirota K, Yoshimura T, Esumi K (2003) Effect of dilution on aqueous dispersion of drug particles. Colloids Surfaces B Biointerfaces 27 :177–180.

Vega FE, Jackson MA, McGuire MR (1999) Germination of conidia and blastospores of *Paecilomyces fumosoroseus* on the cuticle of the silverleaf whitefly, *Bemisia argentifolii*. Mycopathologia 147:33–35.

Wickham H, (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

5. COMPARATIVE RNASEQ ANALYSIS OF THE INSECT-PATHOGENIC FUNGUS *Metarhizium anisopliae* REVEALS SPECIFIC TRANSCRIPTOME SIGNATURES OF FILAMENTOUS AND YEAST LIKE DEVELOPMENT

Natasha Sant´Anna Iwanicki[1*]; Italo Delalibera Júnior[1]; Jørgen Eilenberg[2]; Henrik H. De Fine Licht[2]

1 Department of Entomology and Acarology, Escola Superior de Agricultura “Luiz de Queiroz”, University of São Paulo (ESALQ-USP), Av. Pádua Dias, 11, C.P. 9, Piracicaba, SP CEP 13418-900, Brazil

2 Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark.

*Corresponding author: Natasha S. A. Iwanicki E-mail address: natasha.iwanicki@usp.br Department of Entomology and Acarology, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

Abstract

The fungus *Metarhizium anisopliae* is a facultative insect pathogen used as biological control agent of several agricultural pests worldwide. It is a dimorphic fungus that is able to display two growth morphologies, a filamentous phase with formation of hyphae and a yeast-like phase with formation of single-celled blastospores. Blastospores play an important role for *M. anisopliae* pathogenicity during disease development. They are formed solely in the hemolymph of infected insects as a fungal strategy to quickly multiply and colonize the insect´s body. Here, we use comparative genome-wide transcriptome analyses to determine changes in gene expression between the filamentous and blastospore growth phases in vitro to characterize physiological changes and metabolic signatures associated with *M. anisopliae* dimorphism. Our results show a clear molecular distinction between the blastospore and mycelial phases. In total 6.4% (n=696) out of 10,981 predicted genes in *M. anisopliae* were differentially expressed between the two phases with a fold-change > 4. The main physiological processes associated with up-regulated gene content in the single-celled yeast-like blastospores during liquid fermentation were oxidative stress, amino acid metabolism, respiration processes, transmembrane transport and production of secondary metabolites. In contrast, the up-regulated gene content in hyphae were associated with increased growth metabolism and cell wall re-organization, which underlines the specific functions and altered growth morphology of *M. anisopliae* blastospores and hyphae, respectively. Our study revealed significant transcriptomic differences between the metabolism of blastospores and hyphae. These findings illustrate important aspects of fungal morphogenesis in *M. anisopliae* and highlight the main metabolic activities of each propagule under *in vitro* growth conditions.

Keywords: Fungal morphogenesis, Entomopathogenic fungi, Hypocreales, Differentially expressed genes (DEGs), Blastospores

5.1. Introduction

Fungi show a high degree of phenotypic plasticity with fungal cells exhibiting a diverse array of shapes and sizes. Part of this plasticity in fungal phenotypes is a consequence of the indeterminate growth of fungi, but also manifests itself as polyphenisms in the form of discrete and distinct types of fungal structures such as hyphae, conidia, dividing single-cells etc. The various types of fungal structures are important at different stages of fungal growth and reproduction, and especially transitions between yeast-hyphal dimorphisms are significant for the virulence of pathogenic fungi [1]. For the human fungal pathogens *Histoplasma capsulatum* and *Paracoccidioides brasiliensis,* the yeast phase are involved in the infection processes [2, 3] whereas the human pathogen *Candida albicans* and the plant pathogen

Ophiostoma novo-ulmi are dimorphic fungi where the yeast phase and the mycelium phase are involved in pathogenic growth [4, 5]. However, our understanding of the underlying genetic mechanisms of how fungal pathogens switch between phenotypes and growth forms is rather limited and primarily restricted to a few mostly human pathogenic fungi where fungal dimorphism is thermally regulated.

Fungi from the genus *Metarhizium* are highly diverse and many show a remarkable degree of phenotypic plasticity. Depending on external stimuli and species involved, *Metarhizium* can grow inside insects as entomopathogens [6, 7] inside plants as primarily root endophytes [8, 9], and they can grow as hyphae in the soil connecting insect carcasses and plants [10]. The *Metarhizium* species *M. anisopliae* and *M. brunneum* are widely used as biological control agents against pest insects and mites. The fungi are cultivated on solid substrates such as rice for 10-14 days where after infectious, uniform, hydrophobic conidia are harvested and formulated into a dry powder-based product. This is not very cost-effective and for several fungi employed as biological control agents, submerged culture fermentation has been considered as a more optimal method for the production of infective propagules [11, 12].

In addition to hyphae and conidia, *Metarhizium* morphogenesis also includes microsclerotia and blastospores. The former is an overwintering structure made of often melanized compact hyphal aggregates that can be induced in carbon-rich submerged liquid cultures [13, 14]. Microsclerotia are considered desiccation tolerant and able to produce infective conidia at specific environmental conditions when applied in the field [15]. Blastospores are thin-walled, pleomorphic, hydrophilic single fungal cells that can be induced after only 2-3 days of liquid fermentation. Following germination, both conidia and blastospores are able to penetrate the cuticle using mechanical and enzymatic force or by natural openings of the insects [16, 17]. Once inside the insect, *Metarhizium* proliferates in the hemocoel as single-celled yeast-like structures that are also termed blastospores. Compared to aerial conidia, blastospores have been found to be more virulent against susceptible hosts [11, 16, 17], although less desiccation tolerant [13]. This makes blastopores an attractive alternative to conidia for biological control but it is unclear exactly why blastospores are more virulent. Blastospores generally germinate within 2-8 hours compared to

12-24 hours for conidia [16, 17], which is an attractive trait for applied purposes and could explain the increased virulence compared to conidia.

In the present study, we aimed to investigate physiological changes and metabolic signatures of the fungal growth phases, blastospores and hyphae, of the entomopathogenic fungus *M. anisopliae s. str.* Specifically, we hypothesized whether changes in gene expression can be related to i) phenotypic differentiation and growing processes in hyphae and blastospores, ii) potential differences in fungal cell-wall metabolism, iii) responses in cellular respiration and oxidative stress to liquid and solid media, iv) genes involved in arthropod pathogenicity that are differentially expressed between hyphae and blastospores, and v) specific classes of biosynthesis genes involved in secondary metabolism produced by each fungal structure.

5.2. Material and Methods

5.2.1. Fungal material and laboratory culturing

The present study investigated the fungus *Metarhizium anisopliae* sensu stricto (ss) using the isolate ESALQ4676 originated from soil of a rainforest biome in native vegetation in Alagoas state, 9 51'7,30"S, 36 20'0,40"W in Brazil. The fungus was isolated using an insect-baiting method using *Galleria melonella* (Lepidoptera: Pyralidae) as insect bait. A conidial monospore culture was obtained by growing the fungus in potato dextrose agar (PDA, Difco, Sparks, MD, USA) for three weeks in a growth chamber at 26°C and 12:12 h photoperiod. A fungal stock culture was established by preserving sporulating agar chunks immersed in a sterile 10% glycerol solution at -80 °C. The isolate is deposited in the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) with the accession number ESALQ4676.

Fungi were grown on a modified Adamek medium [65] with the following nutritional composition per liter: 80 g yeast extract, 40 g cornsteep liquor (Sigma®, St. Louis, USA), minerals, trace metals and vitamins adapted from Jackson's medium [12] at the following concentrations per liter: KH_2PO_4, 2.5 g; $CaCl_2.2H_2O$, 1.0 g; $MgSO_4.7H_2O$, 0.83 g; $FeSO_4.7H_2O$, 0.3 g ; $CoCl_2.6H_2O$, 29.6 mg; $MnSO_4.H_2O$, 12.8 mg; $ZnSO_4.7H_2O$, 11.2 mg; 0.2 mg each of thiamin, riboflavin, pantothenate, niacin,

pyridoxamine, thioctic acid; and 0.02 mg each of folic acid, biotin, and vitamin B12. The medium was amended with 140g L^{-1} of glucose solutions that were autoclaved separately. Sterile solutions of vitamins and metals were added to the autoclaved medium before pH was adjusted to 6.8. Growth and formation of mycelial hyphae during the filamentous phase of the fungus (Figure 1a) and hyphal bodies (blastospores) in the single-celled yeast-like state (Figure 1b) where induced by growing *M. anisopliae* ESALQ4676 on solid and in liquid modified Adamek medium, respectively. Thus identical nutritional compositions were used to grow mycelial hyphae and blastospores, with the only difference that 15 g L^{-1} of agar were added to solidify the medium used for inducing hyphal growth.

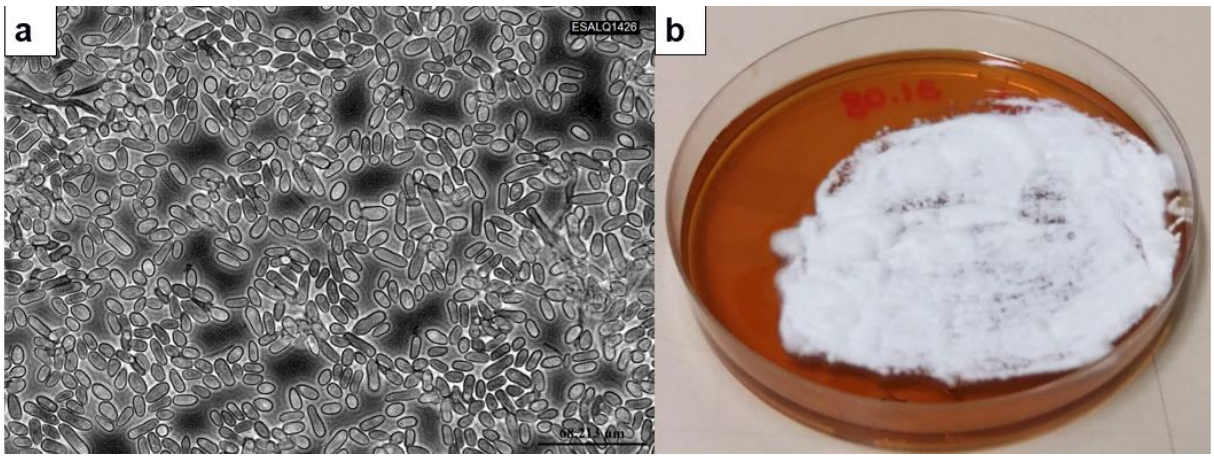


Figure 1. Phase-contrast microscopic image of *Metarhizium anisopliae* (ESALQ4676) blastospores produced in liquid culture (4 days of culture) (magnification: 400x) (a) and mycelium of *M. anisopliae* grown in modified Adamek medium (5 days of growing) (b).

5.2.1. Induction of hyphal and blastospore formation *in vitro*

Conidia of *M. anisopliae* ESALQ4676 were obtained by growing agar chunks from the monosporic stock culture in Petri dishes containing potato dextrose agar in a concentration of 39g per liter of distilled water (PDA, Difco, Sparks, MD, USA) for ten days at 26 °C and 12:12 h photoperiod. Conidia were harvested by washing Petri dishes containing actively sporulating fungal cultures with 10 mL of a sterile aqueous

solution with 0.02% polyoxyethylene sorbitan monooleate (Tween® 80, Sigma). A conidial suspension of 5×10^6 conidia mL^{-1} was used to inoculate 50 mL liquid modified Adamek medium in 250-mL baffled Erlenmeyer flasks giving a final concentration in the culture broth of 5×10^5 conidia mL^{-1} (i.e., 10% v/v inoculum). Liquid cultures were incubated at 28 °C in a rotatory incubator shaker and 350 rpm for 72 hours. Four Erlenmeyer flasks were inoculated and each considered a biological replicate. A volume of 120uL of the 5×10^6 conidia mL^{-1} suspension was spread with sterile Drigalski handles in Petri dishes (9 cm diameter) containing solid modified Adamek medium. Four fungal cultures on solid media in separate petri dishes were incubated at 28 °C for 5 days with a 12:12 h photoperiod, and each considered as one biological replicate.

5.2.2. RNA extraction and sequencing

Blastospores were harvested by filtering three-day old fungal cultures grown in liquid modified Adamek medium. According to laboratory experiments, at this time, the blastospore population was at the end of the exponential growth phase, in this way it was possible to obtain large amount of cells for this study. Total culture broth of each replicate were filtered in a vacuum pump coupled to a Buchner funnel lined with disk filter paper with 7 cm diameter and 11µ pore sizes (Whatman®, n°1) to remove hyphae. To verify that the filtrate contained only blastospores, each replicate was examined on microscope slides at 400x magnification using optic microscopy. To separate blastospores from the culture medium, 30mL of filtrate were added to 50mL Falcon® tubes and centrifuged at 2500 rpm, 4 °C. for 5 minutes. The supernatant was discarded and the blastospore pellet was quickly transferred with a pre-cooled scoop to a pre-cooled porcelain mortar, before immediately adding liquid nitrogen and macerating the pellet with a pre-cooled pestle. The resulting powder were not allowed to thaw and transferred to an Eppendorf tube containing 1mL of TRIzol® and kept on ice. A previous study was carried out to determine the moment of expressive mycelial growth in agar medium, but without the presence of conidia for RNA extraction. Then, mycelial hyphae were harvested from five day old fungal cultures grown on modified Adamek agar media [65]. Hyphae were removed from the medium with a sterilized and pre-cooled spatula and placed immediately in an

Eppendorf tube containing 1mL of TRIzol® and kept on ice. Care was taken to avoid collecting the modified Adamek medium when scraping off the hyphae.

Total RNA was extracted from fungal samples immersed in TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. Eppendorf tubes containing 1mL of TRIzol® with either blastospores or hyphae were incubated for 5 minutes at room temperature, before homogenizing the samples by pipetting up and down. This was followed by centrifugation for 5 minutes at 12000x G at 4 °C. The supernatant was transferred to a new clean Eppendorf tube and the samples were homogenized for 5 minutes in a tissue homogenizer to break the fungal cell walls. 200µL of chloroform were added to samples following agitation for 15 seconds and incubated at room temperature for 5 min. Then, another centrifugation was performed (12,000 x spin, 15 minutes at 4 °C) to separate the mixture and total RNA was precipitated from the upper aqueous phase with half a volume isopropanol (0.5 ml isopropoanol per 1 mL of TRIzol®) and centrifugation . The pellet was washed with 1 mL of 75% ethanol and placed to dry for 30 minutes at room temperature, followed by resuspension of total RNA in 20 uL of pre-cooled DEPC-treated water. Total RNA was quantified fluorometrically using a Qubit® (Invitrogen) and the purity and quality evaluated in a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA). The RNA integrity was estimated with 1% agarose-formaldehyde gel capillary electrophoresis using a Bioanalyzer (Agilent), and only samples with a RNA integrity measure (RIN) higher than 8 were used.

Messenger RNA libraries were prepared with Illumina TruSeq Stranded mRNA Library Prep kit (Illumina Inc., San DieGo, CA) and quantified with qPCR using the Illumina KAPA Library Quantification kit. Samples were sequenced with Illumina HiSeq 2500 technology, which yielded at least 20 million 100-bp paired-end reads per library. Library preparation and sequencing were performed by “Laboratório Multiusuários Centralizado de Genômica Funcional Aplicada à Agropecuária e Agroenergia” in Piracicaba-SP, Brazil.

5.2.3. Mapping of RNA-Seq reads and quantitative differential expression analysis

The quality of the raw reads before and after quality and adaptor trimming was assessed using the fastQC [66] program. Illumina adapters and low-quality sequences were removed using Trimmomatic V0.32 [67] with the following options: HEADCROP:7 TRAILING:20 MINLEN:36. Quality trimmed reads were aligned to the reference genome (*M. anisopliae* sensu stricto ARSEF549 from NCBI) using HISAT2 [68] First, we used the python scripts included in the HISAT2 package: extract_splice.py and extract_exons.py, to extract the splice-site and exon information's from the annotation file, respectively. Then, we built the indexes for the reference genome with the program hisat2-build with the options: --ss and –exon, to provide outputs from splice sites and exons, respectively. Finally, we aligned RNA-seq reads to the reference genome with the program hisat2 with the options: –dta and –p 8. Gene quantification were performed with StringTie v1.3.3 [68] using gene annotations from *M. anisopliae* ARSEF549 strain reference genome information. The stringtie program were used with the following options: -b, -B and –G. The gene count matrix were obtained with the python script: prepDE.py, provided by John Hopkins University, center for computational biology, CCB (http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual#deseq). The gene count matrix was used as input file to the differential expression analysis that was conducted using DESeq2 [69] from the statistical software R [70] We chose a conservative approach to designate differential expression to avoid false positives, so only genes with a false discovery rate (FDR) adjusted p-values <0.001 and $\log_2$ fold change (FC) > 4, for up-regulated genes and $\log_2$FC < -4, for down-regulated genes were considered differentially expressed. Genes were considered exclusively expressed in either blastospores or hyphae when all the biological samples from one fungal structure showed expression values in the same direction (up or down), while all biological replicate samples for the other fungal structure had no reads mapped to that same gene. Diagnostic plots (MA-plot and Volcano-plot) are provided in supplementary material (Additional file 10). Individual gene expression was not re-validated by qPCR because previous studies have shown extremely close correlation between qPCR and RNAseq data [71-74], our biological samples are robustly replicated and being highly similar within treatments and clearly distinct between treatments (Figure 2), and there is little evidence that qPCR analyses of a few specific genes of the same samples will add any new utility to our data or change the

major conclusions drawn from the much larger groups of genes analyzed in the RNAseq dataset. Heatmaps of differentially expressed genes were made with the web application “shinyheatmap” [75] with the following parameters: apply clustering: column, Distance metric: Euclidian; Linkage algorithm: complete.

5.2.4. Gene-set enrichment analysis

Gene set enrichment analysis (GSEA) is a software that determines whether a priori defined set of genes is statistically significant between two biological states [18]. GSEA rank genesets by enrichment magnitude and indicate classes of genes that are over-represented in geneset. As recommended for RNA-seq datasets, GSEA was used in the GSEAPreranked mode with a user provided list of all genes pre-ranked according to a defined metric that could be the $\log_2$ fold change, adjusted *p-value* or inverse *p-value* and a list of gene sets. Then, GSEAPreranked calculates an enrichment score by matching genes from gene sets to those in the user ranked list. Next, the gene set’s enrichment score shows how often members of that gene set occur at the top or bottom of the ranked data set. In this study, we used GSEAPreranked mode with gene sets categorized by gene ontology (GO), protein family domain (PFAM), and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway annotation. The metric used in GSEA input file was the multiplied the sign of fold change by its inverse *p-value*. We used the *p-value* provided as an output of DESeq2. When the *p-value* output from DESeq2 was “0”, the “0” value was replaced by artificially high or low values “+1E+308” or “-1E+308” for up and down-regulated genes, respectively, according to the sign of fold change. The parameter adopted for running the GSEAPreranked for KEGG, GO and PFAM terms were: minlengh 10 and maxlengh 500, enrichment statistic: “classic” and FDR-correction for multiple testing < 0.25 for enriched gene sets. This high threshold is recommended by GSEA software and the explanation for that is “*An FDR of 25% indicates that the result is likely to be valid 3 out of 4 times, which is reasonable in the setting of exploratory discovery where one is interested in finding candidate hypothesis to be further validated as a results of future research*” more details can be access in GSEA official site The web server REVIGO [76] was used to analyze GO terms in the categories: *biological process, cellular component* and *molecular function*.

5.2.5. Secondary metabolites

We identified genes from different families of secondary metabolism pathways classified by Donzelli and Krasnoff (2016) [58], which are based on orthology, phylogenetic analysis and conservation of gene organization around them. The following families were investigated: Nonribosomal peptide synthetase pathway (NRPS), Polyketides synthases (PKS), Hybrid PKS-NRPS (HPN) and Terpenoids biosynthesis (TER). Genes differentially expressed between blastospores and hyphae were filtered by false discovery rate (FDR) adjusted *p-values* were <0.001 and $\log_2$ fold change (FC) > 4 or -4 as described above. Functional annotation of differentially expressed genes according to Donzelli and Krasnoff (2016) [58] are provided Additional file 9

5.3. Results

5.3.1. RNAseq data statistics and reproducibility

To compare genome-wide expression profiles of blastospores and hyphae of *M. anisopliae* (ESALQ4676), a total of 115 million paired-end 100-bp quality-checked reads from four replicate blastospores and four replicate hyphal samples (between 13-16 million paired-end reads per sample), were obtained. The percentage of quality filtered reads that mapped to the *M. anisopliae* reference genome (ARSEF549) were 94% for blastospores and 84% for hyphae (Table 1). Clustering analysis showed that 98% of sample variation was represented by differences between treatments and consequently a high similarity of biological samples within treatment (blastospores vs. hyphae) (Figure 2).

Table 1. Summary of *M. anisopliae* RNA-Seq read filtering and mapping. Values represents the values for each of the four biological replicates for blastospores (BL) and hyphae (H), respectively.

Sample	Clean paired reads	Mapped reads (%)	Unmapped reads (%)	Unique match (%)
BL1	15,949,141	15,049,609 (94.3%)	899,531 (5.7%)	9011677 (56%)
BL2	14,305,193	13,555,600 (94.7%)	749,592 (5.3%)	8079589 (56.4%)
BL3	13,170,251	12,466,959 (94.6%)	703,291 (5.4%)	8022903 (60.9%)
BL4	16,858,276	15,990,074 (94.8%)	868,201 (5.2%)	8704384 (51.63%)
H1	13,555,682	11,481,662 (84.7%)	2074,019 (15.3%)	7258528 (53.54%)
H2	13,470,151	11,320,314 (84%)	2149,836 (16%)	7007145 (52.09%)
H3	14,050,046	11,872,288 (84.5%)	2177,757 (15.5%)	7255368 (51.63%)
H4	13,988,895	11,673,732 (83.4%)	2315,162 (17.6%)	7410046 (52.9%)

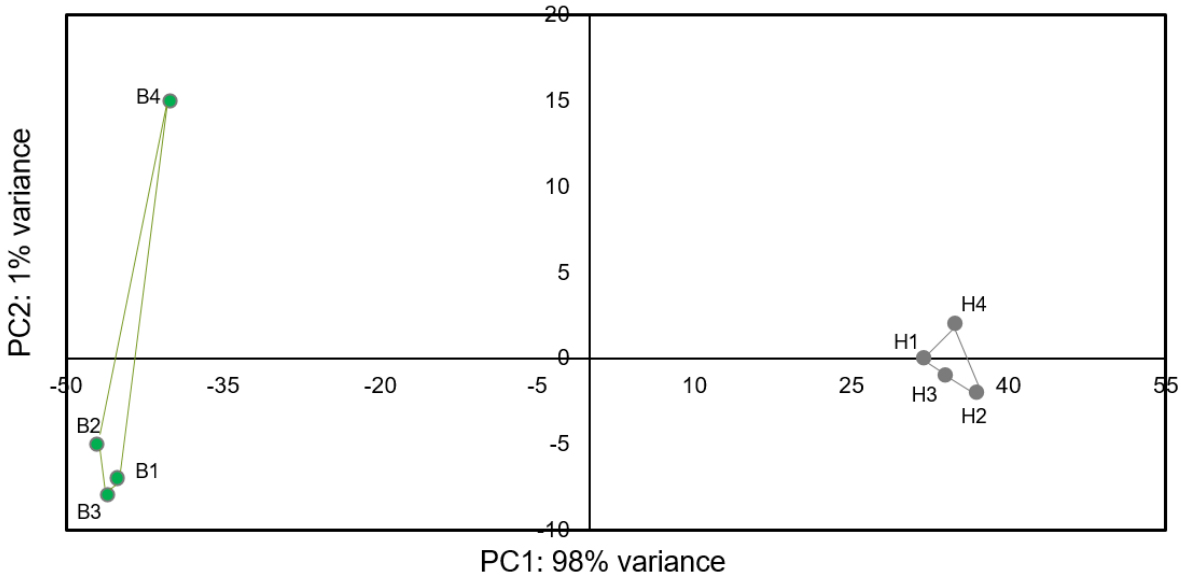


Figure 2. Principal component analysis of regularized-logarithmic (rlog) transformed gene counts of blastospores (B1-4) and hyphal samples (H1-4). Blastospores and hyphae samples are represented by green and grey dots, respectively.

From 10,891 genes annotated in the *M. anisopliae* genome (ARSEF 549) (Additional file 1), 696 genes were differentially expressed between blastospores and hyphae (FDR adjusted $p < 0.001$, $Log_2FC > 4$ or < -4). Of these, 240 genes were up-

regulated in blastospores and 456 were up-regulated in hyphae (Figure 3, 4 and Additional file 2). Moreover, we found 48 genes exclusively and differentially expressed in blastospores, and 97 genes exclusively expressed in hyphae (Additional file 3). A set of 647 genes in the reference genome were not expressed in any of the two fungal growth phases analyzed here.

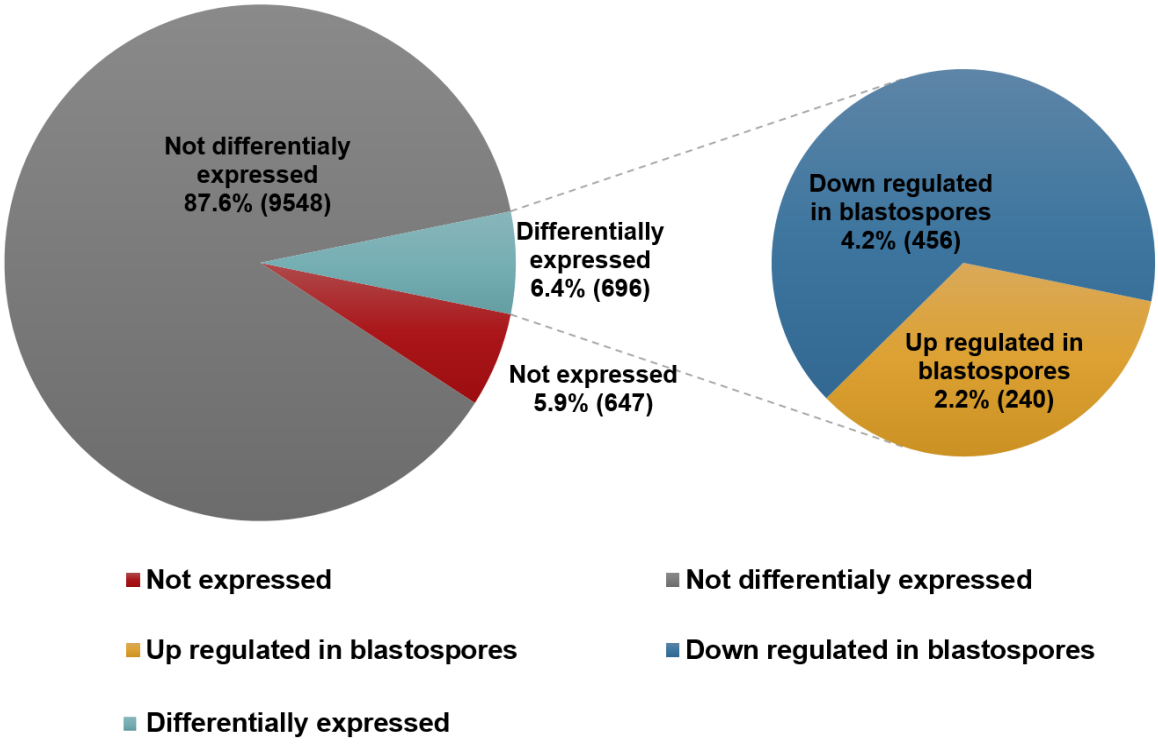


Figure 3. Number of genes differentially expressed in blastospores vs. hyphae. The percentage of genes that are not expressed, not differentially expressed and differentially expressed are shown in the left circle. The right circle shows the percentage of down and up-regulated genes in blastospores out of the differentially expressed genes.

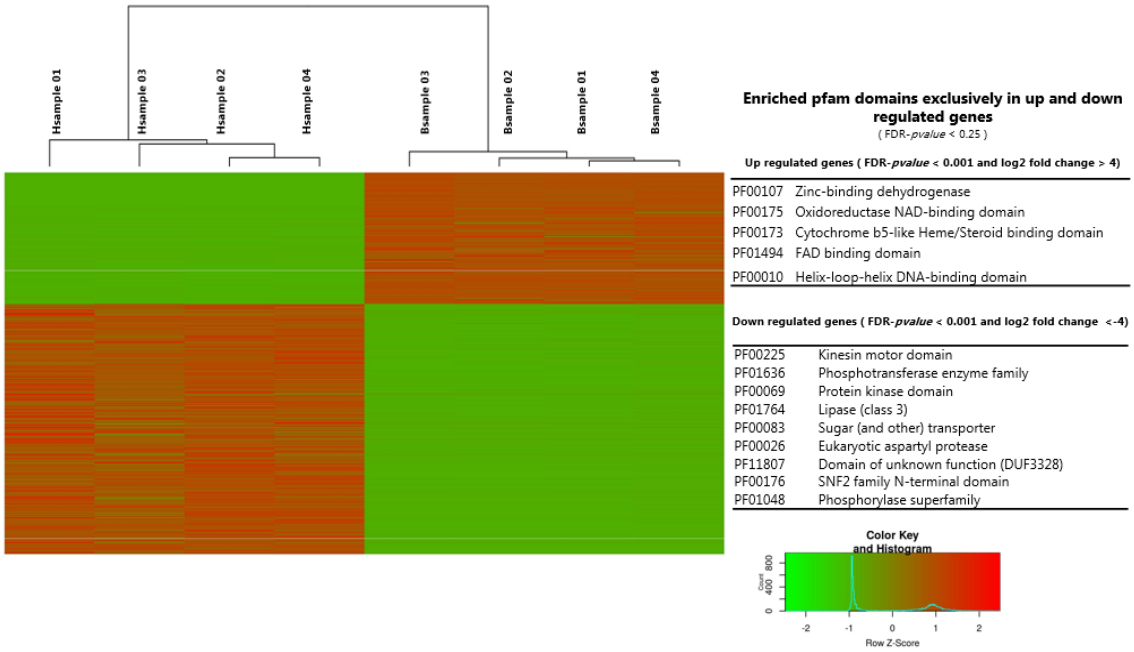


Figure 4. Heat map of the 696 genes differentially expressed (FDR-*pvalue* < 0.001 and log2 fold change > 4 or -4), with 240 up and 456 down-regulated genes in blastospores, respectively. The enriched exclusively pfam domains assigned to up and down-regulated genes are presented in the right panel (Additional file 3).

5.3.3. Gene set enrichment analyses

Gene Ontology (GO) terms could be assigned to 66% (465/696) of the differentially expressed genes. To functionally characterize the set of genes significantly up-regulated in blastospores and hyphae we carried out GO-term gene set enrichment analyses using GSEA [18]. In blastospores, 69 GO terms were significantly enriched (FDR adjusted $p < 0.25$), of these, 15 were assigned to biological processes, eight to cellular components and 46 to molecular function (Additional file 4). The largest set of enriched genes among the biological processes were assigned to: *metabolic process* (GO:0008152), *Transcription, DNA-templated* (GO:0006351), and *transmembrane transport* (GO:0055085) (Figure 5a). Thirty-three GO terms were found to be significantly enriched in hyphae compared to blastospores (FDR adjusted $p < 0.25$), with 13 assigned to biological processes (figure 5b), five to cellular components and 15 to molecular functions (Additional file 4).

