ENTOMOPATHOGENIC FUNGI FOR THE CONTROL OF ARTHROPOD PESTS

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ENTOMOPATHOGENIC FUNGI FOR THE CONTROL OF ARTHROPOD PESTS

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EDITORIAL
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Editorial: Entomopathogenic Fungi for the Control of Arthropod Pests

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Editorial on the Research Topic

Entomopathogenic Fungi for the Control of Arthropod Pests

Biological control has stood out, particularly in recent years, as a sustainable and environmental-friendly method to control arthropod pests that cause significant economic losses in agriculture, and negatively impact animal and human health. This Research Topic encompasses the contribution of eleven original research articles/reviews that demonstrate the use of entomopathogenic fungi (EPF), especially *Metarhizium anisopliae* and *Beauveria bassiana,* to control arthropod pests with relevance in agriculture (*Athalia rosae*, *Solenopsis invicta* and *Oedalus asiaticus*), animal health (*Stomoxys calcitrans, Rhipicephalus microplus* and *Haemaphysalis longicornis*), and public health (*Anopheles* sp. and *Aedes aegypti* mosquitoes). In addition, this collection includes two original research articles that expand our knowledge on the molecular mechanisms underpinning blastospores' and microsclerotia development and provide insights into microsclerotia tolerance to ultraviolet B (UV-B) radiation and heat.

The virulence of EPF depends on the infectivity of their conidia or blastospores, propagules with distinct morphological and physiological characteristics (Mascarin et al., 2019) that in turn present advantages and disadvantages to their use (de Paula et al., 2021). In this Research Topic, Paixão et al. describe two other *Metarhizium robertsii* propagules (microsclerotia and mycelial pellets) that are morphologically similar but differ in biomass production and tolerance to UV-B radiation and heat. This study also characterized the ultrastructure and gene expression pattern involved in microsclerotial differentiation.

Blastospores of *B. bassiana* are promising propagules for pest control. Their production is conducted via liquid fermentation with high glucose concentration and high aeration. However, the mechanisms behind the development of these propagules are not completely understood. Mascarin et al. conducted an RNAseq-based transcriptomic study of *B. bassiana* blastospores and showed that a higher proportion of genes were downregulated when the fungus was grown under high glucose than under low glucose concentrations. However, other genes related to the antioxidant response, calcium transport, conidiation, and osmosensory signaling, were highly upregulated in high glucose concentrations. These molecular findings provide new knowledge on blastospore development and may help facilitate the industrial production of *B. bassiana* blastospores for a wide range of pest control applications.

The mechanism of EPF infection of a host is complex and while some advancements have been made, there is still much more that needs to be uncovered to improve biological control efforts. On

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this end, recent findings indicate that the Msn2 transcription factor regulates the acaricidal virulence in the fungal pathogen *B. bassiana*. Loss of the Msn2 transcription factor in *B. bassiana* mutants caused reduced production of protease, which may have contributed to the inability of the mutant strain to breach the cuticle of the tick *R. microplus* (Muniz et al.). Two other articles reported the use of EPF against ticks (Alonso-Dias and Fernández-Salas and Lee et al.). The first is a review on the acaricidal effect of fungi to ticks with relevance for Mexico's cattle livestock, particularly *R. microplus*, *Rhipicephalus annulatus*, and *Amblyomma mixtum*. These authors highlighted that epidemiological and environmental aspects are important for the EPF acaricidal efficacy. In turn, Lee et al. analyzed the molecular interactions at the transcriptional level between the longhorned tick *H. longicornis* and *M. anisopliae*. These authors reported that both fungus and tick genes were mostly up-regulated at the early stages of infection. This suggests that while the fungus starts the infection process, the tick is actively mounting a defense response.

The stable fly, *Stomoxys calcitrans,* is another arthropod, along with ticks, that significantly affects the livestock industry. A study by Baleba et al. assessed the infection of the stable fly by *M. anisopliae* and provided detailed information on how fungal infection affects the feeding, fecundity, and fertility of this harmful fly. Among eleven fungal strains screened, one of them was identified as the most virulent, with potential to be developed as a biopesticide agent against this fly.

Entomopathogenic fungi are also regarded as potential biocontrol agents against major arthropod vectors of human pathogens. Two articles in this Research Topic describe new efforts on this front, providing a potential new delivery method and identifying new fungal species with biocontrol potential. Here, Reyes-Villanueva et al. described the contact rate of wild *A. aegypti* females with fungus-infected males, providing support to a mosquito control strategy via the release of fungus-infected males. Meanwhile, Accoti et al. conducted an environmental screen and described five fungi with pathogenicity against two major mosquito vectors.

Smaller in size, in comparison to the other arthropod pests described above, the fire ant, *S. invicta*, is a major urban and agricultural pest. A study by Wei et al. examined the temporal gene expression profiles of chemosensory and odorant binding proteins (CSPs and OBPs, respectively) in response to infection by *B. bassiana*. Their study describes the dynamic gene expression of these two gene families, providing insights into the mechanisms that might mediate detection of microbial pathogens, and would trigger grooming and nest sanitation. In a study that evaluated the use of EPF against another hymenopteran, the sawfly *A. rosae*, Zanchi et al. demonstrated that this insect pest is resistant to *B. bassiana* infection, with low incidence of mycelial growth and sporulation from *A. rosae* cadavers. Furthermore, their results revealed that clerodanoids, compounds with antimicrobial activity adsorbed by *A. rosae* adults from host plants, are unlikely to be responsible for their resistance against this EPF.

Entomopathogenic fungi are often tested in association with plant-derived metabolites in an effort to increase their lethality against insect pests. In this regard, plant-derived oils can provide added protection to the propagule and maintain EPF virulence. In such study, Li et al. evaluated the synergistic control effect of *artemisia sieversiana* crude extracts with *M. anisopliae* on *O. asiaticus*, a major pest in northern China. By using different doses of *M. anisopliae* and crude extracts of *A. sieversiana,* singly and in combination, and by analyzing four insect enzymes, the authors demonstrated that *A. sieversiana* effectively increases *M. anisopliae* virulence.

The review articles summarize the current state of the art and the original articles included in this Research Topic provide much-needed new knowledge in this area of research and will improve the use of EPF as biocontrol agents against important arthropod pests. We thank all authors for their contribution and participation in this Research Topic. We are also grateful to all reviewers and editors, whose participation during the publication process made the development of this Research Topic possible.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

de Paula, A. R., Silva, L. E. I., Ribeiro, A., da Silva, G. A., Silva, C. P., Butt, T. M., et al. (2021). Metarhizium Anisopliae Blastospores Are Highly Virulent to Adult *Aedes aegypti*, an Important Arbovirus Vector. *Parasites Vectors* 14, 555. doi:10.1186/s13071-021-05055-z

Mascarin, G. M., Lopes, R. B., Delalibera, Í., Fernandes, É. K. K., Luz, C., and 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. doi:10.1016/j.jip.2018.01.001

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ORIGINAL RESEARCH
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Infection of the Stable Fly, *Stomoxys calcitrans*, L. 1758 (Diptera: Muscidae) by the Entomopathogenic Fungi *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) Negatively Affects Its Survival, Feeding Propensity, Fecundity, Fertility, and Fitness Parameters

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Entomopathogenic fungi can cause substantial mortality in harmful insects. Before killing the insect, these pathogens start by negatively affecting the biological parameters of the host. Prior to our study, the information about how fungal exposure affects the biological parameters of the stable fly, *Stomoxys calcitrans* was still elusive. Therefore, we aimed to assess the infection of *S. calcitrans* with some *Metarhizium anisopliae* strains, and their impact on feeding, fecundity, fertility and other life-history traits of this fly. Among the 11 *M. anisopliae* strains screened, we identified ICIPE 30 as the most virulent strain against *S. calcitrans*. We observed that the infectivity of this strain was sex and age-dependent. Infected male *S. calcitrans* died earlier than their counterpart females. Older infected *S. calcitrans* died faster than infected young ones. Also, male and female *S. calcitrans* successfully transmitted ICIPE 30 conidia to their mates. We demonstrated that infection by ICIPE 30 extended the feeding time of *S. calcitrans* and consequently reduced the feeding probability of the fly and the amount of blood taken. Using a dual test oviposition bioassay, we determined that uninfected gravid female *S. calcitrans* avoided laying eggs on substrates amended with ICIPE 30 conidia. We showed that these conidia could lower the hatchability of the eggs deposited by gravid females. Using, a no-choice test, we showed that gravid female *S. calcitrans* infected with ICIPE 30 laid fewer eggs than uninfected females and those eggs hatched less. Using 11 strains of *M. anisopliae* and four high concentrations of ICIPE 30 conidia, we verified that *S. calcitrans* larvae were not susceptible to fungal infection. Further, we showed that though these larvae were tolerant to fungal infection, there was a significant effect on their fitness, with contaminated larvae having a small bodyweight coupled with longer developmental time as compared to uncontaminated larvae. Our study provides detailed information on how fungal infection

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affects the biology of *S. calcitrans* and the potential of using *M. anisopliae* ICIPE 30 as a biopesticide to reduce the fly population. Such knowledge can assist in developing fungal-based control strategies against this harmful fly.

Keywords: *Metarhizium anisopliae*, *Stomoxys calcitrans*, biological parameters, pre-lethal effects, ICIPE 30

INTRODUCTION

Metarhizium anisopliae (Hypocreales: Clavicipitaceae) is a ubiquitous entomopathogenic fungus infecting a wide range of insect hosts and used for biological control (Brunner-Mendoza et al., 2019). Its mode of action involves attachment to the host's cuticle, germination, epicuticle penetration and dissemination inside the insect body as hyphae (Ortiz-Urquiza and Keyhani, 2013). This infection mechanism is facilitated by a group of enzymes including hydrolases, proteases, chitinases and lipases (Brunner-Mendoza et al., 2019). When proliferating inside insect tissues, blastospores produce toxic molecules (e.g., destruxins) that induce pathogenesis, paralysis, cellular alterations and dysfunction of the middle intestine, malpighian tubules, and muscle tissues (Samuels et al., 1988). These cascades of events ultimately provoke insect death 3–7 days after infection (Mondal et al., 2016). It has been demonstrated that the speed with which an insect succumbs from fungal infection could depend on the fungal strain (Valero-Jiménez et al., 2014), insect species, insect sex, and age (Maniania and Odulaja, 1998). However, before the death occurs, several pre-lethal reactions including the reduction in development, feeding propensity, and reproduction can be observed in infected insects.

