UNIVERSIDADE FEDERAL DE GOIÁS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO

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Fatores abióticos, tais como luz e anoxia, durante o crescimento melhoram a virulência e outras qualidades fenotípicas em *Metarhizium robertsii*

Abiotic factors, such as light and anoxia, during the growth improves virulence and other phenotypical qualities in *Metarhizium robertsii*

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ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE DISSERTAÇÃO DE ARIEL DE SOUZA OLIVEIRA - Aos doze dias do mês de março do ano de 2018 (12/03/2018), às 14:00 horas, reuniram-se os componentes da Banca Examinadora: Profs. Drs. DRAUZIO EDUARDO NARETTO RANGEL, JOSÉ DANIEL GONÇALVES VIEIRA e GILBERTO ÚBIDA LEITE BRAGA, para, sob a presidência da primeira, e em sessão pública realizada no INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA, procederem à avaliação da defesa de dissertação intitulada: **"FATORES ABIÓTICOS, TAIS COMO LUZ E ANOXIA, DURANTE O CRESCIMENTO MELHORAM A VIRULÊNCIA E OUTRAS QUALIDADES FENOTÍPICAS EM *Metarhizium robertsii*"**, em nível de **MESTRADO**, de autoria de **ARIEL DE SOUZA OLIVEIRA**, discente do PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO, da Universidade Federal de Goiás. A sessão foi aberta pelo Orientador, Prof. Dr. DRAUZIO EDUARDO NARETTO RANGEL, que fez a apresentação formal dos membros da Banca e orientou o Candidato sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida ao autor da dissertação que, em 30 minutos procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca arguiu o Candidato, tendo-se adotado o sistema de diálogo seqüencial. Terminada a fase de arguição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1492/2017 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Biologia da Relação Parasito-Hospedeiro a Banca, em sessão secreta, expressou seu Julgamento, considerando o candidato **Aprovado** ou **Reprovado**:

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Em face do resultado obtido, a Banca Examinadora considerou o candidato HABILITADO (**Habilitado ou não Habilitado**), cumprindo todos os requisitos para fins de obtenção do título de **MESTRE EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO**, pela Universidade Federal de Goiás. Cumpridas as formalidades de pauta, às 16 h 15 min, a presidência da mesa encerrou esta sessão de defesa de dissertação e para constar eu, JOSÉ CLEMENTINO DE OLIVEIRA NETO, secretário do Programa de Pós-Graduação em Biologia da Relação Parasito-Hospedeiro, lavrei a presente Ata que depois de lida e aprovada, será assinada pelos membros da Banca Examinadora e por mim em duas vias de igual teor.
A Banca Examinadora aprovou a seguinte alteração no título da Dissertação:
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3. **Dr. Gilberto Úbida Leite Braga**

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SUMMARY

RESUMO

As condições de luz, bem como hipoxia e anoxia durante o crescimento do fungo, causa várias mudanças fisiológicas em seu metabolismo, germinação, crescimento micelial e produção de conídios. Neste estudo, conídios do fungo entomopatogênico *Metarhizium robertsii* foram produzidos em meio ágar dextrose batata (PDA) no escuro; sob luz branca; sob luz azul; sob luz vermelha; sob hipoxia contínua; anoxia transitória; sob normoxia; e em meio mínimo (meio Czapek sem sacarose) suplementado com 3 % de lactose (MML) no escuro. A produção de conídios para cada tratamento, assim como a velocidade de germinação dos conídios e virulência do fungo para o inseto *Tenebrio molitor* foram avaliados. O fungo crescido sob luz azul produziu mais conídios que o fungo crescido no escuro. A produção de conídios do fungo crescido sob luz branca e vermelha foi similar ao obtido no escuro, e o MML ofereceu menor produção de conídios. Conídios produzidos em MML ou em meio PDA sob luz branca ou azul, germinaram mais rápido que conídios produzidos em meio PDA no escuro ou sob luz vermelha. Conídios produzidos em meio PDA sob luz branca foram mais virulentos que conídios produzidos no escuro, sob luz azul ou sob luz vermelha. O fungo crescido sob hipoxia ou anoxia transitória produziu quantidade similar de conídios e que germinaram com a mesma velocidade que o fungo crescido sob normoxia. Conídios produzidos sob anoxia transitória foram mais virulentos que conídios produzidos sob normoxia e hipoxia, contudo, foram menos virulentos que conídios produzidos em MML.

Palavras-chave: fungo entomopatogênico, virulência, anoxia, fotobiologia de fungos

ABSTRACT

Light conditions, as well as hypoxia and anoxia during fungal growth, cause several physiological changes in their metabolism, germination, mycelial growth, and conidial production. In this study, conidia of the entomopathogenic fungi *Metarhizium robertsii* were produced on potato dextrose agar (PDA) medium in the dark; under white light; under blue light; under red light; under continuous hypoxia; transient anoxia, under normoxia; and minimum medium (Czapek medium without sucrose) supplemented with 3 % lactose (MML) in the dark. The conidial production for each treatment as well as the speed of conidial germination, and virulence to the insect *Tenebrio molitor* were evaluated. The fungus grown under blue light produced more conidia than the fungus grown in the dark. The conidial production of the fungus grown under white and red light were similar to that obtained in the dark, and MML afforded the least conidial production. Conidia produced on MML or on PDA medium under white or blue light germinated faster than conidia produced on PDA medium in the dark or under red light. Conidia produced on PDA medium under white light were more virulent than conidia produced in the dark, under blue light or red light. The fungus grown under hypoxia or transient anoxia produced similar amounts of conidia and germinated at the same speed than the fungus grown under normoxia,. Conidia produced under transient anoxia were more virulent than conidia produced under normoxia and hypoxia, however, were less virulent than conidia produced on MML.

Keywords: entomopathogenic fungus, virulence, anoxia, fungal photobiology

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CHAPTER 1 – INTRODUCTION

Biological control of insects

In the search for new alternatives to control insect pests in agriculture, predatory organisms and insect parasites have been used in biological control programs. Biological control, in addition to reducing insect populations that cause great economic damage to agriculture, is a more sustainable alternative than pesticides. These chemicals, when used irresponsibly, can cause ecological imbalance and health problems to individuals involved in their preparation and application in the field.

Within the group of living organisms used in biological control, microorganisms are very important, because it is known that all insect pests carry at least one disease (Alves 1998). The most studied microorganisms used in pest control are entomopathogenic bacteria, viruses, and fungi. In Brazil, fungi are the most used in microbial pest control (Mascarin & Pauli 2010).

Entomopathogenic fungi

Entomopathogenic fungi are fungi capable of infecting, parasitizing, and killing their host insect. A great advantage of these microorganisms in the field is the easy dispersion of their spores into the environment by wind and rain, as well as the direct contamination of healthy insects when they come into contact with a corpse mummified by the fungus (Li et al. 2010; Zimmermann 2007). Unlike other microorganisms used for biological control, fungi do not need to be ingested to cause disease (Alves 1998) and can infect different stages of host development (Cook et al. 1996; McCoy & Tigano-Milani 1992).

The first insect defense barrier is the cuticle, a component rich in chitin and other degradation resistant proteins, as well as fatty acids with antimicrobial properties (St. Leger et al. 1991). When intact, this physical barrier is able to prevent infection by several microorganisms that do not have a cuticle penetration system (e.g., bacteria, protozoa, and viruses) requiring other means of infection. However, penetration through this structure is the main route of infection used by fungi (Lu & St Leger 2016). The

three main genera of entomopathogenic fungi used in Latin America are *Beauveria*, *Metarhizium*, and *Lecanicillium* (Mascarin 2010).

For the infection process, the conidia of the entomopathogenic fungus must adhere, using proteins called adhesins and hydrophobins, and germinate on the surface of their host (Ortiz-Urquiza & Keyhani 2013). On the insect cuticle, the germinative tube forms and later the appressory becomes a clamp-like structure for penetration. In addition to the mechanical force exerted by the clamp against the cuticle (Roberts & St. Leger 2004), the appressory also produces extracellular hydrolytic and detoxifying enzymes (proteases, lipases, chitinases, catalases, esterases), which aid in the degradation of the cuticle and posterior penetration of the fungus inside the insect (St. Leger et al. 1996; Ortiz-Urquiza & Keyhani 2013). When it penetrates the internal cavity called the hemocele, the structures called hyphal bodies form in the hemolymph that, along with absorbing nutrients, produce mycotoxins (secondary metabolites) responsible for acting against the host's immune system to facilitate establishment of the fungus in the insect (Roberts, 1966; Roberts & St. Leger 2004). After the death of the host and depletion of its nutrient reserve, hyphae are emitted through the joints and natural openings of the corpse, so that the fungus returns to the external environment and begins the process of conidiogenesis, forming new conidia to infect other insects and continuing the life cycle of the fungus (Ortiz-Urquiza & Keyhani 2015).

The fungus *Metarhizium* spp.

According to Alves (1998), the fungi of the genus *Metarhizium* produce hyaline hyphae with uninucleate and cylindrical conidia. At the beginning, only two species of the genus, *M. anisopliae* and *M. flavoviride* were accepted. *M. anisopliae* was divided into two other varieties according to the size of the conidium: *M. anisopliae var. anisopliae* with short spore of 5-8 μm length, and *M. anisopliae var. majus* with spores of 10-14 μm, which my reach up to 16 μm in length. *M. flavoviride* differs from *M. anisopliae* because it presents larger conidia and ranges from yellowish green to grayish, whereas *M. anisopliae* produces dark green conidia. There are currently 64 species of the genus *Metarhizium* (www.indexfungorum.org); the most known are *M. anisopliae, M. acridum, M. album, M. flavoviride,* and *M. robertsii*.

The advances of molecular biology now make it possible to identify the high genetic variability of *Metarhizium* species isolated from different geographic regions,

and from different host insects (Humber et al. 2014; Kepler et al. 2014). The association is now known to exist between genotypes of *Metarhizium* fungi from temperate and cold regions related to the environment, and of tropical and subtropical regions related to their preferred host insect (Bidochka et al. 2001; Bidochka & Small 2005).