Pfam terms could be assigned to 61% (427/696) of differentially expressed genes. Using the same GSEA-methodology as described above, 28 pfam terms were significantly enriched in blastospores (Additional file 4). Five of these, (Zinc-binding dehydrogenase (PF00107), Oxidoreductase NAD-binding domain (PF00175), Cytochrome b5-like Heme/Steroid binding domain (PF00173), FAD binding domain (PF01494), and Helix-loop-helix DNA-binding domain (PF00010) are exclusively found among up-regulated genes in blastospores (Figure 4 and Additional file 5). A total of 18 pfam terms were significantly enriched among up-regulated genes in hyphae (Additional file 4), of which nine were exclusively present among the up-regulated genes (PF00225:Kinesin motor domain, PF01636:Phosphotransferase enzyme family, PF00069:Protein kinase domain, PF01764:Lipase (class 3), PF00083:Sugar (and other) transporter, PF00026:Eukaryotic aspartyl protease, PF11807:Domain of unknown function (DUF3328), PF00176:SNF2 family N-terminal domain, PF01048:Phosphorylase superfamily)(Figure 4 and Additional file 5). To explore patterns of metabolic pathway regulation between blastospores and hyphae we attempted to assign KEGG terms, which resulted in KEGG annotation of 15% (110/696) of the differentially expressed genes. In total 26 KEGG terms were enriched in blastospores of which 16 are exclusively found among up-regulated genes in blastospores (Table 2, Additional file 4 and 5) and only a single KEGG-pathway was exclusively and significantly enriched among up-regulated in hyphae (ko00513: *Various types of Nglycan biosynthesis)* (Additional file 5).

To complement these gene set enrichment analyses, we further analyzed functional annotations of the differentially expressed gene set in relation to the five hypotheses outlined above, which we detail in the paragraphs below.

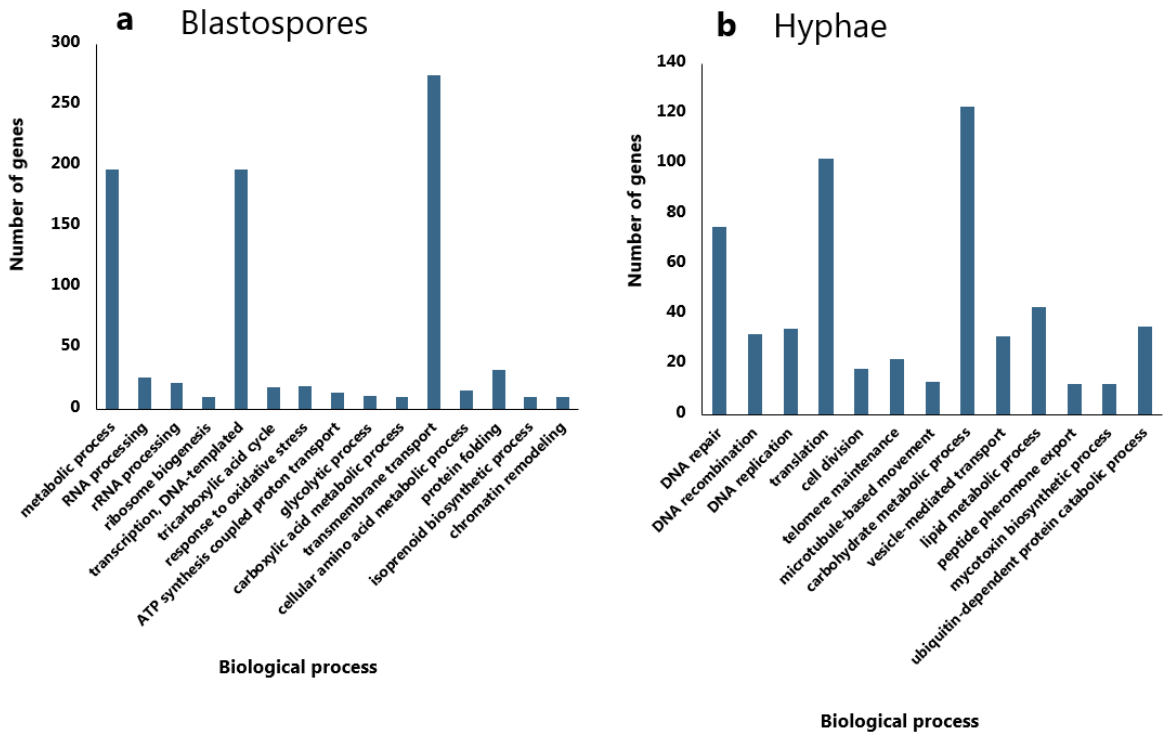


Figure 5. Enriched gene ontology (GO) terms for the *biological process* category based on GSEA analysis (See text for details) in blastospores (5a) and hyphae (5b) (FDR- *p-value* < 0.25) (Additional file 4).

Table 2. KEGG pathway terms significantly enriched among up-regulated genes in blastospores (Additional file 5).

KEGG	Pathway
ko00010	Glycolysis / Gluconeogenesis
ko00071	Fatty acid degradation
ko00190	Oxidative phosphorylation
ko00260	Glycine, serine and threonine metabolism
ko00310	Lysine degradation
ko00340	Histidine metabolism
ko00350	Tyrosine metabolism
ko00360	Phenylalanine metabolism
ko00380	Tryptophan metabolism
ko00410	betaAlanine metabolism
ko00620	Pyruvate metabolism
ko00630	Glyoxylate and dicarboxylate metabolism
ko01100	Metabolic pathways
ko01110	Biosynthesis of secondary metabolites
ko01130	Biosynthesis of antibiotics
ko01200	Carbon metabolism

5.3.4. Cellular growth and DNA homeostasis

We analyzed the putative function of genes differentially expressed between blastospores and hyphae for signatures of cellular growth and DNA homeostasis. In hyphae, we found six out of 13 significantly enriched GO terms in the biological processes category, which were related to cell division and DNA activities. These terms represent *DNA replication* (GO:0006260), *DNA translation* (GO:0006412), *DNA recombination* (GO:0006310), *DNA repair* (GO:0006281), *cell division* (GO:0051301) and *telomere maintenance* (GO:0000723) (figure 5B). This is also evident in the significantly enriched KEGG-pathways in hyphae such as *DNA replication* (ko:03030), *DNA Mismatch repair* (ko:03430), *Base excision repair* (ko:03410), *Homologous recombination* (ko03440), *Nucleotide excision repair* (ko:03420) and *Pyrimidine metabolism* (ko:00240) (Additional file 4).

The increased DNA-related activity could indicate actively growing cells, and we therefore also identified for enriched GO terms in hyphae involved in fungal growth and cell proliferation, like *microtubule* (GO:0005874), *microtubule motor activity* (GO:0003777) and *microtubule-based movement* (GO:0007018). Microtubules represent polymers of tubulins that are components of the cell cytoskeleton that undergo rearrangements during cell growth. Some of the significantly enriched pfam terms found in hyphae is in concordance with these findings, such as the kinesin motor domain (PF00225) that function in proteins that moves along microtubules and is related to mitosis, meiosis and transportation of cellular components, and the protein kinase domain (PF00069), involved in cellular division, differentiation, and cytoskeletal rearrangement. Additionally, we found two genes, exclusively and highly expressed in hyphae involved in conidiophore formation and differentiation of conidiation structures, conidiospore surface protein ($\log_2$ fold change -6.3, FDR-*pvalue* < 0.001, KID70618) and conidiation-specific protein ($\log_2$ fold change -6.6, FDR-*pvalue* < 0.001, KID63680) (Additional file 3).

To maintain active growth, heterotrophic organisms like fungi need to break down nutritional substrates into smaller molecules that can be absorbed into the cell. In hyphae, we observed a high number of up-regulated genes involved in transportation of substances in membrane-bound vesicle (Figure 5B). These findings are illustrated by the significantly enriched GO term: *vesicle-mediate-transport*

(GO:0016192) and pfam term: *Sugar (and other) transporter* (PF00083) in hyphae. In blastospores, we similarly found the significantly enriched GO term *Transmembrane transport* (GO:0055085), which indicates active transport of substance across external or intracellular membranes. The main metabolic pathway that provides energy for fungal cells is glycolysis and the TCA-cycle. These pathways were significantly enriched in blastospores (FDR-*pvalue* < 0.25), emphasizing that genes facilitating an increased metabolic rate and energy consumption are up-regulated in blastospores compared to hyphae. Significant enrichment in blastospores of the glyoxylate and dicarboxylate metabolism pathways (KEGG: ko00630, FDR-*pvalue* < 0.25) further supports this inference (Additional file 4). This pathway is important for assimilation of alternative carbon sources like two carbon substances and fatty acids. Specifically, two well-known glyoxylate cycle intermediates, isocitrate lyase (ICL, pfam: PF00463) and malate synthase (MLS, pfam: PF01274), that convert 2-carbon compounds like acetate and hydrolytic products of fatty acids [19] were significantly up-regulated in blastospores (Two ICL genes in *M. anisoplaie* ARSEF 549, $\log_2$ fold change 5.4 and 1.8, FDR-*pvalue* < 0.001, KID66430 and KID66042, respectively, and MLS, KID70056, $\log_2$ fold change 3.0, FDR-*pvalue* < 0.001).

5.3.5. Cell wall metabolism

The main components of Ascomycete cell walls are polymers such as chitin, that make up the inner layer of the wall, and alfa/beta glucans and galactomannoproteins that comprise the gel-like polymers in the outer cell wall layer [20]. Differences in cell wall composition between *M. anisopliae* blastospores and hyphae was evident from the significant enrichment in hyphae of the GO category *carbohydrate metabolism* (GO:0005975) and KEGG pathway *Various types of Nglycan biosynthesis* (ko:00513) (Additional file 4). These classifications contain many genes related to cell wall metabolism, and we therefore expected to find gene expression differences in the glycoside hydrolases (GH), that synthetize or degrade cell wall components (Figure 6, Additional file 6). Enzymes in GH family 18 (PF00704), GH family 16 (PF00722), and GH family 3 (PF00933) were the largest and most differentially regulated group of GH's between blastospores and hyphae (Figure 6). We identified 25 GH family 18 genes that includes chitinases from group

18 and class III, which degrade chitin and were expressed both in hyphae and blastospores (Figure 6). The second largest group of GH in *M. anisopliae* is GH family 16, which include enzymes involved mainly in degradation of cell wall polymers of glucans (Figure 6). In blastospores, glycoside hydrolases that include beta-1,3-endoglucanase (KID71664) and Concanavalin A-like lectin/glucanas (KID71072) were up-regulated, whereas in hyphae cell wall glucanosyltransferase, endo-1,3(4)-beta-glucanase, extracellular cell wall glucanase, glucan 1,3-beta-glucosidas and one gene assigned to Concanavalin A-like lectin/glucanas (KID68560) were up-regulated (Figure 6, Additional file 6). The essential component of fungal cell walls, chitin, is synthesized by chitin synthases and members of GH family 3 (PF00933), which were differentially regulated between balstospores and hyphae (Figure 6, Additional file 6). Chitin synthase is activated by N-acetylglucosamine and uses Uridin diphospho-N-acetylglucosamine (UDPGlcNAc) to produce chitin [20, 21]. That chitin synthesis is primarily taking place in hyphae is further supported by the chitin synthase enzymes Chitin synthase 1 (PF01644), including chitin synthase class I and II, and Myosin_head (PF00063), including chitin synthase class V and VII, which were up-regulated in *M. anisopliae* hyphae but not in blastospores (Figure 6, Additional file 6). Analysis of the GH family 27 (PF16499) showed that alpha-galactosidase and alpha-N-acetylgalactosaminidase were up-regulated in blastospores and hyphae, respectively, whereas GH family 19 (PF0365) alpha-1,3-glucanases were exclusively up-regulated in hyphae (Figure 6, Additional file 6).

Specific cell wall proteins such as hydrophobins and adhesin are important for creating an hydrophobic surface that can provide protection to fungal cells and adherence to substrate or host [22,23], exemplified by the adhesin proteins MAD 1 (KID69933) and MAD 2 (KID69968) that were highly expressed in hyphae ($\log_2$ fold change 4.0 and 7.8, FDR-adjusted $p < 0.001$, respectively). Out of four genes encoding hydrophobin proteins in the *M. anisopliae,* one was significantly expressed in blastospores (KID65917) and another hydrophobin gene in hyphae (KID65291) ($\log_2$ fold change 4.1 and $\log_2$ fold change 6.1, FDR-adjusted $p < 0.001$, respectively)

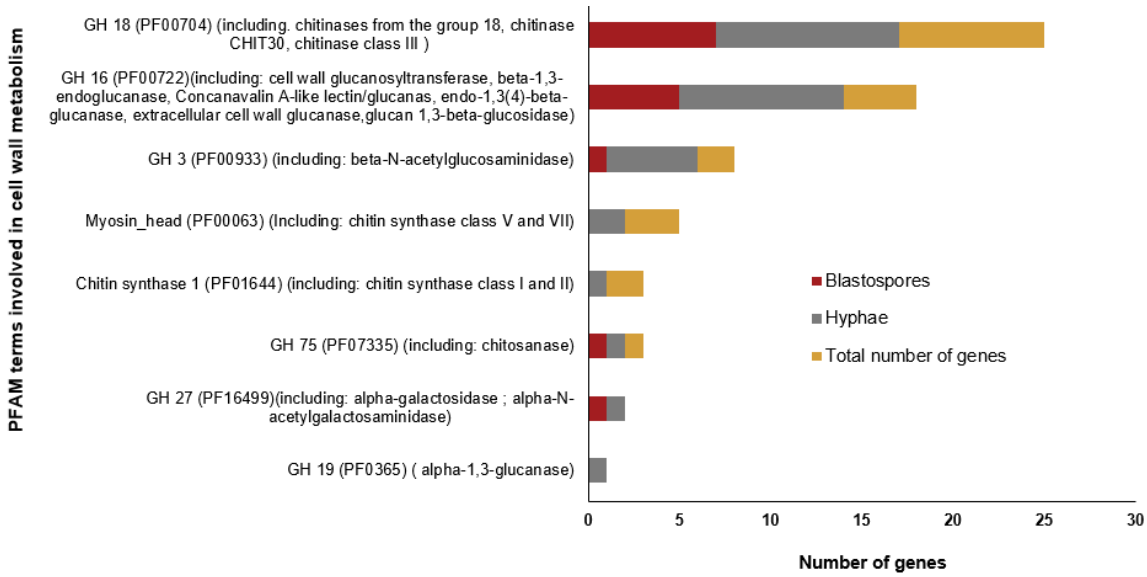


Figure 6. Number of genes with PFAM terms involved in cell wall metabolism. Up-regulated genes are represented by dark red bar for blastospores and grey bars for hyphae, FDR-*pvalue* < 0.001 (Additional file 6).

5.3.6. Cellular respiration and oxidative stress

Fungal respiration processes appear to be highly activated in blastopores produced under liquid growth conditions, as exemplified by the significantly enriched GO categories: *tricarboxylic acid cycle* (GO:0006099), *glycolytic process* (GO:0006096), and *ATP synthesis coupled proton transport* (GO:0015986) (Figure 5a). The pfam domains representing mitochondria protein family's (PF00153) and Acyl-CoA dehydrogenase protein family domains (PF00441, PF02770, and PF02771) were similarly enriched among up-regulated genes in blastospores (Additional file 4). The mitochondrial respiratory mechanism constitutes the main intracellular source of reactive oxygen species (ROS). However, ROS accumulation within the cell trigger the cell responses to oxidative stress, which was evident in blastospores by the significant enrichment of GO category *response to oxidative stress* (GO:0006979) (Figure 5a).

5.3.7. Expression of virulence factors

Because blastospores and hyphae differ in their insect infectivity, we investigated whether there were expression differences in pathogenicity-related genes between hyphae and blastospores. Certain protease enzymes are important for entomopathogenic fungi during penetration of the insect cuticle, and especially the subtilisin-like serine proteases (pfam: PF00082) are well known to be implicated in entomopathogenicity. Two out of 49 genes with PF00082 in *M. anisopliae* genome were up-regulated in hyphae, the proteinase K-like, Pr1K (log_2 fold change: -8.5, FDR-adjusted $p < 0.001$, KID64661) and subtilisin-like serine protease, Pr1C (log_2 fold change: -4.6, FDR-adjusted $p < 0.001$, KID70176). In blastospores, only a single but different subtilisin-like serine protease Pr1C were up-regulated (log_2 fold change: 4.2, FDR-adjusted $p < 0.001$, protein product: KID65298) (Additional file 8). Another group of proteases well known to be involved in pathogenicity of fungi are trypsin-like serine proteases (pfam:PF00089) [24]. Ten trypsin-proteases, out of 24 genes in the ARSEF 549 *M. anisopliae* genome containing domain PF00089 were expressed in hyphae, being four of them significantly and highly expressed (log_2 fold change – 6.6, - 7.8, -5.2, -7.2, FDR-*pvalue* < 0.001, protein products: KID60022, KID63483, KID70241 and KID70499 respectively), whereas no trypsin-proteases were differentially expressed in blastospores (Additional file 8).

When entomopathogenic fungi grow inside insects they have to cope with the host immune response in the hemolymph. Fungi overcome host defense either by developing cryptic growth forms, like blastospores, that are partially masked from host defense responses or by producing immunomodulating molecules that suppress the host defense [22] Blastospores of *M. robertsii* express the collagen-like protein MCL1, which is a well know protein that provides an antiadhesive protective coat that masks beta-glucan components of the cell wall and hinders detection by the host hemocytes [25]. In our in-vitro experiments, the two identified MCL1 proteins in *M. anisopliae* (protein product: KID63518 and KID71631) were both up-regulated in blastospores, and one of them were highly expressed (log_2 fold change 8.2, FDR-adjusted $p < 0.001$). Phosphoketolase, is an essential enzyme in the phosphoketolase pathway involved in sugar metabolism and required for full virulence in *Metarhizium* spp. This enzyme is usually highly expressed by

Metarhizium species when grown in trehalose-rich insect haemolymph but poorly induced by insect cuticle [26]. Here we found a phosphoketolase (protein product: KID62449) up-regulated in blastospores (log_2 fold change 3.8, FDR-*pvalue* < 0.001).

5.3.8. Secondary metabolites

Many fungi produce high amounts of secondary metabolites important for pathogenicity, and one of the best known types of secondary compounds produced by *M. anisopliae* during fermentation are dextrusins. In the present experiments both blastospores and hyphae expressed the gene destruxin synthetase (KID59658, gene: MAN_10464; Mean (± SE) of sample normalized count data for hyphae: 1522 ± 20 and blastospores: 1476 ± 67) (Additional file 1), but there was no difference in expression between the two fungal structures (log_2 fold change -0.04, FDR-adjusted p = 0.61). The KEGG pathway *Biosynthesis of secondary metabolites* (ko01110) were enriched in blastospores and a higher number of significantly expressed secondary metabolite related genes were observed in blastospores (n=12) compared to hyphae (n=7) indicating that blastospores produce more secondary metabolites than hyphae (Figure 7) at the time it was harvested from the liquid culture medium. A gene annotated as Nonribosomal Peptide synthetase-like (NPLs) (MAN_01071/ KID71472) where exclusively expressed in hyphae, whereas two genes related to Hybrid polyketide-nonribosomal peptide synthetases (HPNs) (MAN_01651/KID69137, MAN_09390/ KID61106) where exclusively expressed in blastospores (Figure 7). Although expression occur in both blastospores and hyphae, Nonribosomal Peptide Synthetases (NRPSs) where primarily expressed in hyphae (n = 3), whereas Terpene biosynthetic gene families (TERs) where primarily expressed in blastospores (N = 8) (Figure 7, Additional file 9).

Cytochrome P450 is a large group of proteins that among other functions are involved in conversion of hydrophobic intermediates of primary and secondary metabolic pathways and detoxification processes that sustain fungal growth under stressing conditions. Out of 103 Cytochrome P450 genes (PF00067) in *M. anisopliae,* we found 34 genes differentially expressed between blastospores and hyphae (log_2 fold change >4 or < -4, FDR-adjusted p < 0.001) (Additional file 11). These where divided evenly with 16 up-regulated in blastospores and 17 up-

regulated in hyphae. Many of these Cythocrome P450 were among the most highly expressed genes as exemplified by their presence on the list of the top 50 up-regulated genes in blastospores (Additional file 7)

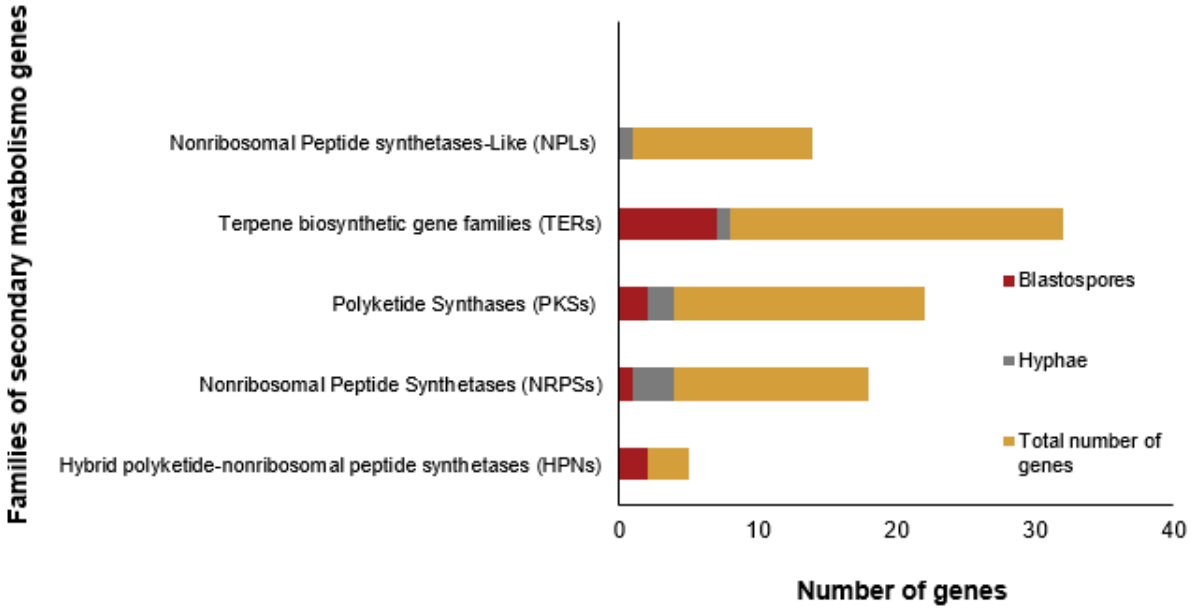


Figure 7. Number of genes in families of biosynthetic genes involved in secondary metabolism of *Metarhizium*. Up-regulated genes are represented by dark red bar for blastospores and grey bars for hyphae, FDR-*pvalue* < 0.001, $\log_2$FC > 4 (Additional file 9).

5.4. Discussion

Here we compared the transcriptomic profile of blastospores and hyphae induced by growth in liquid and solid medium, respectively, of the dimorphic entomopathogenic fungus *M. anisopliae*. Hyphae and blastospores were grown under identical nutritional conditions and the media thus only differed in oxygen levels, water composition, viscosity and agitation. In total 6.4% (n=696) out of the 10,981 predicted genes in *M. anisopliae* were significantly differentially expressed between blastospores and hyphae with a fold-change > 4. Functional annotation and gene enrichment analyses highlighted that the main physiological processes that were up-regulated in the single-celled yeast-like blastospores during liquid fermentation were: oxidative stress, amino acid metabolism, respiration processes, transmembrane transport and production of secondary metabolites. In contrast,

hyphae had molecular signatures of increased growth metabolism and cell wall re-organization, which underlines the different growth morphology of *M. anisopliae* blastospores and hyphae.

Differential growth of hyphae and blastospores

Hyphae and blastospores show clear differences in their pattern of growth. Hyphae exhibit polarized growth whereas blastospores grow isotropically until cell division by budding [27]. The three principal components of polar growth characteristic for fungal hyphae, are vesicles, responsible for the enzyme supply for cell wall synthesis, the cytoskeleton that provides a scaffold for vesicle transport and cell wall polymers that sets the shape of the cells [20]. Several genes from all three components were highly expressed in *M. anisopliae* hyphae (Figure 4 and 5). This is similar to other pathogenic dimorphic fungi (Table 3), and consistent with hyphal cell walls being more rigid than single-celled yeast-like growth forms in *Metarhizium*.

The main components of Ascomycete cell walls are chitin, alfa/beta glucans and galactomannoproteins [20]. These polymers are synthesized and broken by a broad group of enzymes named Glycoside hydrolases (GH). One group of GHs important for cell wall modulation and involved in chitin degradation, are the chitinases that break β-1,4-linkages. These enzymes are found in two GH families GH18 and GH19 with distinct catalytic mechanisms [28, 29]. The GH18 family chitinases are the most common in fungi [30], and we discovered three GH18 chitinases significantly up-regulated in hyphae while only a single GH18 chitinase were significantly up-regulated in blastospores (Additional file 6). This is consistent with previously reported low activity of chitinases in *M. anisopliae* blastospores compared to mycelium [28]. However, in blastospores a beta-1,3-endoglucanase (GH16), which is involved in degradation of glucan polymers where highly expressed (Additional file 6). The thinner characteristics of *M. anisopliae* blastospore cell walls compared with aerial and submerged conidia [31] is consistent with the differential expression of cell-wall related GH enzymes (Figure 6). This is likely explained by the submerged growth of blastospores in liquid medium allowing increased flexibility and permeability of the cell wall [32]. These cell wall modifications enhance nutrient flux

and are almost certainly induced in response to different nutritional and environmental conditions.

Seven distinct classes of chitin synthases belonging to two families have been identified in fungi based on amino acid sequences [33]. Here we found classes I, II, V and VII expressed in hyphae and blastospores, where class V and VII are primarily involved in synthesis of chitin at the tip of growing hyphae and during conidiogenesis and not present in most yeast species [34]. When infectious conidia are produced by *M. anisopliae* they develop from conidiophores formed in the hyphal growth phase and not directly from blastospores. Consistent with this, we observed up-regulated expression of the enzyme beta-N-acetylglucosaminidase (gene MAN_04359, KID67601) involved in chitin synthesis pathways and genes related to conidiophore and conidiogenesis differentiation exclusively in hyphae.

The synthesis of new biological material in hyphae correlates with increased activity of cell wall metabolism, but also with high activity of genes associated with DNA and carbohydrate metabolism. However, these findings do not agree with the fact that the main energetic metabolic pathway like: TCA-cycle, glycolysis and glyoxylate cycle are all up-regulated in blastospores, as well as amino acid metabolic pathways. The glyoxylate cycle is activated by many fungi when non-fermentable carbon sources and 2-carbon compounds are provided [35]. Following injection of conidia, *M. anisopliae* up regulate the glyoxylate cycle during growth within insect hemocytes but repress the glyoxylate cycle when the fungus evades hemocytes and proliferate in the hemolymph [19]. This suggests that *M. anisopliae* can hydrolyse and extract energy from intracellular lipids during growth within insect haemocytes [19]. In our experiment, *M. anisopliae* blastospores were grown in rich glucose medium (140g/L) with two protein sources, yeast extract and cornsteep liquor. The finding that the glyoxylate cycle, glycolysis and TCA-cycle is up-regulated in blastospores, indicates that energy for cell metabolism comes from different sources of substrate and not only glucose. The glyoxylate cycle is known to be activated in bacteria under oxidative stress [36], and although we found that blastospores are under oxidative stress, the correlation with the glyoxylate cycle is to the best of our knowledge unknown in fungi.

When hyphae are growing and multiplying (i.e. consuming energy and building new biological material), we expected the major metabolic cycles to be up-regulated

in hyphae and not in blastospores. Instead, the observed transcriptome pattern indicates that not all the energy produced by blastospore metabolic activity is converted into new biological material in the form of new growth, otherwise we would have expected DNA associated activities to be equally up-regulated in blastospores and hyphae. These results could be partially explained by continuous polarized growth of hyphae as long as conditions are favorable, whereas yeast-like growth of blastospores is determined and ends when development is complete [20]. Microscopic analysis of slides from each biological replicate at the moment of RNA extraction revealed that few blastospores were actively replicating. This could be due to beginning physio-chemical changes in the media or that some cells had reached the final size and stopped cell division as the moment in which these cells were harvest from medium consisted with the end of exponential phase. These differences in growth correlates with low chitinase expression in blastospores compared to hyphae, where in contrast hyphal growth involves constant degradation and synthesis of the external and internal septal cell walls. Increased expression of cell wall organization proteins in hyphae has also been reported for other dimorphic fungal pathogens such as the human pathogen *Paracoccidioides brasiliensis* [37] and the phytopathogenic fungus *Ustilago maydis* [38] when compared to their yeast phases, respectively. The composition of cell walls is fundamentally different between yeast and mycelial forms in many fungi [39, 40, 41, table 3], and our RNAseq data is consistent with this also being the case between *M. anisopliae* blastospores and hyphae.

Oxidative stress in blastospores during submerged growth

Oxidative stress is caused by intracellular accumulation of reactive oxygen species (ROS) or changes in cellular redox stability. External environmental causes for ROS formation are: ionizing radiation, visible light, temperature shifts, oxygen exposure and UV radiation. Cellular defense against oxidative stress involves enzymatic and non-enzymatic detoxification mechanism aiming to remove ROS and keep the redox balance [42]. In our study, the high agitation speed (350rpm) provided to culture flasks and high glucose concentration of the medium (140g/L) result in high dissolved oxygen levels [43, 44], which could expose blastospores to an increased

oxygen triggering formation and ROS. Another possible explanation for this physiological response would be the constitutive expression of antioxidant enzymes as a defense mechanism against the stressful conditions posed in the hemolymph of insects, environment in which blastospores are naturally produced, [105,106]. This host's ROS-mediated immune response is supported by increased expression of antioxidant catalases enzymes involved in the response to oxidative stress (GO:0006979) in blastospores. These catalase enzymes break down the ROS hydrogen peroxide (H_2O_2) into H_2O and O_2, consistent with blastospores being more stressed by ROS than hyphae in our experiments.

The mitochondrial respiratory chain constitutes the main intracellular source of ROS in most tissues [45]. Indeed, the high activity of the TCA-cycle and respiration processes in blastospores could also be related to ROS production. An increase in intracellular ROS levels have been shown to induce morphogenesis of other fungi (Table 3), and also in propagules under aerated liquid culture conditions such as in the formation of microsclerotia in the entomopathogenic fungus *Metarhizium rileyi* [46], hyphal to yeast transitions in *P. brasiliensis* (Nunes et al., 2005), and the formation of sclerotia in phytopatogenic fungi [47]. These findings could indicate a role for ROS in blastopore morphogenesis, but the high levels of intracellular ROS would need to be tightly regulated because of the toxicity of radicals. Proteins from the versatile group of Cytochrome P450 are among other functions also involved in detoxification of ROS, and for example, we observed comparatively more Cytochrome P450's and monooxygenases among the list of the top 50 most up-regulated genes in. This list also contained the important copper oxidase enzyme laccase, which can provide protection against oxidative stress caused by oxygen radicals in fungi [48]. The laccase of the white rot fungus, *Pichia pastoris,* significantly enhances scavenging of intracellular H_2O_2 and lipid oxidative damage by stimulating production of glutathione-based antioxidants [49]. Glutathione is an important antioxidant in fungi, and we found a glutathione S-transferases among the top 50 most up-regulated genes (log2FC: 10.5, FDR-*pvalue* < 0.001, protein product: KID61097). This enzyme is involved in detoxification of reactive electrophilic compounds by catalyzing their conjugation to glutathione, indicating that laccase has an important indirect role for protecting blastospores against oxidative stress. The transcriptome profile of blastospores indicate that ROS may be involved in

blastospore formation but also as a stressor that force blastospores to constantly try to maintain cell homeostasis and probably excrete cytotoxic components produced during oxidative process. Additionally, these conditions can be found in insect hemolymph and the response to oxidative stress may be a constitutive condition pose by liquid culture medium that to some extent mimics hemolymph environment.