Several laboratory and field trials have demonstrated the lethal and pre-lethal effects of *M. anisopliae* infection in insects. In blood-feeding insects, this fungus is known to cause high mortality in immature and adult stages. For instance, *M. anisopliae* has proven to reduce survival of different larval stages of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* (Diptera: Culicidae) (Greenfield et al., 2015; Ravindran et al., 2016). In *Phlebotomus papatasi* (Diptera: Psychodidae), *M. anisopliae* reduces adult emergence when applied to larval food (Zayed et al., 2013; Alkhaibari et al., 2017). Adults of the tsetse fly, *Glossina morsitans* (Diptera: Glossinidae) that emerge from *M. anisopliae*-infected pupae suffer from high mortality (Kaaya and Munyinyi, 1995). Maniania (2002) found that in the field *M. anisopliae* can reduce the population of *Glossina* spp. by 82.4%. Laboratory-based bioassay revealed high mortality in *Anopheles gambiae* and *C. quinquefasciatus* owing to *M. anisopliae* infection (Scholte et al., 2003). In *Meccus pallidipennis* (Hemiptera: Reduviidae), a vector of *Trypanosoma cruzi*, Flores-Villegas et al. (2016) observed that individuals treated with *M. anisopliae* died sooner than untreated individuals. In addition to this lethal effect, infection by *M. anisopliae* is also known to induce pre-lethal effects on hematophagous insects. This has been shown in *An. gambiae* (Scholte et al., 2003) and *Ae. aegyptis* (Paula et al., 2011); where a reduction of feeding and reproduction was reported after exposure to *M. anisopliae*. Here, we studied the infection of *M. anisopliae* in the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae) and its consequences on the feeding, fecundity, fertility, and life-history traits of this fly.

S. calcitrans is a cosmopolitan blood-feeding dipteran involved in the mechanical transmission of viruses (e.g., West Nile fever virus, Rift Valley fever virus), bacteria (e.g., *Bacillus anthracis*, *Pasteurella multocida*), protozoa (e.g., *Trypanosoma evansi*, *Besnoitia besnoit*), and helminths (e.g., *Habronema microstoma*, *Dirofilaria repens*) in various hosts including cattle, camels, horses, dogs, and humans (Baldacchino et al., 2013). During its high infestation periods, *S. calcitrans* can induce a reduction of 40–60% in milk yield and 19% in cattle weight gain (Walker, 1990; Carn, 1996) as a result of the nuisance caused to livestock. In cattle industries, losses attributed to *S. calcitrans* are estimated to be around $2.2 billion annually (Taylor et al., 2012). As all holometabolous insects, the development of *S. calcitrans* goes through an egg, three larval instars (with the size and morphology varying across the three instars (see Friesen et al., 2015), pupae and adult stages. The development of *S. calcitrans* occurs in herbivorous dung (Baleba et al., 2019) and rotting organic matter such as silage, hay, grass clippings, and garden compost (Cook et al., 2018).

The use of the entomopathogenic fungus *M. anisopliae* as a control agent against *S. calcitrans* has already been investigated in various studies; with results showing the high susceptibility of adults, but not larvae (Moraes et al., 2008). The *M. anisopliae* strain Ma135 was reported to kill more than 90% of *S. calcitrans* adults (López-Sánchez et al., 2012). When aspersed on dairy cattle, Cruz-Vazquez et al. (2015) established that the *M. anisopliae* strain Ma134 reduce populations of S. calcitrans by 73%. To our knowledge, there are no studies in the literature addressing the pre-lethal effect of *M. anisopliae* infection in *S. calcitrans* or the influence of the age or sex of the fly. Moreover, there is no evidence of the horizontal transmission of *M. anisopliae* conidia in *S. calcitrans*. Thus, our main aim was to study the lethal and pre-lethal effect of *M. anisopliae* infection in *S. calcitrans*. Specifically, we tested whether: (1) infection of *S. calcitrans* by *M. anisopliae* conidia would be sex- and age-dependent; (2) *M. anisopliae* conidia would be horizontally transmitted in *S. calcitrans*; (3) *M. anisopliae* infection would negatively impact the feeding propensity, fecundity and fertility of *S. calcitrans*; (4) there would be a trade-off between the tolerance of *S. calcitrans* larvae to *M. anisopliae* infection and their larval life-history traits.

MATERIALS AND METHODS

M. anisopliae Strain Culture

The eleven strains of *M. anisopliae* used in our experiment were obtained from the *icipe*'s Arthropod Germplasm Center (Mweke

et al., 2018; Akutse et al., 2020). The strains were cultured on Sabouraud Dextrose Agar (SDA) medium using 90-mm Petri dishes and maintained in the darkness at 25°C. Two weeks after the start of the culture, we harvested conidia of each strain by scraping the surface of the sporulated cultures using a sterile spatula. We suspended conidia of the different strains in 10 ml of distilled water with 0.05% Triton X-100 in universal bottles containing 3–5 glass beads (3 mm in diameter per bottle) each. The mixture was then vortexed for 5 min at 700 rpm to homogenize the suspension. Using an improved Neubauer haemocytometer under the light microscope, we determined the conidia concentration of each strain suspension following the protocol described by Lacey (2012). Before each bioassay, we tested the ability of conidia to germinate by spreading 100 µl of each strain suspension (titrated at 3×10^6 conidia ml^{-1}) on SDA plate. We sealed the inoculated plates with Parafilm membrane and incubated them in complete darkness at 25°C. At 18 h post-incubation, we flooded the plates with lactophenol aniline cotton blue to stop the germination process and stain the spore to ease their visibility for counting. Following this, we determined the number of conidia that germinated by counting 100 randomly selected conidia beneath each coverslip under a light microscope (400×). Conidium was considered as germinated if the length of its germ-tube was at least twice its diameter (Lacey, 2012). For each strain, we used five plates as replicates.

S. calcitrans Colony

Individuals of *S. calcitrans* used in all our experiment were obtained from the "International Center of Insect Physiology and Ecology (*icipe*)" Animal Rearing and Quarantine Unit (ARCU) in Nairobi, Kenya (1° 13′ 12″ S, 36° 52′ 48″ E; ≈ 1,600 m above sea level) colony. This colony was established and maintained as described in Baleba et al. (2019). Briefly, wild individuals of *S. calcitrans* were captured at *icipe* campus using Vavoua traps (Laveissière and Grebaut, 1990), maintained inside a cage (75 cm × 60 cm × 45 cm) and fed twice per day (800 and 1,600 h) on defibrinated bovine blood poured on moistened cotton to initiate reproduction. Once gravid females were obtained, we exposed them to rabbit dung (fermented in a plastic bag for 1 week) placed in plastic containers (21.5 cm × 14.5 cm × 7.4 cm) for oviposition. After 24 h, we transferred the exposed containers to another cage (75 cm × 60 cm × 45 cm), and we monitored the development of the larval and pupal stages until adult emergence. We fed emerged adults with bovine blood and repeated the previously described above. We reared all the insects and performed our experiments in a laboratory under buffered conditions of 25 ± 5°C 65 ± 5% relative humidity, and 12L:12D photoperiod.

Effect of *M. anisopliae* Infection on the Survival of *S. calcitrans* Adults

We determined the pathogenicity and virulence of 11 strains of *M. anisopliae* on *S. calcitrans* adults following the contamination protocol used by Wamiti et al. (2018) on *Glossina fuscipes fuscipes* (Diptera: Glossinidae). In this protocol, the contamination device (**Supplementary Figure 1A**) is comprised of a cylindrical plastic tube (95 mm × 48 mm) which has an inner part covered by a velvet carpet material impregnated with fungal dried conidia. Adult flies were gently introduced into the contamination device and allowed to pick conidia. After this period of exposure, flies were gently removed, and transferred in another cylindrical plastic tube free of conidia. In all our experiment, we used 0.1 g of conidia evenly spread on the velvet carpet, and exposed flies to conidia for 10 min. After transferring the fungus-exposed flies in a clean cylindrical plastic tube, we provided them with blood and recorded the number of dead flies daily for 7 days. We removed cadavers found inside the plastic tube using sterilized forceps and incubated them in Petri dishes containing moistened filter paper to assess the outgrowth of the applied fungal conidia (**Supplementary Figure 1B**). We used fungus-free flies as control. For each treatment, we used 10 flies and replicated the experiment five times.

Effect of Sex and Age on *M. anisopliae* ICIPE 30 Infectivity in *S. calcitrans*

We used newly emerged (24 h old) males and females [differentiated based on the size of the two compound eyes that are smaller and more widely separated in females (dioptic) than in males (holoptic)] to see whether the pathogenicity of *M. anisopliae* varied between the sex of *S. calcitrans*. In the earlier experiment, we identified ICIPE 30 as the most virulent *M. anisopliae* strain against *S. calcitrans* (see results section). Here, we used this strain (0.1 g of dried conidia) to contaminate 10 males and 10 females following the protocol previously described above (Wamiti et al., 2018). As a control, we used unexposed males and females. For the age effect bioassay, we used only 10 exposed female flies (to account for any bias resulting from sex effect) of 1, 7, and 14 days old. The control groups consisted of fungus-free flies of 1, 7, and 14 days old. In both bioassays, we provided each group with bovine blood and recorded individual mortalities daily for 7 days. To confirm whether the death of the flies was caused by *M. anispoliae* ICIPE 30 infection, we placed dead flies in Petri dishes (9 cm) containing moistened filter paper to initiate fungal sporulation on the cadaver surfaces. We replicated each experiment five times.