This genetic variability of the genus *Metarhizium* is very important to control of pests, because only *M. anisopliae* species has as host range of more than 200 species of insects (Li et al. 2010). In Brazil, several companies produce *Metarhizium* for the control of agricultural insect pests, mainly for sugarcane for the control of *Mahanarva* spp. since 2001 (Roberts & St. Leger 2004).

The entomopathogenic fungus *Metarhizium robertsii* is a common inhabitant of the soil in several regions of the planet. In addition to being pathogenic to insects, it also has the capacity to colonize the rhizosphere, which is the region of the soil influenced by the roots with maximum microbial activity, and is influenced by excretions roots and soil microorganisms (Atlas & Bartha 1998). This fungus adheres to the roots of the plant and assists its development by transferring nitrogen from insect corpses to the roots (Sasan & Bidochka 2012; Behie et al. 2015). Two different adhesin genes cooperate for this adaptation, MAD1 and MAD2; the first one is responsible for the adhesion of the conidia on the insect cuticle and the second for the adhesion on the plant root (Wang & St Leger 2007). In addition to *Metarhizium robertsii*, other entomopathogenic fungi, such as *Acremonium* spp., *Beauveria* spp., *Cladosporium* spp., *Clonostachys* spp., and *Isaria* spp., also demonstrate some capacity of beneficial interaction with plants, which goes beyond the control of insect pests (Vega 2008).

Virulence in entomopathogenic fungi

Virulence in invertebrate pathology is defined by the “power to produce disease of an organism” and is a term that quantifies the degree of pathogenicity within a group or species of microorganism (Shapiro-Ilan et al. 2005). The virulence of an insect pathogen can be manipulated in the laboratory in several ways, such as repeated passages through susceptible insects (Daoust & Roberts 1982); growth of the fungus under certain conditions that improve virulence (Ibrahim et al. 2002; Rangel et al. 2008; Rangel et al. 2015; Shah et al. 2005); insertion or manipulation of genes that improve virulence (Ortiz-Urquiza & Keyhani 2015; Pava-Ripoll et al. 2008; Wang & Feng

2014); and screening of isolates within a pathogen species for differential virulence (Alston et al. 2005; Fernandes et al. 2011; Keyser et al. 2017).

The virulence of entomopathogenic fungal conidia is strongly influenced by the medium where the mycelial growth occurs (Ibrahim et al. 2002; Rangel et al. 2008, Shah et al. 2005); however, continuous subculture in artificial media may reduce virulence (Lacey 2012; Shah et al. 2007; Vega & Kaya 2012). In addition, according to Daoust and Roberts (1982), many species of entomopathogenic fungi are composed of morphologically distinct variants that differ significantly in their ability to produce pathogenesis in a susceptible host.

The growth of entomopathogenic fungi under some physical or chemical conditions produces conidia with increased germination speed and, consequently, endow greater virulence (Braga et al. 2001; Altrea et al. 1978; Altre et al. 1999; Rangel et al. 2008). This is the case with the nutrient stress generated by the minimal medium (Czapek medium without sucrose), which stimulates the production of conidia by *Metarhizium robertsii* with high germination speed. This is due to the much greater amount of trehalose and mannitol inside these conidia, compared to the amount of these sugars from the same fungus produced on rich medium (potato dextrose agar supplemented with yeast extract - PDAY). Therefore, the increased accumulation of these carbohydrates in dormant conidia should accelerate germination acting as endogenous sugars (Rangel et al. 2008).

The fungus *Metarhizium robertsii* (ARSEF 2575) grown in minimal medium or in minimal medium with 3 % lactose (MML), produces conidia twice as virulent as the conidia produced in PDAY medium. In addition to germinating faster, growth in MML also produces conidia with better insect adhesion than conidia produced in rich medium (Rangel et al. 2008).

Other stress conditions during growth may also help increase the virulence of entomopathogenic fungi, such as exposure to osmotic stress (Safavi et al. 2007; Rangel et al. 2008), UV-A radiation (Rangel et al. 2008), higher concentration of oxygen (Miranda-Hernández et al. 2014), and blue light (Alves et al. 1980). This is not always related to the increase in the germination speed.

In addition to host defenses, environmental factors may directly affect the development of entomopathogenic fungi, impairing their field use in the biological control of insect pests. For example, conidia exposed to solar radiation endure high temperature and direct incidence of ultraviolet B (UV-B) radiation, which may increase

the germination time or even cause inactivation of conidia (Braga et al. 2015; Rangel et al. 2015). Consequently, the virulence of conidia is reduced due to the negative effect on germination. Light radiation can stimulate, be indifferent, or impair the development of fungi, depending on the microorganism, its developmental state, and the type and amount of radiation. Temperature can also act on pathogens, affecting their production, viability, and pathogenicity (Alves et al. 1984).

In addition to the environmental factors and host defenses, storage conditions of the micopesticide on the shelf may also impair the viability and virulence of the conidia of the entomopathogenic fungi, thus compromising the success of the final product (Daoust & Roberts 1983; Mascarin & Pauli 2010). The technique used to produce the fungus, product formulation, field dosages, and shelf life are also important topics that affect the efficiency of entomopathogenic fungus used in the biological control of agricultural pests (Mascarin & Pauli 2010).

Therefore, it is necessary to better understand the effect of these factors and to modify the production process to physiologically manipulate increased virulence of an entomopathogenic fungus, to facilitate a greater chance of success of the micopesticide in the field. With the objective of increasing the production of conidia and with greater virulence, this work investigates the effects of different light colors and oxygen deprivation on the development of the fungus *Metarhizium robertsii* and its virulence in the laboratory for the insect *Tenebrio molitor*.

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CHAPTER 2

Metarhizium robertsii illuminated during mycelial growth produces conidia with increased germination speed and virulence[1]

Abstract

Light conditions during fungal growth are well known to cause several physiological adaptations in the conidia produced. In this study, conidia of the entomopathogenic fungi *Metarhizium robertsii* were produced on: 1) potato dextrose agar (PDA) medium in the dark; 2) PDA medium under white light (4.98 W m^{-2}); 3) PDA medium under blue light (4.8 W m^{-2}); 4) PDA medium under red light (2.8 W m^{-2}); and 5) minimum medium (Czapek medium without sucrose) supplemented with 3 % lactose (MML) in the dark. The conidial production, the speed of conidial germination, and the virulence to the insect *Tenebrio molitor* (Coleoptera: Tenebrionidae) were evaluated. Conidia produced on MML or PDA medium under white or blue light germinated faster than conidia produced on PDA medium in the dark. Conidia produced under red light germinated slower than conidia produced on PDA medium in the dark. Conidia produced on MML were the most virulent, followed by conidia produced on PDA medium under white light. The fungus grown under blue light produced more conidia than the fungus grown in the dark. The quantity of conidia produced for the fungus grown in the dark, under white, and red light was similar. The MML afforded the least conidial production. In conclusion, white light produced conidia that germinated faster and killed the insects faster; in addition, blue light afforded the highest conidial production.

Key Words: entomopathogenic fungi; fungal photobiology; fungal nutritional stress; virulence; germination speed; conidial production

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Introduction

Illumination conditions during fungal growth are well known to influence conidial production (Fanelli et al., 2012; Mooney and Yager, 1990; Sanchez-Murillo et al., 2004; Zhang et al., 2009), sexual and asexual development (Ruger-Herreros et al., 2011), spore discharge and dispersal (Page, 1962), secondary metabolites (Bayram et al., 2008; Fischer, 2008), pigment formation (Avalos and Lim_on, 2015), and tolerance to UV-B radiation (Brancini et al., 2016; de Menezes et al., 2015; Rangel et al., 2015, 2011) and heat (Rangel et al., 2015, 2011).

Light is one of the many signals that fungi use to perceive and to interact with the environment, providing critical information about their habitat (Corrochano, 2007; Herrera-Estrella and Horwitz, 2007). Three light-sensing systems have been described in fungi. 1) Blue-light sensing is performed by a flavin chromophore-binding domain named LOV (for Light, Oxygen, or Voltage). A fungal LOV domain photosensor, first identified and named WHITE COLLAR 1 (WC-1) in *Neurospora crassa*, is present throughout most of the kingdom, suggesting an ancient origin. In all species examined, WC-1 physically interacts with a second protein, WHITE COLLAR 2 (WC-2), that contains a zinc finger DNA-binding domain such that the complex can act as a light-sensitive transcription factor. In addition to the LOV domain proteins, fungal blue light sensing may also be mediated by the cryptochrome/photolyase family of proteins. 2) Red-light sensing is achieved by phytochrome photoreceptors that sense red and far-red light through a linear tetrapyrrole chromophore. 3) Rhodopsins, which sense blue-green light, are embedded in seven-transmembrane domains and a central region where a retinal chromophore is bound (Corrochano and Galland, 2016; Fuller et al., 2015; Idnurm and Crosson, 2009; Purschwitz et al., 2006; Qiu et al., 2014).

A direct association has been recognized between specific protein photosensors and the capacity to cause disease by pathogenic bacteria and fungi (Bonomi et al., 2016; Hevia et al., 2016, 2015; Idnurm and Crosson, 2009; Idnurm and Heitman, 2005; Kennis and Crosson, 2007; Moriconi et al., 2013; Ricci et al., 2015; Rio-Alvarez et al., 2014; Ruiz-Roldan et al., 2008; Swartz et al., 2007); consequently, certain pathogens use these photosensors, which, depending on the pathogen and host, can induce virulence genes making the pathogen more or less virulent. For example, the blue light photoreceptors are known to be required for virulence in microbes (Idnurm and Crosson, 2009).