The transcriptome profiles highlighted differences in the transport of substances between hyphae and blastospores, where transmembrane transport was more active in blastospores whereas vesicle transport of substance was more pronounced in hyphae. Increased transport activity across the outer membrane has also been observed in the yeast phase of three human fungal pathogens: *Histoplasma capsulatum* [3], *Paracoccidioides brasiliensis* [50], and *Penicillium marneffei* [51]. The outer membrane transporters functions in the absorption of nutrients, export of toxic molecules, maintenance of cell turgor, cell development, and maintenance of ion and pH homeostasis [52]. A high number of functional categories related to amino acid metabolism and respiration metabolism were significantly up-regulated in blastospores suggesting that the high activity of membrane transporters in blastospores could be partially explained by increased amino acid metabolism, glycolytic processes and the export of toxic molecules in response to oxidative stress.

Although transmembrane transport is present in hyphae, genes involved in vesicle-mediated transport were significantly up-regulated in hyphae compared to blastospores. During mycelial growth, not all hyphal cells in the mycelium are in direct contact with the substrate and resources are usually reached at the tips of hyphae, sometimes following branching of the hyphae depending on fungal species. Different from the multi-cellular hyphae, yeast-like growth is characterized by the cells being in constant direct contact with the substrate and resources are reached through growth [20]. The vesicle mediated transport play an important role for delivering nutrients to cells that are not in direct contact with the substrate, in order to supply enzymes for cell wall synthesis and to transport material across cell wall [53]. Thus, the vesicle mediated transport is potentially more important for mycelium development then for the yeast-like phase of blastospores in *M. anisopliae*.

Secreted metabolites and virulence related enzymes

The main group of genes involved in secondary metabolite production up-regulated in blastospores were the terpenoids (Figure 7). Four (M-TER10, M-TER11, M-TER26 and M-TER31) out of seven terpenoid families found up-regulated in blastospores (Figure 7), belong to a conserved cluster that closely resemble those involved in indole diterpene production in other Ascomycetes [54]. Some indole diterpenes are known to have insecticidal effects through ion channel modulation [55, 56], and indole-diterpenes from sclerotia of species in the genera *Aspergillus* and *Penicillium* have biological activity against insects [57]. Although indole diterpenes have been isolated from Clavicipitaceaen fungi, their chemical details have not been worked out in *Metarhizium* [58]. Two other families of terpenoides expressed in blastospores, M-TER33 (terpene cyclase) and MTER43 (UbiA prenyltransferase) are homologues to *Aspergillus nidulans* genes ausL and ausN, which are responsible for catalysing terpene cyclization and C-alkylation of the 3,5-dimethylorsellinic acid in the meroterpenol austinol pathway [57, 59]. Only a single tryptophan dimethylallyltransferase terpene (M-TER29) were up-regulated in hyphae (Figure 7), which catalyses the first step of ergot alkaloid biosynthesis that are mycotoxins toxic to animals and are important in pharmaceutical industry [59].

A similar pattern of extensive expression in blastospores compared to hyphae was also seen for hybrid polyketide-nonribosomal peptide synthetases (PKS-NRPS) (Figure 7). The PKS-NRPS, M-HPN2, expressed in blastospores correspond to NGS1 synthetase related to biosynthesis of the NG-391 in *M. robertsii* [60]. NG-391 is a mutagenic mycotoxin first identified in *Fusarium* species. This toxin is highly produced in the exponential growth phase of *M. robertsii* mycelium, but detected in low quantities during the stationary growth phase in liquid culture and in early phases of insect infection. NG-391 is not detected in *M. robertsii* conidia and is suggested to be developmentally regulated [60]. Our finding is to the best of our knowledge the first to relate NGS1 expression in *M. anisopliae* blastospores when grown in liquid broth. Although an NGS1 knock-out mutant of *M. robertsii* did not reduce virulence of *M. robertsii* conidia towards *Spodoptera exigua* [60], the specific expression in blastospores is consistent with fungal growth related expression. Transcriptome patterns also indicated that blastospores may be producing terpenoid substances, because two polyketide synthases (PKSs) conserved within *Metarhizium* (M-PKS24

and M-PKS28) were up-regulated in blastospores (Figure 7), [58]. These two PKSs have similar chemical functions with two polyprenyl transferases (M-TER43 and M-TER33), which we also found to be up-regulated in blastospores (M-TER43: $\log_2$ fold change 5.4, FDR-*pvalue* < 0.001 and M-TER3: $\log_2$ fold change 4.2, FDR-*pvalue* < 0.001). Several different biosynthesis gene families of secondary metabolites are up-regulated in blastospores, which is consistent with blastospores produced during liquid in-vitro growth expressing a distinct repertoire of secondary metabolites. This secondary metabolite transcriptome profile almost certainly plays a role in the high insect-pathogenic potential of blastospores.

Species of *Metarhizium* not only rely on secondary metabolites but also produces several secreted proteins that are important during pathogenesis. Especially, subtilisin-like serine proteases are important during conidial germination and appressorial penetration of the insect cuticle [61]. In the present study hyphae expressed two subtilisin-like serine proteases (Pr1C and Pr1K) and three trypsin-like serine proteases, whereas blastospores only up-regulated a single subtilisin-like serine protease (Pr1C). Since our experiments are exclusively performed during *in-vitro* growth, we interpret the expression of these proteases in hyphae and blastospores to be related with acquisition of proteinaceous nutrients from the substrate [62]. Our study revealed that blastospores produce the collagen-like protein MCL1 when grown in liquid culture medium (Additional file 1). The MCL1 protein provides an antiadhesive protective coat that masks beta-glucan components of the blastospore cell wall and thereby hinder hemocytes from killing the fungal cells during growth in insect hemolymph [25]. Since this protein is also expressed in blastospores grown during *in-vitro* cultivation in liquid media with agitation, it supports that blastospores of *M. anisopliae* produced during *in-vivo* and *in-vitro* growth are comparable and that MCL1 does not require insect hemolymph to induce expression.

Furthermore, the insect-pathogenic fungus *M. anisopliae* produces a group of mycotoxins named destruxins, which are crucial for colonization of the insect body and have insecticidal effects against many pests [62,63]. Although we found no difference in gene expression of the destruxins, both hyphae and blastospore express them in solid and liquid medium, respectively (KID59658, gene: MAN_10464, Additional file 1). These findings suggest that destruxins are partially constitutively expressed or have additional functions to pathogenicity. The production

of destruxins in liquid culture is well known [64], but our data suggests that blastopores could be a potential candidate for exploring production of destruxins for toxicity studies. This might be especially relevant for the chemical and pest management industry, although the amount and the types of destruxins produced by blastospores and the chemical and biological characterization would need to be worked out first.

Considerations for in-vitro production of M. anisopliae blastospores

The main fungal propagule used in biological control programs worldwide is conidia. However, commercial interest in industrial-scale blastospore production has increased during the last years due to numerous advantages of blastospores and the production process compared to industrial scale production of conidia on high-quality solid substrates such as rice. Our transcriptome study contributes to clarify some aspects of blastopore metabolism when grown in liquid culture, such as oxidative stress can be one of the morphogenetic factors inducing the formation and maintenance of these cells in liquid medium. Therefore, it will be essential that blastospores produced in industrial bioreactors are provided with appropriate oxygen levels to maintain the desired metabolism profile. The oxygen level could be manipulated mainly by injection of oxygen or air flow inside of bioreactors or by increasing the rotation speed of liquid cultures. We have confirmed that the transcriptomic profiles corroborate a thinner cell wall of blastospores with less components compared to hyphal cell walls. These traits must be taken into account during blastospore manipulation for industrial processes, for example via formulations and drying process that decrease cell wall damage. Finally, we have shown that blastospores produce secondary metabolites, but very little is currently known and future studies to explore their role as insecticidal compounds could for example start with the group of indole terpenoids. In conclusion, we consider that blastospores can be industrially prospected, not only to make products for use in biological control programs but also for the industrial production of substances for other commercial use.

References

1. David Moore. Development of Form. In: Cambridge, editor. Fungal Morphogenesis. 1998. p. 246–391.

2. Felipe MSS, Andrade R V., Petrofeza SS, Maranhão AQ, Torres FAG, Albuquerque P, et al. Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. Yeast. 2003;20:263–71.

3. Hwang L, Hocking-Murray D, Bahrami AK, Andersson M, Jasper Rine and AS. Identifying phase-specific genes in the Fungal Pathogen *Histoplasma capsulatum* using a genomic shotgun microarray. Mol Biol Cell. 2003;14 June:2314–26.

4. Gow N a. R, Brown AJP, Odds FC. Fungal morphogenesis and host invasion. Curr Opin Microbiol. 2002;5:366–71.

5. Comeau AM, Dufour J, Bouvet GF, Jacobi V, Nigg M, Henrissat B, et al. Functional annotation of the *Ophiostoma novo-ulmi* genome: Insights into the phytopathogenicity of the fungal agent of Dutch elm disease. Genome Biol Evol. 2015;7:410–30.

6. Shahid AA, Rao AQ, Bakhsh A, Husnain T. Entomopathogenic fungi as biological controllers: New insights into their virulence and pathogenicity. Arch Biol Sci. 2012;64:21–42.

7. Boomsma JJ, Jensen AB, Meyling N V, Eilenberg J. Evolutionary interaction networks of insect pathogenic fungi. Annu Rev Entomol. 2014;59 October 2013:467–85. doi:10.1146/annurev-ento-011613-162054.

8. Sasan RK, Bidochka MJ. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. Am J Bot. 2012;99:101–7. doi:10.3732/ajb.1100136.

9. Elena GJ, Beatriz PJ, Alejandro P, E LR. *Metarhizium anisopliae* (Metschnikoff) Sorokin Promotes Growth and Has Endophytic Activity in Tomato Plants. Adv Biol Res. 2011;5:22–7.

10. Behie SW, Zelisko PM, Bidochka MJ. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science. 2012;336:1576–7. doi:10.1126/science.1222289.

11. Mascarin GM, Jackson MA, Kobori NN, Behle RW, Delalibera JI. Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. J Invertebr Pathol. 2015;127:11–20. doi:10.1016/j.jip.2014.12.001.

12. Jackson MA, Mcguire MR, Lacey LA, Wraight SP. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycol Res. 1997;101:35–41.

13. Jaronski ST. Mass Production of Entomopathogenic Fungi: State of the Art. Mass Prod Benef Org Invertebr Entomopathog. 2013; November 2013:357–413.

14. Mascarin GM, Kobori NN, de Jesus Vital RC, Jackson MA, Quintela ED. Production of microsclerotia by Brazilian strains of *Metarhizium* spp. using submerged liquid culture fermentation. World J Microbiol Biotechnol. 2014;30:1583–90.

15. Goble T a., Gardescu S, Jackson M a., Hajek AE. Evaluating *Metarhizium brunneum* F52 microsclerotia in hydromulch formulations using different tackifiers under forest and orchard conditions. BioControl. 2017. doi:10.1007/s10526-017-9835-7.

16. Alkhaibari AM, Carolino AT, Yavasoglu SI, Maffeis T, Mattoso TC, Bull JC, et al. *Metarhizium brunneum* Blastospore Pathogenesis in *Aedes aegypti* Larvae: Attack on Several Fronts Accelerates Mortality. PLoS Pathog. 2016;12.

17. Bernardo CC, Barreto LP, Silva CDSRE, Luz C, Arruda W, Everton KK. Conidia and blastospores of *Metarhizium* spp. and *Beauveria bassiana* s.l.: Their development during theinfection process and virulence against the tick *Rhipicephalus microplus*. Ticks Tick Borne Dis. 2018;9:1334–42. doi:10.1016/j.ttbdis.2018.06.001.

18. Subramanian A, Subramanian A, Tamayo P, Tamayo P, Mootha VK, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102:15545–50. doi:10.1073/pnas.0506580102.

19. Padilla-Guerrero IE, Barelli L, González-Hernández GA, Torres-Guzmán JC, Bidochka MJ. Flexible metabolism in *Metarhizium anisopliae* and *Beauveria bassiana*: Role of the glyoxylate cycle during insect pathogenesis. Microbiology. 2011;157:199–208.

20. Gow NAR. The growing fungus. London: Chapman & Hall; 1995.

21. Elieh-Ali-Komi D, Hamblin MR. Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. Int J Adv Res. 2015;344:1173–8.

22. Bidochka MJ, St Leger RJ, Joshi L, Roberts DW. The rodlet layer from aerial and submerged conidia of the entomopathogenic fungus *Beauveria bassiana* contains hydrophobin. Mycol Res. 1995;99:403–6. doi:10.1016/S0953-7562(09)80637-0.

23. Silva WOB, Santi L, Schrank A, Vainstein MH. *Metarhizium anisopliae* lipolytic activity plays a pivotal role in *Rhipicephalus* (*Boophilus*) *microplus* infection. Fungal Biol. 2010;114. doi:10.1016/j.mycres.2009.08.003.

24. Dubovenko AG, Dunaevsky YE, Belozersky MA, Oppert B, Lord JC, Elpidina EN. Trypsin-like proteins of the fungi as possible markers of pathogenicity. Fungal Biol. 2010;114:151–9. doi:10.1016/j.funbio.2009.11.004

25. Wang C, St Leger RJ. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci U S A. 2006;103:6647–52.

26. Duan Z, Shang Y, Gao Q, Zheng P, Wang C. A phosphoketolase Mpk1 of bacterial origin is adaptively required for full virulence in the insect-pathogenic fungus *Metarhizium anis*opliae. Environ Microbiol. 2009;11:2351–60.

27. Fang W, Fernandes ÉKK, Roberts DW, Bidochka MJ, St. Leger RJ. A laccase exclusively expressed by *Metarhizium anisopliae* during isotropic growth is involved in pigmentation, tolerance to abiotic stresses and virulence. Fungal Genet Biol. 2010;47:602–7. doi:10.1016/j.fgb.2010.03.011.

28. Junges Â, Boldo JT, Souza BK, Guedes RLM, Sbaraini N, Kmetzsch L, et al. Genomic analyses and transcriptional profiles of the glycoside hydrolase family 18 genes of the entomopathogenic fungus *Metarhizium anisopliae*. PLoS One. 2014;9.

29. Mondal S, Baksi S, Koris A, Vatai G. Journey of enzymes in entomopathogenic fungi. Pacific Sci Rev A Nat Sci Eng. 2016;18:85–99. doi:10.1016/j.psra.2016.10.001.

30. Hartl L, Zach S, Seidl-Seiboth V. Fungal chitinases: Diversity, mechanistic properties and biotechnological potential. Appl Microbiol Biotechnol. 2012;93:533–43.

31. Leland JE, Mullins DE, Vaughan LJ, Warren HL. Effects of media composition on submerged culture spores of the entomopathogenic fungus, *Metarhizium anisopliae* var*. acridum* Part 1: Effects of media osmolality on cell wall characteristics, carbohydrate concentrations, drying stability, and pathogenicity. Biocontrol Sci Technol. 2005;15:393–409. doi:10.1080/09583150400016910.

32. Wang C, Hu G, St Leger RJ. Differential gene expression by *Metarhizium anisopliae* growing in root exudate and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fungal Genet Biol. 2005;42:704–18. doi:10.1016/j.fgb.2005.04.006.

33. Morozov AA, Likhoshway Y V. Evolutionary history of the chitin synthases of eukaryotes. Glycobiology. 2016;26:635–9.

34. Fernandes C, Gow NAR, Gonçalves T. The importance of subclasses of chitin synthase enzymes with myosin-like domains for the fitness of fungi. Fungal Biol Rev. 2016;30:1–14.

35. Lorenz MC, Fink GR. The glyoxylate cycle is required for fungal virulence. Nature. 2001;412:83–6.

36. Ahn S, Jung J, Jang IA, Madsen EL, Park W. Role of glyoxylate shunt in oxidative stress response. J Biol Chem. 2016;291:11928–38.

37. Andrade RV, Paes HC, Nicola AM, de Carvalho MJA, Fachin AL, Cardoso RS, et al. Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells. BMC Genomics. 2006;7 ii:1–13.

38. Robledo-Briones M, Ruiz-Herrera J. Regulation of genes involved in cell wall synthesis and structure during *Ustilago maydis* dimorphism. FEMS Yeast Res. 2013;13:74–84.

39. Domer JE, Hamilton JG, Harkin JC. Walls of the Yeastlike and capsulatum Comparative Study of the Cell Walls of the Yeastlike and Mycelial Phases of Histoplasma capsulatum. 1967;94:466–74.

40. Kanetsuna F, Carbonell LM. Cell wall glucans of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. J Bacteriol. 1970;101:675–80.

41. Chattaway FW, Bishop R, Holmes MR, Odds FC, Barlow a J. Enzyme activities associated with carbohydrate synthesis and breakdown in the yeast and mycelial forms of *Candida albicans*. J Gen Microbiol. 1973;75:97–109.

42. Mager WH, de Boer AH, Siderius MH, Voss HP. Cellular responses to oxidative and osmotic stress. Cell Stress Chaperones. 2000;5:73–5. doi:10.1379/1466-1268(2000)005<0073:CRTOAO>2.0.CO;2.

43. Giese H, Azizan A, Kammel A, Liao A, Peter CP, Fonseca J a., et al. Liquid films on shake flask walls explain increasing maximum oxygen transfer capacities with elevating viscosity. Biotechnol Bioeng. 2014;111:295–308.

44. Mascarin GM, Jackson M a., Kobori NN, Behle RW, Dunlap C a., Delalibera JI. Glucose concentration alters dissolved oxygen levels in liquid cultures of *Beauveria bassiana* and affects formation and bioefficacy of blastospores. Appl Microbiol Biotechnol. 2015;99:6653–65.

45. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552:335–44.

46. Song Z, Yin Y, Jiang S, Liu J, Chen H, Wang Z. Comparative transcriptome analysis of microsclerotia development in *Nomuraea rileyi*. BMC Genomics. 2013;14:411. doi:10.1186/1471-2164-14-411.

47. Papapostolou I, Georgiou CD. Superoxide radical is involved in the sclerotial differentiation of filamentous phytopathogenic fungi: identification of a fungal xanthine oxidase. Fungal Biol. 2010;114:387–95. doi:10.1016/j.funbio.2010.01.010.

48. Thurston CF. The structure and function of fungal laccases. Microbiology. 2019;1 May:19–26.

49. Yang et al., 2012 - expression of the laccase

50. Bastos KP, Bailão AM, Borges CL, Faria FP, Felipe MSS, Silva MG, et al. The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process. BMC Microbiol. 2007;7:29. doi:10.1186/1471-2180-7-29.

51. Yang E, Chow WN, Wang G, Woo PCY, Lau SKP, Yuen KY, et al. Signature Gene Expression Reveals Novel Clues to the Molecular Mechanisms of Dimorphic Transition in *Penicillium marneffei*. PLoS Genet. 2014;10.

52. Kschischo M, Ramos J, Sychrová H. Membrane Transport in Yeast, An Introduction. In: Ramos J, Sychrová H, Kschischo M, editors. Yeast Membrane Transport. Cham: Springer International Publishing; 2016. p. 1–10. doi:10.1007/978-3-319-25304-6_1.

53. Casadevall A, Nosanchuk JD, Williamson P, Rodrigues ML. Vesicular transport across the fungal cell wall. Trends Microbiol. 2009;17:158–62.

54. Wang JB, St. Leger RJ, Wang C. Advances in Genomics of Entomopathogenic Fungi. Adv Genet. 2016;94:67–105.

55. Knaus HG, McManus OB, Schmalhofer W a., Garcia-Calvo M, Helms LMH, Sanchez M, et al. Tremorgenic Indole Alkaloids Potently Inhibit Smooth Muscle High-Conductance Calcium-Activated Potassium Channels. Biochemistry. 1994;33:5819–28.

56. Smith MM, Warren V a., Thomas BS, Brochu RM, Ertel E a., Rohrer S, et al. Nodulisporic acid opens insect glutamate-gated chloride channels: Identification of a new high affinity modulator. Biochemistry. 2000;39:5543–54.

57. Lo HC, Entwistle R, Guo CJ, Ahuja M, Szewczyk E, Hung JH, et al. Two separate gene clusters encode the biosynthetic pathway for the meroterpenoids austinol and dehydroaustinol in *Aspergillus nidulans*. J Am Chem Soc. 2012;134:4709–20.

58. Donzelli BGG, Krasnoff SB. Chapter Ten - Molecular Genetics of Secondary Chemistry in Metarhizium Fungi. In: Lovett B, Leger RJ St., editors. Genetics and Molecular Biology of Entomopathogenic Fungi. Academic Press; 2016. p. 365–436. doi:https://doi.org/10.1016/bs.adgen.2016.01.005.

59. Young C a., Schardl CL, Panaccione DG, Florea S, Takach JE, Charlton ND, et al. Genetics, genomics and evolution of ergot alkaloid diversity. Toxins (Basel). 2015;7:1273–302.

60. Donzelli BGG, Krasnoff SB, Churchill ACL, Vandenberg JD, Gibson DM. Identification of a hybrid PKS-NRPS required for the biosynthesis of NG-391 in *Metarhizium robertsii*. Curr Genet. 2010;56. doi:10.1007/s00294-010-0288-0.

61. St. Leger RJ, Joshi L, Bidochka MJ, Rizzo NW, Roberts DW. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl Environ Microbiol. 1996;62:1257–64.

61. Bagga S, Hu G, Screen SE, St Leger RJ. Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene. 2004;324.

62. Sowjanya SK, Padmaja V, Murthy Y. Insecticidal activity of destruxin, a mycotoxin from *Metarhizium anisopliae* (Hypocreales), against *Spodoptera litura* (Lepidoptera: Noctuidae) larval stages. Pest Manag Sci. 2008;64:119–25.

63. Hu QB, An XC, Jin FL, Freed S, Ren SX. Toxicities of destruxins against *Bemisia tabaci* and its natural enemy, *Serangium japonicum*. Toxicon. 2009;53:115–21. doi:10.1016/j.toxicon.2008.10.019.

64. Am., Khiri-Besheli, Bambay B, Cameron S, Deadman ML, Butt TM. Inter- and intra-specific variation in destruxin production by insect pathogenic *Metarhizium* spp., and its significance to pathogenesis. Mycol Res. 2000;104:447–52

65. Iwanicki NSA, Ferreira BDO, Mascarin GM, Júnior ÍD. Modified Adamek's medium renders high yields of *Metarhizium robertsii* blastospores that are desiccation tolerant and infective to cattle-tick larvae. Fungal Biol. 2018;:1–8.

66. Simon Andrews. FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

67. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20.

68. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016;11:1650–67. doi:10.1038/nprot.2016.095.

69. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550. doi:10.1186/s13059-014-0550-8.

70. R: A language and environment for statistical computing. 2017. http://www.r-project.org/.

71. Griffith M, Griffith OL, Mwenifumbo J, Goya R, Morrissy a S, et al. (2010) Alternative expression analysis by RNA sequencing. Nat Methods 7: 843–847. doi:10.1038/nmeth.1503.

72. Asmann YW, Klee EW, Thompson EA, Perez E a, Middha S, et al. (2009) 3' tag digital gene expression profiling of human brain and universal reference RNA using Illumina Genome Analyzer. BMC Genomics 10: 531. doi:10.1186/1471-2164-10-531.

73. Wu AR, Neff NF, Kalisky T, Dalerba P, Treutlein B, et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11: 41–46. doi:10.1038/nmeth.2694.

74 Shi Y, He M (2014) Differential gene expression identified by RNA-Seq and qPCR in two sizes of pearl oyster (*Pinctada fucata*). Gene 538: 313–322. doi:10.1016/j.gene.2014.01.031.

75. Khomtchouk BB, Hennessy JR, Wahlestedt C. Shinyheatmap: Ultra fast low memory heatmap web interface for big data genomics. PLoS One. 2017;12:1–9.

76. Supek F, Bošnjak M, Škunca N, Šmuc T. Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011;6.

77. Sbaraini N, Guedes RLM, Andreis FC, Junges Â, de Morais GL, Vainstein MH, et al. Secondary metabolite gene clusters in the entomopathogen fungus *Metarhizium anisopliae*: genome identification and patterns of expression in a cuticle infection model. BMC Genomics. 2016;17:736. doi:10.1186/s12864-016-3067-6.

78. Bidochka MJ, Pfeifer TA, Khachatourians GG. Development of the entomopathogenic fungus *Beauveria bassiana* in liquid cultures. Mycopathologia. 1987;99:77–83. doi:10.1007/BF00436909.

79.Tartar, A., Shapiro, A.M., Scharf, D.W. et al. Mycopathologia (2005) 160: 303. https://doi.org/10.1007/s11046-005-0134-y

80. Xiao G, Ying S-H, Zheng P, Wang Z-L, Zhang S, Xie X-Q, et al. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci Rep. 2012;2.

81. Ortiz-Urquiza A, Keyhani NO. Chapter Six - Molecular Genetics of *Beauveria bassiana* Infection of Insects. In: Lovett B, Leger RJ St., editors. Genetics and Molecular Biology of Entomopathogenic Fungi. Academic Press; 2016. p. 165–249. doi:https://doi.org/10.1016/bs.adgen.2015.11.003.

82. Wang, Z. L., Zhang, L. B., Ying, S. H., & Feng, M. G. (2013). Catalases play differentiated roles in the adaptation of a fungal entomopathogen to environmental stresses. Environmental Microbiology, 15, 409e418. http://dx.doi.org/10.1111/j.1462-2920.2012.02848.x

83. Nigg M, Laroche J, Landry CR, Bernier L. RNAseq Analysis Highlights Specific Transcriptome Signatures of Yeast and Mycelial Growth Phases in the Dutch Elm Disease Fungus *Ophiostoma novo-ulmi.* Genes|Genomes|Genetics. 2015;5:2487–95. doi:10.1534/g3.115.021022.

84. Jensen EC, Ogg C, Nickerson KW. Lipoxygenase inhibitors shift the yeast/mycelium dimorphism in *Ceratocystis ulmi*. Appl Environ Microbiol. 1992;58:2505–8.

85. Naruzawa ES, Bernier L. Control of yeast-mycelium dimorphism invitro in Dutch elm disease fungi by manipulation of specific external stimuli. Fungal Biol. 2014;118:872–84. doi:10.1016/j.funbio.2014.07.006.

86. Sbaraini N, Andreis FC, Thompson CE, Guedes RLM, Junges Â, Campos T, et al. Genome-wide analysis of secondary metabolite gene clusters in *Ophiostoma_ulmi* and *Ophiostoma novo-ulmi* reveals a fujikurin-like gene cluster with a putative role in infection. Front Microbiol. 2017;8 JUN:1–12.

87. García-Pedrajas MD, Baeza-Montañez L, Gold SE. Regulation of Ustila*go maydis* Dimorphism, Sporulation, and Pathogenic Development by a Transcription Factor with a Highly Conserved APSES Domain . Mol Plant-Microbe Interact. 2010;23:211–22.

88. Sánchez-Martínez C, Pérez-Martín J. Dimorphism in fungal pathogens: *Candida albicans* and ustilago maydis - Similar inputs, different outputs. Curr Opin Microbiol. 2001;4:214–21.

89. Robledo-Briones M, Ruiz-Herrera J. Regulation of genes involved in cell wall synthesis and structure during *Ustilago maydis* dimorphism. FEMS Yeast Res. 2013;13:74–84.

90. Banuett F, Herskowitz I. Morphological Transitions in the Life Cycle of *Ustilago maydis* and Their Genetic Control by the a and b Loci. Experimental Mycology. 1994;18:247–66.

91. Kunst L, Klose J, Kronstad JW. The putative phospholipase Lip2 counteracts oxidative damage and influences the virulence of *Ustilago maydis*. 2016;8:210–21.

92. Bölker M, Basse CW, Schirawski J. Ustilago maydis secondary metabolism-From genomics to biochemistry. Fungal Genet Biol. 2008;45 SUPPL. 1:88–93.

93. Albuquerque P, Casadevall A. Quorum sensing in fungi--a review. Med Mycol. 2012;50:337–45. doi:10.3109/13693786.2011.652201.

94. François LM, Duncan W, Bernhard H. *Candida albicans* pathogenicity mechanisms. Virulence. 2013;4:119–28. doi:10.4161/viru.22913.

95. Ruiz-Herrera J, Victoria Elorza M, Valentín E, Sentandreu R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. FEMS Yeast Res. 2006;6:14–29.

96. Whiteway M, Bachewich C. Morphogenesis in *Candida albicans* . Annu Rev Microbiol. 2007;61:529–53.

97. Dantas ADS, Day A, Ikeh M, Kos I, Achan B, Quinn J. Oxidative stress responses in the human fungal pathogen, *Candida albicans*. Biomolecules. 2015;5:142–65.

98. Dufour N, Rao RP. Secondary metabolites and other small molecules as intercellular pathogenic signals. FEMS Microbiol Lett. 2011;314:10–7.

99. Nunes LR, Oliveira RC De, Batista D, Schmidt V, Marques R, Eliana M, et al. Transcriptome Analysis of *Paracoccidioides brasiliensis* Cells Undergoing Mycelium-to-Yeast Transition. Eukaryot Cell. 2005;4:2115–28.

100. Tomazett PK, Cruz AHDS, Bonfim SMRC, Soares CMDA, Pereira M. The cell wall of *Paracoccidioides brasiliensis*: insights from its transcriptome. Genet Mol Res. 2005;4:309–25. http://www.ncbi.nlm.nih.gov/pubmed/16110448.

101. Albuquerque P, Kyaw CM, Saldanha RR, Brigido MM, Felipe MSS, Silva-Pereira I. Pbhyd1 and Pbhyd2: Two mycelium-specific hydrophobin genes from the dimorphic fungus *Paracoccidioides brasiliensis*. Fungal Genet Biol. 2004;41:510–20.

102. Cooper CR, Vanittanakom N. Insights into the pathogenicity of *Penicillium marneffei*. Future Microbiol. 2008;3:43–55.

103. Boyce KJ. Control of morphogenesis and actin localization by the *Penicillium marneffei* RAC homolog. J Cell Sci. 2003;116:1249–60.

104. Tam EWT, Tsang CC, Lau SKP, Woo PCY. Polyketides, toxins and pigments *in Penicillium marneffei*. Toxins (Basel). 2015;7:4421–36.

105. Dubovskii I, Grizanova E, Chertkova E, et al. Generation of reactive oxygen species and activity of antioxidants in hemolymph of the moth larvae Galleria mellonella (L.) (Lepidoptera: piralidae) at development of the process of encapsulation. J Evol Biochem Physiol. 2010a;46:35–43.