Horizontal Transmission of *M. anisopliae* ICIPE 30 Conidia by *S. calcitrans*

Before testing whether *M. anisopliae* ICIPE 30-exposed males and females could transmit conidia to their conspecific mates, we aimed to determine whether the number of conidia carried by *S. calcitrans* individuals could vary between sex and across time. To do so, we chilled 5 males and 5 females (2 days old) in ice for 2–3 min to induce a coma. Using fine sterilized forceps, we gently placed the immobilized flies inside the cylindrical plastic tube (on top of velvet carpet containing 0.1 g of ICIPE 30). After recovered from the coma, we allowed the flies to walk on conidia for 30 min, then individually introduced them inside universal bottles containing 2–5 glass beads and 1 ml of sterile distilled water with 0.05% Triton X-100. The bottles with the exposed flies were thereafter vortexed for 5 min (to remove conidia from the insect's body) and estimated the number of conidia carried by each individual using the Neubauer haemocytometer. To

assess how the number of conidia carried by each individual be across time, we transferred the exposed male and female flies in a cleaned cage (15 cm × 15 cm × 20 cm), waited for 2, 4, 6, and 8 h before proceeding with the conidia quantification as previously described.

With a slight modification, we followed the protocol described by Maniania et al. (2013) to perform the horizontal transmission assay. We contaminated 5 males (donors) with 0.1 g of *M. anisopliae* ICIPE 30 conidia for 10 min then transferred them into another clean cage (15 cm × 15 cm × 20 cm). Four hours after this process, we transferred these males inside a clean cylindrical plastic tube and paired them with 5 fungus-free females (receivers). We use the same protocol to pair fungus-exposed females (donors) with fungus-free males (receivers). We considered fungus-free males and females as control. In all the treatments, we provided our flies with blood and recorded their mortality daily for 7 days. To confirm whether the dead in both sexes was induced by *M. anisopliae* infection, we placed separately the dead bodies of male flies in Petri dishes (9 cm) containing moistened filter paper to later assess fungal growth on the cadaver surfaces. We used five replicates in all the bioassays.

Impact of *M. anisopliae* ICIPE 30 Infection on the Feeding Propensity of *S. calcitrans*

Here, using the previous contamination device, we exposed female *S. calcitrans* (2 days old) with the *M. anisopliae* strain ICIPE 30. To determine the effect of this fungal infection on the feeding propensity of *S. calcitrans*, we recorded three parameters, namely (1) the feeding duration, (2) the proportion of blood-fed, and (3) the amount of blood consumed. We determined the feeding duration by recording the time taken by an individual fly to get engorged after inserting its proboscis into the blood source (**Supplementary Figure 2B**). The proportion of blood-fed corresponded to the number of flies (in a group of 10 individuals) that managed to take blood after 60 s of their exposure to the blood exposition. The amount of blood consumed per fly was estimated as the difference in their weight, after (**Supplementary Figure 2C**) and before (**Supplementary Figure 2A**) the blood meal. As a control, we used fungus-free individuals. We collected all the data 2, 3, and 4 days after fungal infection. The feeding duration and the amount of blood consumed data were obtained from 30 fungus-exposed and fungus-free female flies; while the proportion of blood-fed data were from 5 groups of 10 individuals each.

Influence of *M. anisopliae* ICIPE 30 on Gravid Female *S. calcitrans* Reproduction Traits

To elucidate whether *M. anisopliae* ICIPE 30 could affect the reproduction of *S. calcitrans*, we used (1) egg-laying decision, (2) fecundity and (3) fertility as proxies. To test the effect of *M. anisopliae* ICIPE 30 on *S. calcitrans* egg-laying decision, we conducted two oviposition choice bioassays (**Supplementary Figure 3A**). In the first bioassay, we exposed 10 gravid female *S. calcitrans* to two Petri dishes (Diameter: 5.5 cm) containing each, only 50 g of rabbit dung to see whether they will lay the same number of eggs on both Petri dishes. For the second bioassay, we presented rabbit dung supplemented with 0.1 g of *M. anisopliae* conidia (strain ICIPE 30) and rabbit dung only (control) to 10 gravid female *S. calcitrans* to see whether these females will select either substrate preferentially. In both bioassays, we used 10 replicates, and for each replicate, we counted the number of eggs laid on each substrate after 24 h and determined their ability to hatch 5 days after egg deposition (by counting the number of larvae found on each substrate).

To assess the effect of *M. anisopliae* ICIPE 30 on the fecundity (number of eggs laid) and fertility (number of eggs hatched) of *S. calcitrans*, we performed two no-choice oviposition bioassays (**Supplementary Figures 3B**i,ii). To do so, following the previously described protocol, we exposed 30 females (4 days old) to *M. anisopliae* strain ICIPE 30 and transferred them individually inside cages (15 cm × 15 cm × 20 cm) containing 2 males to allow mating. We supplied these flies with blood daily and once females become gravid, we provided them with a Petri dish containing rabbit dung for oviposition. As a control, we used fungus-free gravid female *S. calcitrans*. We recorded the number of eggs laid on each substrate daily until the female succumbs from fungal infection. To assess the fertility of eggs laid by infected and uninfected gravid female *S. calcitrans*, we determined their hatchability by counting the number of larvae found on each substrate 5 days after the egg deposition.

Trade-Offs Between *M. anisopliae* ICIPE 30 Infection Tolerance and Life-History Traits in *S. calcitrans* Larvae

It has been reported previously that *S. calcitrans* larvae are not susceptible to *M. anisopliae* infection (Moraes et al., 2008). To test this, we infected second larval instar of this fly with the same 11 strains of *M. anisopliae* as described above. For each strain, we sprayed 10 larvae (placed on a Petri dish) with 10 ml of suspension at the concentration of 2×10^8 conidia ml^{-1} using a Burgerjon's spray tower (Burgerjon, 1956). After spraying, we transferred the contaminated larvae in transparent plastic cups of 200 ml prior containing 50 g of rabbit dung. As a control group, we used larvae treated with sterile distilled water containing 0.05% Triton X-100. We recorded the number of dead larvae daily until pupation. We carried out all the treatments in five times. We observed that most infected *S. calcitrans* larvae (90 %) managed to reach the pupal stage. Therefore, in a subsequent bioassay, we aimed to challenge these larvae with higher concentrations of *M. anisopliae*. As previously described, we contaminated 10 *S. calcitrans* larvae with four increasing conidia concentrations (3×10^8, 4×10^8, 5×10^8, and 6×10^8 conidia ml^{-1}) of the strain ICIPE 30 and recorded the number of dead larvae daily until pupation.

To test whether the tolerance to *M. anisopliae* ICIPE 30 infection in *S. calcitrans* larvae could impact their fitness parameters, we contaminated 10 individuals of each *S. calcitrans* larval instar (L1, L2, and L3) with 10 ml of ICIPE 30 concentrated at 2×10^8 conidia ml^{-1}. It is indicated that life stages that undergo metamorphosis (occasioning the change in size and morphology) should be treated independently when studying

their responses to biotic stresses (McCormick and Gagliano, 2009; Kingsolver et al., 2011; Ezeakacha and Yee, 2019). We followed the contaminated larvae daily until the adult stage by recording the following life-history fitness parameters: (1) pupation time, (2) larval weight, (3) pupation rate, (4) pupal weight, (5) emergence percentage, (6) emergence time, and (7) adult weight. Larval, pupal, and adult weight data were collected as described in Baleba et al. (2020). For the weight parameter, we weighed all the larvae individually, as well as pupae and adults that emerged from contaminated larvae. We recorded larval weight 2, 4, and 6 days after fungal contamination in the individuals from L1 and L2 instars. While in individuals from the L3 instar (close to the pupal stage), we recorded weight only 2 days after contamination. As a control, we used L1, L2, and L3 individuals sprayed with sterile distilled water containing 0.05% Triton X-100. We replicated this experiment five times.

Data Analysis

We conducted all the statistical analysis in the R environment for statistical computing (version 3.6.3) (R Core Team., 2020) and grouped all the graphs in Adobe Illustrator CC 2017(version 21.0). Before conduct the analysis, we subjected mortality data to Abbot's correction (Abbot, 1925).

For the bioassay aiming to study the effect of the 11 strains of *M. anisopliae* on the *S. calcitrans* survival, we performed Kaplan–Meier survival analysis with the Mantel–Cox log-rank chi-squared test using the R package "survival" (Therneau, 2015) to see how the survival of *S. calcitrans* adults varied as a result to exposure to the different fungus strains. Owing to the normal distribution (Shapiro–Wilk test: $P > 0.05$) and the homoscedasticity (Bartlett's test: $P > 0.05$) of the median lethal time data, we ran the analysis of variance (ANOVA) followed by the Student–Neuman–Keuls (SNK) *post-hoc* multiple comparison tests to see how this parameter varied across the 11 strains. For the same reason, we performed the ANOVA followed by the SNK *post-hoc* tests to compare the proportion of alive *S. calcitrans* (at 7th day of our bioassay) across the 11 strains.

In the experiment testing the effect of sex and age on *M. anisopliae* infectivity, we used the Kaplan–Meier survival analysis with the Mantel–Cox log-rank chi-squared test to elucidate how these factors affected the infectivity of *M. anisopliae*. We employed the unpaired *t*-test to compare the median lethal time between the sexes of *S. calcitrans*. To determine whether this parameter could vary across the three ages of *S. calcitrans* (1, 7, and 14 days), we performed the ANOVA followed by the SNK *post-hoc* tests. Using the same analysis, we compared the number of alive *S. calcitrans* (at 7th day of our bioassay) across the sex and the ages.

For the experiment aiming to test whether, in *S. calcitrans*, *M. anisopliae* conidia could be transferred from one sex to another, we used the Kaplan–Meier survival analysis with the Mantel–Cox log-rank chi-squared test to see whether the survival of the fungus-donor, fungus-receiver, and fungus-free (control) *S. calcitrans* could significantly vary. We ran the unpaired *t*-test to compare the median lethal time between fungus-donor and fungus-receiver flies. We performed the ANOVA followed by the SNK *post-hoc* tests to compare the number of fungus-donor, fungus-receiver, and fungus-free *S. calcitrans* that were still alive at the end of our experiment (7th day).