The first study that associated light with virulence examined the fungus *Histoplasma capsulatum*, which become less virulent when the cultures were grown under light exposure (Campbell and Berliner, 1973). Other studies suggest links between light exposure and virulence in insect pathogens, for example, conidia of *Metarhizium anisopliae* formed under blue light are more pathogenic towards *Galleria mellonella* larvae (Alves et al., 1980). Blue light also significantly affected the virulence of the plant pathogen *Colletotrichum acutatum* to hot-pepper fruit. In this system, the highest level of host-plant infection occurred under blue light and it was two times more virulent than that observed under darknessor white-, red-, green-light conditions (Yu et al., 2013). Another relationship between light sensing and virulence is found in the *Cryptococcus neoformans*, a cause of fatal meningitis in humans, in which a wc-1 homolog is required for virulence. In this fungus, light regulates the mating process of the fungus via the WHITE COLLAR homologs Bwc1 and Bwc2. Mutation of these genes causes a reduction in tolerance to UV light, and also the strain becomes less virulent to a mouse when the disease is induced by fungal inhalation (Idnurm and Heitman, 2005). The wc-1 homolog of *Fusarium oxysporum*, a soilborne plant pathogen that causes economically important losses of a wide variety of crops and has been described as an emerging human pathogen, is also required for full virulence in mouse, but surprisingly has no effect on virulence in tomato roots (Ruiz-Roldan et al., 2008). Moreover, the deletion of the wc-1 ortholog leads to attenuated virulence in the gray mold pathogen *Botrytis cinerea* (Canessa et al., 2013). However, virulence of *Aspergillus fumigatus* in a murine model is variable across isolates and is independent of light response (Fuller et al., 2016).

A connection between growth under visible light versus virulence has also been established in the bacterium *Brucella abortus*, where exposure to light increased the enzymatic activity of the light, oxygen, or voltage (LOV) histidine kinase, and this, in turn, increased virulence of the bacterium (Kennis and Crosson, 2007; Swartz et al., 2007). *B. abortus* is ten times more virulent upon exposure to visible light than bacteria that were not exposed (Kennis and Crosson, 2007; Swartz et al., 2007).

Light conditions during growth and conidiogenesis of the insect-pathogenic fungus *M. anisopliae* has been studied for a long time (Alves et al., 1980; Roberts and Campbell, 1977; Vouk and Klas, 1932). However, little is known about whether light conditions influence the conidial germination speed and virulence in insect-pathogenic fungal species. The genus *Metarhizium* is frequently used as mycoinsecticides in many

countries to control insect pests in agriculture (Alston et al., 2005; Faria and Wraight, 2007; Li et al., 2010). In this study, conidia of the entomopathogenic fungi *Metarhizium robertsii* were produced: 1) on potato dextrose agar (PDA) medium in the dark (control); 2) on PDA medium under white light; 3) on PDA medium under blue light; and 3) on PDA medium under red light. We evaluated the conidial production of each treatment as well as the speed of conidial germination and virulence to the insect *Tenebrio molitor* (Coleoptera: Tenebrionidae). The conidial production, germination speed, and virulence of the above treatments were compared with conidia produced on minimum medium (Czapek medium without sucrose) supplemented with 3 % lactose (MML) in the dark because this nutritive stress condition is known to induce the production of conidia with higher germination rate and higher virulence towards the insect *T. molitor* than conidia produced on rich PDA medium supplemented with yeast extract (Rangel et al., 2008a).

Materials and methods

Fungal isolate

M. robertsii (ARSEF 2575) was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Robert W. Holley Center for Agriculture & Health, Ithaca, NY, USA). Stock cultures were maintained at 4 ºC in test tubes on slants of potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, USA) adjusted to pH 6.9.

Conidial production and harvesting

Conidia were produced on 23 ml of the following media: PDA (Difco, Sparks, MD, USA), or on a basal medium for growth under nutritive stress (MML = Czapek Dox Agar without sucrose) [$NaNO_3$ 0.2 %, K_2HPO_4 0.1 %, $MgSO_4$ 0.05 %, KCl 0.05 %, $FeSO_4$ 0.001 %, lactose (Sigma) 3.0 %, and Bacto Agar 1.5 % (Becton, Dickinson and CO, Sparks, MD, USA)] in 95 mm polystyrene Petri dishes. Growth under nutritive stress is the positive control, a condition that will always induce *M. robertsii* (ARSEF 2575) to produce conidia with higher tolerance to UV-B radiation and heat (Rangel et al., 2006, 2008b, 2015, 2012, 2011), as well as more virulent to the insects (Rangel et

al., 2008a) than conidia produced on PDAY medium. The pH of all media was adjusted to 6.9. A conidial suspension (100 ml of 10^7 conidia ml^{-1}) was inoculated evenly with a glass spreader onto agar media. The cultures were incubated at 26 ± 1 ºC and approximately 90 % relative humidity (RH) for 14 d. Three different batches of conidia were produced.

For white light treatment, the Petri dishes with cultures on PDA medium with lids in place, in a single layer (not stacked) were maintained under continuous light provided by two 15W cool white Philips (TL-D 15W/75-650) broad-spectrum fluorescent light bulbs suspended 25 cm above the samples. A sheet of 0.13-mm cellulose diacetate covered the plates to avoid medium dehydration. The integrated irradiance of the lamps that passed through the diacetate film plus the Petri dish lid was 4.98 W m^{-2} (Fig. 1) and 2230 lux. The irradiance was measured by an Ocean Optics USB 2000 spectroradiometer (Dunedin, FL). The illuminance (in lux) of the incubators were measured with an Onset HOBO® data logger U12-012.

For blue- or red-light treatment, the Petri dishes with cultures on PDA medium with lids in place, in a single layer (not stacked), were maintained under continuous blue or red light provided by two incubators that were adjusted to enable incubation of the cultures under different wavelengths of light. Incubator 1 contained four Color Led LLUM® E27 5W (Jinli Lighting Co., China), set in the blue wavelength providing an integrated irradiance of 4.8 W m^{-2} and 645.5 lux (Fig. 1). Incubator 2 contained four Color Led LLUM® E27 5W, set in the red wavelength providing an integrated irradiance of 2.8 W m^{-2} and 53 lux (Fig. 1). The distance between the LEDs and the agar plates was 6 cm and the temperature of the incubators were adjusted to 26 ºC. The spectral irradiances (in W m^{-2}) of the incubators were measured with a spectroradiometer Ocean Optics (Dunedin, FL) Model USB 2000 + Rad connected to a laptop, and the illuminance (in lux) of the incubators were measured with an Onset HOBO® data logger U12-012. The LEDs did not produce any detected heat.

For dark treatments, all Petri dishes were maintained in the same incubator as the light treatment, but the Petri dishes were kept inside a plastic box covered with a thick black cloth sleeve.

For germination experiments, conidia from minimal medium plus 3 % lactose (MML) were collected after 14 d of growth by flooding one plate with 10 ml of a solution of Tween 80 (0.01 % v/v). Conidia from PDA medium produced under white light, blue light, red light, or in the dark treatments were collected after 14 d of growth

with one pass of a microbiological loop (Decon Labs, Inc. PA, USA) and transferred to 10 ml of sterile Tween 80 (0.01 % v/v) in screw cap Pyrex tubes (20 × 125 mm). The quantity of conidia collected were enough to make a suspension of 10^5 conidia ml^{-1}. The suspensions were shaken vigorously using a vortex, and 40 µl were inoculated (dropped, but not spread) on the center of the PDA medium. All suspensions were used immediately after preparation for each of the conditions, i.e., dark, white light, blue light, red light, and MML.

For virulence bioassay experiments, conidia from 4 Petri dishes from each treatment (PDA medium produced under white light, blue light, red light, or in the dark treatments) were collected after 14 d with a sterile polyethylene cell lifter (Costar 3008, Corning International, Corning, NY, USA) to liberate the conidia from the mycelial mat. For the experiments of conidia produced MML, conidia were collected from 20 Petri dishes after 14 d of growth and harvested as described above.

The mass of conidia was transferred to 10 ml of sterile Tween 80 (0.1 % v/v) in sterile 50 ml Erlenmeyer. The first suspension was then filtered using two layers of sterile gauze to remove the mycelial mat. The concentration of the conidial suspensions were adjusted, based on haemocytometer counts, to 1×10^5 and 1×10^7 conidia ml^{-1}.

Conidial germination

The speeds of germination of conidia produced on the different treatments were assessed by inoculating 40 µl (10^5 conidia ml^{-1}) of the suspension on 5 ml PDA medium in polystyrene Petri dishes (35 × 10 mm) accordingly to Rangel et al. (2004). The plates were kept at 28 ± 1 ºC in the dark, this temperature is optimal for germination and growth for this fungal isolate (Rangel et al., 2010). The germinations were observed at 2, 4, 6, 8, 10, 12, and 14 h after the conidial suspension was inoculated on the medium. After each incubation time, the agar plate at the point of the inoculation was stained with a drop of Methyl Blue solution (Braga et al., 2002). The drop was covered with a circular (15 mm diameter) coverslip (Vidrobras, Brazil) and examined under light microscope at 400 × magnification. Conidia presenting a germ tube longer than the length of the conidia were considered to have germinated accordingly to Braga et al. (2001b). At least 300 conidia per plate were evaluated, and the percent of germination was calculated as described by Braga et al. (2001a). All germinated or non-germinated conidia were counted on a single coverslip. The scanning pattern for counting was

around the margin of the conidial suspension drop, which is an area commonly less populated by conidia. Each treatment was repeated three times with a fresh batch of conidia produced for each repetition.

Virulence bioassays

Fifty *T. molitor* (Vidaproteína Indústria e Comércio Ltda, Nerópolis, GO, Brazil) were used for each treatment. The weight average ± standard deviation of the insects was 101 ± 9.34 mg per insect. The insects were dipped for 20 s into 10 ml of each conidial suspension in 50 ml sterile Nalgon plastic beakers or into sterile Tween 80 [0.1 % (v/v) solution] as a control. The insects were then removed from the suspensions by sterile gauze filtration and excess moisture removed by allowing the larvae to crawl on dry sterile filter paper for 30 s. The insects were maintained in 60 × 15 mm polystyrene Petri dishes, ten insects per dish, inside hermetic plastic boxes lined with wet filter paper to maintain RH at approximately 90 %. The plastic boxes were held at 26 ± 1 ºC for 14 d in the dark. The insects were maintained without food. Insect mortality was recorded daily. Dead insects were removed daily and placed in sterile Petri dishes and kept in humid chambers lined with moist filter paper. Cadavers were monitored for 14 d for fungal growth until forming a layer of fungal outgrowth on the cadavers. Four complete trials (repetitions) were conducted using a different culture batch and insect population for each trial. *T. molitor* is routinely used as a model host insect for studies with insect pathogenic fungi (Shah et al., 2005).