106. Dubovskiy I, Kryukov VY, Benkovskaya G, et al. Activity of the detoxificative enzyme system and encapsulation rate in the Colorado potato beetle Leptinotarsa decemlineata (Say) larvae under organophosphorus insecticide treatment and entomopathogenic fungus Metharizium anisopliae (Metsch.) infection. Euroasian Entomol J. 2010b;9:577–582

Appendix

Appendix A - Dataset with genes exclusively expressed in hyphae (H) or blastospores (B)

Gene name	Protein name	Log_2FC	Hyphae (H) / Blast (B)
MAN_00440	2OG-Fe(II) oxygenase superfamily protein, partial	-10.23896189	H
MAN_07863	Allergen V5/Tpx-1-related protein, partial	-8.483560921	H
MAN_06055	amino acid permease 2, partial	-9.711196418	H
MAN_08864	Ankyrin repeat-containing domain protein, partial	-5.438834958	H
MAN_04624	Ankyrin repeat-containing domain protein, partial	-9.279653523	H
MAN_09472	ATP synthase beta chain precursor, partial	-9.61816507	H
MAN_00907	benzoate 4-monooxygenase cytochrome P450, partial	-8.514325844	H
MAN_09465	Beta-ketoacyl synthase, partial	-7.120428191	H
MAN_06105	chitinase, partial	-8.271737202	H
MAN_07621	choline binding protein D, partial	-7.026871734	H
MAN_02928	choline dehydrogenase, partial	-6.488080982	H
MAN_06851	conidiation-specific protein 10, partial	-6.624798348	H
MAN_00217	conidiospore surface protein, partial	-6.393183963	H
MAN_07738	cytochrome P450 3A31, partial	-9.002111737	H
MAN_03562	cytochrome P450 4F8, partial	-6.125706786	H
MAN_06358	extracellular dihydrogeodin oxidase/laccase, partial	-6.469496209	H
MAN_07745	fatty acid synthase subunit beta dehydratase, partial	-7.321927613	H
MAN_09721	Floculation protein FLO1, partial	-9.415325298	H
MAN_09183	Glucose-methanol-choline oxidoreductase, partial	-6.430260273	H
MAN_05753	glutathione S-transferase, partial	-6.535805115	H
MAN_07916	glycosyl transferase, partial	-8.542661933	H
MAN_06097	G-protein coupled receptor, partial	-8.741596239	H
MAN_06852	HHE domain containing protein, partial	-6.127807451	H
MAN_00154	hypothetical protein MAN_00154, partial	-9.012124545	H
MAN_00414	hypothetical protein MAN_00414, partial	-7.084356247	H
MAN_00833	hypothetical protein MAN_00833, partial	-9.371685343	H
MAN_00896	hypothetical protein MAN_00896, partial	-6.192097533	H
MAN_01933	hypothetical protein MAN_01933, partial	-10.5354731	H
MAN_01962	hypothetical protein MAN_01962, partial	-6.582064263	H
MAN_04613	hypothetical protein MAN_04613, partial	-7.129893971	H
MAN_05237	hypothetical protein MAN_05237, partial	-10.06688943	H
MAN_05239	hypothetical protein MAN_05239, partial	-6.862576433	H
MAN_05270	hypothetical protein MAN_05270, partial	-8.545484395	H
MAN_05515	hypothetical protein MAN_05515, partial	-8.064236342	H
MAN_05759	hypothetical protein MAN_05759, partial	-10.23426126	H
MAN_06025	hypothetical protein MAN_06025, partial	-6.919434223	H
MAN_06033	hypothetical protein MAN_06033, partial	-5.296741835	H
MAN_06770	hypothetical protein MAN_06770, partial	-6.029954163	H
MAN_07015	hypothetical protein MAN_07015, partial	-6.394414279	H

MAN_07148	hypothetical protein MAN_07148, partial	-8.774710601	H
MAN_07166	hypothetical protein MAN_07166, partial	-8.599140275	H
MAN_07256	hypothetical protein MAN_07256, partial	-6.984852251	H
MAN_07598	hypothetical protein MAN_07598, partial	-7.095074269	H
MAN_07853	hypothetical protein MAN_07853, partial	-8.534752711	H
MAN_07875	hypothetical protein MAN_07875, partial	-8.794629223	H
MAN_09130	hypothetical protein MAN_09130, partial	-5.39239182	H
MAN_09675	hypothetical protein MAN_09675, partial	-6.558270842	H
MAN_09438	Inositol monophosphatase, partial	-6.698263795	H
MAN_09226	Intradiol ring-cleavage dioxygenase, core, partial	-10.54430377	H
MAN_07732	lipase 3 precursor, partial	-6.83516526	H
MAN_10474	Major facilitator superfamily domain, general substrate transporter, partial	-6.367931234	H
MAN_10832	Major facilitator superfamily domain, general substrate transporter, partial	-7.54803636	H
MAN_00283	Major facilitator superfamily domain, general substrate transporter, partial	-7.601465684	H
MAN_02412	Major facilitator superfamily domain, general substrate transporter, partial	-8.551437443	H
MAN_05698	methyltransferase domain-containing protein, partial	-9.955679814	H
MAN_05750	methyltransferase, partial	-9.805610984	H
MAN_09673	mucoidy inhibitor-like protein, partial	-5.85773511	H
MAN_08919	NACHT and Ankyrin domain protein, partial	-5.401031444	H
MAN_07141	nacht nucleoside triphosphatase, partial	-8.532911928	H
MAN_09281	NAD(P)(+)-binding proteins & short chain dehydrogenase, partial	-7.21265585	H
MAN_01603	nitroreductase family protein, partial	-8.66942188	H
MAN_05757	non ribosomal peptide synthase, partial	-5.786763391	H
MAN_08857	non-ribosomal peptide synthetase, partial	-6.770801227	H
MAN_00207	Nucleoside phosphorylase domain protein, partial	-7.523817073	H
MAN_07915	O-methyl transferase B, partial	-5.828706869	H
MAN_00098	Peptidase cysteine/serine, trypsin-like protein, partial	-7.227016188	H
MAN_05751	Peptidase M19, renal dipeptidase, partial	-11.44952749	H
MAN_01922	Peptidase S28, partial	-5.66618727	H
MAN_09693	Peptidase S28, partial	-6.63931937	H
MAN_06654	Peptidase S8, partial	-7.496524572	H
MAN_05329	phosphotransferase family protein, partial	-7.434782253	H
MAN_06329	Pregnancy-associated plasma protein-A, partial	-6.591101851	H
MAN_09223	proline rich protein 5MeD, partial	-7.891025767	H
MAN_07734	protein kinase-like protein, partial	-6.84549034	H
MAN_03765	Pyruvate/Phosphoenolpyruvate kinase, partial	-8.528754313	H
MAN_06835	subtilisin-like protease PR1K, partial	-8.564077051	H
MAN_03821	succinate-semialdehyde dehydrogenase NADP+, partial	-6.055014695	H
MAN_04947	Taurine catabolism dioxygenase TauD/TfdA, partial	-8.834369405	H
MAN_01935	transferase, partial	-6.269210905	H
MAN_00882	Transmembrane protein SKG6, partial	-8.106734684	H

MAN_01639	tripeptidyl peptidase A, partial	-6.632236318	H
MAN_01640	tripeptidyl peptidase A, partial	-8.181653343	H
MAN_02755	Trypsin- protease, partial	-5.245896986	H
MAN_10272	Trypsin- protease, partial	-6.662120738	H
MAN_07684	trypsin-like protease, partial	-7.859012722	H
MAN_06560	Tryptophan halogenase, partial	-5.745891951	H
MAN_00908	Tryptophan halogenase, partial	-6.558451417	H
MAN_07807	WD40/YVTN repeat-like-containing domain protein, partial	-8.805729726	H
MAN_06149	Alcohol dehydrogenase superfamily, zinc-type, partial	10.71210952	B
MAN_00742	allantoate permease, partial	6.559287647	B
MAN_02739	alpha/beta hydrolase, partial	5.662039916	B
MAN_01650	alpha/beta hydrolase, partial	9.70955388	B
MAN_10283	alpha-galactosidase, partial	6.914681068	B
MAN_10031	ankyrin repeat domain protein 17 isoform a, partial	6.916661314	B
MAN_00054	CAP domain protein, partial	5.988507113	B
MAN_09808	chitooligosaccharide oxidase, partial	12.92904631	B
MAN_06331	cyclohexanone monooxygenase, partial	10.16341354	B
MAN_02742	cytochrome P450, partial	7.204864027	B
MAN_01655	Cytochrome P450, partial	11.01357438	B
MAN_01649	eEF-1B gamma subunit-like protein, partial	6.907745789	B
MAN_05762	Esterase/lipase, partial	6.336246164	B
MAN_00291	hypothetical protein MAN_00291, partial	5.497659616	B
MAN_01768	hypothetical protein MAN_01768, partial	7.05140339	B
MAN_02936	hypothetical protein MAN_02936, partial	5.331719171	B
MAN_04667	hypothetical protein MAN_04667, partial	7.71637468	B
MAN_05742	hypothetical protein MAN_05742, partial	8.631514895	B
MAN_06161	hypothetical protein MAN_06161, partial	5.876893711	B
MAN_06617	hypothetical protein MAN_06617, partial	5.216850255	B
MAN_09073	hypothetical protein MAN_09073, partial	5.735378542	B
MAN_09148	hypothetical protein MAN_09148, partial	5.08161113	B
MAN_09408	hypothetical protein MAN_09408, partial	5.286932724	B
MAN_10529	hypothetical protein MAN_10529, partial	6.529189679	B
MAN_10477	laccase, partial	13.91529035	B
MAN_09757	Major facilitator superfamily domain, general substrate transporter, partial	10.92297318	B
MAN_06201	Monooxygenase, FAD-binding protein, partial	6.577083941	B
MAN_01644	NAD(P)-binding domain protein, partial	9.560938193	B
MAN_04170	NmrA family protein, partial	5.591733849	B
MAN_00019	Outer membrane protein, beta-barrel, partial	9.793626095	B
MAN_06146	oxidoreductase, FAD-binding protein, partial	13.00423262	B
MAN_04672	oxidoreductase, short chain dehydrogenase/reductase family, partial	8.714065566	B
MAN_04558	oxidosqualene:lanosterol cyclase, partial	5.069513648	B
MAN_04597	P450 monooxygenase, partial	8.808230868	B
MAN_04588	P450 monooxygenase, partial	10.91611752	B

MAN_04589	P450 monooxygenase, partial	11.37591299	B
MAN_04590	P450 monooxygenase, partial	12.59441644	B
MAN_06813	Peptidase S8/S53, subtilisin/kexin/sedolisin, partial	7.162619245	B
MAN_01619	polyketide synthase, partial	5.383938136	B
MAN_09372	polyketide synthetase PksP, partial	6.897514582	B
MAN_04171	porphyromonas-type peptidyl-arginine deiminase superfamily, partial	10.60162483	B
MAN_06029	protein phosphatase regulatory subunit Gac1, partial	11.26501407	B
MAN_02681	Ribonuclease III, partial	9.137722278	B
MAN_10264	small secreted protein, partial	11.78879066	B
MAN_01654	succinate semialdehyde dehydrogenase, partial	7.744298357	B
MAN_06164	#N/D	4.36265204	B
MAN_08129	#N/D	4.535490026	B
MAN_01861	#N/D	4.691922159	B

Appendix B - Enriched Gene ontology (GO), Protein families (PFAM) and KEGG (ko pathway) terms in hyphae (Spreadsheet 1) and blastospores (Spreadsheet 2) resulted from Gene set enrichment analysis.

	GO – Gene ontology - Hyphae
	Biological Process
GO:0007018	microtubule-based movement
GO:0006260	DNA replication
GO:0005975	carbohydrate metabolic process
GO:0051301	cell division
GO:0006310	DNA recombination
GO:0006281	DNA repair
GO:0016192	vesicle-mediated transport
GO:0043386	mycotoxin biosynthetic process
GO:0006511	ubiquitin-dependent protein catabolic process
GO:0006629	lipid metabolic process
GO:0000770	peptide pheromone export
GO:0006412	translation
GO:0000723	telomere maintenance
	Celular component
GO:0005874	microtubule
GO:0005622	intracellular
GO:0005789	endoplasmic reticulum membrane
GO:0005576	extracellular region
GO:0005840	ribosome
	Molecular function

GO:0003777	microtubule motor activity
GO:0030246	carbohydrate binding
GO:0004672	protein kinase activity
GO:0008017	microtubule binding
GO:0003678	DNA helicase activity
GO:0003887	DNA-directed DNA polymerase activity
GO:0003684	damaged DNA binding
GO:0003735	structural constituent of ribosome
GO:0004674	protein serine/threonine kinase activity
GO:0022891	substrate-specific transmembrane transporter activity
GO:0004725	protein tyrosine phosphatase activity
GO:0046982	protein heterodimerization activity
GO:0004298	threonine-type endopeptidase activity
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0004190	aspartic-type endopeptidase activity
	PFAM – Protein family - Hyphae
PF00225	Kinesin motor domain
PF01636	Phosphotransferase enzyme family
PF00069	Protein kinase domain
PF14214	Helitron helicase-like domain at N-terminus
PF01822	WSC domain
PF01764	Lipase (class 3)
PF05970	PIF1-like helicase
PF06985	Heterokaryon incompatibility protein (HET)
PF08022	FAD-binding domain
PF00083	Sugar (and other) transporter
PF01794	Ferric reductase like transmembrane component
PF00026	Eukaryotic aspartyl protease
PF11807	Domain of unknown function (DUF3328)
PF08030	Ferric reductase NAD binding domain
PF12796	Ankyrin repeats (3 copies)
PF00646	F-box domain
PF00176	SNF2 family N-terminal domain
PF01048	Phosphorylase superfamily
	ko Pathway - Hyphae
KO03030	DNA replication
KO03430	Mismatch repair
KO03410	Base excision repair
KO03440	Homologous recombination
KO03420	Nucleotide excision repair
KO04111	Cell cycle yeast
KO00240	Pyrimidine metabolism
KO04145	Phagosome
KO03050	Proteasome

KO04144	Endocytosis
KO00513	Various types of Nglycan biosynthesis
KO00500	Starch and sucrose metabolism
KO04141	Protein processing in endoplasmic reticulum
KO04113	Meiosis yeast

	GO – Gene ontology - Blastospores
	Biological Process
GO:0006979	response to oxidative stress
GO:0008152	metabolic process
GO:0008299	isoprenoid biosynthetic process
GO:0042254	ribosome biogenesis
GO:0006364	rRNA processing
GO:0055085	transmembrane transport
GO:0006099	tricarboxylic acid cycle
GO:0006096	glycolytic process
GO:0015986	ATP synthesis coupled proton transport
GO:0006351	transcription, DNA-templated
GO:0006338	chromatin remodeling
GO:0006457	protein folding
GO:0006396	RNA processing
GO:0019752	carboxylic acid metabolic process
GO:0006520	cellular amino acid metabolic process
	Celular component
GO:0005730	nucleolus
GO:0005737	cytoplasm
GO:0005739	mitochondrion
GO:0005743	mitochondrial inner membrane
GO:0005634	nucleus
GO:0005852	eukaryotic translation initiation factor 3 complex
GO:0019013	viral nucleocapsid
GO:0030529	intracellular ribonucleoprotein complex
	Molecular function
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding
GO:0009055	electron carrier activity
GO:0020037	heme binding
GO:0051213	dioxygenase activity
GO:0003899	DNA-directed 5'-3' RNA polymerase activity
GO:0016491	oxidoreductase activity
GO:0048038	quinone binding
GO:0051537	2 iron, 2 sulfur cluster binding
GO:0051539	4 iron, 4 sulfur cluster binding

GO:0000287	magnesium ion binding
GO:0048037	cofactor binding
GO:0003746	translation elongation factor activity
GO:0003743	translation initiation factor activity
GO:0008080	N-acetyltransferase activity
GO:0003723	RNA binding
GO:0008168	methyltransferase activity
GO:0005506	iron ion binding
GO:0050661	NADP binding
GO:0046872	metal ion binding
GO:0010181	FMN binding
GO:0030170	pyridoxal phosphate binding
GO:0071949	FAD binding
GO:0051287	NAD binding
GO:0050660	flavin adenine dinucleotide binding
GO:0008270	zinc ion binding
GO:0003700	transcription factor activity, sequence-specific DNA binding
GO:0003824	catalytic activity
GO:0005215	transporter activity
GO:0005507	copper ion binding
GO:0015171	amino acid transmembrane transporter activity
GO:0016831	carboxy-lyase activity
GO:0016874	ligase activity
GO:0008483	transaminase activity
GO:0004497	monooxygenase activity
GO:0004386	helicase activity
GO:0016829	lyase activity
GO:0046983	protein dimerization activity
GO:0050662	coenzyme binding
GO:0003676	nucleic acid binding
GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups
GO:0003677	DNA binding
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
GO:0016887	ATPase activity

PFAM – Protein family - Blastospores

PF04082	Fungal specific transcription factor domain
PF00153	Mitochondrial carrier protein
PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
PF00107	Zinc-binding dehydrogenase

PF00291	Pyridoxal-phosphate dependent enzyme
PF00175	Oxidoreductase NAD-binding domain
PF08240	Alcohol dehydrogenase GroES-like domain
PF03144	Elongation factor Tu domain 2
PF07992	Pyridine nucleotide-disulphide oxidoreductase
PF00173	Cytochrome b5-like Heme/Steroid binding domain
PF00076	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)
PF00441	Acyl-CoA dehydrogenase, C-terminal domain
PF02770	Acyl-CoA dehydrogenase, middle domain
PF00118	TCP-1/cpn60 chaperonin family
PF01494	FAD binding domain
PF00248	Aldo/keto reductase family
PF00067	Cytochrome P450
PF00106	short chain dehydrogenase
PF00270	DEAD/DEAH box helicase
PF12697	Alpha/beta hydrolase family
PF02771	Acyl-CoA dehydrogenase, N-terminal domain
PF00009	Elongation factor Tu GTP binding domain
PF01565	FAD binding domain
PF00010	Helix-loop-helix DNA-binding domain
PF01370	NAD dependent epimerase/dehydratase family
PF05368	NmrA-like family
PF00005	ABC transporter
PF00082	Subtilase family

ko Pathway - Blastospores

KO01110	Biosynthesis of secondary metabolites
KO01100	Metabolic pathways
KO01130	Biosynthesis of antibiotics
KO01200	Carbon metabolism
KO00020	Citrate cycle (TCA cycle)
KO03008	Ribosome biogenesis in eukaryotes
KO00190	Oxidative phosphorylation
KO00071	Fatty acid degradation
KO00630	Glyoxylate and dicarboxylate metabolism
KO00620	Pyruvate metabolism
KO00380	Tryptophan metabolism
KO00010	Glycolysis / Gluconeogenesis
KO01230	Biosynthesis of amino acids
KO00350	Tyrosine metabolism
KO00410	betaAlanine metabolism
KO00310	Lysine degradation
KO00280	Valine, leucine and isoleucine degradation
KO01212	Fatty acid metabolism

KO00650	Butanoate metabolism
KO01210	2Oxocarboxylic acid metabolism
KO00270	Cysteine and methionine metabolism
KO00360	Phenylalanine metabolism
KO04146	Peroxisome
KO00220	Arginine biosynthesis
KO00340	Histidine metabolism
KO00260	Glycine, serine and threonine metabolism

Appendix C - Genes grouped by Glycoside hydrolase (GH) family and protein family

PFAM: PF00704 : Glyco_hydro_18 : Glycoside hydrolase					
Gene	**log2FoldChange**	**padj**	**Protein product**	**Protein name**	**Cross-reference (Pfam)**
MAN_06105	-8.271737202	3.87389E-14	KID65094.1	chitinase, partial	PF00704;
MAN_10487	-6.738260186	4.02648E-13	KID59681.1	endo-N-acetyl-beta-D-glucosaminidase precursor, partial	PF00704;
MAN_02125	-4.771245712	1.9685E-233	KID69611.1	chitinase 18-11, partial	PF00704;
MAN_07138	-2.761129963	9.15225E-16	KID63967.1	class III chitinase ChiA2, partial	PF00704;
MAN_09429	-2.169155255	3.9007E-05	KID61145.1	chitinase 18-15, partial	PF00704;
MAN_01345	-1.530795049	2.66109E-07	KID71746.1	chitinase, partial	PF00704;
MAN_05741	-1.42306436	9.69289E-12	KID66082.1	chitinase, partial	PF00734;PF00704;
MAN_09099	-1.335166032	3.25611E-27	KID61334.1	alkaline phosphatase, partial	PF00704;
MAN_07104	-1.201022178	7.54426E-34	KID63933.1	chitinase 18-4, partial	PF00704;
MAN_05824	-1.037198624	4.88388E-08	KID64813.1	chitinase 18-3, partial	PF00704;
MAN_02751	-0.858684964	0.100593754	KID70237.1	Glycoside hydrolase, subgroup, catalytic core, partial	PF00187;PF00704;PF01476;
MAN_05179	0.050597479	0.861431275	KID65520.1	chitinase CHIT30, partial	PF00704;
MAN_05584	0.645428256	0.000125405	KID65925.1	chitinase, partial	PF00704;
MAN_09585	0.782507393	2.22624E-24	KID60857.1	chitinase 18-7, partial	PF00704;
MAN_01370	0.896930379	1.35391E-17	KID71771.1	chitinase, partial	PF00704;
MAN_10565	1.273719698	0.00609564	KID59624.1	class III chitinase ChiA2, partial	PF00704;
MAN_00174	1.440573312	9.88665E-14	KID70575.1	chitinase, partial	PF00704;
MAN_00045	2.020696156	1.70119E-16	KID70446.1	Cellulose-binding domain, fungal, partial	PF00734;PF00704;
MAN_10732	3.213523454	2.56362E-07	KID59427.1	Glycoside hydrolase, chitinase active site protein, partial	PF00704;
MAN_10405	5.155878242	0	KID59764.1	chitinase 18-18, partial	PF00704;
MAN_10050	6.057838676	NA	KID60233.1	chitinase, partial	PF00187;PF00704;
MAN_02753	NA	NA	KID70239.1	chitinase, partial	PF00704;
MAN_07219	NA	NA	KID64048.1	glycoside hydrolase family 18 protein,	PF00704;PF01476;

				partial	
MAN_10554	NA	NA	KID59613.1	chitinase 18-11, partial	PF00704;
MAN_10724	NA	NA	KID59419.1	Glycoside hydrolase, subgroup, catalytic core, partial	PF00704;PF01476;

PFAM: PF03659 : Glyco_hydro_19 : Glycoside hydrolase

Gene	log2FoldChange	padj	Protein product	Protein name	Cross-reference (Pfam)
MAN_07765	-0.808633896	8.76405E-07	KID62549.1	alpha-1,3-glucanase, partial	PF03659;

PFAM: PF07335 : Glyco_hydro_75 : Glycoside hydrolase

Gene	log2FoldChange	padj	Protein product	Protein name	Cross-reference (Pfam)
MAN_01994	-5.491978592	0.001352262	KID69480.1	chitosanase, partial	PF07335;
MAN_00041	-1.730664416	1.9193E-53	KID70442.1	Fungal chitosanase, partial	PF07335;
MAN_01279	3.207888802	1.6341E-180	KID71680.1	chitosanase precursor, partial	PF07335;

PFAM: PF16499 : Glycoside hydrolase family 27 / PF00652: Ricin_B_lectin

Gene	log2FoldChange	padj	Protein product	Protein name	Cross-reference (Pfam)
MAN_06828	-2.823373516	4.20958E-29	KID64654.1	alpha-N-acetylgalactosaminidase, partial	PF16499;PF00652;
MAN_07264	1.069968657	0.000453136	KID63063.1	alpha-galactosidase, partial	PF16499;

PFAM: PF00933: Glycoside hydrolase family Glyco_hydro_3

Gene	log2FoldChange	padj	Protein product	Protein name	Cross-reference (Pfam)
MAN_03066	-2.934550906	6.6404E-128	KID68210.1	Glycoside hydrolase, family 3, partial	PF14310;PF00933;PF01915;
MAN_07632	1.990817556	6.50599E-87	KID63431.1	Glycoside hydrolase, family 3, partial	PF00933;
MAN_07819	-1.342610667	9.51037E-74	KID62603.1	Glycoside hydrolase, family 3, partial	PF14310;PF00933;PF01915;PF07691;
MAN_03578	-0.968472091	1.32953E-11	KID68722.1	Glycoside hydrolase, family 3, partial	PF14310;PF00933;PF01915;
MAN_04359	-0.338644271	1.91116E-07	KID67601.1	beta-N-acetylglucosaminidase, partial	PF00933;
MAN_09007	-0.534886944	8.32268E-05	KID61242.1	Glycoside hydrolase, family 3, partial	PF14310;PF00933;PF01915;

MAN_07023	0.186126524	0.006549758	KID63852.1	Glycoside hydrolase, family 3, partial	PF14310;PF00933;PF01915;
MAN_08274	0.804709125	0.015383855	KID62270.1	Glycoside hydrolase, family 3, partial	PF00933;

PFAM: PF01644: Chitin synthase: Chitin_synth_1 (PF01644); Chitin_synth_1N (PF08407)					
Gene	log2FoldChange	padj	Protein product	Protein name	Cross-reference (Pfam)
MAN_01349	-0.754536178	3.83459E-20	KID71750.1	class 2 chitin synthase, partial	PF01644;PF08407;
MAN_00898	0.11378953	0.284203551	KID71299.1	chitin synthase class I, partial	PF01644;PF08407;
MAN_01089	-0.049964846	0.516227115	KID71490.1	chitin synthase 1, partial	PF01644;PF08407;

PFAM: PF00063 Myosin_head					
Gene	**log2FoldChange**	**padj**	**Protein product**	**Protein name**	**Cross-reference (Pfam)**
MAN_10807	-1.139148484	1.43516E-46	KID59315.1	myosin type II heavy chain, partial	PF00063;PF02736;PF01576;
MAN_08582	-0.867287713	4.03132E-27	KID61903.1	class VII chitin synthase, partial	PF00173;PF08766;PF00063;
MAN_08581	-0.717873409	1.35275E-19	KID61902.1	class V chitin synthase, partial	PF00173;PF08766;PF00063;
MAN_07078	-0.382726137	2.67948E-07	KID63907.1	Myosin head, motor domain protein, partial	PF01843;PF00612;PF00063;
MAN_05871	0.282780847	0.00774386	KID64860.1	myosin-5, partial	PF00063;PF06017;PF00018;

PFAM: PF00722 : Glyco_hydro_16					
Gene	**log2FoldChange**	**padj**	**Protein product**	**Protein name**	**Cross-reference (Pfam)**
MAN_01263	5.043468706	0	KID71664.1	beta-1,3-endoglucanase, partial	PF00722;
MAN_08250	0.562899496	2.51686E-10	KID62246.1	cell wall glucanase (Utr2), partial	PF00722;
MAN_04205	-0.349119742	0.000501131	KID67447.1	cell wall glucanosyltransferase Mwg1, partial	PF00722;
MAN_09520	-0.005828222	0.986926316	KID60792.1	cell wall glucanosyltransferase Mwg1, partial	PF00722;
MAN_03626	-4.935257567	0	KID68770.1	cell wall glucanosyltransferase Mwg2, partial	PF00722;
MAN_03416	-6.166017571	4.68331E-65	KID68560.1	Concanavalin A-like lectin/glucanase,	PF00722;

				partial	
MAN_02846	-1.133064362	3.74102E-36	KID67990.1	Concanavalin A-like lectin/glucanase, partial	PF00722;
MAN_02282	-0.866606985	0.005399682	KID69768.1	Concanavalin A-like lectin/glucanase, partial	PF00722;
MAN_00671	7.144828171	0	KID71072.1	Concanavalin A-like lectin/glucanase, partial	PF00722;PF01822;
MAN_10545	NA	NA	KID59604.1	Concanavalin A-like lectin/glucanase, partial	PF00722;
MAN_03658	0.403279936	0.105485412	KID68802.1	endo-1,3(4)-beta-glucanase, partial	PF00722;
MAN_03452	-0.670060425	1.04589E-13	KID68596.1	extracellular cell wall glucanase Crf1, partial	PF00722;
MAN_10435	-1.945535933	2.20186E-46	KID59794.1	glucan 1,3-beta-glucosidase, partial	PF00722;
MAN_00636	-3.670586634	9.37214E-96	KID71037.1	glucan endo-1,3-beta-glucosidase A1-like protein, partial	PF00722;
MAN_04534	0.605749546	0.000471847	KID66253.1	glycoside hydrolase family 16, partial	PF00722;
MAN_05496	1.782530366	1.44685E-63	KID65837.1	glycoside hydrolase family 16, partial	PF00722;
MAN_05722	-1.716722899	8.68657E-87	KID66063.1	glycosyl hydrolase family 16, partial	PF00722;
MAN_10232	-0.60965574	2.83245E-07	KID59982.1	gram-negative bacteria-binding protein 1 precursor, partial	PF00722;

Appendix D - Dataset with top 50 genes up-regulated in hyphae and blastospores

Top 50 genes up regulated in Hyphae				
Gene name	**log2FoldChange**	**padj**	**Protein name**	**Protein product**
MAN_05751	-11.4495	2.01279E-27	Peptidase M19, renal dipeptidase, partial	KID66092
MAN_09226	-10.5443	8.33797E-24	Intradiol ring-cleavage dioxygenase, core, partial	KID61461
MAN_01933	-10.5355	4.78128E-23	hypothetical protein MAN_01933, partial	KID69419
MAN_00440	-10.239	2.60227E-22	2OG-Fe(II) oxygenase superfamily protein, partial	KID70841
MAN_05759	-10.2343	2.31964E-22	hypothetical protein MAN_05759, partial	KID66100
MAN_05237	-10.0669	7.88834E-22	hypothetical protein MAN_05237, partial	KID65578
MAN_05698	-9.95568	3.5016E-21	methyltransferase domain-containing protein, partial	KID66039
MAN_05750	-9.80561	1.50928E-20	methyltransferase, partial	KID66091
MAN_06055	-9.7112	1.06633E-19	amino acid permease 2, partial	KID65044
MAN_09472	-9.61817	1.5647E-19	ATP synthase beta chain precursor, partial	KID61188
MAN_09721	-9.41533	1.59867E-18	Floculation protein FLO1, partial	KID60586
MAN_00833	-9.37169	9.53002E-19	hypothetical protein MAN_00833, partial	KID71234
MAN_04624	-9.27965	4.28953E-18	Ankyrin repeat-containing domain protein, partial	KID66343
MAN_07196	-9.25457	7.3161E-27	glycosyltransferase family 31 protein, partial	KID64025
MAN_00154	-9.01212	3.6615E-17	hypothetical protein MAN_00154, partial	KID70555
MAN_07738	-9.00211	3.75429E-17	cytochrome P450 3A31, partial	KID63537
MAN_06364	-8.99001	8.40573E-26	HC-toxin synthetase, partial	KID65353
MAN_08123	-8.85816	2.95146E-18	hypothetical protein MAN_08123, partial	KID62907
MAN_04947	-8.83437	5.35093E-16	Taurine catabolism dioxygenase TauD/TfdA, partial	KID66666
MAN_09213	-8.81057	1.22968E-09	nonribosomal peptide synthase, partial	KID61448
MAN_07807	-8.80573	2.86298E-16	WD40/YVTN repeat-like-containing domain	KID62591

			protein, partial	
MAN_07875	-8.79463	2.52771E-09	hypothetical protein MAN_07875, partial	KID62659
MAN_07148	-8.77471	8.64923E-16	hypothetical protein MAN_07148, partial	KID63977
MAN_06097	-8.7416	3.94716E-16	G-protein coupled receptor, partial	KID65086
MAN_06374	-8.74061	NA	Phenylacetic acid degradation-related protein, partial	KID65363
MAN_10206	-8.72256	2.5173E-17	hypothetical protein MAN_10206, partial	KID59956
MAN_01603	-8.66942	6.79491E-16	nitroreductase family protein, partial	KID69089
MAN_03024	-8.64289	NA	leucoanthocyanidin dioxygenase, partial	KID68168
MAN_07166	-8.59914	1.15076E-14	hypothetical protein MAN_07166, partial	KID63995
MAN_00091	-8.59066	3.92381E-11	nacht nucleoside triphosphatase, partial	KID70492
MAN_06835	-8.56408	2.8111E-15	subtilisin-like protease PR1K, partial	KID64661
MAN_02412	-8.55144	3.82053E-15	Major facilitator superfamily domain, general substrate transporter, partial	KID69898
MAN_05270	-8.54548	4.63553E-15	hypothetical protein MAN_05270, partial	KID65611
MAN_07916	-8.54266	4.2328E-15	glycosyl transferase, partial	KID62700
MAN_06024	-8.53974	NA	Amino acid adenylation, partial	KID65013
MAN_07853	-8.53475	2.36251E-15	hypothetical protein MAN_07853, partial	KID62637
MAN_07141	-8.53291	1.26343E-14	nacht nucleoside triphosphatase, partial	KID63970
MAN_03765	-8.52875	6.01293E-15	Pyruvate/Phosphoenolpyruvate kinase, partial	KID67007
MAN_00907	-8.51433	6.0003E-15	benzoate 4-monooxygenase cytochrome P450, partial	KID71308
MAN_07863	-8.48356	9.43669E-15	Allergen V5/Tpx-1-related protein, partial	KID62647
MAN_06372	-8.44847	NA	macrophomate synthase, partial	KID65361
MAN_05610	-8.446	NA	aspartic peptidase A1, partial	KID65951
MAN_01997	-8.38658	NA	phosphatidylglycerol / phosphatidylinositol transfer protein, partial	KID69483
MAN_09096	-8.38101	0.000358119	hypothetical protein MAN_09096, partial	KID61331
MAN_01835	-8.34972	3.06168E-13	Mannose-binding lectin, partial	KID69321
MAN_00121	-8.34571	2.90382E-07	hypothetical protein MAN_00121, partial	KID70522