In the feeding propensity test, we used the unpaired Wilcoxon test to compare the feeding time of infected and uninfected flies. Owing to the binary nature of the feeding proportion data (engorged vs. not engorged) we performed a generalized linear model (GLM) with binomial distribution followed by the analysis of deviance (with the chi-squared test) to see how the proportion of blood-fed flies varied between infected and uninfected flies. We executed the unpaired *t*-test to compare the amount of blood taken by infected and uninfected flies.

For the experiment testing the effect of *M. anisopliae* on *S. calcitrans* reproduction, we used the paired *t*-test to compare the number of eggs laid by gravid females *S. calcitrans* on the two Petri dishes containing only rabbit dung. We used the same statistical analysis to compare the number of eggs laid by these females on Petri dishes with and without *M. anisopliae* ICIPE 30 dried conidia. The Egg hatchability data were binary (hatched vs. unhatched); therefore, we used a GLM with binomial distribution and analysis of deviance (with chi-squared test) to see how this parameter varied between substrates with and without conidia. To compare the number of eggs laid by infected and uninfected gravid females *S. calcitrans*, we used an unpaired *t*-test. We compared the hatchability the eggs produced by these females, using a GLM with binomial distribution and analysis of deviance (with chi-squared test).

For the data from the bioassay testing the effect of *M. anisopliae* on the survival of *S. calcitrans* larvae, we performed the ANOVA test to compare the proportion of larvae that pupated across the different *M. anisopliae* strains and the ICIPE 30 concentrations. In the experiment testing the effect of *M. anisopliae* infection on the life-history parameters of three different larval instars of *S. calcitrans*, we subjected data from pupation time, larval weight, pupal weight, emergence time, and adult weight to the normality and homogeneity tests. In case the data of a particular parameter were normally distributed (Shapiro–Wilk test: $P > 0.05$) and their variances were homogeneous (Bartlett's test: $P > 0.05$), we used the unpaired *t*-test to see how this parameter varied between infected and uninfected larvae. When these two assumptions were not fulfilled, we used the unpaired Wilcoxon test. We analyzed the pupation and emergence percentage data using a GLM with binomial distribution and analysis of deviance (with chi-squared test).

Statistical significance was noted at $P < 0.05$ and its strength was represented with asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, and $^{****}P < 0.001$).

RESULTS

Effect of *M. anisopliae* Infection on the Survival of *S. calcitrans* Adults

All the 11 strains of *M. anisopliae* used in our study possessed a germination percentage above 90% (**Figure 1A**). As time progressed, the proportion of *S. calcitrans* surviving from *M. anisopliae* infection reduced with a significant difference across

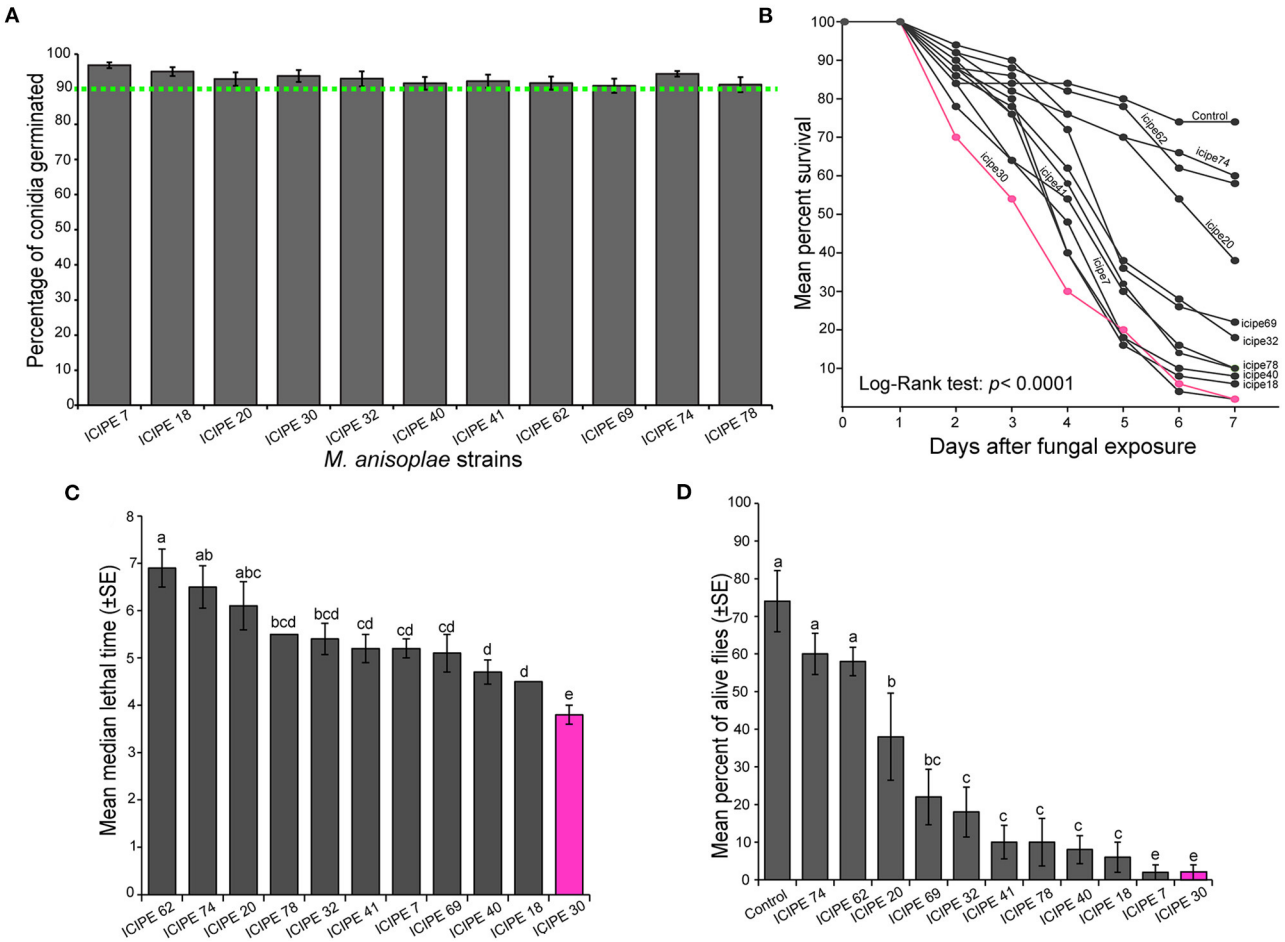

FIGURE 1 | Virulence of different *Metarhizium anisopliae* strains toward *Stomoxys calcitrans*. **(A)** Bar chart showing the mean percentage germination of the 11 *M. anisopliae* strains used in all our experiment. **(B)** Kaplan–Meier curve showing survivorship over time in the adult of *S. calcitrans* across the different *M. anisopliae* strains (Mantel–Cox log-rank χ^2 test, $P < 0.05$, $n = 5$). **(C)** Bar chart illustrating the mean median lethal time (LT_{50}) in *S. calcitrans* adults infected by the different *M. anisopliae* strains. **(D)** Bar chart depicting the mean percentage of *S. calcitrans* adults alive 7 days after fungal infection, across the different *M. anisopliae* strains (ANOVA followed by SNK *post-hoc* test; $p < 0.05$, $n = 5$). Error bars indicate the standard error of the mean. Bars with different lowercase letters are significantly different from each other.

the strains (**Figure 1B**; log-rank test, $\chi^2 = 50.3$, df $= 11$, $P < 0.0001$). The median lethal time [**Figure 1C**; One-way ANOVA: $F_{(10-44)} = 7.79$, $P < 0.0001$] and the proportion of alive *S. calcitrans* after the 7 days post-infection [**Figure 1D**; One-way ANOVA: $F_{(11-47)} = 17.5, P < 0.0001$] significantly differed across the 11 strains of *M. anisopliae*. Of all the *S. calcitrans* individuals infected with 11 strains of *M. anisopliae*, only those infected with the strain ICIPE 30 had simultaneously, lower median lethal time (**Figure 1C**) and lower proportion of alive individuals (20%) at the end of our experiment (**Figure 1D**). Although at the end of our experiment, the proportion of alive *S. calcitrans* infected with the strain ICIPE 7 was similar to that of *S. calcitrans* infected with the strain ICIPE 30 (**Figure 1D**), the strain ICIPE 7 took the longest time (>5 days) to kill half individuals of *S. calcitrans* as compared to the strain ICIPE 30 (<4 days) (**Figure 1C**). Therefore, we considered the strain ICIPE 30 as the most potent and virulent for *S. calcitrans*.

Effect of *S. calcitrans* Sex and Age on *M. anisopliae* ICIPE 30 Infectivity

In both sex of *S. calcitrans*, the survival over time of uninfected (males and females) and infected (males and females) individuals significantly varied (**Figure 2A**i; log-rank test, $\chi^2 = 11.8$, df $= 3$, $P = 0.008$). But the pairwise comparison using the log-rank test revealed that the survival of infected males and infected females over time did not significantly differ ($P = 0.26$). This was also true for uninfected males and uninfected females ($P = 0.39$). The mean median lethal time of infected female *S. calcitrans* was significantly higher than that of infected males (**Figure 2A**ii; $U = 22$, $P = 0.04$). At the end of our experiment, infected flies of both sexes had a significantly lower proportion of alive individuals as compared to that uninfected flies [**Figure 2A**iii; One-way ANOVA: $F_{(3-16)} = 27.27$, $P < 0.0001$]. But this proportion was similar between infected males and females; and uninfected males and females.