Measurement of conidial production

To measure conidial production under different conditions, three agar plugs (per plate) were removed with a cork borer (5 mm diam) at places on the medium surface with an even coverage of conidia, and the conidia were suspended in 1 ml sterile Tween 80 (0.1 %) solution. Conidial concentrations were determined by haemocytometer counts. Each experiment was performed on three different dates, and each experiment used a new batch of cultures.

Statistical analyses

Germination assay. The effect of treatment on percent germination was assessed with analysis of variance of a one-way factorial. Significance levels of pair-wise mean comparisons among treatments were controlled for experiment-wise type I error using the Tukey method with overall $\alpha = 0.05$. Separate analyses were computed for germination from 2 to 14 h.

Virulence bioassays. The effects of treatments and trial replication on percentage mortality of the host insect on days 1-14 after inoculation were assessed using a one-way factorial. Significance levels of pair-wise mean comparisons among treatments were controlled for experiment-wise type I error using the Tukey method with overall $\alpha = 0.10$.

Measurement of conidial production. The effect of treatment on the measurement of conidia production was assessed with analysis of variance of a one-way factorial. Significance levels of pair-wise mean comparisons among treatments were controlled for experiment-wise type I error using the Tukey method with overall $\alpha = 0.05$.

All analyses were carried out in the free statistical program Sisvar (Ferreira, 1999, 2011).

Results and Discussion

The *M. robertsii* genome harbors orthologues of blue and red fungal photoreceptor proteins (Gao et al., 2011). The BLAST searches were made against the *M. robertsii* genome ARSEF 23, which is the strain used by Gao. White collar-1 was found and is identified by the same name with accession number MAA_04453. White collar-2 has been annotated in the genome as cutinase protein palindrome-binding protein, and its accession number is MAA_07440. Phytochrome was annotated in the genome as signal transduction response regulator, receiver domain protein with an accession number MAA_02298. This composition of photoreceptors resembles that found in *Aspergillus nidulans* (Purschwitz et al., 2006, 2008), with the exception of opsins, because there is no hit in the *Metarhizium* genome against the *N. crassa* NOP-1

protein nor against CarO protein of *Fusarium fujikuroi*, so it is possible that *M. robertsii* has no opsins. Indeed, *M. robertsii* (ARSEF 2575) responded well to white, blue, and red light. We did not expose it to green light because the fungus did not respond to this light (data not published) and, consequently, agreed with the information that *Metarhizium* genome has no opsins.

White light induced the production of conidia that germinated faster (Fig. 2) and, consequently, were more virulent to *Tenebrio molitor* (Fig. 3). In addition, the germination speed and virulence of conidia produced under white light was similar to that of conidia produced under nutritive stress (MML), which is known to produce conidia of *M. anisopliae* (Ibrahim et al., 2002; Shah et al., 2005) and *M. robertsii* (Rangel et al., 2008a) with enhanced germination speed as well as virulence towards insects. It is well established that the germination speed of the conidium influences its virulence, and, consequently, in general conidia that germinate faster are more virulent to insects (Altre et al., 1999; Hassan et al., 1989; Ibrahim et al., 2002; Rangel et al., 2008a), suggesting that virulence greatly depends on the speed of infection.

Conidia produced under blue light germinated faster than conidia produced in the dark (Fig. 2); conversely, conidia produced under red light germinated slower than conidia produced in the dark (Fig. 2). Intriguingly, the virulence of conidia produced under blue and red light was similar to that of conidia produced in the dark (Fig. 3). It is known that when performing experiments with only one interaction (just the fungus versus its growth condition, for example only germination as found in Fig. 2), the response is stronger from all different treatments (Rangel et al., 2008b). However, when the fungus has an interaction with the insect-host, there are multiple interactions and, consequently, a weaker response (Rangel et al., 2008a) as also found in Fig. 3. Conidia that germinates faster definitively kills its host faster, as found by conidia produced on MML, but the weaker response in virulence may reduced the differences between both treatments (Fig. 3).

The fungus grown under blue light produced more conidia than the fungus grown in the dark, under red light, and on MML. The conidial production of the fungus grown in the dark and under white and red light was similar (Fig. 4A). The MML medium afforded the least conidial production (Fig. 4A). Conidiation, however, is not important for pathogenesis per se, but rather the ability to induce spore formation, which is important for the mass production of the biocontrol agent.

Growth under red light produced less conidia than growth under blue light. In addition, conidia produced under red light germinated slower than conidia produced under blue light. It is worth mentioning that, in these experiments, conidial production was conducted under different light conditions, but the tests for germination and virulence of conidia produced under blue and red light were done in the dark (Figs. 2 and 3). Different studies have already been done with conidia produced in the dark and the germination rates of conidia produced in the dark were done under blue and red light (Röhrig et al., 2013). One of the mechanism that make conidia germinate faster is higher trehalose accumulations inside conidia (Rangel et al., 2008a, 2006, 2008b), because there is a circadian control of trehalose synthase in *N. crassa*, which suggests a link between light and stress responses (Shinohara et al., 2002). Therefore, we hypothesize that conidia of *M. robertsii* produced under blue and white light accumulated higher quantity of trehalose inside and consequently germinated faster. In addition, red light has deleterious effects towards tolerance to UV-B radiation, for example, colonies of *Metarhizium acridum* that have been briefly exposed to white or blue/UV-A light show an increased tolerance to UV-B radiation, but colonies kept in the dark or exposed to red light were less tolerant to UV-B radiation (Brancini et al., 2016). Moreover, colonies previously stimulated with white or blue/UV-A light showed a reduced growth delay after UV-B exposure compared to colonies exposed either to red light or kept in the dark (Brancini et al., 2016).

In addition, the culture morphology and color differed drastically for each treatment (Fig. 4B). The cultures produced under white and blue light were darker than the cultures produced in the dark (Fig. 4B). The culture grown under red light was also a lighter green than the culture grown in the dark (Fig. 4B). Blue light is known to improve conidial production of *M. anisopliae* (Alves et al., 1980) and *Paecilomyces fumosoroseus*, currently *Isaria fumosorosea* (Sanchez-Murillo et al., 2004), as well as the fungus *Pyrenophora semeniperda*, which is a biological control agent of grass weeds (Campbell et al., 2003). However, different species may perceive and behave differently to the diverse wavelengths, for example, *Fusarium verticillioides* produces more conidia under red light than blue light (Fanelli et al., 2012). Conidial production under red light was similar to the production in the dark. Similarly, colonies of *A. fumigatus* grown under dark and red light were identical (Fuller et al., 2013).

In conclusion, light conditions such as white and blue light produced conidia that germinated faster. Conidia produced under white light also killed the insects faster. In

addition, blue light afforded the highest conidial production. Nutritive stress caused by minimal medium (MM) or minimal medium supplemented with 3 % lactose (MML) is known to induce higher virulence and germination speed (Rangel et al., 2008a) as well as higher tolerance to UV-B radiation and heat (Rangel et al., 2006, 2008b, 2015, 2012, 2011). The faster germination speed of conidia produced on MML is due to its higher accumulation of trehalose and mannitol inside conidia (Rangel, 2011; Rangel et al., 2006, 2008b, 2015), therefore, the mobilization of these two carbohydrates during the conidial awakening allows faster germination than when conidia is produced on rich PDA medium, which accumulate very little amounts of trehalose and mannitol (Rangel et al., 2008b). Therefore, this medium was used as control to know if any light condition will surpass the virulence and germination speed caused by MML.

It is well established that white light during mycelial growth induces higher conidial tolerance to UV-B radiation and heat in *M. robertsii* (Rangel et al., 2015). In this study, mycelial growth under white light also stimulated the production of conidia that are more virulent to the insects. Therefore, light could be used as a method to improve virulence and stress tolerance of *M. robertsii* conidia. Other entomopathogenic fungi such as *Beauveria bassiana* may respond to light similar to *Metarhizium* because it has been found that blue- and red-light photoreceptors are present and are essential for virulence and stress responses (Qiu et al., 2014; Tong et al., 2017).

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LEGENDS FOR THE FIGURES

Figure 1. Spectral irradiances of the lamp setups used. For white light treatment, the Petri dishes with cultures on PDA medium with lids in place, in a single layer (not stacked) were maintained under continuous light provided by two 15W cool white Philips (TL-D 15W/75-650) broad-spectrum fluorescent light bulbs suspended at a distance of 25 cm above the samples. The integrated irradiance of the lamps that passed through the diacetate film plus the Petri dish lid was 4.98 W m^{-2}. For blue- or red-light treatment, the Petri dishes with cultures on PDA medium with lids in place, in a single layer (not stacked) were maintained under continuous blue or red light provided by two incubators. Incubator 1 contained four Color Led LLUM® E27 5W (Jinli Lighting Co., China), set in the blue wavelength providing an integrated irradiance of 4.8 W m^{-2}. Incubator 2 contained four Color Led LLUM® E27 5W set in the red wavelength providing an integrated irradiance of 2.8 W m^{-2}. In both blue and red incubators, the distance between the LEDs and the agar plates were 6 cm, and the temperature of the incubators were 26 °C.

Figure 2. Germination of *Metarhizium robertsii* (ARSEF 2575) on PDA medium. The treatments were: 1) Conidia produced on PDA medium in the dark (Dark); 2) Conidia produced on PDA medium under white light (White light); 3) Conidia produced on PDA medium under blue light (Blue light); 4) Conidia produced on PDA medium under red light (Red light); 5) Conidia produced on minimum medium plus 3 % lactose according to Rangel et al. (2008) grown in the dark (MML). A conidial suspension produced from each treatment were inoculated on PDA medium and incubated in the dark at 28 °C. Each treatment was repeated at least three times with a fresh batch of conidia produced for each repetition. Error bars are standard error of at least three independent experiments. Means with the same letter are not significantly different ($p < 0.05$).