MAN_10205	-8.31737	1.08246E-05	Major facilitator superfamily domain, general substrate transporter, partial	KID59955
MAN_09214	-8.27742	NA	Non-robosomal peptide synthetase, partial	KID61449
MAN_09186	-8.27643	NA	Major facilitator superfamily domain, general substrate transporter, partial	KID61421
MAN_06105	-8.27174	3.87389E-14	chitinase, partial	KID65094

Top 50 genes up regulated in Blastospores

Gene name	**log2FoldChange**	**padj**	**Protein name**	**Protein code**
MAN_10477	13.91529035	2.57739E-41	laccase, partial	KID59671
MAN_06146	13.00423262	3.02565E-36	oxidoreductase, FAD-binding protein, partial	KID65135
MAN_09808	12.92904631	7.65189E-36	chitooligosaccharide oxidase, partial	KID60673
MAN_04590	12.59441644	4.67392E-34	P450 monooxygenase, partial	KID66309
MAN_09382	12.02732112	6.87804E-17	hypothetical protein MAN_09382, partial	KID61098
MAN_10264	11.78879066	6.44735E-30	small secreted protein, partial	KID60014
MAN_09389	11.45261363	9.7062E-120	methyltransferase SirN-like protein, partial	KID61105
MAN_07950	11.45082216	6.3993E-146	hypothetical protein MAN_07950, partial	KID62734
MAN_04589	11.37591299	7.1242E-28	P450 monooxygenase, partial	KID66308
MAN_09390	11.29333622	2.8305E-214	Beta-ketoacyl synthase, partial	KID61106
MAN_06029	11.26501407	2.69403E-27	protein phosphatase regulatory subunit Gac1, partial	KID65018
MAN_09388	11.25643797	2.42452E-22	Cytochrome P450, partial	KID61104
MAN_05612	11.22663994	1.24557E-78	Nucleotide sugar dehydrogenase, partial	KID65953
MAN_06392	11.15580383	0	hypothetical protein MAN_06392, partial	KID64218
MAN_09398	11.10041699	0	UbiA prenyltransferase family, partial	KID61114
MAN_09391	11.07822832	0	alpha/beta hydrolase, partial	KID61107
MAN_01655	11.01357438	3.98324E-26	Cytochrome P450, partial	KID69141
MAN_09757	10.92297318	1.18086E-25	Major facilitator superfamily domain, general substrate transporter, partial	KID60622
MAN_04588	10.91611752	1.17221E-25	P450 monooxygenase, partial	KID66307
MAN_06393	10.8817116	0	WSC domain containing protein, partial	KID64219

MAN_09384	10.81836787	1.5534E-169	steroid monooxygenase, partial	KID61100
MAN_09397	10.77604343	2.0602E-244	cytochrome P450 oxidoreductase OrdA-like protein, partial	KID61113
MAN_09385	10.75740724	0	acetate-CoA ligase, partial	KID61101
MAN_09395	10.74934095	0	phytanoyl-CoA dioxygenase family protein, partial	KID61111
MAN_10475	10.74645419	3.5462E-221	conidial pigment polyketide synthase PksP/Alb1, partial	KID59669
MAN_06149	10.71210952	1.7129E-24	Alcohol dehydrogenase superfamily, zinc-type, partial	KID65138
MAN_09380	10.61228301	3.3341E-197	O-methyltransferase, family 2, partial	KID61096
MAN_04171	10.60162483	3.3169E-24	porphyromonas-type peptidyl-arginine deiminase superfamily, partial	KID67413
MAN_09381	10.58854612	0	Glutathione S-transferase protein, partial	KID61097
MAN_09383	10.50614863	2.92243E-89	hypothetical protein MAN_09383, partial	KID61099
MAN_06331	10.16341354	2.96538E-22	cyclohexanone monooxygenase, partial	KID65320
MAN_05613	10.12392176	2.949E-163	glycosyl transferase, group 2 family protein, partial	KID65954
MAN_09730	10.11507627	3.56852E-22	hypothetical protein MAN_09730, partial	KID60595
MAN_09392	10.00950215	9.5747E-208	Dimeric alpha-beta barrel, partial	KID61108
MAN_00019	9.793626095	1.28798E-20	Outer membrane protein, beta-barrel, partial	KID70420
MAN_01650	9.70955388	3.24362E-20	alpha/beta hydrolase, partial	KID69136
MAN_07788	9.650178427	2.73808E-39	hypothetical protein MAN_07788, partial	KID62572
MAN_01644	9.560938193	1.24986E-19	NAD(P)-binding domain protein, partial	KID69130
MAN_04596	9.420363191	1.45512E-19	FAD dependent monooxygenase, partial	KID66315
MAN_06006	9.350566592	6.0084E-130	NAD(P)-binding domain protein, partial	KID64995
MAN_02861	9.308885771	0	NAD(P)-binding domain protein, partial	KID68005
MAN_04592	9.295390598	3.18167E-45	geranylgeranyl diphosphate synthase, partial	KID66311
MAN_02681	9.137722278	1.23388E-17	Ribonuclease III, partial	KID70167
MAN_10647	8.902270522	0	metalloprotease MEP1, partial	KID59477
MAN_00992	8.816253439	0	pantothenate transporter liz1, partial	KID71393
MAN_04597	8.808230868	1.55601E-16	P450 monooxygenase, partial	KID66316
MAN_04672	8.714065566	5.87681E-16	oxidoreductase, short chain dehydrogenase/reductase family, partial	KID66391
MAN_00979	8.660748325	4.10274E-97	hypothetical protein MAN_00979, partial	KID71380
MAN_00814	8.639048902	0	UDP-glucuronosyl/UDP-glucosyltransferase, partial	KID71215
MAN_05742	8.631514895	1.06448E-15	hypothetical protein MAN_05742, partial	KID66083

Appendix F. Dataset with specific protein families involved in virulence factors

Gene	log2FoldChange	Protein names - Pr1 (PF00082)	Cross-reference (Pfam)
MAN_06835	-8.564077051	Subtilisin-like protease PR1K (Fragment)	PF05922;PF00082;
MAN_02690	-4.660778109	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF02225;PF00082;
MAN_09650	-2.995987503	Subtilisin-like protease (Fragment)	PF06280;PF00082;
MAN_10829	-2.798036302	Subtilisin-like protease PR1I (Fragment)	PF05922;PF00082;
MAN_05645	-2.791568594	S8 family Peptidase (Fragment)	PF00082;
MAN_03640	-1.763893488	Tripeptidyl peptidase (Fragment)	PF00082;PF09286;
MAN_01993	-1.392124076	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;PF09286;
MAN_01788	-1.334101661	Peptidase S8, subtilisin-related protein (Fragment)	PF00082;
MAN_00158	-1.051184185	Subtilisin-like protease Pr1B (Fragment)	PF05922;PF00082;
MAN_01617	-0.967304728	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;
MAN_03501	-0.679721926	Subtilisin-like protease Pr1A (Fragment)	PF05922;PF00082;
MAN_10377	-0.165498814	Alkaline serine protease AorO (Fragment)	PF00082;PF09286;
MAN_07446	-0.138276781	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;
MAN_00139	-0.125454334	Peptidase S8, subtilisin-related protein (Fragment)	PF00082;
MAN_09813	-0.095723896	Subtilisin-like protease PR1E (Fragment)	PF00082;
MAN_03835	-0.033883672	Subtilisin-like protease (Fragment)	PF00082;
MAN_08447	-0.024981685	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;
MAN_05588	-0.023763984	Subtilisin-like protease PR1H (Fragment)	PF05922;PF00082;
MAN_04715	0.000216838	Intracellular serine protease (Fragment)	PF00082;
MAN_06710	0.209708941	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;
MAN_00268	0.231068339	Kexin-like protease (Fragment)	PF01483;PF00082;
MAN_08835	0.320055201	Subtilisin-like protease PR1G (Fragment)	PF05922;PF00082;
MAN_09316	0.342703924	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;
MAN_08220	0.43119623	Subtilisin-like serine protease PR1J (Fragment)	PF05922;PF00082;
MAN_06009	0.445868271	Subtilisin-like protease (Fragment)	PF06280;PF02225;PF00082;
MAN_10478	1.40986057	Peptidase S8, subtilisin-related protein (Fragment)	PF00082;

MAN_01941	1.798863162	Subtilisin-like protease PR1E (Fragment)	PF00082;
MAN_10648	1.860636095	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;
MAN_06916	2.443466454	Tripeptidyl-peptidase 1 (Fragment)	PF00082;PF09286;
MAN_06594	2.487141896	Subtilisin-like serine protease (Fragment)	PF00082;
MAN_07651	2.681000618	Tripeptidyl peptidase (Fragment)	PF00082;PF09286;
MAN_07668	2.927004937	Subtilisin-like protease PR1F (Fragment)	PF00082;
MAN_07805	3.180212952	Subtilisin-like protease PR1E (Fragment)	PF00082;
MAN_01940	3.271722734	Subtilisin-like protease PR1F (Fragment)	PF00082;
MAN_06767	3.351003597	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;PF09286;
MAN_06309	4.228122776	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;
MAN_06813	7.162619245	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;
MAN_00058	NA	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF00082;
MAN_00876	NA	KP-43 peptidase (Fragment)	PF00082;
MAN_00878	NA	Serine protease (Fragment)	PF05922;PF00082;
MAN_01671	NA	Subtilisin-like serine protease PR1C (Fragment)	PF00082;
MAN_01833	NA	Subtilisin-like serine protease PR1D (Fragment)	PF05922;PF00082;
MAN_04670	NA	Subtilisin like protein (Fragment)	PF00082;
MAN_05643	NA	Peptidase S8, subtilisin-related protein (Fragment)	PF00082;
MAN_09413	NA	Peptidase S8/S53, subtilisin/kexin/sedolisin (Fragment)	PF05922;PF00082;
MAN_09683	NA	Subtilisin-like serine protease PR1J (Fragment)	PF05922;PF00082;
MAN_10200	NA	Subtilisin (Fragment)	PF00082;
MAN_10814	NA	Subtilisin-like serine protease PR1C (Fragment)	PF06280;PF00082;

Gene	log2FoldChange	Protein name - Trypsin (PF00089)	Cross-reference (Pfam)
MAN_07684	-7.859012722	trypsin-like protease, partial	PF00089;
MAN_00098	-7.227016188	Peptidase cysteine/serine, trypsin-like protein, partial	PF00089;
MAN_10272	-6.662120738	Trypsin- protease, partial	PF00089;
MAN_02755	-5.245896986	Trypsin- protease, partial	PF00089;
MAN_05738	-3.671000451	peptidase S1 and S6, chymotrypsin/Hap, partial	PF00089;
MAN_04570	-3.321924314	extracellular trypsin protease, partial	PF00089;
MAN_10512	-2.825081814	Peptidase S1/S6, chymotrypsin/Hap, partial	PF00089;
MAN_03928	-2.802431228	trypsin delta/gamma, partial	PF00089;
MAN_06561	-1.303167869	V8-like Glu-specific endopeptidase, partial	PF00089;
MAN_01776	-1.07473382	V8-like Glu-specific endopeptidase, partial	PF00089;
MAN_01066	-0.138386138	Trypsin-like serine protease, partial	PF00089;
MAN_08799	0.55766993	Peptidase S1/S6, chymotrypsin/Hap, partial	PF00089;
MAN_06794	0.7872653	trypsin-related protease, partial	PF00089;
MAN_00410	1.01276626	extracellular metalloprotease, partial	PF00089;
MAN_10461	2.209513112	Peptidase S1/S6, chymotrypsin/Hap, partial	PF00089;
MAN_09260	2.487141896	trypsin- protease, partial	PF00089;
MAN_01210	3.577993819	trypsin precursor, partial	PF00089;
MAN_01927	3.873507727	Trypsin-related protease, partial	PF00089;
MAN_04524	NA	extracellular trypsin protease, partial	PF00089;
MAN_06045	NA	Trypsin- protease, partial	PF00089;
MAN_06558	NA	trypsin-like protease, partial	PF00089;
MAN_07125	NA	trypsin-like protease, partial	PF00089;
MAN_07843	NA	Peptidase cysteine/serine, trypsin-like protein, partial	PF00089;

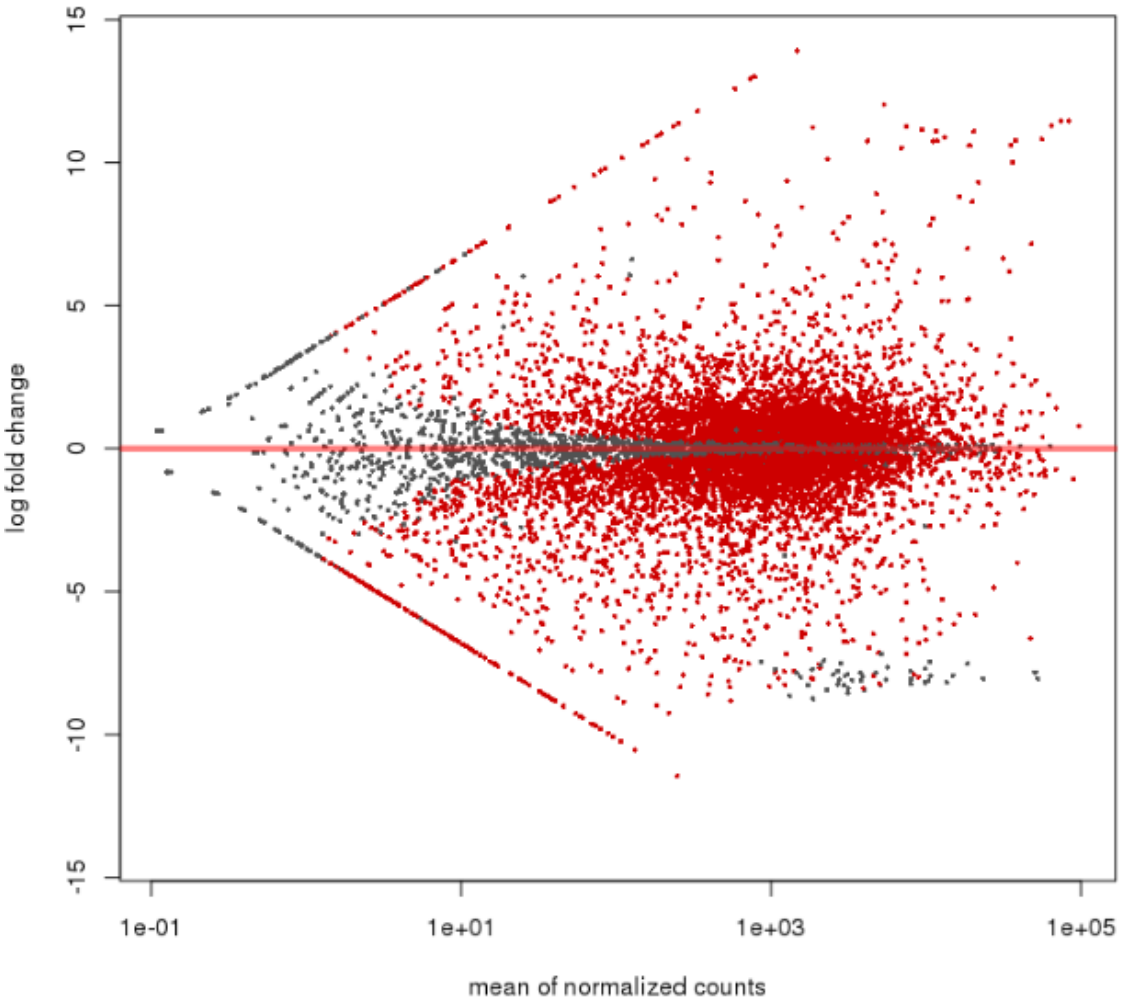
Appendix G. Dataset with families of biosynthetic genes involved in secondary metabolism of Metarhizium. Genes were previously filtered by FDR-pvalue < 0.001, log2FC > 4 or < -4 and then assigned to respectively families

UP/DOWN regulated	Family	Protein name	Gene name	domain structure
UP	M-HPN2	hybrid PKS-NRPS protein, partial	MAN_01651	KS AT DH cMT KR PP C A T R
UP	M-HPN7	Beta-ketoacyl synthase, partial	MAN_09390	KS AT DH cMT KR PP C A T R
UP	M-NRPS25	AMP-dependent synthetase/ligase, partial	MAN_01587	TC ATTC
UP	M-PKS24	polyketide synthase, partial	MAN_09812	SAT KS AT PT PP cMT
UP	M-PKS28	conidial pigment polyketide synthase PksP/Alb1, partial	MAN_10475	SAT KS AT PT PP PP TE
UP	M-TER10	prenyl transferase, partial	MAN_04594	Indole diterpene
UP	M-TER11	geranylgeranyl diphosphate synthase, partial	MAN_04592	Indole diterpene
UP	M-TER26	P450 monooxygenase, partial	MAN_04590	Indole diterpene
UP	M-TER31	AtmB protein, partial	MAN_04593	Indole diterpene
UP	M-TER33	AtmB protein, partial	MAN_09811	M-PKS24 pathway
UP	M-TER43	UbiA prenyltransferase family protein, partial	MAN_09810	M-PKS24 cluster
UP	M-TER44	UbiA prenyltransferase family, partial	MAN_09398	fma-TC ortholog
DOWN	M-NPL3A	Male sterility, NAD-binding protein, partial	MAN_01071	A T R
DOWN	M-NRPS1	non-ribosomal peptide synthetase, partial	MAN_08857	ATC ATC C AT
DOWN	M-NRPS23	non ribosomal peptide synthase, partial	MAN_05757	ATC ATC
DOWN	M-NRPS26	HC-toxin synthetase, partial	MAN_06364	ATC A
DOWN	M-PKS14	Beta-ketoacyl synthase, partial	MAN_01934	KS AT DH ER KR PP
DOWN	M-PKS16	Beta-ketoacyl synthase, partial	MAN_09465	KS AT DH cMT KR PP
DOWN	M-TER29	dimethylallyl tryptophan synthase GliD1, partial	MAN_05697	Xpta ortholog

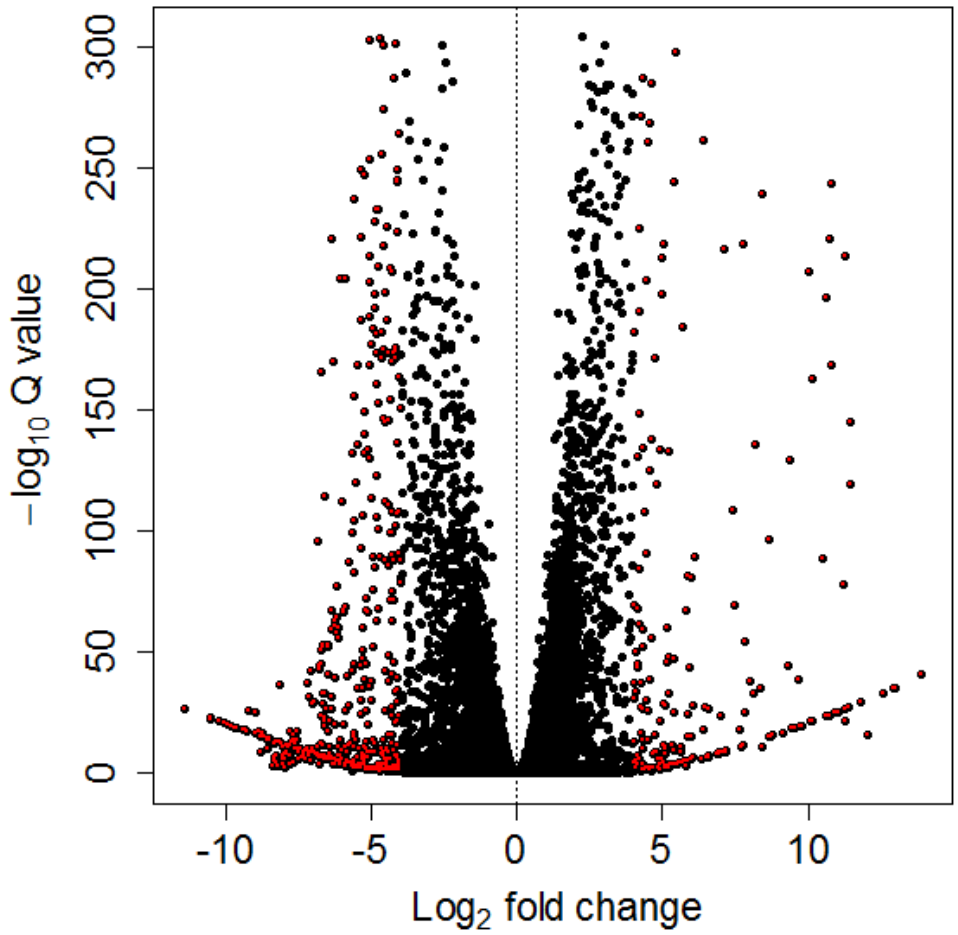
Legend

Polyketide Synthases (PKSs)
Hybrid polyketide-nonribosomal peptide synthetases (HPNs)
Terpene biosynthetic gene families (TER)
Nonribosomal Peptide synthetases-Like (NPLs)
Nonribosomal Peptide Synthetases (NRPSs)

Appendix H. Diagnostic plots of RNA-Seq data: MA-Plot and Volcano-plot



MA-plot of RNA-Seq data of *Metarhizium anisopliae* (ESALQ4676) blastospores vs. hyphae. This graphic was generated by the plotMA function in DESeq2. Each dot is represented by one gene.The data represents all four biological samples of blastospores and hyphae.



Volcano plot of RNA-Seq data of *Metarhizium anisopliae* (ESALQ4676) blastospores vs. hyphae. Each dot is represented by one gene. The red points indicate genes of interest that display large-magnitude fold-changes (x-axis) and high statistical significance (-log10 of pvalue, y-axis). The data represents all four biological samples of blastospores and hyphae.

6. TRANSCRIPTOMICS OF *Metarhizium rileyi* YEAST-LIKE PHASES: UNIQUE SIGNATURES ASSOCIATED TO GROWTH METABOLISM, VIRULENCE AND THEIR IMPLICATIONS FOR MORPHOGENESIS

Natasha Sant´Anna Iwanicki[1*]; Italo Delalibera Júnior[1]; Jørgen Eilenberg[2]; Henrik H. De Fine Licht[2]

1 Department of Entomology and Acarology, Escola Superior de Agricultura “Luiz de Queiroz”, University of São Paulo (ESALQ-USP), Av. Pádua Dias, 11, C.P. 9, Piracicaba, SP CEP 13418-900, Brazil

2 Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark.

*Corresponding author: Natasha S. A. Iwanicki E-mail address: natasha.iwanicki@usp.br Department of Entomology and Acarology, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

Abstract

Metarhizium rileyi is an entomopathogenic fungus with a specialist host range that distinguishes it from other *Metarhizium* species. This species is also unique for the initial yeast growth in solid medium. The lack of knowledge concerning the main metabolism and genetic signatures of the *M. rileyi* yeast-phase is one of the bottlenecks of large-scale production of *M. rileyi* by the biocontrol industry. In the present work, we used transcriptomic analysis to document the main biological processes and up-regulated genes involved in the induction and maintenance of *M. rileyi* yeast-like phases to clarify genomics signatures of the yeast-like phase in solid and liquid medium. We showed that the yeast-like phase produced in liquid medium activates a series of specific genes related to signal transduction, and specific membrane transporters related to iron acquisition. We showed that oxidative stress and activation of specific heat shock proteins are key factors involved in formation of these cells. On the other hand, the yeast-like phase grown in solid medium activates a set of unique genes, not found in other *Metarhizium* spp, including specific membrane proteins and several virulence factors. Our data therefore clarified the main metabolism required for yeast-like cells growth in liquid medium and identified candidate genes that will serve as a basis for future research on optimizing *M. rileyi* production. The fact that the yeast-phase in solid medium activates several genes associated with virulence factors opens new opportunities to explore this yeast-like single-celled stage in biological control programs.

Keywords: Blastospores, Liquid culture fermentation, Fungal dimorphism Entomopathogenic fungi

6.1. Introduction

Entomopathogenic fungi of the genus *Metarhizium* (Ascomycetes: Hypocrealeans: Clavicipitaceae) have been used worldwide in the biological control of pests. In Brazil aerial conidia of *Metarhizium anisopliae* have been successfully applied to more than 2 million hectares of sugarcane every year (Parra, 2014) while in Europe, it is *Metarhizium brunneum* that is more commonly used (Fischhoff et al. 2017).

Metarhizium spp. present dimorphic growth, that is, depending on the conditions in which they are cultivated, they can grow either in the filamentous

or in the yeast-like form (usually referred to as blastospores when grown artificially in liquid culture). Such cell dimorphism represents a strategy developed by many fungi to maximize their survival in various environments, so that they may produce infective or dispersal structures and search for nutrients (Gauthier 2015). In nature, yeast-like cells are produced during the fungal infection cycle in insect hemolymph, as a response to environmental changes from host-tissue to the liquid hemolymph that has high osmolarity (Lovett and Leger, 2015). Unique features of yeast-like cells, such as specific cell wall surface epitopes, thinner cell wall and a collagenous protective coat (Mlc1) enable them to remain undetected by the host immune system (Wang and St. Leger, 2006). Therefore, yeast-like cells function is dispersion and colonization of the fungi inside the insect body (Lu and St. Leger, 2016).

Metarhizium spp. are produced industrially mainly in a biphasic system where fungal biomass (mycelia, blastospores and/or submerged conidia) cultivated initially in liquid culture media are then inoculated in solid substrate (grains such as rice or wheat) to shorten the production time of conidia. However, blastospores are infective and can be used as the active ingredient of biopesticides. Therefore, it is crucial for the biological control industry to have an inexpensive liquid medium that supports high blastospore yield of *Metarhizium*, that will then be used either for the biphasic production of conidia or to be commercialized directly as blastospore-based biological control agent.

While the *M. anisopliae* and *M. brunneum* biphasic production is well established in the industry and conidia-based biopesticides of these species are commercialized worldwide, *M. rileyi* is more fastidious, and is not stable for continuous production by solid substrate fermentation. Although *M. rileyi* belongs to the same genus as *M. anisopliae* and *M. brunneum*, it has very peculiar characteristics including its development in culture medium. Compared to *M. anisopliae* and *M. brunneum*, *M. rileyi* is a nutritionally demanding fungus that takes longer to be cultivated (Goettel and Roberts 1991). *M. rileyi* produces yeast-like cells from aerial conidia in solid agar medium prior to switching to hyphae (Pendland and Boucias, 1997) while *M. anisopliae* and *M. brunneum* do not. *M. anisopliae* and *M. brunneum* conidia in solid media germinate directly to form hyphae and mycelium. Actually, *M. rileyi* is the only *Metarhizium* species that produce yeast like cells in solid medium and the mechanisms underlying

the genetic features involved in formation of this morphotype by *M. rileyi* are still unknown.

The cellular dimorphism of *M. rileyi* depends on the expression of specific genes and metabolic processes; In recent years, genome-wide transcriptomic and protein analysis has been applied to elucidate these processes (Boucias et al. 2016, Song et al. 2013, 2016). In the first comparative transcriptome study, in which Song et al. (2013) aimed to enhance the fermentation process of *M. rileyi* microsclerotia, the authors demonstrated that a large number of genes related to their formation is involved in the response to oxidative stress. Later, by applying knockout gene techniques, the same authors showed that microsclerotia formation was mediated by two mitogen-activated protein kinases, Hog1 and Slt2-type as mutants for these genes delayed germination and vegetative growth and were more sensitive to various stresses (Song et al. 2016). The way to overcome the bottlenecks in the industrial production of fungi is to first understand the main metabolism involved in the induction and maintenance of the desired fungus structure in culture medium. Then this knowledge can be applied to improve the production system by optimizing the conditions that will stimulate the expression of metabolic pathways and genes related to fermentation. Furthermore, candidate genes related to blastospore production may serve as molecular markers for selecting high-productive strains in the future.

In order to provide a basis for optimizing *M. rileyi* blastospore production process we, in the present work, identified the main biological processes and changes in gene expression between the yeast and filamentous growth in liquid and solid media. Comparing transcriptomes allowed us to clearly determine which genes are active in each growth form specifically related to nutrient transport, cell wall and membrane components, cell growth, transcriptional factors and morphogenesis of yeast-phases grown in solid medium.

6.2. Materials and Methods

6.2.1. Fungal material

The present study investigated the fungus *Metarhizium rileyi* using the isolate ESALQ4948 originated from infected *Anticarsia gemmatalis* larvae collected in the city Itaara from the Rio Grande do Sul State in Brazil. The isolate is deposited in the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) with the accession number ESALQ4948. This isolate was chosen as it in previous studies conducted by our team showed that it produces high amounts of blastospores in liquid culture medium (data not published). A conidial monospore culture was obtained by growing the fungus in SMAY (agar (15g/L), maltose (40 g/L), Bacto® neopeptone (10g/L) yeast extract (20g/L)) for two weeks in a growth chamber at 28°C and 12:12 h photoperiod. A fungal stock culture was established from preserving sporulating agar chunks immersed in a sterile 10% glycerol solution at -80 °C.

Induction of filamentous and yeast growth

Hyphae and yeast-like cells induced in liquid medium were grown on modified Jackson's medium (Jaronski and Jackson, 2012) with the following nutritional composition per liter: 45 g yeast extract, minerals, trace metals and vitamins at the following concentrations per liter: KH_2PO_4, 2.5 g; $CaCl_2.2H_2O$, 1.0 g; $MgSO_4.7H_2O$, 0.83 g; $FeSO_4.7H_2O$, 0.3 g ; $CoCl_2.6H_2O$, 29.6 mg; $MnSO_4.H_2O$, 12.8 mg; $ZnSO_4.7H_2O$, 11.2 mg; 0.2 mg each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid; and 0.02 mg each of folic acid, biotin, and vitamin B12. The medium was amended with 45g L^{-1} of glucose solutions that were autoclaved separately. Sterile solutions of vitamins and metals were added to the autoclaved medium before pH was adjusted to 5.5. Growth and formation of hyphae during the filamentous phase of the fungus and yeast-like state were induced by growing *M. rileyi* ESALQ4948 on solid and in liquid Jackson´s medium, respectively. Thus identical nutritional compositions were used to grow mycelial hyphae and yeast-like state in liquid medium, with the only difference that 15 g L^{-1} of agar were added to solidify the medium used for inducing hyphal growth. To induce growth of the yeast-like state in solid medium, we used the SMAY medium (previously described) as this medium provides excellent conditions to this morphotype development.