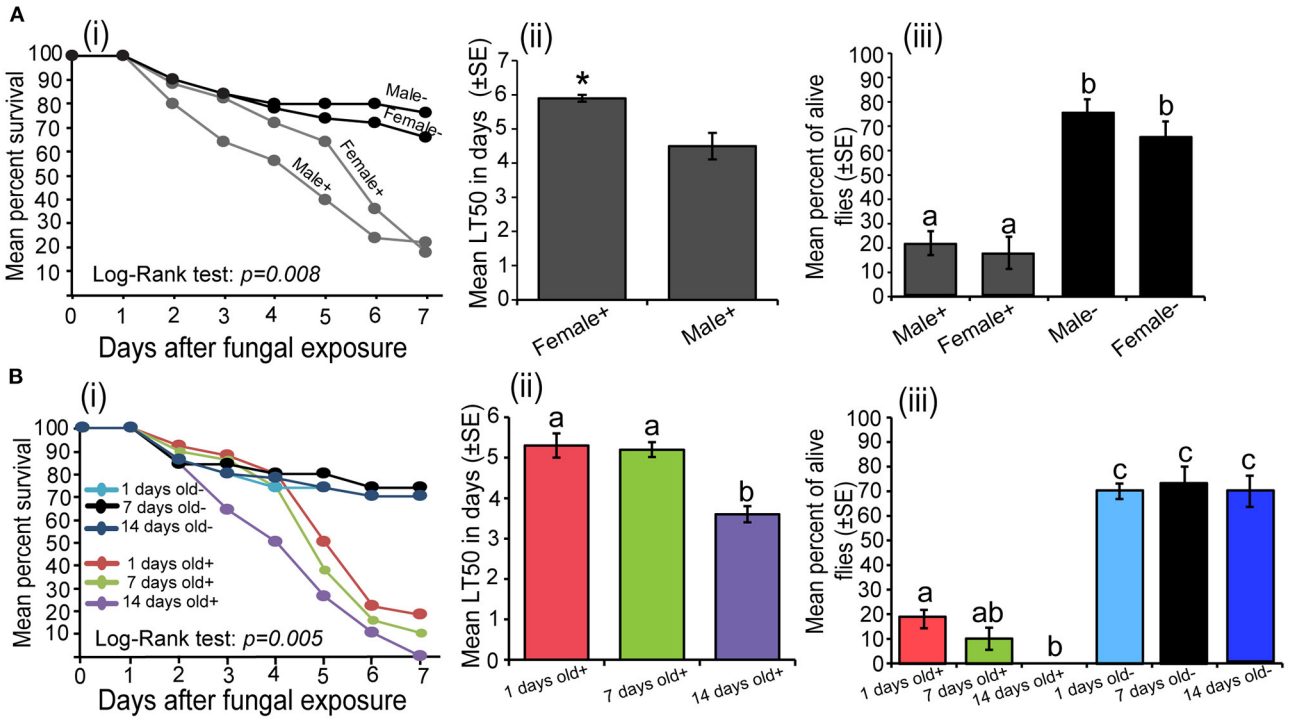

FIGURE 2 | (A) Influence of the *Stomoxys calcitrans* sex on *Metarhizium anisopliae* ICIPE 30 infectivity: (i) Kaplan–Meier curve showing survivorship over time of infected (males and females) and uninfected (males and females) *S. calcitrans* (Mantel–Cox log-rank χ^2 test, $P < 0.05$, $n = 5$), (ii) Bar chart illustrating the mean median lethal time (LT_{50}) between infected males and females of *S. calcitrans* (unpaired *t*-test, $^*p < 0.05$, $n = 5$), (iii) Bar chart depicting the mean percentages of individual alive across the sex of infected and uninfected *S. calcitrans* the 7th day of our bioassay (ANOVA followed by SNK *post-hoc* test; $p < 0.05$, $n = 5$). **(B)** Influence of the *S. calcitrans* age on *M. anisopliae* ICIPE 30 infectivity: (i) Kaplan–Meier curve showing survivorship over time of infected (1, 7, and 14 days old) and uninfected (1, 7, and 14 days old) *S. calcitrans* (Mantel–Cox log-rank χ^2 test, $P < 0.05$, $n = 5$), (ii) Bar chart illustrating the mean median lethal time (LT_{50}) across the age of infected *S. calcitrans* (ANOVA followed by SNK *post-hoc* test; $p < 0.05$, $n = 5$), (iii) Bar chart showing the mean percentages of individual alive across the age of infected and uninfected *S. calcitrans* at the 7th day of our bioassay (ANOVA followed by SNK *post-hoc* test; $p < 0.05$, $n = 5$). The signs + and – denote infected and uninfected individuals, respectively. Error bars indicate the standard error of the mean. Bars with different lowercase letters are significantly different from each other.

The survival of *S. calcitrans* over time significantly changed across the different age of flies (log-rank test, $\chi^2 = 16.9$, df = 5, $P = 0.005$); with 14-days old infected possessing the lower survival rate (**Figure 2B**i). As compared to 1 and 7 days old, 14-days old infected flies had a smaller median lethal time [**Figure 2B**ii; One-way ANOVA: $F_{(2-12)} = 16.65$, $P < 0.001$]. Regardless of the age, the proportion of alive flies obtained 7 days after contamination was significantly lower in infected flies and as compared to uninfected flies [**Figure 2B**iii; One-way ANOVA: $F_{(5-24)} = 59.39$, $P < 0.0001$]. In infected flies, this proportion was significantly lower in 14 days old flies followed by 7 and 1-day old flies (**Figure 2B**iii).

Horizontal Transmission of *M. anisopliae* ICIPE 30 Conidia by *S. calcitrans*

The amount of *M. anisopliae* ICIPE 30 conidia carried by *S. calcitrans* significantly varied across time ($P < 0.0001$) with no variations between sex ($P = 0.051$). This amount was higher directly after the contamination process; but 2 h later, it drastically dropped with no significant change even 8 h after the fly's contamination (**Figure 3A**). We found that 4 h after exposure to *M. anisopliae* ICIPE 30 conidia, fungus-exposed flies (donors) were still able to contaminate fungus-free flies (receivers). When we contaminated *S. calcitrans* males (donors) and associated them with fungus-free females (receivers), the survival of these flies over time significantly reduced as compared to those of males and females maintained uncontaminated throughout the bioassay (**Figure 3B**i; log-rank test, $\chi^2 = 26.2$, df = 3, $P < 0.0001$). The median lethal time of male donors was significantly lower as compared to that of female receivers (**Figure 3B**ii; $t = 3.6$, df = 6, $P = 0.013$). Both male donors and female receivers had reduced proportion of alive individuals at the 7th-day post-contamination as compared to males and females maintained uncontaminated [**Figure 3B**iii; One-way ANOVA: $F_{(3-14)} = 16.29$, $P < 0.0001$]. We obtained the same result pattern when we contaminated females (donors) and associated them with fungus-free males (receivers). The survival of female donors and male receivers significantly reduced over time as compared to the survival of uncontaminated males and females (**Figure 3C**i; log-rank test, $\chi^2 = 16.2$, df = 3, $P < 0.001$). Female donors had a lower mean median lethal time as compared to that of male receivers (**Figure 3C**ii, $U = 2$, $P = 0.026$). The proportion of female