Figure 3. Virulence of *Metarhizium robertsii* (ARSEF 2575) to *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae). The treatments are: 1) *T. molitor* larvae immersed only in Tween 80 0.1 % (Negative Control); 2) *T. molitor* larvae immersed in suspension of conidia produced on PDA medium in the dark (Dark); 3) *T. molitor* larvae immersed in suspension of conidia produced on PDA medium under white light (white light); 4) *T.*

molitor larvae immersed in suspension of conidia produced on PDA medium under blue light (Blue light) ; 5) *T. molitor* larvae immersed in suspension of conidia produced on PDA medium under red light (Red light) ; 6) *T. molitor* larvae immersed in suspension of conidia produced on minimum medium plus 3 % lactose according to Rangel et al. (2008) grown in the dark (MML). All bioassay treatments were carried out in the dark at 26 °C. A) Conidial concentration at 1×10^5. B) Conidial concentration at at 1×10^7. Each treatment was repeated at least four times with a fresh batch of conidia produced for each repetition. Error bars are standard error of at least four independent experiments. Means with the same letter are not significantly different ($p < 0.10$).

Figure 4. A) Conidial production of *Metarhizium robertsii* (ARSEF 2575) on PDA medium. The conidial production treatments were: 1) PDA medium in the dark (Dark); 2) PDA medium under white light (White light); 3) PDA medium under blue light (Blue light); 4) PDA medium under red light (Red light); 5) Minimum medium plus 3 % lactose according to Rangel et al. (2008) grown in the dark (MML). This experiment was performed four times using four different batches of cultures. B) Eight-day old cultures. Error bars are standard error of at least four independent experiments. Means with the same letter are not significantly different ($p < 0.05$).