Conidia of *M. rileyi* ESALQ4948 were obtained by growing agar chunks from the monosporic stock culture in Petri dishes containing SMAY for fourteen days at 28 °C and 12:12 h photoperiod. Conidia were harvested by washing Petri dishes containing actively sporulating fungal cultures with 10 mL of a sterile aqueous solution with 0.02% polyoxyethylene sorbitan monooleate (Tween® 80, Sigma). A conidial suspension of 5×10^6 conidia mL^{-1} was used to inoculate 50 mL liquid Jackson´s medium in 250-mL baffled Erlenmeyer flasks giving a final concentration in the culture broth of 5×10^5 conidia mL^{-1} (i.e., 10% v/v inoculum). Liquid cultures were incubated at 28°C in a rotatory incubator shaker and 350 rpm for 72 hours. Four Erlenmeyer flasks were inoculated and each considered a biological replicate.

A volume of 120uL of the 5×10^6 conidia mL^{-1} suspension was spread with sterile Drigalski handles in Petri dishes (9 cm diameter) containing solid Jackson´s medium (to induce hyphae growth) or SMAY medium (to induce yeast-like phase growth). Four fungal cultures, for each solid media in separate Petri dishes were incubated at 28 °C for 6 days to obtain mycelial hyphae and 3 days to obtain yeast-like state, with a 12:12 h photoperiod. Each Petri dishes was considered as one biological replicate.

6.2.2. RNA extraction and sequencing

Yeast-like cells from liquid culture were harvested by filtering three-day old fungal cultures grown in liquid Jackson´s medium. Total culture broth of each replicate were filtered in a vacuum pump coupled to a Buchner funnel lined with disk filter paper with 7 cm diameter and 11µ pore sizes (Whatman®, n°1) to remove hyphae. To verify that the filtrate contained only yeast-like cells, each replicate was examined on microscope slides at 400x magnification using optic microscopy. To separate yeast-like cells from the culture medium, 30mL of filtrate were added to 50mL Falcon® tubes and centrifuged at 2500 rpm, 4°C. for 5 minutes. The supernatant was discarded and the yeast-like cells pellet was quickly transferred with a pre-cooled scoop to a pre-cooled porcelain mortar, before immediately adding liquid nitrogen and macerating the pellet with a pre-cooled pestle. The resulting powder were not allowed to thaw and transferred to an Eppendorf tube containing 1mL of TRIzol® and kept on ice. Mycelial hyphae

and yeast-like cells grown in solid medium were harvested from six and three days old fungal cultures respectively grown on Jackson´s agar and SMAY medium respectively. Hyphae and yeast-like cells were removed from the medium with a sterilized and pre-cooled spatula and placed immediately in an Eppendorf tube containing 1mL of TRIzol® and kept on ice. Care was taken to avoid collecting the solid media when scraping off the hyphae and yeast-like cells.

Total RNA was extracted from fungal samples immersed in TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. Eppendorf tubes containing 1mL of TRIzol® with either yeast-like cells or hyphae were incubated for 5 min at room temperature, before homogenizing the samples by pipetting up and down. This was followed by centrifugation for 5 minutes at 12000x G at 4°C. The supernatant was transferred to a new clean Eppendorf tube and the samples were homogenized for 5 minutes in a tissue homogenizer to break the fungal cell walls. 200µL of chloroform were added to samples following agitation for 15 seconds and incubated at room temperature for 5 min. Then, another centrifugation was performed (12,000 x spin, 15 min at 4 ° C) to separate the mixture and total RNA was precipitated from the upper aqueous phase with half a volume isopropanol (0.5 ml isopropanol per 1 mL of TRIzol®) and centrifugation. The pellet was washed with 1 mL of 75% ethanol and placed to dry for 30 min at room temperature, followed by resuspension of total RNA in 20 uL of pre-cooled DEPC-treated water. Total RNA was quantified fluorometrically using a Qubit® (Invitrogen) and the purity and quality evaluated in a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA). The RNA integrity was estimated with 1% agarose-formaldehyde gel capillary electrophoresis using a Bioanalyzer (Agilent), and only samples with a RNA integrity measure (RIN) higher than 8 were used.

Messenger RNA libraries were prepared with Illumina TruSeq Stranded mRNA Library Prep kit (Illumina Inc., San DieGo, CA) and quantified with qPCR using the Illumina KAPA Library Quantification kit. Samples were sequenced with Illumina HiSeq 2500 technology, which yielded at least 20 million 100-bp paired-end reads per library. Library preparation and sequencing were performed by “Laboratório Multiusuários Centralizado de Genômica Funcional Aplicada à Agropecuária e Agroenergia” in Piracicaba-SP, Brazil.

6.2.3. Mapping of RNA-Seq reads and quantitative differential expression analysis

The quality of the raw reads before and after quality and adaptor trimming was assessed using the fastQC (Babraham Bioinformatics) program. Illumina adapters and low-quality sequences were removed using Trimmomatic V0.32 (Bolger et al. 2014) with the following options: HEADCROP:7 TRAILING:20 MINLEN:36. Quality trimmed reads were aligned to the reference genome (*M. rileyi* RCEF4871 from NCBI) using HISAT2 (Pertea et al. 2016). First, we used the python scripts included in the HISAT2 package: extract_splice.py and extract_exons.py, to extract the splice-site and exon information's from the annotation file, respectively. Then, we built the indexes for the reference genome with the program hisat2-build with the options: --ss and –exon, to provide outputs from splice sites and exons, respectively. Finally, we aligned RNA-seq reads to the reference genome with the program hisat2 with the options: –dta and –p 8. Gene quantification were performed with StringTie v1.3.3 (Pertea et al. 2016) using gene annotations from *M. rileyi* RCEF4871 strain reference genome information. The stringtie program were used with the following options: -b, -B and –G. The gene count matrix were obtained with the python script: prepDE.py, provided by John Hopkins University, center for computational biology, CCB (http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual#deseq). The gene count matrix was used as input file to the differential expression analysis that was conducted using the DESeq2 library (Love et al. 2014) for the statistical software R (R Team Core, 2017). Only genes with a false discovery rate (FDR) adjusted p-values <0.001 and $\log_2$ fold change (FC) > 2, for up-regulated genes and $\log_2$FC < -2, for down-regulated genes were considered differentially expressed. Individual gene expression was not re-validated by qPCR because previous studies have shown extremely close correlation between qPCR and RNAseq data (Griffith et al. 2010, Asmann et al. 2009, Wu et al. 201,), our biological samples are robustly replicated and being highly similar within treatments and clearly distinct between treatments (Figure 2), and there is little evidence that qPCR analyses of a few specific genes of the same samples will

add any new utility to our data or change the major conclusions drawn from the much larger groups of genes analyzed in the RNAseq dataset. Heatmaps of differentially expressed genes were made with the web application “shinyheatmap” (Khomtchouk et al. 2017) with the following parameters: apply clustering: column, Distance metric: Euclidian; Linkage algorithm: complete.

6.2.4. Gene-set enrichment analysis

Gene set enrichment analysis (GSEA) is a software that determines whether a priori defined set of genes is statistically significant between two biological states (Subramanian et al. 2005). GSEA rank genesets by enrichment magnitude and indicate classes of genes that are over-represented in geneset. As recommended for RNA-seq datasets, GSEA was used in the GSEAPreranked mode with a user provided list of all genes pre-ranked according to a defined metric that could be the $\log_2$ fold change, adjusted *p-value* or inverse *p-value* and a list of gene sets. Then, GSEAPreranked calculates an enrichment score by matching genes from gene sets to those in the user ranked list. Next, the gene set’s enrichment score shows how often members of that gene set occur at the top or bottom of the ranked data set. In this study, we used GSEAPreranked mode with gene sets categorized by gene ontology (GO) and protein family domain (PFAM) annotation. The metric used in GSEA input file was the multiplied the sign of fold change by its inverse *p-value*. We used the *p-value* provided as an output of DESeq2. When the *p-value* output from DESeq2 was “0”, the “0” value was replaced by artificially high or low values “+1E+308” or “-1E+308” for up and down-regulated genes, respectively, according to the sign of fold change. The parameter adopted for running the GSEAPreranked for GO and PFAM terms were: minlengh 10 and maxlengh 500, enrichment statistic: “classic” and FDR-correction for multiple testing < 0.25 for enriched gene sets. The unusual high FDR threshold of < 0.25 is recommended by GSEA because it indicates that the result is likely to be valid 3 out of 4 times, which arguably is reasonable for exploratory discovery analysis before future validation (Subramanian et al. 2005). The web server REVIGO (Supek et al. 2011) was used to analyze GO terms in the categories: biological process, cellular component and molecular function.

6.2.5. Orthologous analysis of common genes in *M. rileyi* and related species

To clarify the genetic novelty of *M. rileyi* involved in yeast-phase formation in solid agar medium we estimated orthologous gene groups from 13 fungi species that exhibit dimorphic development, including eight *Metarhizium* species, a closely related species *Pochonia clamydosporia* (Hypocreales: Clavicipitaceae), the entomopathogenic fungus *B. bassiana* (Hypocreales: Cordycipitaceae), the human pathogen *Candida albicans* (Saccharomycetales:Saccharomycetaceae), and plant pathogens *Ustilago maydis* (Ustilaginales: Ustilaginaceae) and *Zymoseptoria tritici* (Capnodiales: Mycosphaerellaceae).

Orthologous gene groups estimation analysis using OrthoFinder program (Emms and Kelly, 2019) with default parameters described in Emms and Kelly., (2019). Orthologous genes up regulated in *M. rileyi* yeast-phase grown in solid medium were identified in other 8 species of *Metarhizium* (*Metarhizium robertsii* strain ARSEF 23 and ARSEF 2575, *Metarhizium anisopliae* strain E6 and ARSEF 549, *Metarhizium album* strain ARSEF 1941, *Metarhizium guizhouense* ARSEF 977, *Metarhizium brunneum* ARSEF 3297, *Metarhizium rileyi* strain Cep018-CH2, *Metarhizium acridum* strain CQMa 102 and *Metarhizium majus* strain ARSEF 297), *P. chlamydosporia*, strain 170 and 123, *B. bassiana*, strain D1-5 and ARSEF JEF-007, *C. albicans*, strain SC5314 and the plant pathogens *U. maydis*, strain 521 and *Z. tritici* strain IPO323. Protein sequences of these genomes were obtained in NCBI database and used as input in Orthofinder. To obtain the evolutionary relationships between orthogroup we analyzed a phylogenetic tree of orthologous genes inferred by the Maximum Likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001) (Ma et al. 2010) and the bootstrap percentage was inferred from 1000 replicates. The alignment and phylogenetic tree were conducted in MEGA6 (Tamura et al. 2013).

6.3. Results

6.3.1. Summary of RNA-Seq

To determine the main genes related to *M. rileyi* yeast-like cells formation in liquid medium and to elucidate the genetic features and metabolism involved in yeast-like cells grown in solid medium, we compared the genome-wide expression profile of yeast-like cells grown in liquid and solid media and hyphae. A total of 165.6 million paired-end 100-bp clean reads were obtained in this experiment. Of this total of reads, 54.7 million represented yeast-like cells from liquid medium (YL) samples, 58.1 million represented yeast-like cells from solid medium (YS) samples and 52.8 million represented hyphae (H) samples of *M. rileyi* (Table 1). The average rate of YL, YS and H reads alignment against the reference genome were 94%, 90% and 94% respectively. Table 1 summarizes the RNA-Seq results.

Table 1. Summary of *M. rileyi* RNA-Seq read filtering and mapping. Numbers represents values of the total of the four biological replicates for yeast-like cells grown in liquid medium (YL) yeast-like cells grown in solid medium (YS) and hyphae (H).

Total samples	**Yeast-like cells - YL (liquid medium)**	**Yeast-like cells - YS (solid medium)**	**Hyphae-H**
Clean reads paired	54,730,140(100%)	58,161,753(100%)	52,888,850(100%)
Mapped reads	51,446,331(94%)	52,700,364 (90%)	49,837,163(94%)
Unmapped reads	3,283,809(6%)	5,461,389(10%)	3,051,687(6%)
Unique match	37,044,152(68%)	35,976,260(62%)	34,624,339 (65%)

Clustering analysis of biological samples from YL, YS and H clearly showed differences in gene expression between treatments (YS, YL and H) (Figure 1). The first principal component differentiated the three treatments and explained 64% of sample variation. The difference found in the gene expression levels may explain general metabolism requirements by *M. rileyi* to produce different phenotypes. Therefore, levels of gene expression of yeast-like cells

grown in solid medium (YS), yeast-like cells grown in liquid medium (YL) and hyphae (H) were compared pairwise.

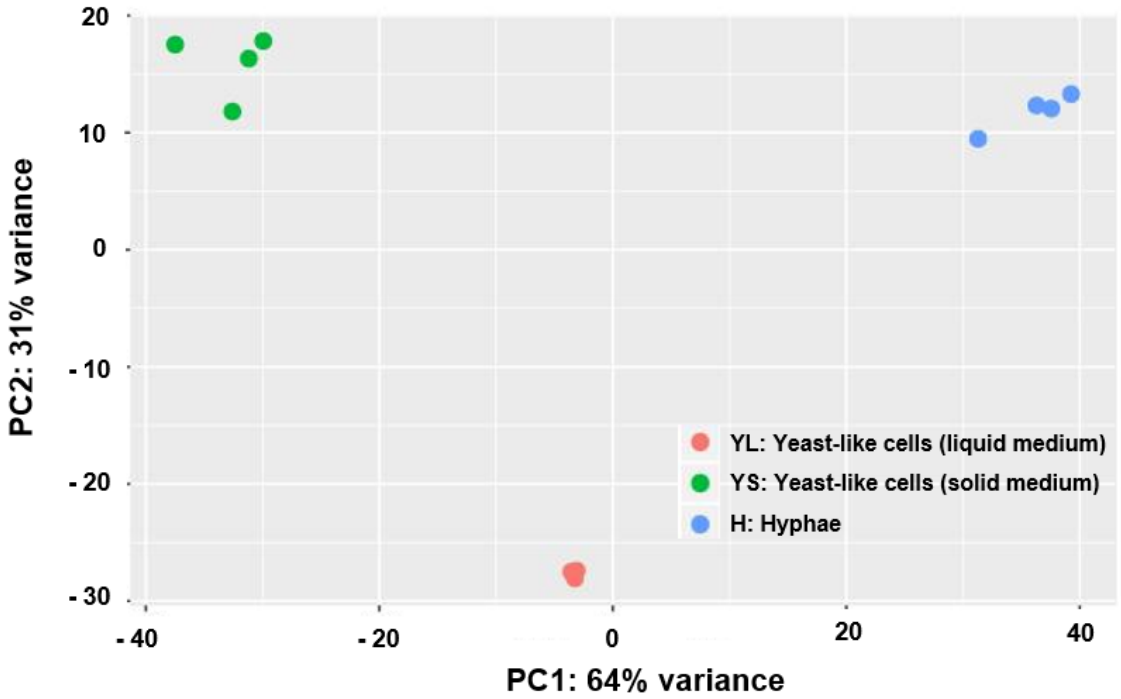


Figure 1. Principal component analysis of regularized-logarithmic (rlog) transformed gene counts of four biological samples of yeast-like cells grown in liquid medium (YL) yeast-like cells grown in solid medium (YS) and hyphal samples (H). YL, YS and H samples are represented by pink, green and blue dots, respectively.

From 8,764 genes annotated in the *M. rileyi* genome (RCEF 4871) (Additional file 1), 1,024 (11.68% of genome), 986 (11.26% of genome) and 893 (10.18% of genome) genes were differentially expressed between YS and H, YS and YL and YL and H (FDR adjusted $p < 0.001$, $Log_2FC > 2$ or < -2) respectively. Of these, 417 genes (4,75% of genome) were up-regulated in YS compared to H and 145 genes (1,65% of genome) compared to YL. Additionally, 186 genes were found up-regulated in YL compared to H. (Figure 2 and Additional file 2). We found 276 (3.14 % of genome), shared genes up-regulated in YS compared to H and YL (Figure 2). The fungus thus activates many more genes during filamentous growth than in the yeast phases in liquid medium (YL) and solid medium (YS). We found 647 genes (7.38% of genome)

up-regulated in H compared to YS and 340 genes (3.87% of genome) compared to YL. A total of 650 genes (7.39% of genome) were down regulated in YL compared to H. (Figure 3).

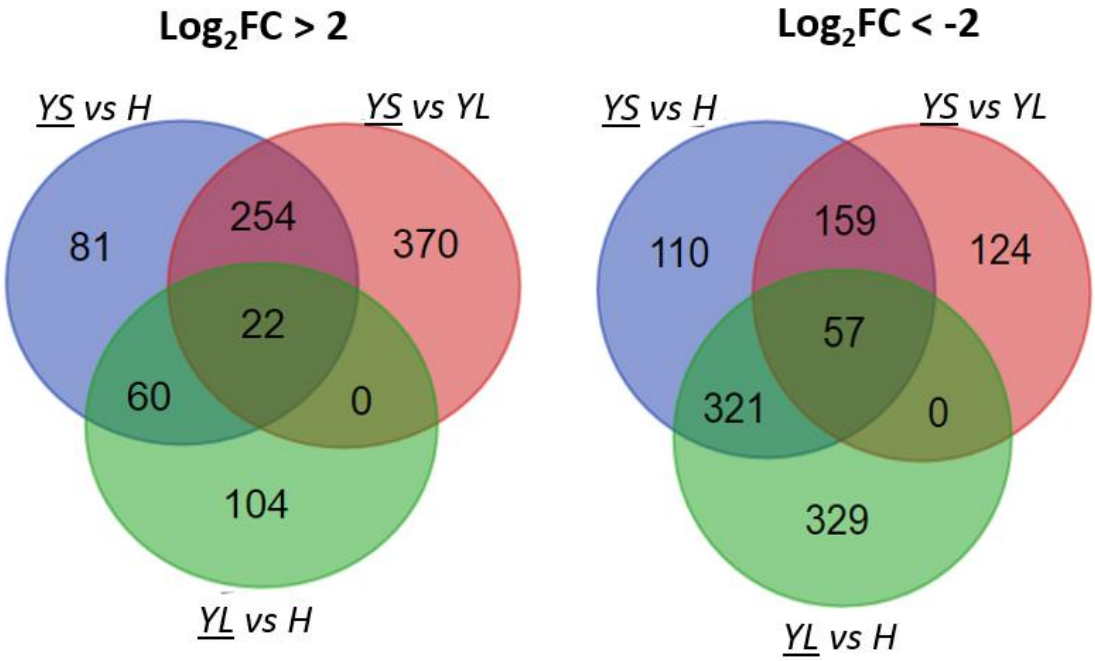


Figure 2. Venn diagrams showing differentially expressed genes, up (left) and down (right) regulated in yeast-like cells produced in liquid (YL), solid (YS) medium and hyphae. Log2FC > 2 or Log_2FC < -2. FDR-*p-value* < 0.001. Subscribed letters indicate phenotype in which genes were up (left) or down (right) regulates

6.3.2. Overall metabolism: Gene set Enrichment analysis of hyphae and yeast-like cells

Hyphae and yeast-like cells are different fungus morphotypes with specific metabolism related to each growth form. Here, we performed a gene set enrichment analysis to clarify the main biological process, cellular component and molecular function gene ontology terms (GO) in yeast-like cells grown in solid (YS) and liquid medium (YL) and hyphae (H). GO terms could be assigned to 5818 (66.3%) out of 8763 genes in *M. rileyi* RCEF4871 genome.

YS *vs* H

We found 13 GO terms in the biological process category significantly enriched in yeast-like cells grown in solid medium (YS). The most enriched

terms were: 1) metabolic process (*GO:0008152*) represented by chemical reactions and pathways such as anabolism and catabolism, related to cell growth, transformation of small molecules, and macromolecular processes such as DNA repair and replication, and protein synthesis and degradation; 2) intracellular protein transport (*GO:0006886*) and vesicle-mediated transport (*GO:0016192*) both involved in the transport of substances within the cell or enclosed in membrane-bounded vesicles; 3) ATP hydrolysis (*GO:0015991*), representing the directed movement of a proton across a membrane and 4) regulation of translational initiation (*GO:0006446*) that represents process that modulates the frequency, rate or extent of translational initiation (Figure 3A, Appendix A). Supporting these findings, the enriched cellular components category in YS, interpreted as the location of the cell where biological processes are taking place, are the cytosol (*GO:0005829*), the Golgi membrane (*GO:0000139*), the endoplasmic reticulum membrane (*GO:0005789*) and the Golgi apparatus (*GO:0005794*) (Additional file 3). Many enriched molecular function categories and protein families also contribute to these findings as they play a role in the biological process shown; they are: the catalytic activity (*GO:0003824*), the signal transducer activity (*GO:0004871*), the peptidase activity (*GO:0008233*), the protein transporter activity (*GO:0008565*), as well as the amino acid, hydrogen ion and substrate-specific transmembrane transporter activity (*GO:0015171*;*GO:0015078*;*GO:0022891*) (Appendix A).

Conversely, we found 14 GO terms in the biological process category significantly enriched in hyphae (H). Most of them were related to cell growth and replication; the two most enriched terms being DNA replication (*GO:0006260*) and translation (*GO:0006412*) (Figure 3 B, Appendix A). Supporting these findings, many enriched terms in cellular component and molecular function category are related to cell growth and replication (Appendix A).

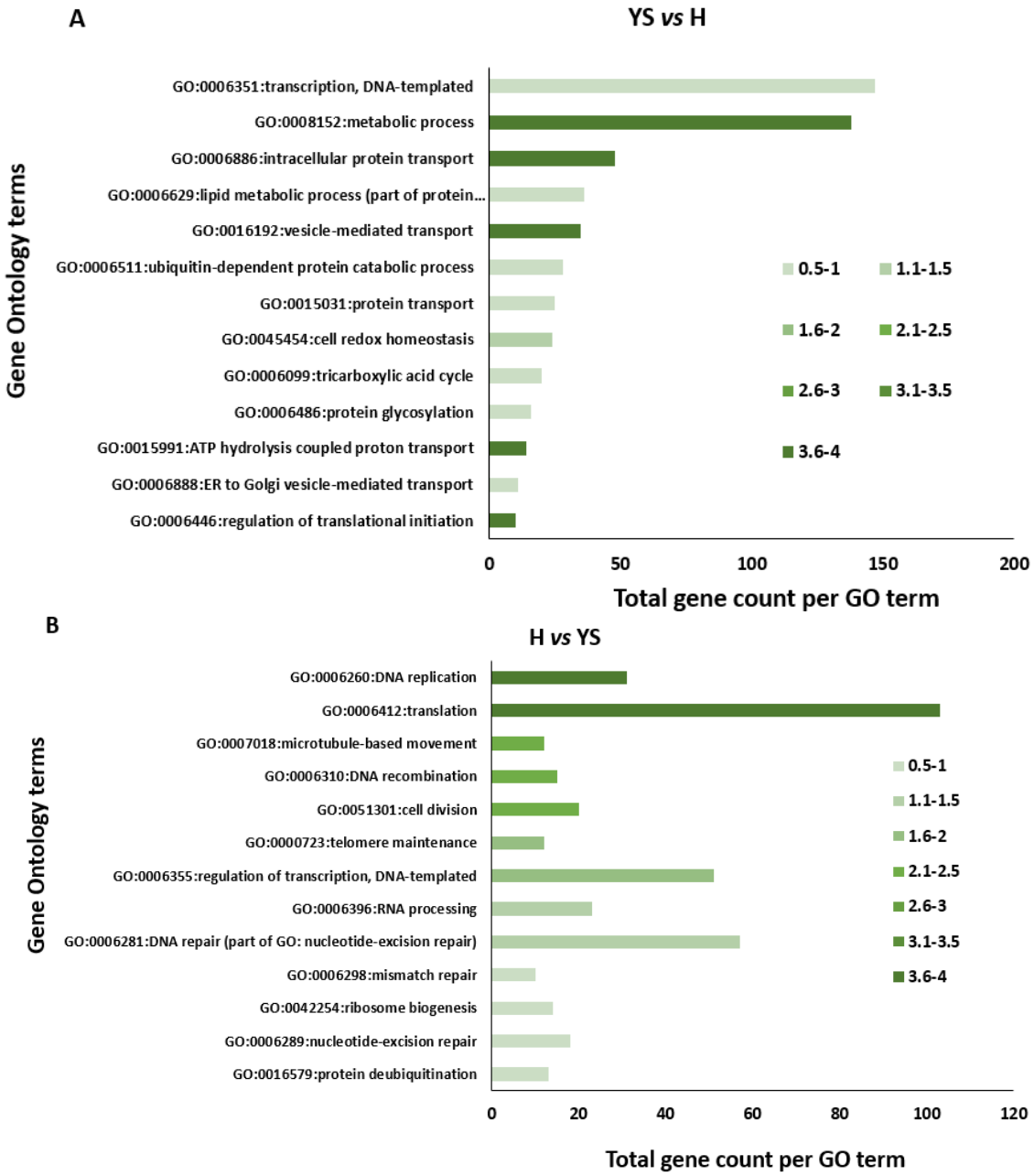


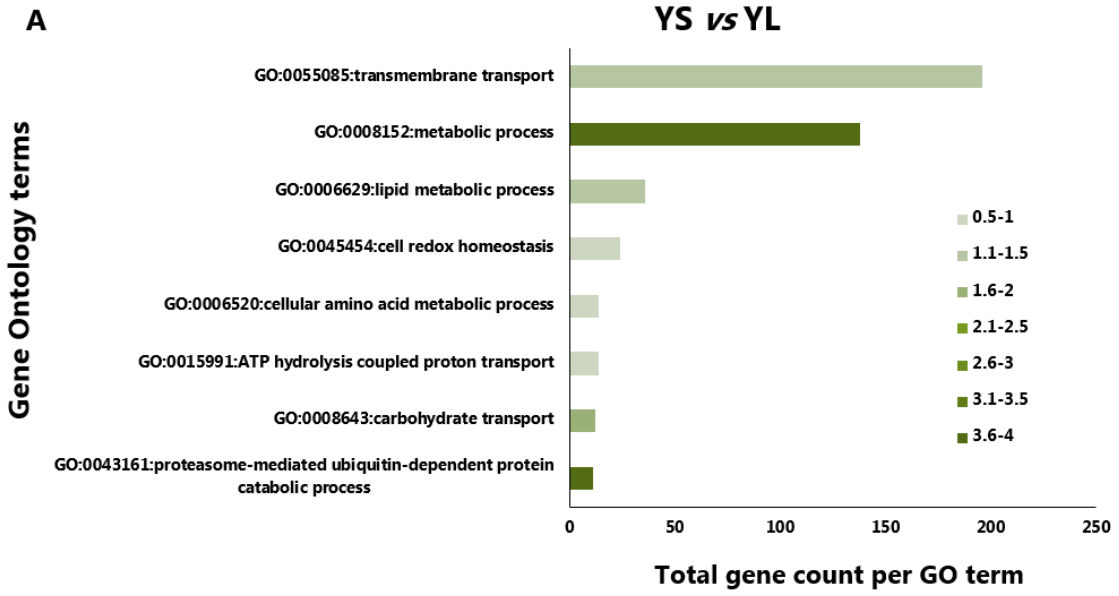
Figure 3. Gene ontology (GO) terms related to biological processes enriched in yeast-like cells grown on solid medium (YS) (A) and Hyphae (H) (B). The color of the bar denotes $-\log_{10}$(FDR-*p-value* from enrichment score). The stronger the color, the more significant is the enrichment.

YS *vs* YL

Here, we found eight GO terms in the biological process category significantly enriched in yeast-like cells grown in solid medium (YS) compared to yeast-like cells grown in liquid medium (YL). The most enriched term was the metabolic process (*GO:0008152*), as described above in the results comparing

enriched GO terms between YS and H. Lipid (*GO:0006629*), amino-acid (*GO:0006520*) and carbohydrate (*GO:0008643*) metabolic processes as well as the cell redox homeostasis (*GO:0045454*) were found to be enriched in YS compared to YL (Figure 4A, Appendix B). Additionally, we found four enriched cellular component categories in BS related to endoplasmatic reticulum (*GO:0005789; GO:0005783*) and Golgi apparatus (*GO:0005794*) (Additional file 3) while enriched molecular functions were related to oxidoreductase (*GO:0016491; GO:0016705; GO:0016810*) metal binding (*GO:0020037; GO:0008270*) and transmembrane transport (*GO:0015171; GO:0022891*) activities (Appendix B).

Conversely, we found 21 GO terms in the biological process category significantly enriched in yeast-like cells grown in liquid culture (YL). A large number of these terms were related to cell growth such as DNA replication (*GO:0006260*), translation (*GO:0006412*) and RNA activities (*GO:0006396*, *GO:006364, GO:0006397*) (Appendix B). The main enriched cellular components were associated with the nucleolus (*GO:0005730*), the cytoplasm (*GO:0005737*) and associated with ribosomes (*GO:0005840*) while the molecular functions were involved in GTP activities (*GO:0005096; GO:0005525; GO:0003924; GO:0008536*) RNA (*GO:0003723; GO:0001104*) and DNA (*GO:0003684; GO:0003899; GO:0005852*) metabolism (Appendix B).