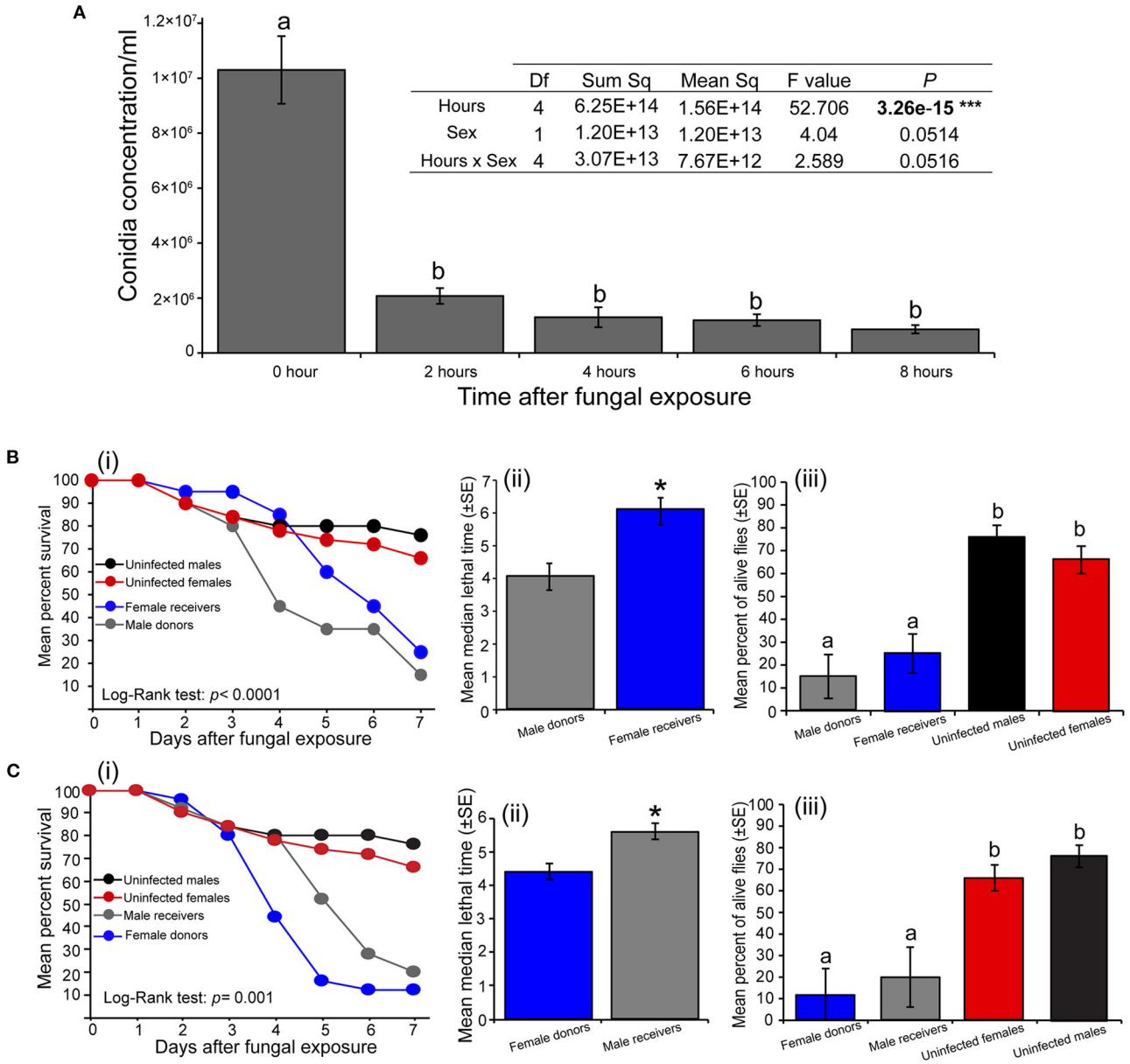

FIGURE 3 | Horizontal transmission of *Metarhizium anisopliae* conidia in *Stomoxys calcitrans*. **(A)** Bar chart showing the variation across the time of conidia load in *S. calcitrans* (one-way ANOVA followed by SNK *post-hoc* test; $P < 0.05$, $^{***}P < 0.001$, $n = 10$). **(B)** Transmission of *M. anisopliae* ICIPE 30 conidia from *S. calcitrans* males (donors) to females (receivers): (i) Kaplan-Meier curve showing survivorship over time of *S. calcitrans* male donors and female receivers (Mantel-Cox log-rank χ^2 test, $P < 0.05$, $n = 5$); (ii) Bar chart illustrating the mean median lethal time (LT_{50}) of male donors and female receivers (unpaired *t*-test, $^{*}P < 0.05$, $n = 5$); (iii) Bar chart illustrating the mean percentage of *S. calcitrans* male donors and female receivers alive the 7th day of our bioassay (one-way ANOVA followed by SNK *post-hoc* test; $P < 0.05$, $n = 5$). **(C)** Transmission of *M. anisopliae* ICIPE 30 conidia from *S. calcitrans* females (donors) to males (receivers): (i) Kaplan–Meier curve showing survivorship over time of *S. calcitrans* female donors and male receivers (Mantel–Cox log-rank χ^2 test, $P < 0.05$, $n = 5$); (ii) Bar chart illustrating the mean median lethal time (LT_{50}) of female donors and male receivers (unpaired *t*-test, $^{*}P < 0.05$, $n = 5$); (iii) Bar chart depicting the mean percentage of *S. calcitrans* female donors and male receivers alive the 7th day of our bioassay (ANOVA followed by SNK *post-hoc* test; $P < 0.05$, $n = 5$). Error bars indicate the standard error of the mean. Letters above error bars indicate a significant difference across the treatment.

donors and male receivers alive at the end of our experiment was significantly lower than those of uncontaminated males and females [**Figure 3C**iii; One-way ANOVA: $F_{(3-17)} = 10.2$, $P < 0.001$].

Effect of *M. anisopliae* ICIPE 30 Infection on the Feeding Propensity of *S. calcitrans*

Infection of *S. calcitrans* by *M. anisopliae* ICIPE 30 significantly altered its feeding propensity. Fourty-eight (48) hours after

fungal exposure, the time taken by *S. calcitrans* to get engorged did not vary between infected and uninfected flies ($t = 0.06$, df $= 67.7$, $P = 0.95$). But, 72 ($t = 2.18$, df $= 65.5$, $P = 0.032$) and 96 ($U = 466$, $P < 0.001$) h after the conidia exposure occurred, infected flies took significantly more time to get engorged as compared to uninfected flies (**Figure 4A**). Sixty (60) seconds after blood exposure, 48-h infected flies and uninfected flies had the same proportion flies that managed to take blood (**Figure 4B**ii; GLM, $\chi^2 = 19.92$, df $= 1$, $P = 0.50$). Nonetheless, this proportion significantly reduced in 72-h (GLM, $\chi^2 = 9.5$, df $= 1$, $P = 0.042$) and 96-h (GLM, $\chi^2 = 25.53$, df $= 1$, $P = 0.004$) infected flies (**Figure 4B**). The amount of blood taken by infected flies at 48 ($t = 4.60$, df $= 61.5$, $P < 0.0001$), 72 ($t = 3.71$, df $= 66$, $P < 0.001$), and 96 ($t = 2.9$, df $= 39.4$, $P < 0.01$) h after fungal exposure was significantly lower as compared to that of uninfected flies (**Figure 4C**).

Influence of *M. anisopliae* ICIPE 30 on *S. calcitrans* Female's Reproduction Traits

In our dual-test oviposition bioassay, when we presented two fungus-free substrates to *S. calcitrans* gravid females, they laid the same number of eggs on both substrates (**Figure 5A**i, $t = 0.83$, df $= 17.9$, $P = 0.41$). However, when we added dried conidia of *M. anisopliae* on one of the substrates, these females laid significantly few numbers of eggs on the fungal-embedded substrate (**Figure 5A**ii; $U = 0$, $P < 0.01$). Consequently, the proportion of eggs deposited on the substrate with dried conidia that hatched was significantly less than that of eggs laid on the substrate without dried conidia (**Figure 5A**iii, GLM; $\chi^2 = 23.07$, df $= 1$, $P < 0.0001$). In the no-choice bioassay, *S. calcitrans* gravid females infected with *M. anisopliae* ICIPE 30 laid a significantly fewer number of eggs than uninfected *S. calcitrans* gravid females (**Figure 5B**i; $t = 5.08$, df $= 11.84$, $P < 0.001$). Also, the hatchability of eggs laid by infected females was significantly lower than that of eggs deposited by uninfected females (**Figure 5B**ii, GLM; $\chi^2 = 26.63$, df $= 1$, $P < 0.0001$).

Fitness Cost Associated With the Tolerance of *M. anisopliae* Infection in *S. calcitrans* Larvae

As demonstrated in other studies, our study showed that *S. calcitrans* larvae are not susceptible to infection with *M. anisopliae* strains used. All the *M. anisopliae* strains-contaminated larvae and uncontaminated larvae (control) had similar pupation percentage [$F_{(11-48)} = 0.42$, $P = 0.94$]. For each treatment, about 90% of larvae reached the pupal stage (**Figure 6A**i). Even at high conidia concentrations, the pupation percentage of contaminated larvae did not reduce [**Figure 6A**ii; $F_{(4-20)} = 0.62$, $P = 0.65$]. Even though the high proportion of contaminated larvae did not succumb from *M. anisopliae* exposure (since some managed to pupate), we demonstrated a negative impact of this contamination on some of their fitness parameters.

We observed that the pupation time was significantly longer in contaminated first (**Figure 6B**i1: $U = 70$, $P < 0.0001$), second (**Figure 6B**ii1: $U = 255.5$, $P < 0.0001$), and third (**Figure 6B**iii1, $U = 346$, $P < 0.0001$) larval instars of *S. calcitrans* as compared to that of their corresponding control (uncontaminated larvae). Also, the weight of contaminated first (**Figure 6B**i2: Day 2: $t = 8.45$, df $= 14.4$, $P < 0.0001$; Day 4: $U = 317$, $P = 0.002$; Day 6: $t = 3.2$, df $= 17.98$, $P = 0.005$), second (**Figure 6B**ii2: Day 2: $t = 5.5$, df $= 14.6$, $P < 0.0001$, Day 4: $t = 3.5$, df $= 18$, $P = 0.002$; Day 6: $t = 2.4$, df $= 15.9$, $P = 0.026$), and third

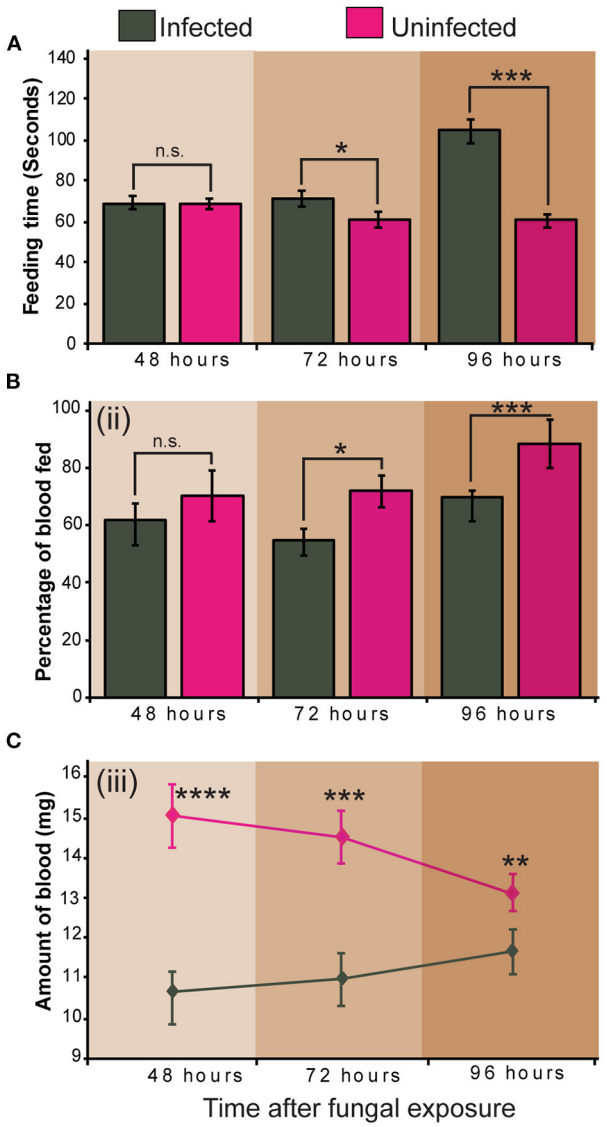

FIGURE 4 | Impact of *Metarhizium anisopliae* infection on *Stomoxys calcitrans* feeding propensity. **(A)** Bar charts showing the mean feeding duration across time in infected and uninfected *S. calcitrans*; **(B)** Bar charts illustrating the mean percentage of infected and uninfected *S. calcitrans* individuals that managed to get engorged in 60 s across time; **(C)** Line graph showing the change of the amount of blood taken by infected and uninfected *S. calcitrans* across time. Error bars indicate the standard error of the mean. Asterisks indicate that mean between infected and uninfected individuals differed significantly (unpaired *t*-test: $^*P < 0.05$; $^{**}P<0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 30$). "n.s" indicates non-significant difference ($P > 0.05$).