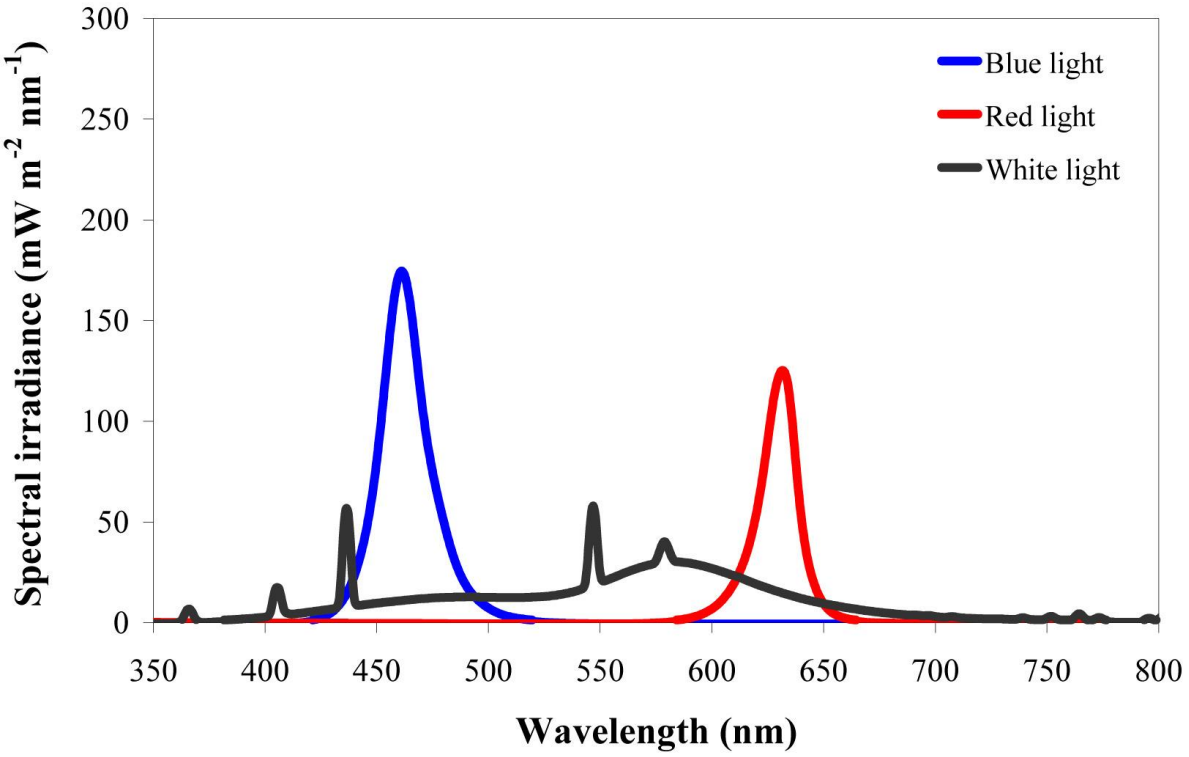


FIGURE 1

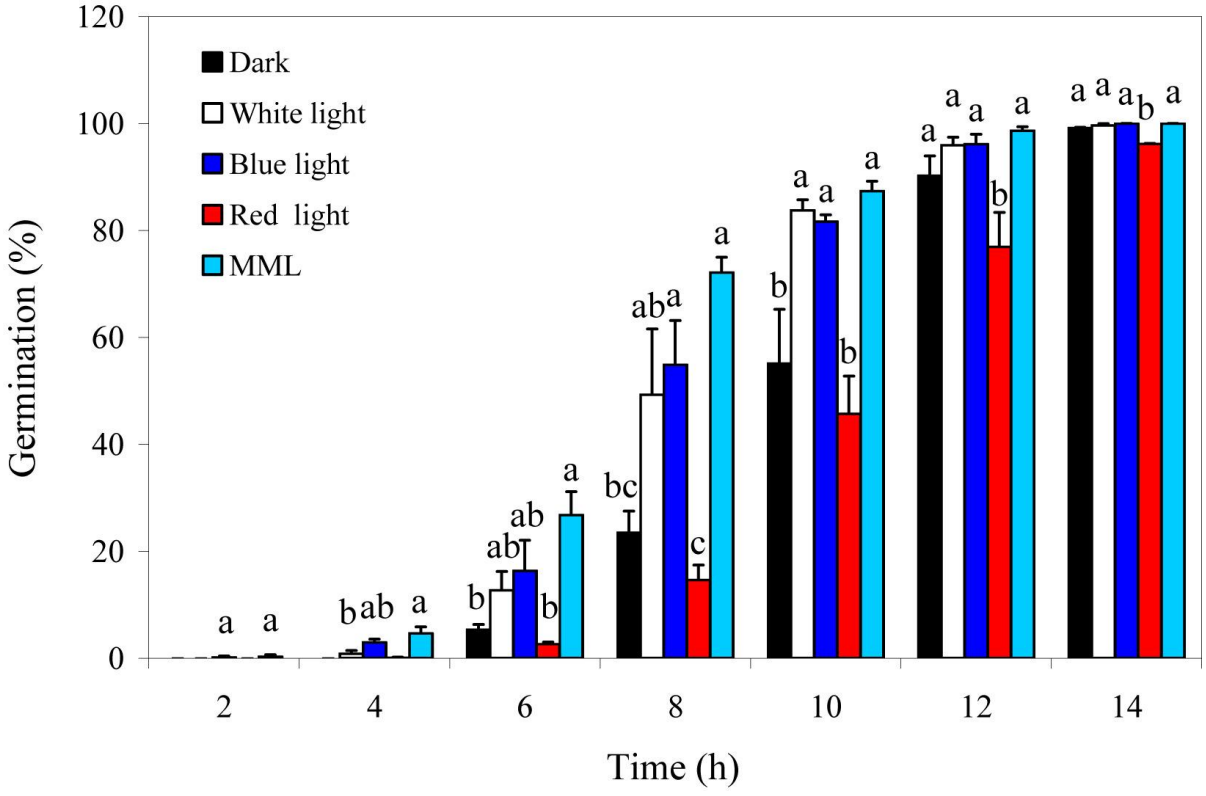


FIGURE 2

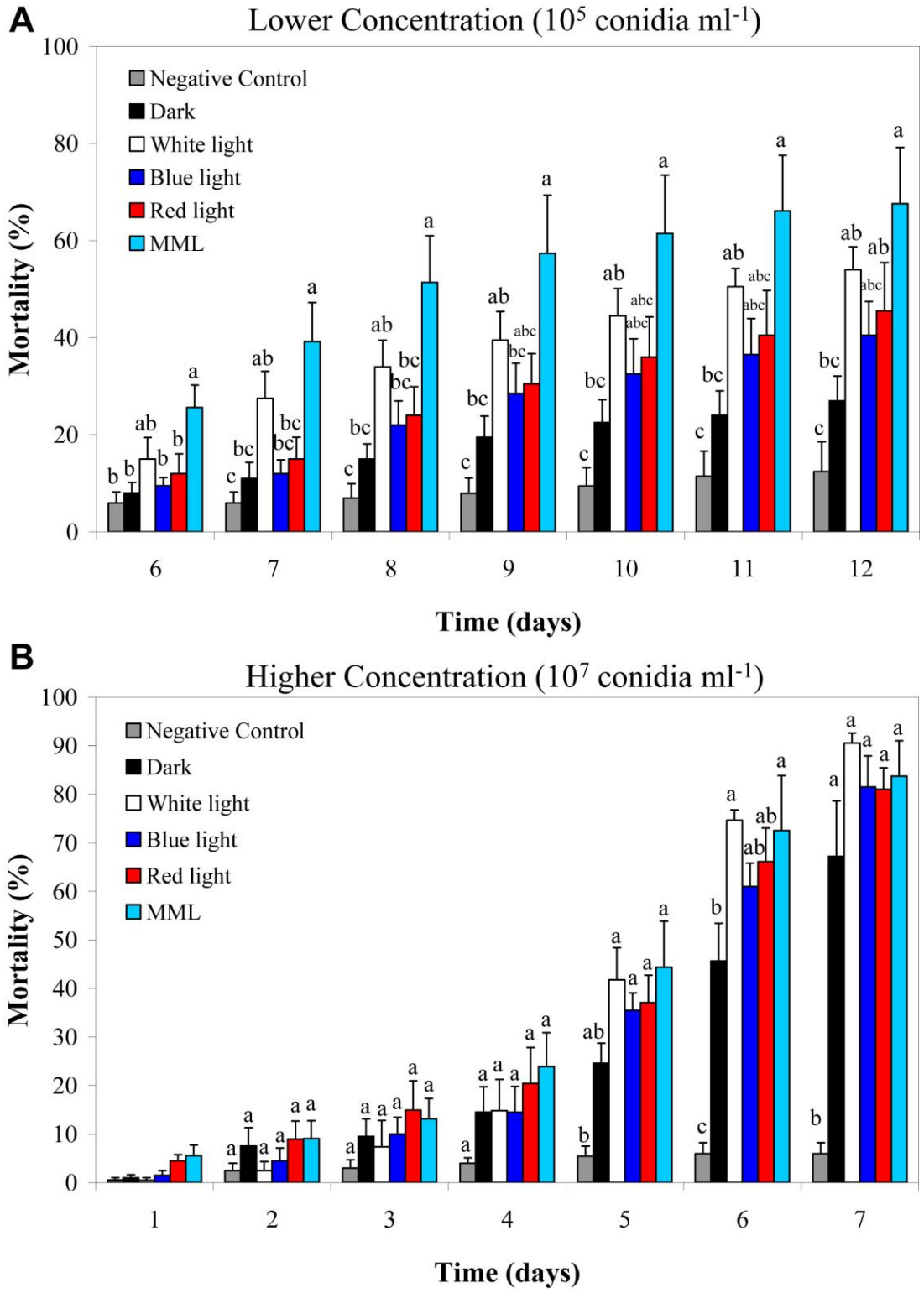
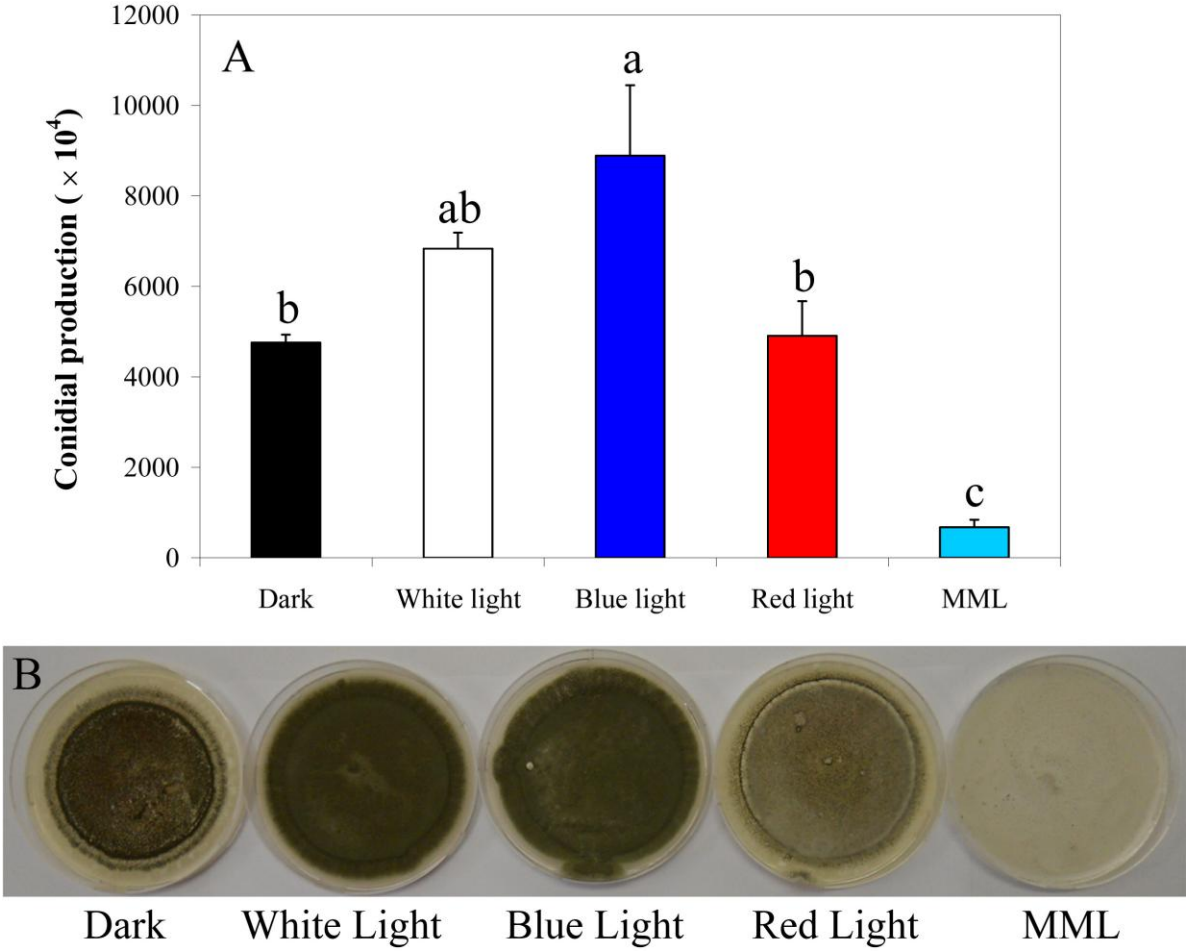

A
Lower Concentration (10^5 conidia ml^-1)
Mortality (%)
Time (days)
Negative Control
Dark
White light
Blue light
Red light
MML
B
Higher Concentration (10^7 conidia ml^-1)
Mortality (%)
Time (days)
Negative Control
Dark
White light
Blue light
Red light
MML

FIGURE 3


A
B
Conidial production (× 10⁴)
12000
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White light
Blue light
Red light
MML
Dark
White Light
Blue Light
Red Light
MML

FIGURE 4

CHAPTER 3

Transient anoxia during *Metarhizium robertsii* growth produces conidia with increased virulence to *Tenebrio molitor*[2]

Abstract

Little is known about the phenotypic effects of hypoxia and transient anoxia on the virulence of an entomopathogenic fungus. Conidia of *Metarhizium robertsii* were produced on: 1) potato dextrose agar medium (PDA) under normoxia; 2) PDA medium under continuous hypoxia; 3) PDA medium under transient anoxia; and 4) minimal medium with lactose (MML) under normoxia. Conidia produced under transient anoxia and produced on MML were the most virulent. Conidia produced under normoxia and hypoxia were the least virulent. Conidial production and germination speed of conidia produced under normoxia, hypoxia, and transient anoxia were similar; however, MML produced less conidia, but germinated faster than any other treatments.

Key Words: entomopathogenic fungus; virulence; normoxia; hypoxia; anoxia; nutritional stress

[2] Coauthored by Ariel S. Oliveira and Drauzio E.N. Rangel (2018) Transient anoxia during *Metarhizium robertsii* growth produces conidia with increased virulence to *Tenebrio molitor*. J. Invertebr. Pathol, doi: 10.1016/j.jip.2018.03.007. Copyright (2018), with permission from Elsevier.

Introduction

Virulence of insect-pathogenic fungus is determined by the environment where the mycelial growth occurs (Hallsworth and Magan, 1994; Ibrahim et al., 2002; Rangel et al., 2008a; Rangel et al., 2015; Shah et al., 2007; Shah et al., 2005). For instance, conidia of *Metarhizium robertsii* produced under nutritive and osmotic stress are more virulent to *Tenebrio molitor* than conidia produced on rich medium (Rangel et al., 2008a). In addition, mycelial growth under white light produces conidia more virulent to *T. molitor* than conidia produced in the dark (Oliveira et al., 2018).

In this study, conidia of *M. robertsii* were produced under normoxia (a condition of having a normal level of oxygen), hypoxia (defined as the reduction of oxygen) or after transient anoxia (a total depletion in the level of oxygen). Fungi frequently encounter oxygen-limiting circumstances in their natural environment, such as soil and during the infection. Filamentous fungi are confronted with low levels of atmospheric oxygen, ranging from 21 in aerobic to 0 % in anoxic habitats (Hillmann et al., 2015). The switch from normoxia to hypoxia or anoxia generally requires massive modification in energy metabolism (Ernst and Tielker, 2009). In addition to the role of oxygen for energy generation, oxygen is also needed by fungi for metabolic steps in the biosynthesis of sterols, unsaturated fatty acids, and some vitamins (Carlile et al., 2001). Recent studies have shown that adaptation to hypoxia contributes to virulence of pathogenic fungi. *Aspergillus fumigatus*, for example, can adapt and continue growth to levels below 0.2 % O_2 when infecting the lungs of immunocompromised patients (Hillmann et al., 2015). Consequently, we hypothesized that hypoxia or transient anoxia during mycelial growth of *Metarhizium robertsii* may produce conidia more virulent to insects.

Materials and methods

Fungal isolate

Metarhizium robertsii (ARSEF 2575) was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Robert W. Holley Center for Agriculture & Health, Ithaca, NY, USA).

Conidial production and harvesting

Conidia were produced on 23 ml of the following media: potato dextrose agar medium (PDA) (Difco, Sparks, MD, USA), or on MML, a basal medium supplemented with lactose according to Oliveira et al. (2018) for growth under nutritive stress. Growth under nutritive stress is the positive control, a condition that induces *M. robertsii* to produce conidia with higher tolerance to UV-B radiation and heat (Rangel et al., 2012; Rangel et al., 2011), and more virulent to insects than conidia produced on PDA medium (Oliveira et al., 2018; Rangel et al., 2008a). The pH of all media was adjusted to 6.9. The cultures were incubated at 26 ± 1 °C for 14 days in the dark. Four different batches of conidia were produced for each experiment.

Conidia of *Metarhizium robertsii* were produced on PDA medium under normoxia, continuous hypoxia (Petri dishes sealed three times with Parafilm®) (Bemis Flexible Packaging, Neenah, WI, USA) as described elsewhere (Fayzalla et al., 2008; Rangel et al., 2015; Shabana et al., 2001), and under transient anoxia. The cultures were grown for 24 h under normoxia and transferred to anaerobic jar with BD GasPak™EZ (260001) Anaerobe Container System with Indicator (Sparks, MD, USA) for five days. According to the GasPak™EZ manufacturer, the amount of carbon dioxide in the anaerobic jar is approximately 13 %, and the mean of oxygen content is less than 0.7 %. After five days the, plates were transferred to normoxia for eight more days.