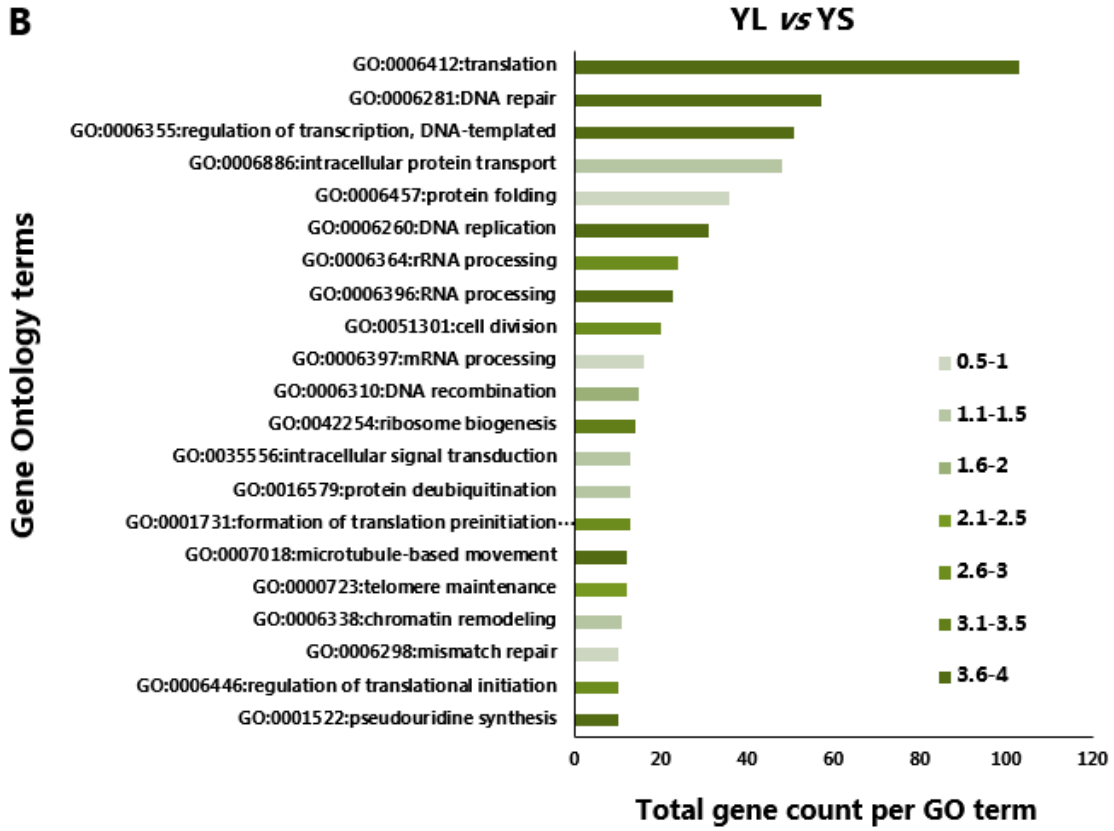
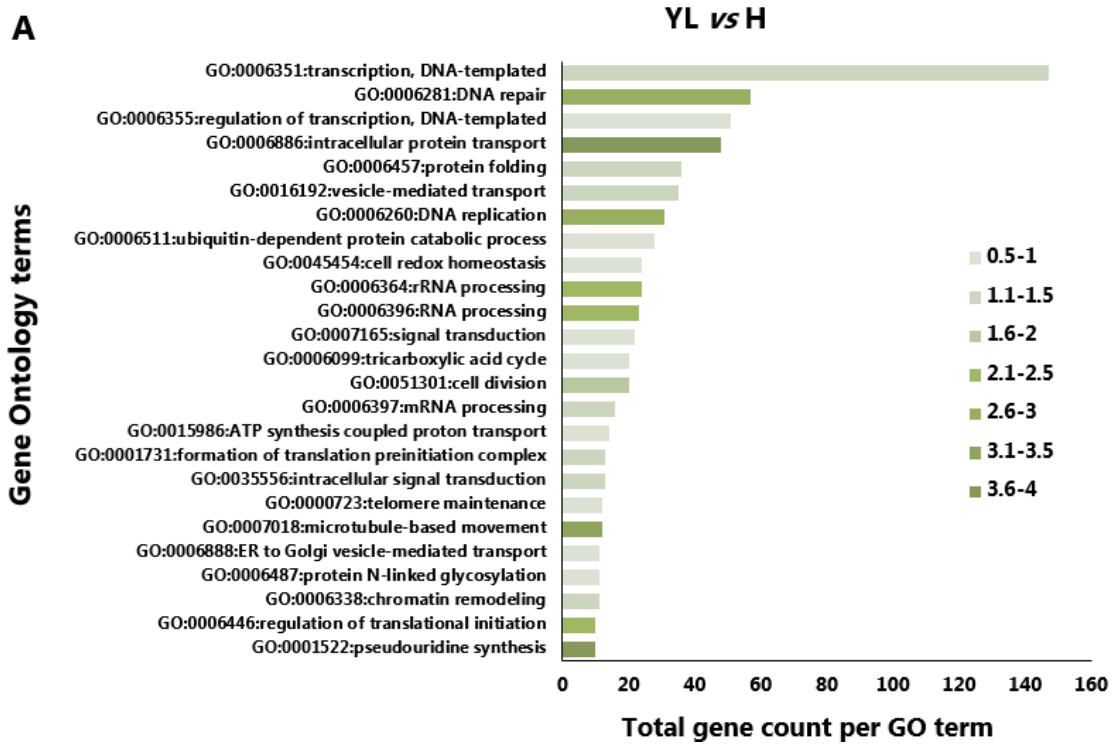


Figure 4. Gene ontology (GO) terms related to biological processes enriched in yeast-like cells grown on solid medium (YS) (A) and liquid medium (YL) (B). The color of the bar denotes –log10(FDR-*p-value* from enrichment score). The stronger the color, the more significant
is the enrichment.

YL *vs* H

Here, we found 25 GO terms in the biological process category significantly enriched in yeast-like cells grown in liquid medium (YL) compared to hyphae (H) (Figure 5A, Appendix C). The enriched terms were related to DNA and RNA activities and signal transduction similar to those we found for YL compared with YS (Figure 4B, Appendix C). The main enriched cellular components were related to the nucleolus (*GO:0005730*), the cytoplasm (*GO:0005737*) ribosomes (*GO:0005840*) and mitochondrion-related terms (*GO:0005743; GO:0005758; GO:0005739*) (Appendix C) while enriched molecular function were similar to those found in BL compared to BS; such as GTP activities (*GO:0005096; GO:0005525; GO:0008536*) RNA (*GO:0003723; GO:0000049*) and DNA (*GO:0003684; GO:0003899; GO:0005852*) activities.

Conversely, we found six GO terms in the biological process category significantly enriched in hyphae (H) compared to yeast-like cells grown in liquid medium (YL), represented mainly by Carbohydrate transport (*GO:0008643; GO:0005975*), transmembrane transport (GO:0055085), proteasome-mediated ubiquitin-dependent protein catabolic process (*GO:0043161*) and autophagy (GO:0006914). The main enriched cellular components were associated with metal binding activity (GO:0020037; GO:0005506), transmembrane movement of substance (GO:0022891; GO:0042626) and endopeptidase activities (*GO:0004190; GO:0004298*) (Appendix C). There was no GO term enriched for molecular function in hyphae.



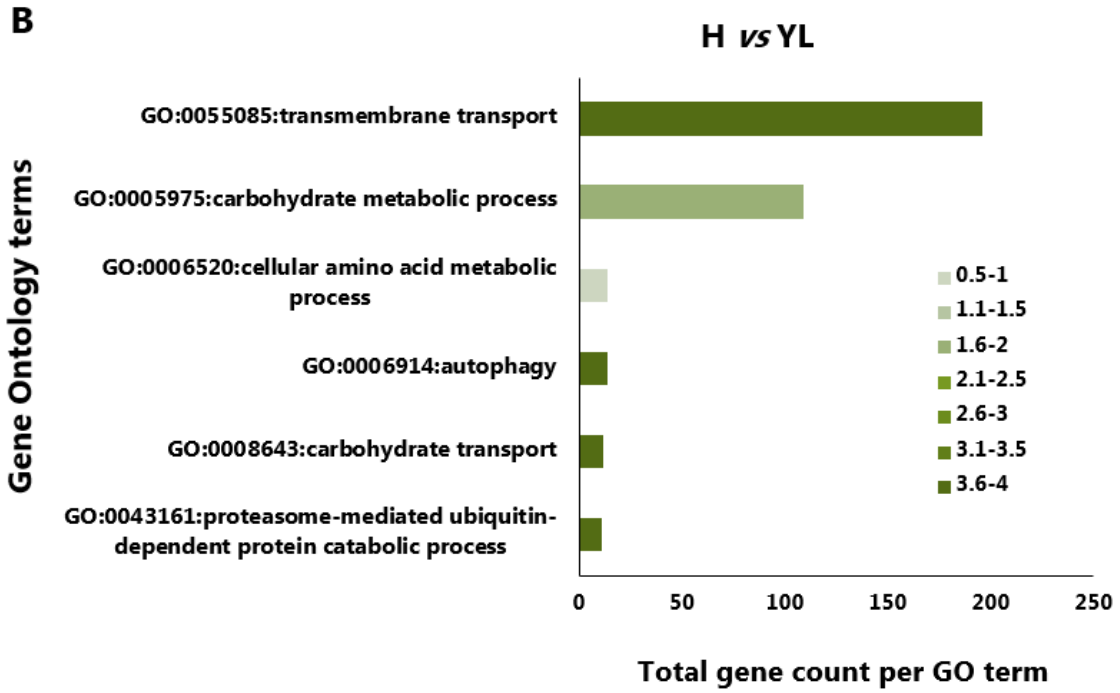


Figure 5. Gene ontology (GO) terms related to biological process enriched in yeast-like cells (YL) grown in liquid medium (A) and hyphae (H)(B). The color of the bar denotes –log10(FDR-*p-value* from enrichment score). The stronger the color, the more significant is the enrichment.

6.3.3. Up-regulated genes and enriched protein families associated with yeast-like cells grown in liquid culture medium

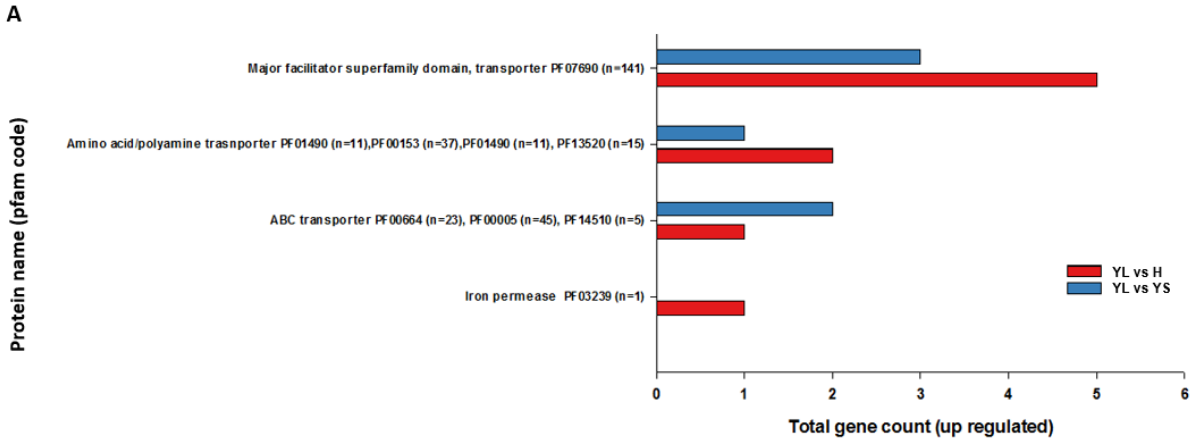
So far we have showed the overall differences between the metabolisms of YL, YS and H by identifying the main GO terms enriched with respect to biological process, cellular components and molecular function. Here, we go a step further by focusing on up regulated genes in YL associated with nutrient transport and absorption, membrane and cell wall components and enriched protein families (pfam). We emphasized those that could be associated with the induction or the maintenance of yeast-like cells growth in liquid culture media.

We found 186 genes up regulated in YL compared to H and 340 genes compared to YS. However, of these, 77 and 131 genes respectively were attributed to “uncharacterized protein” (Additional file 2). Therefore, we focused on those with available functional annotation for protein families (pfam) in which several were attributed to nutrient transport and absorption and membrane and

cell wall components. We emphasize that protein families (pfam) could be assigned to 6185 (70.5%) out of 8763 genes in the *M. rileyi* genome.

The majority of nutrient transporters are expressed at the plasmatic membrane, and their expression levels are strongly regulated by substrate availability (Busto and Wedlich-Soldner, 2019). Here, we found four groups of transporters differently up-regulated in YL (Figure 6A) being the substrate transporter, the Major Facilitator Superfamily Domain (MFS) (pfam: PF07690) the one with most genes up-regulated. Compared to hyphae, we found more genes related to the Major facilitator superfamily domain (MFS) (n=5) and amino acid transporter (n=2) than compared to YS (n=3) and (n=1), respectively. Additionally, we found one gene related to iron permease in YL, up-regulated compared to H. Whilst, compared to YS, we found two up regulated genes related to ABC transport while one compared to H.

The composition of cellular membrane and cell wall and the presence of related-receptors/proteins can vary greatly between fungal structures and can provide clues about their morphogenesis. We found integral membrane proteins and CFEM domain genes up-regulated in YL compared to YS and H. The CFEM domain represents proteins involved in different functional categories such as cell wall biogenesis and integrity and fungal pathogenesis (Zhang et al., 2015). Two genes related to hydrophobins and one to mannosidase, an enzyme which hydrolyses mannose, a fungal cell walls polymer, were up regulated compared to BS whilst two genes related to cell wall glucan synthesis, and one endoglucanase were found up regulated in BL compared to H (Figure 6B).



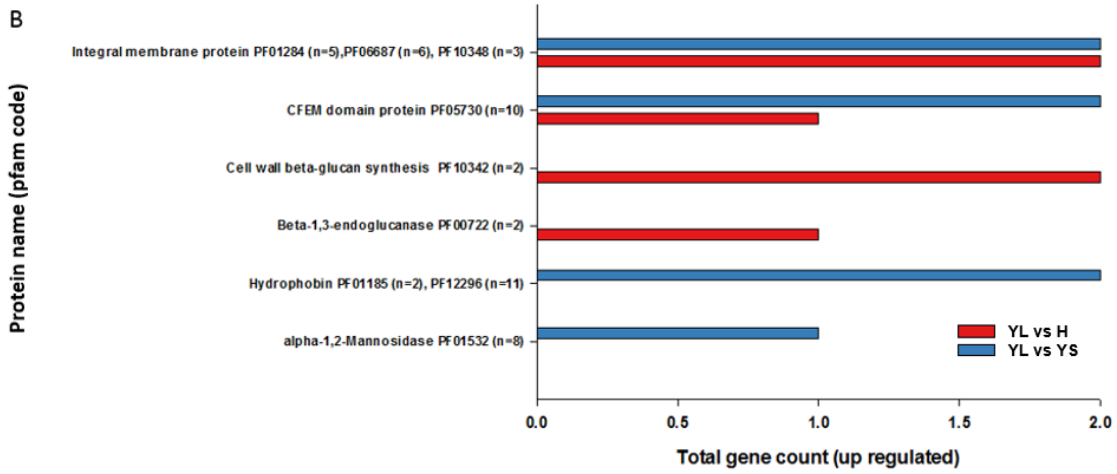


Figure 6. Total up-regulated genes ($Log_2FC > 2$, FDR-*p-value* < 0.001) related to the main protein families of nutrients transporters (A) and cell wall and membrane components (B), in yeast-like cells grown in liquid medium compared to hyphae and yeast-like cells grown in solid medium. In brackets, after pfam code is the number of genes associated to each pfam in the genome of the *Metarhizium rileyi*

Corroborating with the gene ontology enrichment analysis (Figure 5) we found many enriched protein families related to cellular growth in YL compared to YS and H (Additional file 2) (Figure 7). However, we found enriched protein family involved in response to oxidative stress only compared to YS (*PF00085*:Thioredoxin) that were not enriched compared to H (Additional file 2). Supporting these findings, we found up-regulated genes related to oxidative stress in YL compared to YS, such as catalase (gene id: OAA43715), amine oxidase (gene ids: OAA34703, OAA35554), alcohol dehydrogenase (gene ids: OAA36876, OAA41269), Alpha-hydroxy acid dehydrogenase (gene id: OAA36879), monooxygenase (gene id: OAA36869) thioredoxin reductase GliT (gene id: OAA50368), Thioredoxin-like fold protein (gene id: OAA40681) and Dimethylaniline monooxygenase (gene id: OAA40041) (Additional file 2).

Conversely, compared to hyphae we found an enriched protein family related to heat shock protein 60 (Hsp60) (*PF00118*: TCP-1/cpn60 chaperonin family) (Additional file 3) and three genes up- regulated associated with Hsp20 (gene id: OAA50337, OAA49406, OAA34484, pfam: PF00011). while compared to YS we found only one up regulated heat shock protein 70 (gene id:

OAA39357, no pfam assigned). We highlight that any Hsp were found up regulated in YS compared to H (Additional file 2).

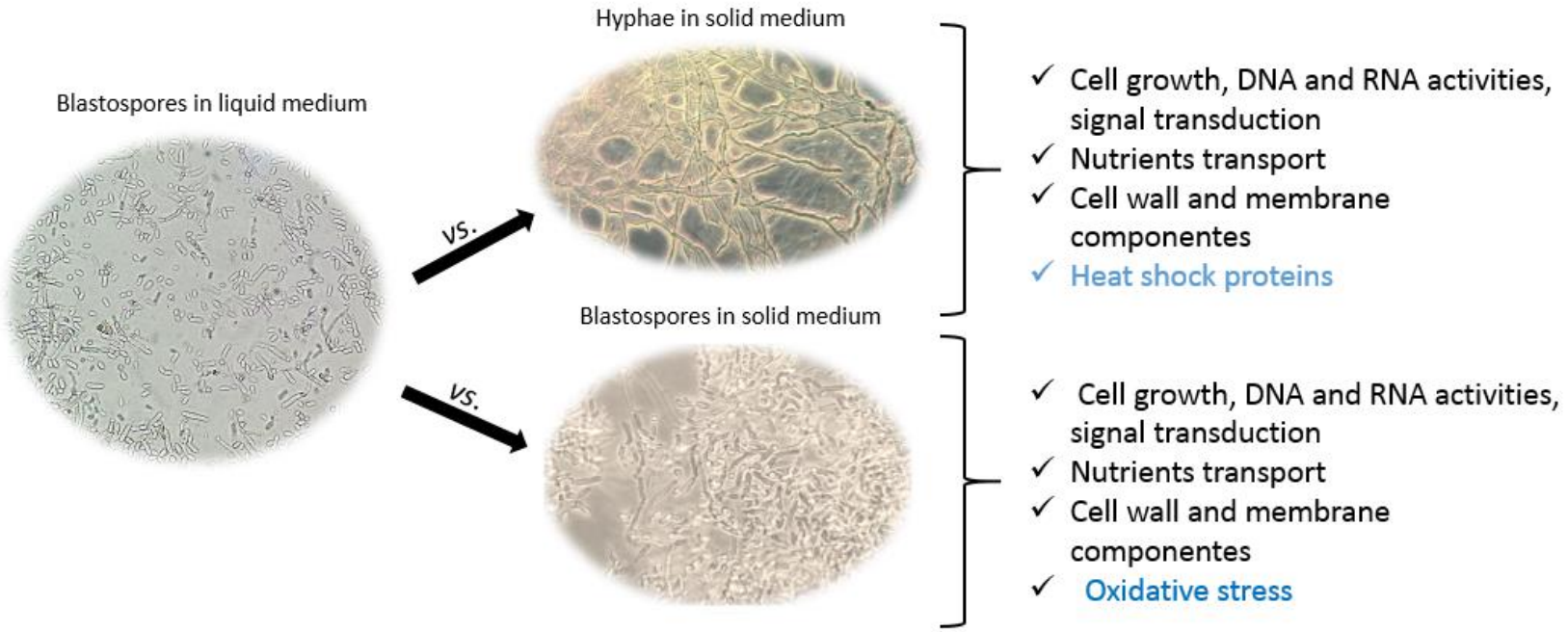


Figure 7. Main metabolic activities found in blastospores grown in liquid medium compared to hyphae and blastospores grown in solid medium based on gene ontology (GO) and protein family (pfam) enrichment analysis. Phase-contrast microscopic image of *Metarhizium rileyi* (ESALQ4948) hyphae produced on Jackson´s solid medium after six days incubated at 28°C and 12h photoperiod (magnification: 200x); Blastospores grown in Jackson´s liquid medium after three days under 350 rpm, 28°C and 12h photoperiod and blastospores grown in SMAY medium after three days and incubated at same conditions as for hyphae (magnification: 400x).

6.3.4. Signature of yeast-like cells in solid culture medium

To elucidate the main signature of yeast-like cells grown in solid medium, we first analyzed the most highly up-regulated genes ($Log_2FC > 4$, FDR-*p-value* < 0.001) and enriched proteins families (pfam) in YS compared to H and YL.

In total we found 66 highly up-regulated genes in YS of which a significant proportion (42.64%) were classified as “Uncharacterized protein” with no available annotation related to protein family (pfam) and gene ontology (GO terms) (Additional file 1 and 2). The remaining 57.64% of genes were related to enzymes that mediate nutrient acquisition such as Trehalase (gene id: OAA35535), Taurine catabolism dioxygenase (gene id: OAA51918), nitroreductase (gene id: OAA51918), amino acid permeases and lipase; to various genes involved in nutrient and substance transport such as Major facilitator superfamily domain, carboxylic acid protein transporter, and oligopeptide transporter and to Metalloproteases such as Peptidase M43 (gene id: OAA35764) and Deuterolysin (gene id: OAA34772) (Additional file 2, Figure 8).

Corroborating these findings, we found 18 enriched protein families in YS compared to hyphae that included proteins related to amino acid acquisition (pfam: *PF01490*; *PF00324*; *PF13520*) and Taurine catabolism dioxygenase (pfam: *PF02668*) (Additional file 3). Conversely, compared to yeast-like cells grown in liquid medium we found 28 enriched protein families related to nutrient transport and acquisition such as Sugar (and other) transporter (pfam: *PF00083*), Major Facilitator Superfamily (*PF07690*), Amino acid permease (*PF13520*) and Transmembrane amino acid transporter protein (*PF01490*) and Fungal specific transcription factor domain (P*F04082*), (Additional file 3).

Next, in order to determine whether common up regulated genes in YS compared do YL and H were *M. rileyi*-specific and to what extent they were exclusively involved in *M. rileyi* YS morphogenesis, we conducted a protein orthology analysis of those genes across other dimorphic fungi including all *Metarhizium* species with available genomes. The orthogroups (genes derived from a common ancestral gene) conserved in the *M. rileyi* and on species that grow as yeast-phase in solid surfaces might provide us some insights about molecular mechanism that trigger formation of YS; Three examples are: *Z. tritici*, *B. bassiana* and *C. albicans*.

A total of 264 orthogroups were assigned to the 276 common up-regulated genes in YS ($Log_2FC > 2$, FDR-*p-value* < 0.001) (Additional file 4). We found

orthogroups shared with *C. albicans* (n=46), *U. mayidis* (n=79), *Z. tritici* (n=126), *B. bassiana* (both isolate) (n=142), *P. chlamydosporia* (both isolate) (n=167) and *Metarhizium* spp. (n=222) (Additional file 4). Furthermore, 29 out of 264 orthogroups were shared only with at least one *Metarhizium* species and three (OG0000924, OG0008162, OG0008163) orthogroups had at least one gene representative in all *Metarhizium* spp. (Additional file 4). One orthogroup was shared only with *B. bassiana* (OG0010358), two shared exclusively with *Z. tritici* (OG0009257, OG0011214) and one shared with *B. bassiana* and *Z. tritici* (OG0008298) exclusively (Additional file 4).

The orthogroup: (OG0008298), shared only with *B. bassiana* and *Z. tritici* was composed of 10 orthologous genes related to aminoacid permeases in which two genes were found for each *B. bassiana* isolate, four genes were from Z. *tritici* and one gene found for each *M. rileyi* isolate (gene id of studied isolate: OAA36856 and part of enriched protein family PF13520: Aminoacid permeases compared to H and YL) (Additional file 4). Nitroredutases and permeases, are enzymes that play a role in the reduction of nitrogen-containing compounds and nutrient acquisition, respectively. We found that *M. rileyi* isolates had in their genomes two orthologous genes related to nitroreductase (gene ids in studied isolate: OAA39368, OAA44961) grouped in orthogroup OG0004805 while all *Metarhizium* species, both isolate of *P. chlamydosporia*, Z. *tritici* and *U. mayidis* had only one gene and any found in *B. bassiana* isolates (Additional file 4). The ML phylogenetic analysis showed that the sequence of the nitroreductase found highly expressed in YS (gene id: OAA39368) compared to H and YL (Figure 8) grouped with that found in *Z. tritici* while the other nitroredutase gene was not differentially expressed in this study grouped with all *Metarhizium* species genes (Appendix D) .

The enzyme Peptidase M43, pregnancy-associated plasma-A was showed to be involved in sporulation, cell wall integrity and virulence factors in *M. robertsii* (Zhou et al., 2018). Here we found that *M. rileyi* had three orthologous genes related to Peptidase M43 in the orthogroup OG0007192 while *M. album, M. brunneum* and *M. robertsii* had only one gene and other *Metarhizium* species had none. One of the *B. bassiana* isolate has two genes in this orthogroup while the other two genes. Additionally, we found a protein named: Pal1 cell morphology (gene is: OAA40599 and orthogroup: OG0000142) regulated in YS. This is a membrane associated

protein involved in the maintenance of cylindrical cellular morphology (Ge et al. 2005). All species had one gene in the OG0000142 ortogroup except by *C. albicans* and *U. mayidis* that has none.

Additionally, YS up-regulate two Heat-labile enterotoxins, one from the orthogroup OG0004298 (gene id: OAA43809) and the other, representing one of the most up regulated gene in YS compared to YL (Log_2FC 12.7, FDR-*pvalue* < 0.001) from the orthogroup OG0000918 (gene id: OAA35032) ; both are enzymes related to virulence. It is noteworthy that *M. rileyi* has three genes in orthogroup OG0000918 while other *Metarhizium* species have only one and the others species none (Additional file 4). Nonetheless, a total of 38 Orthogroups were found to be *M. rileyi*-specific and were not shared with any fungi analyzed in this study such as an unknown cell wall protein (orthogroup: OG0012636, gene id: OAA43728) and the high up-regulated collagen protein (orthogroup: OAA42706, gene id: OG0012597) and (Additional file 4). We found that 2/3 of the collagen protein gene sequence is made up of multiple repeats of a repeat sequence with different GC content. This repeat part of the gene provides high hits (searching against the non-redundant (nr) database on Blast) with insect sequence such as the with the moth *Trichoplusia ni* collagen alpha chain-like isoforms and no or low hit score with other fungi. It is noteworthy that the coding part of this gene is unique to *M. rileyi* and has no hits with any other species. All these findings indicate that the induction of yeast-like cells in solid agar medium requires the expression of genes found exclusively in *M. rileyi*.

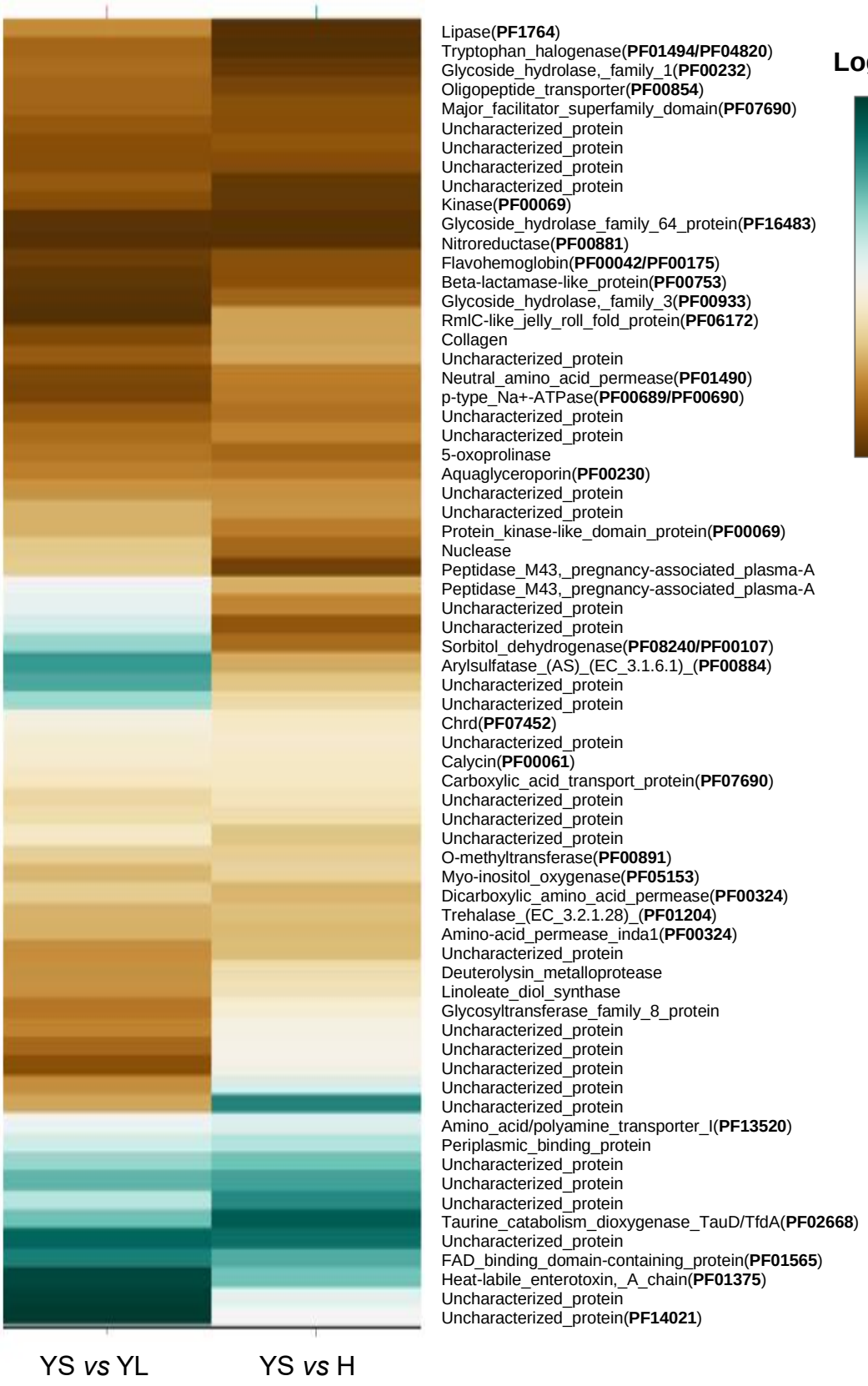


Figure 8. Heatmap of *Metarhizium rileyi* most highly up-regulated genes in yeast-like cells grown on solid medium (YS) compared to yeast-like cells (YL) grown on liquid medium and hyphae (H). Log2FC > 4 and FDR-*p-value* < 0.001*)* (Additional file 2).

6.4. Discussion

M. rileyi is an entomopathogenic fungus with a specialist host range that distinguishes it from other *Metarhizium* species by a different development in culture medium and higher nutritional requirements (Fronza et al. 2017, Pendland and Boucias, 1997). The lack of knowledge concerning the main metabolism and genetic signatures of the unique solid yeast-phase is one of the bottlenecks of large-scale production of *M. rileyi* by the biocontrol industry. In the present work, we used genomic approaches to document the main biological processes and up-regulated genes involved in the induction and maintenance of yeast-like cells grown in liquid medium (YL) and to clarify genomics signatures of yeast-like cells grown in solid medium (YS).

Using gene set enrichment analysis of gene ontology (GO) and of protein families (pfam) we showed that YL up-regulates a set of genes related to cell growth, replication and signal transduction. This clearly shows that an active metabolism is required by yeast-like cells during their growth phase, when yeast-like cells were harvested for mRNA extraction.

One aspect of such activated metabolism is the recognition of an external stimulus through a signal transduction process and translation into an internal trigger for a specific cellular response (Lengeler et al. 2000). Here, the up-regulated genes associated with signal transduction might be involved in transmitting signals to the cell indicating that external conditions are favorable to maintain basal metabolism and to induce cell replication. These candidate genes were grouped in enriched GO terms such as signal transduction (GO:000716) and internal signal transduction (GO:0035556). In fact the involvement of signal transduction in cell development was shown by Fang et al. (2007). For *M. anisopliae,* gene disruption of a protein involved in signal transduction pathways (G protein, cag8) resulted during growth in liquid medium in the formation of irregularly shaped yeast-like cells and in some cases in lysis. Additionally, signal transduction processes are known to regulate dimorphic growth in several fungi such as *Saccharomyces cerevisae*, *Aspergillus nidulans* and *U. maydis* (Lengeler et al. 2000).

To support the intense metabolism of cell growth, we previously showed that liquid-cultivated yeast-like cells of *M. anisopliae* need to import nutrients from the outside environment (data not shown), a process being mediated by various types of membrane transporters (Busto and Wedlich-Soldner, 2019). In fact, our results showed that yeast-like cells activated three types of nutrient transporters compared to hyphae (H) and YS but, only compared to H, it up regulated one iron permease, which is a membrane protein that play a role in uptake extracellular ferrous iron (Fe^{2+}). It is noteworthy that the protein family *PF03239* is associated exclusively to the up regulated iron permease which makes it unique among all up-regulated genes in YL. Therefore, these findings may indicate either that *M. rileyi* YL and H acquire iron using distinct mechanisms or that yeast-like cells have a greater need for this metal as evidenced by the intense growth reported in YL.