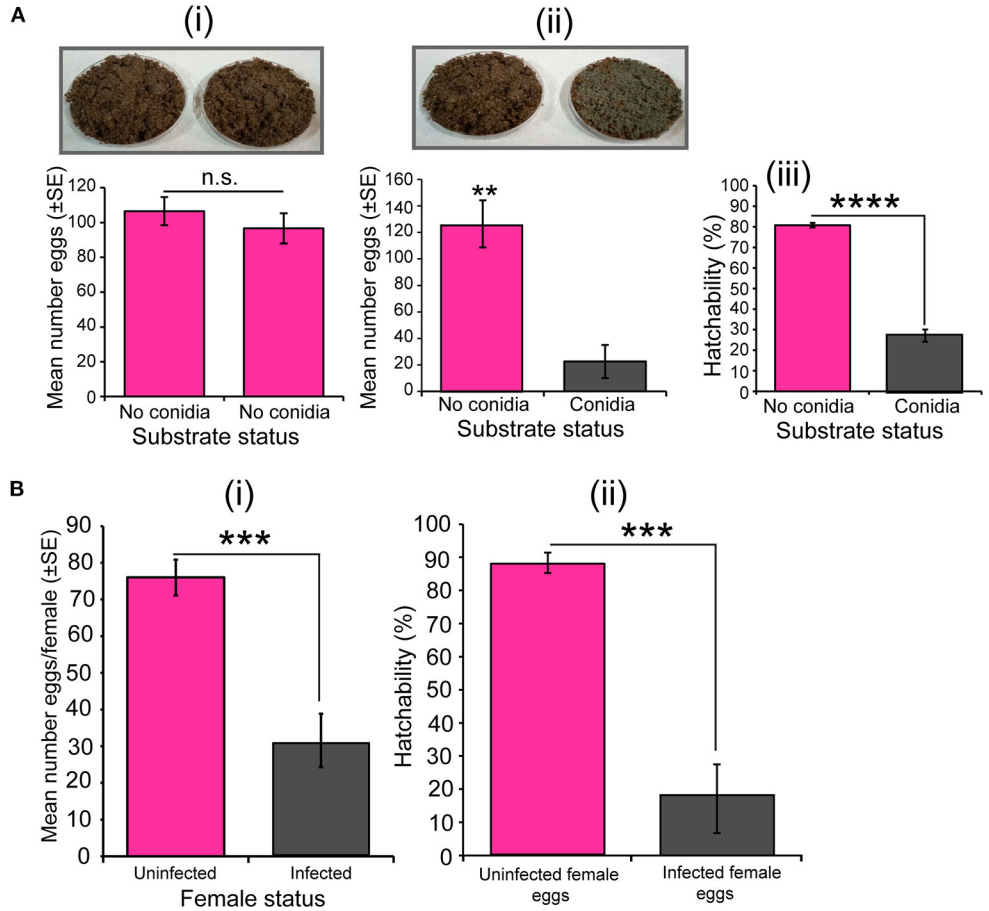

FIGURE 5 | Effect of *Metarhizium anisopliae* on the oviposition decision, fecundity, and egg hatchability of *Stomoxys calcitrans*. **(A)** Egg-laying decision bioassay with uninfected gravid female *S. calcitrans*: (i) Bar chart showing the mean number of eggs laid by *S. calcitrans* on substrates without dried conidia of *M. anisopliae* ICIPE 30 (paired *t*-test, $n = 10$); (ii) Bar chart showing the mean number of eggs laid by *S. calcitrans* on substrates with and without dried conidia of *M. anisopliae* ICIPE 30 (paired *t*-test: $p > 0.05$, $n = 10$); (iii) Bar chart illustrating the proportion of eggs hatched from eggs laid of substrates with and without dried conidia of *M. anisopliae* ICIPE 30 (GLM with binomial distribution followed by the analysis of deviance test, $n = 10$). **(B)** Egg-laying assay with single uninfected and infected gravid female *S. calcitrans*. (i) Bar chart illustrating the mean number of eggs laid by uninfected and infected gravid females of *S. calcitrans* (unpaired *t*-test, $n = 30$); (ii) Bar chart showing the mean proportion of eggs that hatched from eggs laid by uninfected and infected of *S. calcitrans* (GLM with binomial distribution followed by the analysis of deviance test). Errors bar on each bar chart indicates the standard error of the mean. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

(**Figure 6B**iii2; $t = 2.97$, df $= 15.4$, $P = 0.009$) larval instar of *S. calcitrans* was smaller than those of uncontaminated instars. The pupation percentage did not significantly change between contaminated and uncontaminated individuals from the first (**Figure 6B**i3; GLM; $\chi^2 = 3.55$, df $= 1$, $P = 0.06$), second (**Figure 6B**ii3; GLM; $\chi^2 = 3.25$, df $= 1$, $P = 0.071$), and third (**Figure 6B**iii3; GLM; $\chi^2 = 2.48$, df $= 1$, $P = 0.11$) larval instars. The pupal weight was significantly reduced in contaminated individuals developed from the first larval instars (**Figure 6B**i4: $t = 3.5$, df $= 80$, $P < 0.001$). However, pupae formed from the contaminated second (**Figure 6B**ii4: $t = -0.73$, df $= 37.66$, $P = 0.46$), and third (**Figure 6B**iii4: $t = -0.89$, df $= 65.51$, $P = 0.37$) larval instars had similar weight with those developed from uncontaminated second and third larval instars respectively. Pupae developed from contaminated and uncontaminated first (**Figure 6B**i5: $U = 1002.5$, $P = 0.12$), second (**Figure 6B**ii5: $U = 229.5$, $P = 0.16$), and third (**Figure 6B**iii5: $U = 262.5$, $P = 0.32$) larval instars formed at the same time. The proportion of adults obtained from contaminated and uncontaminated first (**Figure 6B**i6: GLM; $\chi^2 = 1.46$, df $= 1$, $P = 0.22$) and second (**Figure 6B**ii6: GLM; $\chi^2 = 0.002$, df $= 1$, $P = 0.97$) larval instars was not significantly different; but in the third larval instar, this proportion was significantly higher in uncontaminated larvae (**Figure 6B**iii6: GLM; $\chi^2 = 7.04$, df $= 1$, $P = 0.008$). Adult obtained from contaminated first larval instars had a significant reduced weight as compared to those emerged from uncontaminated first larval instars (**Figure 6B**i7: $t = -3.27$, df $= 68.8$, $P = 0.002$). While those obtained from contaminated and uncontaminated second (**Figure 6B**ii7: $t = -1.06$, df $= 44.2$, $P = 0.29$) and third (**Figure 6B**iii7: $t = 1.27$, df $= 29.45$, $P = 0.21$) larval instars had the same weight. Independently to the larval instars, we numbered 12 deformed adults from contaminated