Measurement of Conidial production

To measure conidial production under different conditions, three agar plugs (per plate) were removed with a 5 mm diam cork borer at places on the medium surface with an even coverage of conidia. Conidia from the three agar plugs were suspended in 1 ml sterile Tween 80 (0.1 %) solution in one microcentrifuge tube. Conidial concentrations were determined by hemocytometer counts. Each experiment was performed on four different dates with a new batch of cultures.

Conidial germination

Conidia from all treatments were harvested after 14 days of growth with one pass

of a microbiological loop (Decon Labs, Inc. PA, USA) and transferred to 10 ml of sterile Tween 80 (0.01 % v/v) in screw cap Pyrex tubes (20 × 125 mm). The quantity of conidia collected was enough for a suspension of 10^5 conidia ml^{-1}. The suspensions were vortexed, and 40 µl were inoculated (dropped, but not spread) onto the center of polystyrene Petri dishes (35 × 10 mm) with 5 ml of PDA medium. The plates were kept at 28 °C in the dark; this temperature is optimal for germination and growth for this fungal isolate (Rangel et al., 2010). The germination was observed 2, 4, 6, 8, 10, 12, and 14 h after conidial inoculation.

Virulence bioassays

For virulence bioassay experiments, conidia from 10 Petri dishes from each treatment were collected after 14 days with a sterile polyethylene cell lifter (Costar 3008, Corning International, Corning, NY, USA) to liberate the conidia from the mycelial mat. For the experiments of conidia produced on MML, conidia were collected using the polyethylene cell lifter from 20 Petri dishes after 14 days of growth and harvested as described above.

Conidia were transferred to 10 ml of sterile Tween 80 (0.1 % v/v) in sterile 50 ml Erlenmeyer. The first suspension was then filtered using two layers of sterile gauze to remove the mycelial mat. The conidial concentration was adjusted to 1×10^5 or 1×10^7 conidia ml^{-1}.

Fifty *Tenebrio molitor* (Vidaproteína Indústria e Comércio Ltda, Nerópolis, GO, Brazil) were used for each treatment. The weight average ± standard deviation of the insects was 101 ± 9.34 mg per insect. The insects were dipped for 20 s into 10 ml of each conidial concentration in 50 ml sterile Nalgon plastic beakers or into sterile Tween 80 [0.1% (v/v) solution] as a control. The insects were then removed from the suspensions by sterile gauze filtration for 30 s. The insects were maintained without food in 60 × 15 mm polystyrene Petri dishes, ten insects per dish, inside plastic boxes lined with wet filter paper to maintain relative humidity at approximately 90 %. The plastic boxes were kept at 26 ± 0.5 °C for 14 d in the dark. Insect mortality was recorded daily and the dead insects were kept in humid chambers lined with moist filter paper and monitored for 14 d for conidiogenesis. Four complete trials (repetitions) were conducted using a different culture batch and insect population for each trial.

Statistical analyses

Data of all experiments were analyzed using analysis of variance of a one-way factorial. Significance levels of pair-wise mean comparisons among treatments were controlled for experiment-wise type I error using the Tukey method with $\alpha = 0.10$. All analyses were carried out using the free statistical program Sisvar (Ferreira, 1999; Ferreira, 2011).

Results

Growth of *Metarhizium robertsii* under normoxia, hypoxia, and transient anoxia produced similar quantity of conidia (Figure 1A) but with different colony morphology and color (Figure 1B). The colony kept under normoxia had dark green (teak color) conidia without a mycelial overlayer (Figure 1B). The colonies from hypoxia and anoxia treatments produced conidia under a copious overlayer of white mycelium (Figure 1B) as described before in Rangel et al. (2004). This mycelial overlayer covered the spores, but it was easily removed before conidial harvesting. In addition, the colony from hypoxia treatment produced hedge-green conidia as illustrated previously in Rangel et al. (2006b), but the colony from anoxia treatment produced conidia with the same color as normoxia (teak color).

Growth on nutritive stress medium (MML) produced five times less conidia (Figure 1A), but it germinated faster than conidia produced under normoxia, hypoxia, and transient anoxia, which germinated at the same speed (Figure 1C). However, at 10 h, conidia produced under hypoxia germinated at the same speed as conidia produced on MML. At 12 h conidia produced on MML germinated at the same speed as conidia produced under normoxia and hypoxia. At this time, only conidia produced under anoxia germinated slower than conidia produced on MML.

Conidia produced under transient anoxia were similarly virulent as conidia produced on MML (Figure 2), a medium known to produce conidia with higher germination rates and virulence (Oliveira et al., 2018; Rangel et al., 2008a). Although, in the smaller concentration, it was found that conidia produced on MML were more virulent in all mortality curve than conidia produced in all other three treatments (Figure 2A). However, at the highest concentration on days 6 and 7, conidia produced under

transient anoxia were more virulent than conidia produced in normoxia and hypoxia, but with similar virulence than conidia produced on MML (Figure 2B). Conidia produced under hypoxic conditions are statistically similar to conidia produced under normoxia, even though, it looks like it killed faster than conidia produced under normoxia (Figure 2A).

Discussion

Little is known about the effects of transient anoxia during mycelial growth on the fungal phenotype and virulence. In this study, transient anoxia produced conidia more virulent than conidia produced under normoxia and hypoxia, and the virulence of conidia produced under transient anoxia were somewhat similar to the virulence of conidia produced on nutritive stress. The MML produced conidia that germinated faster and were more virulent to *T. molitor*; therefore, this medium has been used in these experiments as a positive control for comparison, as described elsewhere (Oliveira et al., 2018; Rangel et al., 2008a; Rangel et al., 2015). Also conidia produced on MML are more tolerant to UV-B radiation and heat (Rangel et al., 2012; Rangel et al., 2011) because of its high accumulation of trehalose and mannitol inside the conidia (Rangel et al., 2006a).

Hypoxia condition produced conidia of *M. robertsii* with similar virulence and germination speed as conidia produced under normoxia. However, conidia of the fungus *Metarhizium lepidiotum* obtained under hypoxic conditions showed significantly lower viability, hydrophobicity, and virulence against *Tenebrio molitor* larvae, compared with those obtained under normal atmospheric conditions (Garcia-Ortiz et al., 2015).

Growth under hypoxia produced similar quantity of conidia as normoxia, similar to found by Rangel et al. (2015); however, in other fungi such as *Pyrenophora semeniperda*, the treatment by sealing the Petri dishes with Parafilm® decreased the conidiogenesis (Campbell et al., 2003). Different phenotypes in fungi is caused by sealing the Petri dishes with Parafilm, which causes a hypoxic condition (Rangel et al., 2015). Sealing stimulates microphorogenesis and inhibits macrophorogenesis in *Phycomyces blakesleeanus* (Corrochano and Cerda-Olmedo, 1988), and reduces red pigmentation, mycelial growth, and suppressed sporulation of the fungus *Alternaria eichhorniae* (Shabana et al., 2001). In addition, growth under hypoxia produces conidia

of *Metarhizium robertsii* more tolerant to heat than conidia produced under normoxia (Rangel et al., 2015).

Conversely, enriched oxygen pulses (e.g. 26 or 30 % O^2) higher than normal atmosphere (21 % O^2) during mycelial growth is known to increase conidial production and conidia with greater germination rate and resistance to thermal and osmotic stress of *Isaria fumosorosea* (Miranda-Hernández et al., 2014) and *Metarhizium lepidiotae* (García-Ortiz et al., 2017; 2018; Tlecuitl-Beristain et al., 2010). In addition, growth above 26 % oxygen pulses produced conidia with faster germination rates, with increased tolerance to heat, UV-B radiation, and osmotic stress, and were more virulent to the insects than conidia produced under normal oxygen levels (García-Ortiz et al., 2017; Garcia-Ortiz et al., 2015; Miranda-Hernández et al., 2014). Consequently, low or high amounts of oxygen significantly impacted the produced conidia, with changes in virulence and other physiological traits.

In conclusion, *M. robertsii* increased virulence to *T. molitor* when its conidia were produced under transient anoxia in comparison to conidia produced under normoxia. Oxygen limitation is linked to a reduction in growth rate in *Aspergillus niger* that coincides with an increase in mannitol production (Diano et al., 2009), and a marked increase in the production of other polyols such as erythritol, xylitol, glycerol, and arabitol (Meijer et al., 2007). Higher accumulation of erythritol (Hallsworth and Magan, 1994) as well as mannitol and trehalose (Rangel et al., 2008a; Rangel et al., 2008b) in conidia is known to improve conidial virulence. In addition, oxygen limitation in *Beauveria bassiana* can induce production of catalase and superoxide dismutase activity, which are enzymes known to protect the fungal cells against oxidative stress (Garza-Lopez et al., 2012). The production and detoxification of reactive oxygen species (ROS) is likely to play a widespread role in signaling and damage at low-oxygen stress (Mustroph et al., 2010). Because the fungus must overcome host defenses that can include oxidative burst, melanization, sequestration of nutrients, antimicrobial compounds, as well as behaviors intended to minimize the ability of microbes to infect such as behavioral fever (Ortiz-Urquiza and Keyhani, 2015), defenses such as polyols and anti-oxidative enzymes must protected and helped conidia produced under transient anoxia to become more virulent.

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LEGENDS FOR THE FIGURES

Figure 1. A) Conidial production; B) Growth on day 14; and C) Germination of *Metarhizium robertsii* (ARSEF 2575) accordingly to the treatments: Conidia produced on PDA medium under normoxia; Conidia produced under hypoxia; Conidia produced under anoxia; Conidia produced under normoxia on minimum medium plus 3 % lactose (MML).