We found strong evidence that oxidative stress is required for YL formation and maintenance. Oxidative stress is an imbalance between free radical such as reactive oxygen species (ROS) and cell antioxidants (Betteridge, 2000). ROS accumulation is commonly associated with harmful effects on cells such as aging of *M. anisopliae* mycelia (Wang et al. 2005). However, it has also been associated with growth and germination in *M. anisopliae* (Hernandez et al. 2010) and with the development of microsclerotia in *M. rileyi* (Song et al. 2013). In this work, we found that YL compared to YS up regulates genes related to the response to oxidative stress such as catalases, monoxygenases and the Thioredoxin protein family *(PF00085*). These are proteins with known antioxidant activity in fungi that participate in the response to oxidative stress (Angelova et al. 2005, Wang et al. 2013, Haijie et al. 2018; Garrido et al. 2002, Ying and Feng, 2012). It should be mentioned that YL formation was induced in liquid medium under high agitation in order to incorporate the oxygen needed for appropriate yeast-like cells growth (Mascarin et al. 2015). Therefore, the oxidative stress responses in YL could be due to high oxygen levels in liquid culture medium. If that were the case, we would expect to find genes and enriched GO terms or Pfam's associated with a higher response to oxidative stress in YL compared to H. However, we only found a higher response compared to YS indicating that oxidative stress is not related to growth conditions. While higher responses to oxidative stress were evidenced in YL compared to YS, we found a higher response in YL than in H related to activation of heat shock proteins. This

finding is supported by the enriched protein family related to heat shock proteins (pfam: *PF00118*: TCP-1/cpn60 chaperonin family – Heat shock protein 60) and three genes up regulated to Heat shock protein 20 (pfam: *PF00011*) found in YL.

Heat shock proteins (Hsp) are broad range proteins that play a role in various cellular responses such as increases in temperatures, stress conditions, growth and cell differentiation (Tiwari et al. 2015, Izzac et al. 2001). The Hsp60 were previously shown to be highly expressed in the yeast phase of the dimorphic human pathogen fungus *Paracoccidioides brasiliensis* compared to its hyphae phase (Izzac et al. 2001). The authors argued that Hsp60 could play a role in morphogenesis but also could be necessary for the maintenance of *P*. *brasiliensis* in host thermal conditions (37°) as the yeast phase is thermally induced in this species. In our study, yeast-like cells and hyphae were cultivated at the same incubation temperature (28°C), thus we would expect Hsp60 to play a role in yeast-like cells morphogenesis or other stress responses that are not related to increased temperatures. In fungi, Hsp60 and small Hsp, such as Hsp20, control osmotic stress (Tiwari et al. 2015). Here, we found an enriched protein family associated to Hsp60 and three Hsp20 genes more up-regulated in YL than in H. The fact that we found more Hsp60 and Hsp20 up regulated in YL than in YS and any up regulated Hsp in YS compared to H may indicate that osmotic stress is involved in YL formation and maintenance but not in YS. Osmotic stress was suggested to prompt blastospore production of *Beauveria bassiana* (Mascarin et al. 2015) and *M. robertsii* (Iwanicki et al. 2018). When cultivated in hyperosmotic conditions, *B. bassiana* produced significantly more blastospores than in normal conditions. However, further studies are needed to identify genes related to major metabolic pathways involved in osmotic stress response, such as the well-known high-osmolarity glycerol (HOG) pathway (Hohmann, 2002).

Stressful conditions, such as osmotic and oxidative stress are first recognized by the plasma membrane, as it is the first cell barrier between a cell and its environment. Therefore, we expected to find different gene expression profiles in YL and in H or YS related to membrane proteins. However, in the present work, we found few differences in the number of up-regulated genes related to cell and membrane components between YL and YS and YL and H. Most of the genes found up regulated in YL were related to cell wall synthesis and degradation that normally

occurs during cell growth. The only exceptions were the two hydrophobins found up-regulated in YL only and not in YS. Hydrophobins are components of the rodlet layer of conidia cell surfaces, which play a role in virulence and resistance to environmental stress and are not commonly found in yeast cells (Bayry et al. 2012). However, our findings that two hydrophobins were found up regulated in YL but not in YS indicate that the former has a more hydrophobic cell surface and is more resistant to environmental stress than YS (Bayry et al. 2012).

In fact, our results clearly showed that yeast-like cells produced in solid (YS) and liquid (YL) medium are distinct *M. rileyi* yeast-like forms that activate a set of different genes. Nonetheless, we showed that YL up-regulated almost twice as many genes as YS (n = 340) or hyphae (n=186). We expected to find the opposite, as hyphae are multicellular and have a more complex metabolism than unicellular yeast. Morphologically, yeast-like cells formed in solid media are significantly different from yeast-like cells formed in liquid media. While the former resemble pieces of hyphae, the latter resemble much smaller, more rounded yeast cells. These characteristics may be a result of the physical condition of the culture (air versus water) and the osmolarity of the medium.

The orthology analysis showed strong evidence that the induction of yeast-like cells in solid agar medium requires: 1) the expression of genes found exclusively in *M. rileyi*, 2) genes that are not orthologous with *Metarhizium* species, or when they are, they show a higher similarity with any *Metarhizium* species, and 3) activation of orthologous genes from orthogroups in which *M. rileyi* has more genes while other *Metarhizium* spp have only one.

We showed that *M. rileyi* yeast-like cells grown in solid medium highly up-regulated a specific aminoacid permease with orthologous genes exclusively in entomopathogenic *B. bassiana* and phytopathogenic *Z. tritici* fungi. Amino acid permease, as membrane proteins, may have two functions: transport of amino acids into the cell or assessors that trigger signals in response to external amino acids (Boles and André 2004). In the case of *B. bassiana* and *M. rileyi*, the activation of this protein in yeast-like cells may be related to the detection of amino acids in insect hemolymph and therefore be interpreted as a constitutive response of this cell type. Nonetheless, in *M. rileyi* this amino acid permease might play a role in signaling of a quorum-sensing system involved in switching from the yeast-like cells to the mycelial

phase *in vivo* at late stage of infection (Boucias et al. 2016). It is through this membrane protein that yeast-like cells detect an amino acid shortage in hemolymph and therefore signal the induction of hypha formation. Conversely, in *Z. tritici* this amino acid may be involved in ability of yeast-like cells (blastospores) to use amino acids extracted from the surface of wheat leaves (Fransisco et al. 2019).

Besides amino acid permease, we found that *M. rileyi* has two nitroreductases in its genome while all *Metarhizum* spp. have only one. One of these nitroreductases, not differentially expressed, showed a similar sequence to those with other *Metarhiizum* spp while the one that was up-regulated in YS had a higher similarity to a nitroreductase found in *Z. tritici*. This may be related to a unique ability of *Z. tritici* and *M. rileyi* to degrade specific nitric compounds present on the surface of wheat leaves and on the solid culture medium respectively. Degraded compounds may be required for the induction and maintenance of the yeast phase in those species. Nonetheless, the specific amino acid permease previously discussed might be involved in transporting those degraded compounds by nitroreductase or by signaling the presence of specific compounds. However, the complementary action of these genes in the process of induction of yeast-like cells in solid medium should be elucidated in future research.

We found that the *M. rileyi* yeast-like cells growth form might be mediated by other membrane proteins in addition to amino acid permease such as Pa11 or the metalloproteases Peptidase M43. The Pa11 protein is associated with the maintenance of cylindrical cellular morphology and was previously shown to be involved in cellular morphogenesis and cell wall integrity in fission yeast (Ge et al., 2005). Additionally, a recent study showed that one *M. robertsii* Peptidase (M43) is involved in sporulation, cell wall integrity and virulence (Zhou et al., 2018). Here, the higher number of orthologous genes related to Peptidase M43 in *M. rileyi* (n=3) compared to one gene found in only three *Metarhizium* spp. and the fact that we found two of these enzymes highly up regulated in YS, might indicate the specific new role of these genes in yeast-like cells morphogenesis and virulence. In addition to genes associated with nutrient acquisition, membrane proteins and with signaling, we found several genes related to virulence highly up-regulated in YS which lead us to consider the expression of virulence genes one of the main signatures of YS.

In the present work, we expected to find more genes related to virulence factors in YL than in YS. This was because to some extent the liquid medium mimics the insect hemolymph, which as far as we know, is the only environment where *Metarhizium* spp yeast-like cells are naturally produced. However, we found strong evidence that YS more closely resembles the yeast-like cells naturally produced within the insect than does YL. For example, a trehalase enzyme was found much more up-regulated in YS compared to YL or to H. This enzyme is responsible for degrading trehalose, the main sugar component of insect hemolymph (Wyatt 1961) and is not present in the SMAY culture medium. Another example of a potential virulence factor is the collagen-like protein that has no orthologues genes in *Metarhizium* spp. and other fungi. However, it's tempting to suggest that the collagen-like protein function is analogous to the Collagen-Like protein (Mcl) expressed in *M. robertsii* yeast-like cells (Wang and Leger, 2006). Mcl protein masks antigenic components of the cell wall of yeast-like cells that allows it to evade the insect's immune response (Wang and Leger, 2006). Pendland and Boucias (1998) speculated that particular epitope found in *M. rileyi* cell wall hyphal bodies may have the unique "ability" to "mimic" host cell molecules and thus be not recognized by hemocytes in host hemolymph. The collagen-like protein found exclusively in *M. rileyi* may be one of the epitope previously mentioned by the authors. Additionally, the higher similarity of the repeat sequence of collagen-like protein gene with lepidopteran species may be related to specificity of this species to lepidopteran and has been investigated by our team. Nonetheless the close proximity of coding region to the repeat sequence could indicate that the gene has the capacity to be or has been fast evolving.

The fact that yeast-like cells produces a large number of genes related to virulence factors, without a need of hemolymph to induce it, is a strong indication of the potential of this unexplored propagule for pest control. *B. bassiana* yeast-phase induced in solid medium has shown a higher virulence (lower medium lethal concentration and lethal time) than conidia against *Diatraea sacchalais* larvae, an economically important sugar cane pest (Alves et al. 2002). However, the virulence of *M. rileyi* yeast-like cells produced in solid medium has never been demonstrated in any pest. While the ecological function of yeast-like cells in *Z. tritici* has been proposed as a strategy to increase initial inocula on leaf surface (Fransisco et al.

2019) our findings indicate that *M. rileyi* yeast-like cells might be formed not only in hemolymph but also in non-liquid parts of the insect.

It is important to note that some enriched protein families found in YS such as Taurine catabolism dioxygenase (PF02668) are probably enriched due to the medium composition with taurine amino-acid from neopeptone protein source in the SMAY medium, but not present in media used to grown YL and H. Therefore, Taurine catabolism dioxygenase protein family could not be attributed to specific signatures of YS. Another fact that we wish to emphasize is that a very high number of common up-regulated genes (42.64%) in YS compared to YL and H was associated with “Uncharacterized protein”. Therefore, annotation of these genes should be addressed in future research to have a better understanding of the formation of this fungal structure in solid medium.

In summary, we have shown that yeast-like cells produced in liquid medium activate a series of specific genes related to signal transduction, that might be associated with cell growth as well as activation of specific membrane transporters related to iron acquisition. To the best of our knowledge, this is the first report on application of a high-throughput mRNA sequencing analysis to elucidate gene expression of *M. rileyi* blastospores. We showed that oxidative stress and activation of specific heat shock proteins are key factors involved in BL formation. On the other hand, yeast-like cells grown in solid medium have a unique set of genes associated to enzymes such as an aminoacid permease that are active, and not found in other *Metarhizium* spp. Nonetheless, YS activate a unique nitroreductase, specific membrane proteins and several virulence factors. Our study lays the foundation for further exploration of the main metabolism required for yeast-like cells growth in liquid medium and identified candidate genes that will serve as a basis for future research on optimizing *M. rileyi* yeast-like cells production. The fact that YS produces virulence factors constitutively opens new opportunities to explore yeast-like cells produced in solid medium as a component of biopesticides.

References

Alves SB, Rossi LS, Lopes RB, Tamai MA, Pereira RM (2002) *Beauveria bassiana* yeast phase on agar medium and its pathogenicity against *Diatraea saccharalis*

(Lepidoptera: Crambidae) and *Tetranychus urticae* (Acari: Tetranychidae). J Invertebr Pathol 81:70–77.

Angelova MB, Pashova SB, Spasova BK, Vassilev S V., Slokoska LS (2005) Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol Res 109:150–158.

Asmann YW, Klee EW, Thompson EA, Perez EA, Middha S, et al. (2009) 3' tag digital gene expression profiling of human brain and universal reference RNA using Illumina Genome Analyzer. BMC Genomics 10: 531.

Bayry J, Aimanianda V, Guijarro JI, Sunde M, Latgé JP (2012) Hydrophobins-unique fungal proteins. PLoS Pathog 8:6–9.

Betteridge DJ (2000) What is oxidative stress? Metabolism 49:3–8.

Boles E, André B (2004) Role of transporter-like sensors in glucose and amino acid signalling in yeast. In: Molecular Mechanisms Controlling Transmembrane Transport. Springer Berlin Heidelberg, Berlin, Heidelberg 155–178.

Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20.

Boucias D, Liu S, Meagher R, Baniszewski J (2016) Fungal dimorphism in the entomopathogenic fungus *Metarhizium rileyi*: Detection of an in vivo quorum-sensing system. J Invertebr Pathol 136:100–108.

Busto J V., Wedlich-Söldner R (2019) Integration through separation - The role of lateral membrane segregation in nutrient uptake. Front Cell Dev Biol 7:1–6.

Emms DM, Kelly S (2019) OrthoFinder: Phylogenetic orthology inference for comparative genomics. Genome Biol 20:1–14.

Fang W, Pei Y, Bidochka MJ (2007) A regulator of a G protein signalling (RGS) gene, cag8, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation virulence and hydrophobin synthesis. Microbiology 153:1017–1025.

Fischhoff IR, Keesing F, Ostfeld RS (2017) The tick biocontrol agent *Metarhizium brunneum* (= *M. anisopliae*) (strain F52) does not reduce non-target arthropods. PLoS ONE 12(11): e0187675.

Francisco CS, Ma X, Zwyssig MM, McDonald B a., Palma-Guerrero J (2019) Morphological changes in response to environmental stresses in the fungal plant pathogen *Zymoseptoria tritici*. Sci Rep 9:9642.

Fronza E, Specht A, Heinzen H, de Barros NM (2017) *Metarhizium* (*Nomuraea*) *rileyi* as biological control agent. Biocontrol Sci Technol 27:1243–1264 .

Garrido EO, Grant CM (2002) Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides. Mol Microbiol 43:993–1003.

Gauthier GM (2015) Dimorphism in Fungal Pathogens of Mammals, Plants, and Insects. PLoS Pathog 11:1–7.

Griffith M, Griffith OL, Mwenifumbo J, Goya R, Morrissy a S, et al. (2010) Alternative expression analysis by RNA sequencing. Nat Methods 7: 843–847

Goettel MS, Roberts DW (1991) Mass production formulation and field application of entomopathogenic fungi, p. 232-238. In C.J. Lomer & C. Prior (eds.). Biological control of locusts and grasshoppers. Wallingford, UK, 256p.

Hernandez CEM, Guerrero IEP, Hernandez GAG, Solis ES, Guzman JCT (2010) Catalase over expression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*. Appl Microbiol Biotechnol 87:1033–1044.

Hohmann S (2002) Osmotic Stress Signaling and Osmoadaptation in Yeasts. Microbiology And Molecular Biology Reviews, 66(2):300–372.

Iwanicki NSA, Ferreira BDO, Mascarin GM, Júnior ÍD (2018) Modified Adamek's medium renders high yields of *Metarhizium robertsii* blastospores that are desiccation tolerant and infective to cattle-tick larvae. Fungal Biol 1–8

Jarosnki ST, Jackson MA (2012). Mass production of entomopathogenic Hypocreales. In Lacey LA, ed. Manual of Techniques in Invertebrate pathogoly, 2nd ed. Academic Press, London, UK pg 255-284

Khomtchouk BB, Hennessy JR, Wahlestedt C (2017) Shinyheatmap: Ultra fast low memory heatmap web interface for big data genomics. PLoS One 12:1–9.

Lengeler KB, Davidson RC, D'souza C, Harashima T, Shen W-C et al. (2000) Signal Transduction Cascades Regulating Fungal Development and Virulence. Microbiol Mol Biol Rev 64:746–785.

Lovett B, St. Leger RJ (2014) Stress is the rule rather than the exception for Metarhizium. Curr Genet 61:253–261.

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.

Lu H, Leger RJS (2016) Insect Immunity to Entomopathogenic Fungi.

Ma LJ, van der Does, HC, Borkovich KA, Coleman JJ, Daboussi, MJ, Di Pietro A, Dufresne M, Freitag M, Grabherr M, Henrissat et al. Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. Nature, 464:367–373.

Mascarin GM, Jackson MA, Kobori NN, Behle RW, Júnior ID (2015) Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. J Invertebr Pathol 127:11–20.

Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc 11:1650–67.

Parra J (2014) Biological control in Brazil. Sci Agric 71:345–355.

Pendland JC, Boucias DG (1997) In vitro growth of the entomopathogenic hyphomycete *Nomuraea rileyi*. Mycologia 89:66–71.

Pendland JC, Bouclas DG (1998) Characterization of monoclonal antibodies against cell wall epitopes of the insect pathogenic fungus, *Nomuraea rileyi*: Differential binding to fungal surfaces and cross-reactivity with host hemocytes and basement membrane components. Eur J Cell Biol 75:118–127.

R: A language and environment for statistical computing. 2017. .

Babraham Bioinformatics – FastQC a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Shi Y, He M (2014) Differential gene expression identified by RNA-Seq and qPCR in two sizes of pearl oyster (Pinctada fucata). Gene 538: 313–322.

Subramanian A, Tamayo P, Mootha VK, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 102 (43) 15545-15550.

Song Z, Yin Y, Jiang S, Liu J, Chen H, Wang Z (2013) Comparative transcriptome analysis of microsclerotia development in Nomuraea rileyi. BMC Genomics 14:411.

Song Z, Zhong Q, Yin Y, Shen L, Li Y, Wang Z (2016) The high osmotic response and cell wall integrity pathways cooperate to regulate morphology, microsclerotia development, and virulence in *Metarhizium rileyi*. Sci Rep 6:1–10.

Systems G (2018) crossm Stress Resistance, Fungicide Sensitivity, and Virulence of. 84:1–17

Supek F, Bošnjak M, Škunca N, Šmuc T (2011) Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One 6(7):e21800.

Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30:2725-2729.

Tiwari S, Thakur R, Shankar J (2015) Role of Heat-Shock Proteins in Cellular Function and in the Biology of Fungi. Biotechnol Res Int 2015:1–11.

Wang C, Butt TM, St. Leger RJ (2005) Colony sectorization of *Metarhizium anisopliae* is a sign of ageing. Microbiology 151:3223–3236.

Wang C, St Leger RJ (2006) A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci U S A 103:6647–6652.

Wang M, Zhou Z, Wu J, Ji Z, Zhang J (2018) Comparative transcriptome analysis reveals significant differences in gene expression between appressoria and hyphae in *Colletotrichum gloeosporioides*. Gene 670:63–69.

Wang ZL, Zhang L Bin, Ying SH, Feng MG (2013) Catalases play differentiated roles in the adaptation of a fungal entomopathogen to environmental stresses. Environ Microbiol 15:409–418.

Wu AR, Neff NF, Kalisky T, Dalerba P, Treutlein B, et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11: 41–46.

Whelan S, Goldman N (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Molecular Biology and Evolution 18:691-699.

Wyatt GR (1961) The Biochemistry of Insect Hemolymph. Annu Rev Entomol 6:75–102 .

Ying SH, Feng MG (2011) Integration of Escherichia coli thioredoxin (trxA) into *Beauveria bassiana* enhances the fungal tolerance to the stresses of oxidation, heat and UV-B irradiation. Biol Control 59:255–260.

Zhang ZN, Wu QY, Zhang GZ, Zhu YY, Murphy RW, Liu Z, Zou CG (2015) Systematic analyses reveal uniqueness and origin of the CFEM domain in fungi. Sci Rep 5:1–7.

APPENDIX

Appendix - A. Enriched cellular components and molecular function in yeast-like cell grown in solid medium (YS) and hyphae (H).

Enriched Celular component			
YS		**H**	
GO:0005623	cell		
GO:0005829	cytosol	**GO:0005874**	microtubule
GO:0000139	Golgi membrane	**GO:0005840**	ribosome
GO:0005789	endoplasmic reticulum membrane	**GO:0005730**	nucleolus
		GO:0005743	mitochondrial inner membrane
		GO:0000502	proteasome complex
		GO:0032040	small-subunit processome
		GO:0010494	cytoplasmic stress granule
		GO:0016282	eukaryotic 43S preinitiation complex
		GO:0005852	eukaryotic translation initiation factor 3 complex
		GO:0016592	mediator complex

Enriched Molecular function			
YS		**H**	
GO:0008080	N-acetyltransferase activity	**GO:0003682**	chromatin binding
GO:0015171	amino acid transmembrane transporter activity	**GO:0003735**	structural constituent of ribosome
GO:0051213	dioxygenase activity	**GO:0004497**	monooxygenase activity
GO:0016491	oxidoreductase activity	**GO:0003777**	microtubule motor activity
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	**GO:0004672**	protein kinase activity
GO:0003824	catalytic activity	**GO:0020037**	heme binding
GO:0004871	signal transducer activity	**GO:0008017**	microtubule binding
GO:0005198	structural molecule activity	**GO:0003743**	translation initiation factor activity
GO:0008233	peptidase activity	**GO:0003676**	nucleic acid binding
GO:0008565	protein transporter activity	**GO:0003723**	RNA binding
GO:0051287	NAD binding	**GO:0016705**	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0016746	transferase activity, transferring acyl groups	**GO:0004386**	helicase activity
GO:0008536	Ran GTPase binding	**GO:0003677**	DNA binding
GO:0016874	ligase activity	**GO:0046982**	protein heterodimerization activity
GO:0000287	magnesium ion binding	**GO:0003887**	DNA-directed DNA polymerase activity
GO:0016787	hydrolase activity	**GO:0004499**	N,N-dimethylaniline monooxygenase activity
GO:0008270	zinc ion binding	**GO:0030246**	carbohydrate binding

GO:0003779	actin binding	**GO:0004553**	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0046983	protein dimerization activity	**GO:0031177**	phosphopantetheine binding
GO:0071949	FAD binding	**GO:0005509**	calcium ion binding
GO:0046872	metal ion binding	**GO:0004298**	threonine-type endopeptidase activity
GO:0022891	substrate-specific transmembrane transporter activity	**GO:0004725**	protein tyrosine phosphatase activity
GO:0015078	hydrogen ion transmembrane transporter activity	**GO:0005506**	iron ion binding
		GO:0004519	endonuclease activity
		GO:0004222	metalloendopeptidase activity

Appendix B - Enriched cellular components and molecular function in yeast-like cell grown in solid medium (YS) and in liquid medium (YL).

Enriched celular components			
YS		**YL**	
GO:0005789	endoplasmic reticulum membrane	**GO:0005730**	nucleolus
GO:0005783	endoplasmic reticulum	**GO:0005643**	nuclear pore
GO:0005794	Golgi apparatus	**GO:0005737**	cytoplasm
		GO:0005743	mitochondrial inner membrane
		GO:0005840	ribosome
		GO:0005852	eukaryotic translation initiation factor 3 complex
		GO:0005874	microtubule
		GO:0016282	eukaryotic 43S preinitiation complex
		GO:0016592	mediator complex
		GO:0032040	small-subunit processome
		GO:0033290	eukaryotic 48S preinitiation complex
		GO:0000502	proteasome complex
		GO:0010494	cytoplasmic stress granule
		GO:0019013	viral nucleocapsid
		GO:0030529	intracellular ribonucleoprotein complex
Enriched molecular function			
YS		**YL**	
GO:0005506	iron ion binding	**GO:0003676**	nucleic acid binding
GO:0008080	N-acetyltransferase activity	**GO:0003677**	DNA binding
GO:0015171	amino acid transmembrane transporter activity	**GO:0003682**	chromatin binding
GO:0016491	oxidoreductase activity	**GO:0003684**	damaged DNA binding
GO:0016829	lyase activity	**GO:0003723**	RNA binding
GO:0020037	heme binding	**GO:0003735**	structural constituent of ribosome
GO:0022891	substrate-specific transmembrane transporter	**GO:0003743**	translation initiation factor activity

	activity		
GO:0051213	dioxygenase activity	**GO:0003746**	translation elongation factor activity
GO:0051287	NAD binding	**GO:0003777**	microtubule motor activity
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	**GO:0003887**	DNA-directed DNA polymerase activity
GO:0004298	threonine-type endopeptidase activity	**GO:0003924**	GTPase activity
GO:0008236	serine-type peptidase activity	**GO:0004386**	helicase activity
GO:0008270	zinc ion binding	**GO:0004672**	protein kinase activity
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	**GO:0005096**	GTPase activator activity
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	**GO:0005525**	GTP binding
GO:0016787	hydrolase activity	**GO:0008017**	microtubule binding
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	**GO:0008536**	Ran GTPase binding
GO:0016874	ligase activity	**GO:0001104**	RNA polymerase II transcription cofactor activity
GO:0048037	cofactor binding	**GO:0000049**	tRNA binding
		GO:0000166	nucleotide binding
		GO:0003697	single-stranded DNA binding
		GO:0003899	DNA-directed 5'-3' RNA polymerase activity
		GO:0004499	N,N-dimethylaniline monooxygenase activity
		GO:0004674	protein serine/threonine kinase activity
		GO:0005509	calcium ion binding
		GO:0016887	ATPase activity
		GO:0042802	identical protein binding
		GO:0043022	ribosome binding
		GO:0046982	protein heterodimerization activity
		GO:0051082	unfolded protein binding

Appendix C. Enriched cellular components and molecular function in yeast-like cell grown in liquid medium (YL) and hyphae (H).

Enriched celular components

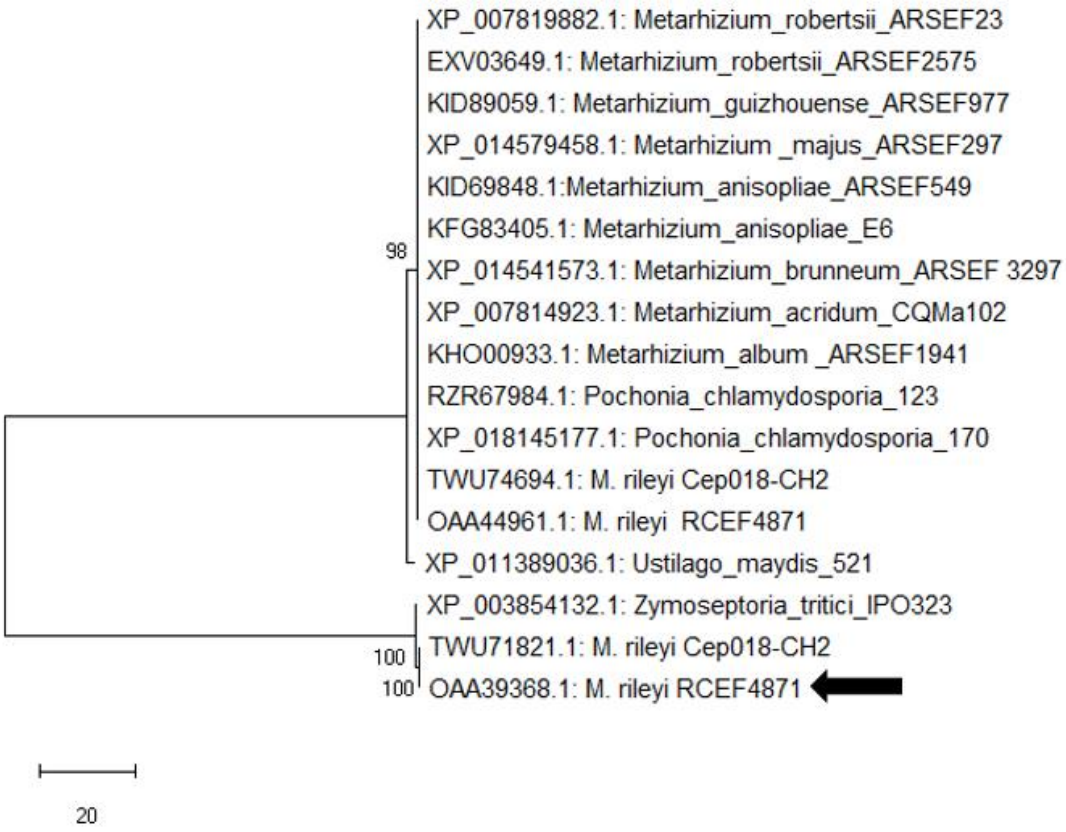
YL		**H**	
GO:0005730	nucleolus	**GO:0004497**	monooxygenase activity
GO:0005829	cytosol	**GO:0020037**	heme binding
GO:0005737	cytoplasm	**GO:0022891**	substrate-specific transmembrane transporter activity
GO:0033290	eukaryotic 48S preinitiation complex	**GO:0004190**	aspartic-type endopeptidase activity

GO:0005852	eukaryotic translation initiation factor 3 complex	**GO:0016491**	oxidoreductase activity
GO:0005739	mitochondrion	**GO:0030246**	carbohydrate binding
GO:0005840	ribosome	**GO:0005506**	iron ion binding
GO:0005643	nuclear pore	**GO:0016787**	hydrolase activity
GO:0019013	viral nucleocapsid	**GO:0016705**	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0016282	eukaryotic 43S preinitiation complex	**GO:0004298**	threonine-type endopeptidase activity
GO:0032040	small-subunit processome	**GO:0042626**	ATPase activity, coupled to transmembrane movement of substances
GO:0005874	microtubule		
GO:0030529	intracellular ribonucleoprotein complex		
GO:0005743	mitochondrial inner membrane		
GO:0005758	mitochondrial intermembrane space		
GO:0005789	endoplasmic reticulum membrane		

Enriched molecular function

YL		H	
GO:0003735	structural constituent of ribosome		
GO:0000049	tRNA binding		
GO:0003676	nucleic acid binding		
GO:0003677	DNA binding		
GO:0003723	RNA binding		
GO:0003746	translation elongation factor activity		
GO:0003777	microtubule motor activity		
GO:0003924	GTPase activity		
GO:0004386	helicase activity		
GO:0005525	GTP binding		
GO:0008017	microtubule binding		
GO:0008536	Ran GTPase binding		
GO:0016887	ATPase activity		
GO:0042802	identical protein binding		
GO:0043022	ribosome binding		
GO:0046872	metal ion binding		
GO:0050660	flavin adenine dinucleotide binding		
GO:0051082	unfolded protein binding		
GO:0051539	4 iron, 4 sulfur cluster binding		
GO:0000166	nucleotide binding		
GO:0003682	chromatin binding		
GO:0003684	damaged DNA binding		
GO:0003697	single-stranded DNA binding		
GO:0003743	translation initiation factor activity		

GO:0003887	DNA-directed DNA polymerase activity
GO:0003899	DNA-directed 5'-3' RNA polymerase activity
GO:0004871	signal transducer activity
GO:0005096	GTPase activator activity
GO:0005509	calcium ion binding
GO:0008168	methyltransferase activity
GO:0008233	peptidase activity
GO:0008565	protein transporter activity
GO:0009055	electron carrier activity
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors
GO:0016746	transferase activity, transferring acyl groups



Appendix D. Maximum Likelihood tree using protein sequences of nitroreductases genes from the Orthogroup: OG0004805. Black arrow indicate the gene up regulated in yeast-phase grown in solid medium. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model (Whelan et al. 2001). Branches are labelled with bootstrap percentage inferred from 1000 replicates (Felsenstein J. 1985). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013)