Figure 2. Virulence of *Metarhizium robertsii* (ARSEF 2575) to *Tenebrio mollitor* larvae (Coleoptera: Tenebrionidae) accordingly to the treatments: 1) Conidia produced on PDA medium under normoxia; 2) Conidia produced under hypoxia; 3) Conidia produced under anoxia; 4) Conidia produced under normoxia on minimum medium plus 3 % lactose (MML).

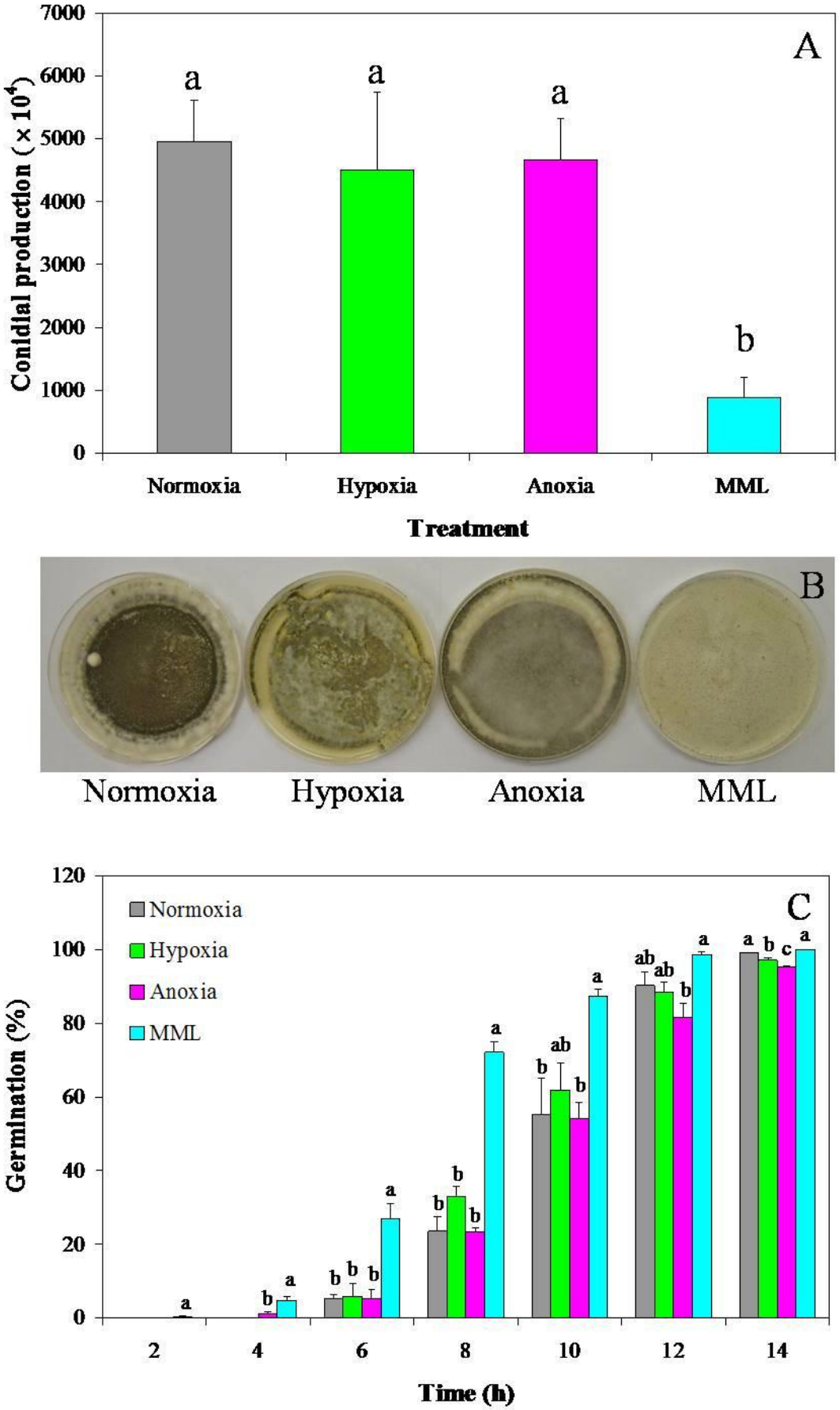
A
Conidial production (×10⁴)
7000
6000
5000
4000
3000
2000
1000
0
a
a
a
b
Normoxia
Hypoxia
Anoxia
MML
Treatment
B
Normoxia
Hypoxia
Anoxia
MML
C
Normoxia
Hypoxia
Anoxia
MML
Germination (%)
120
100
80
60
40
20
0
2
4
6
8
10
12
14
Time (h)

Figure 1.

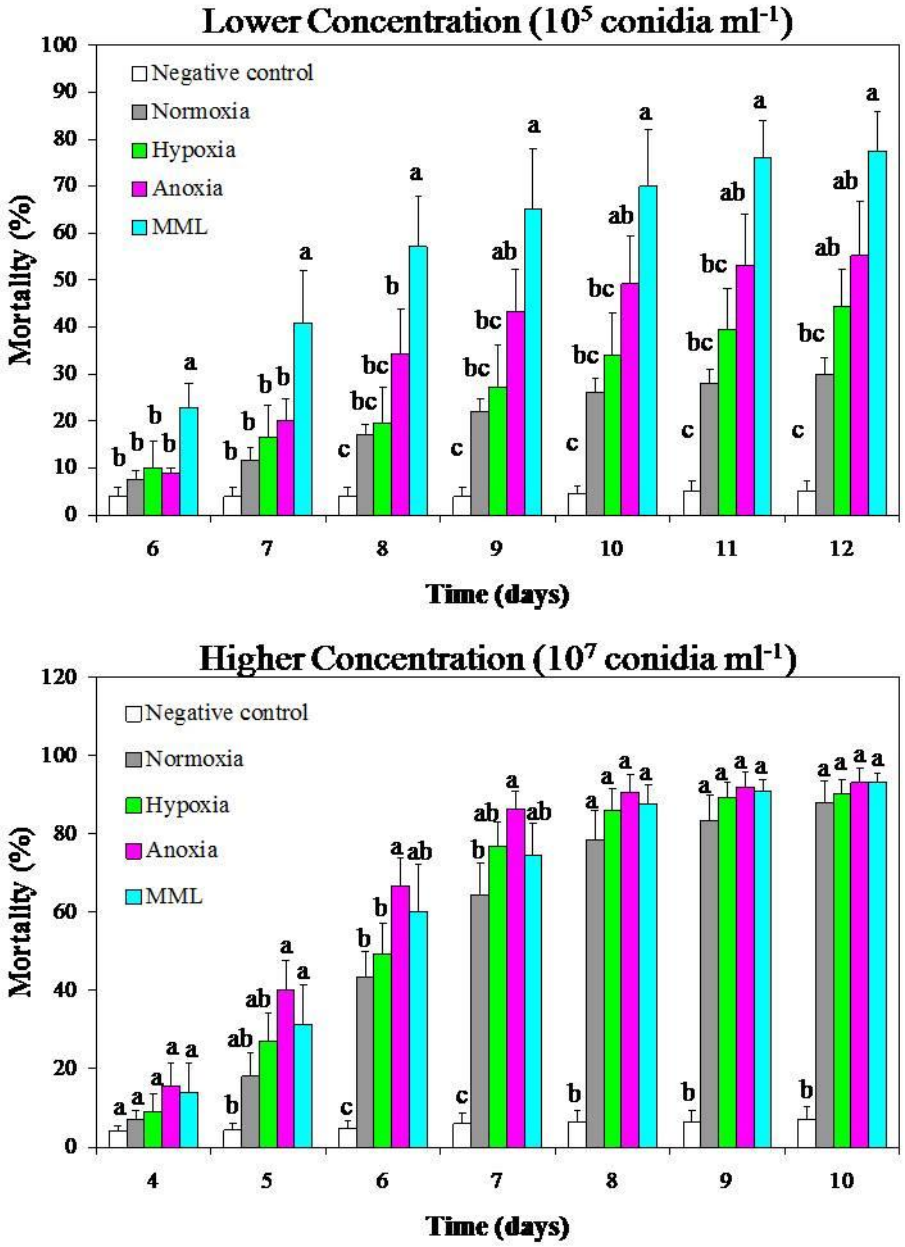
Lower Concentration (10^5 conidia ml^-1)
Negative control
Normoxia
Hypoxia
Anoxia
MML
Mortality (%)
Time (days)
Higher Concentration (10^7 conidia ml^-1)
Negative control
Normoxia
Hypoxia
Anoxia
MML
Mortality (%)
Time (days)

Figure 2

CHAPTER 4 – GENERAL CONCLUSIONS

The studies presented in Chapter 2 (Oliveira et al. 2018) found that mycelial growth under different colors of light had different effects on the viability and virulence of the conidia produced under these conditions. Blue light promoted a higher production of conidia and with faster germination speed. Red light did not increase the production of conidia and delayed the germination time. The virulence to the insect of the conidia produced under blue and red light was similar and not statistically different from the conidia produced in the dark. White light during fungus growth promoted both the production of conidia with higher germination speed and greater virulence to the insect *Tenebrio molitor*.

The studies presented in Chapter 3 (Oliveira & Rangel 2018) showed that oxygen deprivation during growth of the entomopathogenic fungus *M. robertsii* did not alter conidia production and its germination speed. However, transient anoxia condition helped to produce conidia more virulent to the insect.

In both chapters, conidia were produced under nutrient stress which is used as a "positive control" for virulence, as it is known that this condition stimulates the production of *M. robertsii* conidia that are more virulent to *T. molitor* (Rangel et al 2008). Also in this study, the growth of the fungus in minimal medium supplemented with lactose (MML) produced conidia with faster germination and that was more virulent to the insect than the other treatments; however, the conidia production was 7 times lower than the conidia production in PDA medium in the dark.

Another interesting fact of these studies was the effect, of both the different light conditions and hypoxia and transient anoxia, on the fungus phenotype. The observed morphology of the fungus changed in each treatment studied, which, related to the differences in the characteristics studied, is evidence of changes in the natural physiology of the fungus *Metarhizium robertsii*.

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APPENDICES