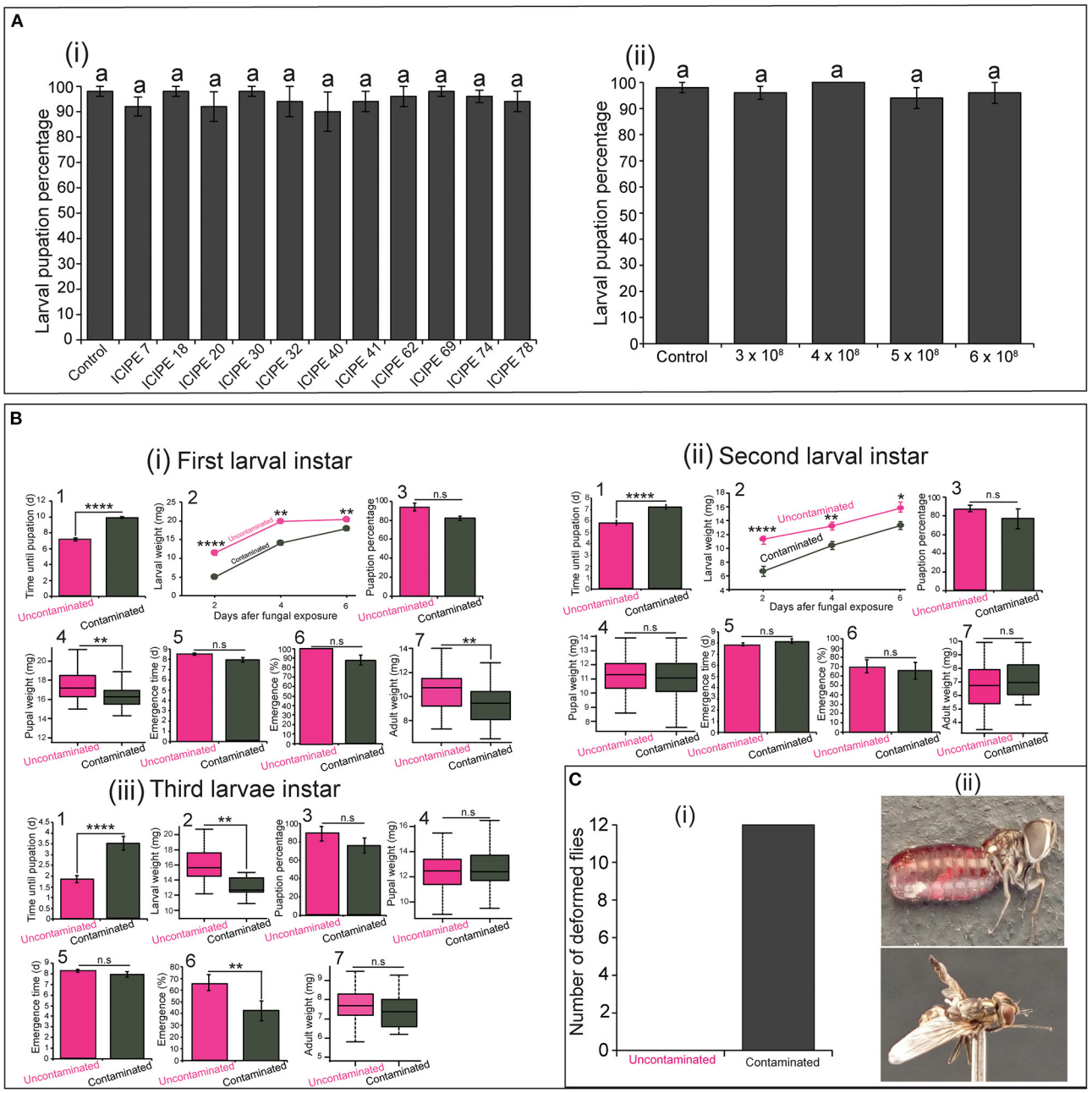

FIGURE 6 | Influence of *Metarhizium anisopliae* infection on the fitness parameters of the three larval instars of *Stomoxys calcitrans*. **(A)** Bar chart showing the mean pupation percentage of *S. calcitrans* larvae contaminated by the 11 strains of *M. anisopliae* (i) and the increasing concentrations of the *M. anisopliae* strain ICIPE 30 (ii) (ANOVA test; $n = 5$). **(B)** Fitness parameters of uncontaminated and contaminated first (i), second (ii), and third (iii) larval instar of *S. calcitrans*: (1) Bar charts depicting the mean pupation time in each uncontaminated and contaminated larval instar of *S. calcitrans* (unpaired Mann–Whitney test); (2) Line graphs showing the variation of weight in each uncontaminated and contaminated larval instar of *S. calcitrans* after 2, 4, and 6 days of infection (unpaired *t*-test test or unpaired Mann–Whitney test); (3) Bar charts illustrating the mean pupation percentage of each uncontaminated and contaminated larval instar of *S. calcitrans* (GLM with binomial distribution followed by the analysis of deviance test, $n = 5$); (4) Box plots showing the pupal weight obtained from each uncontaminated and contaminated larval instar of *S. calcitrans* (unpaired *t*-test); (5) Bart charts illustrating the mean emergence time of *S. calcitrans* adults developed from each uncontaminated and contaminated larval instars (unpaired Mann–Whitney test); (6) Bar charts depicting the emergence percentage of *S. calcitrans* adults developed from each uncontaminated and contaminated larval instars (GLM with binomial distribution followed by the analysis of deviance test, $n = 5$); (7) Box plots showing the weight of *S. calcitrans* adults developed from each uncontaminated and contaminated larval instars (unpaired *t*-test). **(C)** Histogram (i) showing the total number of deformed *S. calcitrans* adults (ii; Original Photo: Steve B. S. Baleba) emerged from contaminated larvae. On each bar chart and line graph, error bars indicate the standard error of the mean. Each box plots shows the median (bold horizontal lines) and whiskers the interquartile range. n.s (non-significant difference): $P > 0.05$; significant difference: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

larvae and no deformed adults from uncontaminated larvae (**Figure 6C**).

DISCUSSION

Our results demonstrate that *M. anisopliae* infection significantly reduces the survival, feeding propensity, fecundity, and fertility of *S. calcitrans*. Also, these findings show that there is a fitness cost associated with the tolerance of *S. calcitrans* larvae to *M. anisopliae* infection.

All the 11 strains of *M. anisopliae* studied killed *S. calcitrans* adults; although the speed and the rate at which this occurred varied significantly among the strains. Several studies have demonstrated the implication of morphological and physiological characteristics of fungal strains on this virulence variation. These characteristics include hyphal growth rate, conidial viability, conidia production, conidia size, enzyme secretion among other factors (Liu et al., 2003; Quesada-Moraga and Vey, 2003; Talaei -Hassanloui et al., 2006). In our study, *M. anisopliae* strain ICIPE 30 rapidly killed (<4 days) half of *S. calcitrans* individuals and appeared to be the most virulent strain. The virulence of this strain has previously been demonstrated in other haematophagous dipterans including *Glossina morsitans morsitans* (Maniania and Odulaja, 1998), *An. gambiae* (Mnyone et al., 2009, 2011) and *Ae. aegypti* (Jemberie et al., 2018). At this stage, there is a need to study the genetic, molecular and physiological mechanisms mediating the virulence of ICIPE 30. Niassy et al. (2013) started by characterizing the chitinase genes (chi2 and chi4) of this strain responsible for the secretion of enzymes that digest the insect cuticle. The overexpression of such genes in *Metarhizium* fungus using bioengineering methods could increase their virulence and this need to be investigated further. For instance, the genetically engineered *M. anisopliae* in which the *cat1* gene has been overexpressed tolerate more exogenous hydrogen peroxide; resulting in the acceleration of its germination and the increase of its virulence (Morales Hernandez et al., 2010).

We demonstrated that the *M. anisopliae* infectivity could be influenced by the sex and the age of *S. calcitrans* individuals. As compared to female *S. calcitrans*, *M. anisopliae* ICIPE 30 killed faster 50 % of male *S. calcitrans* (**Figure 2A**ii). This may indicate that there are differences in the innate immunity of male *S. calcitrans* compared to females. We speculate that female *S. calcitrans* produce more vigorous cellular and humoral immune reactions against *M. anisopliae* blastospore. Using *Drosophila melanogaster* (Diptera: Drosophilidae) and *Beauveria bassiana* (Hypocreales: Cordycipitaceae), Shahrestani et al. (2018) demonstrated the existence of sexual dimorphism in the immune response of insects to fungal infection. Our results contrast with those of Maniania and Odulaja (1998) who showed that females of *G. m. morsitans* and *G. m. centralis* succumb first to ICIPE 30 infection. We attribute this difference to the dissimilarity of the insect species used in our respective studies. Nonetheless, our finding is supported by other studies involving different strains of *M. anisopliae* and fungal species. Kaaya (1989) showed that males of *G. m. morsitans* infected with *M. anisopliae* strains 35-79, 82-82, and 100-82 were more susceptible to the infection than females. In *Musca domestica* (Diptera: Muscidae), males infected with *Entomophthora muscae* (Entomophthorales: Entomophthoraceae) died significantly earlier than females (Mullens, 1985). In addition to this effect of sex, we also found a significant effect of the *S. calcitrans* age on *M. anisopliae* ICIPE 30 infectivity. Fourteen-days old female *S. calcitrans* infected with ICIPE 30 died relatively sooner than females of 1 and 7-days old. This increase of mortality with the age in infected *S. calcitrans* has already been observed in other blood-feeding insects including adult tsetse flies (Maniania and Odulaja, 1998) and *An. gambiae* (Mnyone et al., 2011). The reduction of the immune response to *M. anisopliae* infection could explain why older female *S. calcitrans* died faster. In general, the immune system of animals weakens as they become older. For instance, the melanization which is an immediate immune response to pathogens in arthropods is reduced in old *Ae. aegypti* individuals (Christensen et al., 1986). Enzymes such as phenoloxidases which play a key role in insect immune system decline with the insect age (González-Santoyo and Córdoba-Aguilar, 2012). In *Ae. aegypti*, the high mortality observed in old individuals after their infection with *Escherichia coli* is associated to decrease in the number of hemocytes in their hemolymph (Hillyer et al., 2005). Our results would be explained better by further studies investigating the change that undergoes the immune system of *S. calcitrans* with the age.

We showed that the number of conidia attached to *S. calcitrans* cuticle reduced drastically after the exposure (∼75% within 2 h) (**Figure 3A**). This result could be associated with the fact that, after the fungal exposure, *S. calcitrans* exhibit active grooming behavior, with flies trying to clean as much as possible all their body parts. The conidia found on *S. calcitrans* 8 h after exposure could be from the areas hard to reach (e.g., back of thorax and abdomen) during the active grooming. This reduction of the conidia load over time has also been found in *Ceratitis cosyra* (Diptera: Tephritidae) (Dimbi et al., 2013). We demonstrated that in *S. calcitrans*, 4 h after *M. anisopliae* contamination, conidia of this fungus can still be transmitted horizontally from one sex to another. The survival of females that received ICIPE 30 conidia from contaminated males was significantly lower than that of uncontaminated females (**Figure 3B**). We obtained a similar result with males that received conidia from contaminated females (**Figure 3C**). Horizontal transmission of fungal conidia is also found in other blood-feeding insect species including *G. morsitans* (Maniania et al., 2013), *Triatoma infestans* (Hemiptera: Rediviidae) (Forlani et al., 2011), *An. gambiae* (Scholte et al., 2004) and *Ae. aegyptis* (Diptera: Culicidae) (García-Munguía et al., 2011). We hypothesize that the horizontal transmission of *M. anisopliae* conidia in *S. calcitrans* occurs during courtship events including wing extension and vibration, abdomen drumming and curving, or proboscis extension. Also, the fact that *S. calcitrans* repeatedly mate in a day could increase the probability of an infected individual to transmit conidia to several uninfected mates. This result is important as it demonstrates that ICIPE 30 could be used in an auto-dissemination control strategy to suppress the fly populations. In the field conditions, the incorporation of

this fungal strain into trapping devices that massively catch and release *S. calcitrans* individuals would assist in spreading the fungus inoculum in the environment where the population density of the fly is high. The auto-dissemination control strategy has shown its effectiveness in other control system involving *Aedes albopictus* (Diptera: Culicidae) (Unlu et al., 2017) and this need to be validated under field condition in *S. calcitrans*.

We elucidated that *M. anisopliae* ICIPE 30 infection significantly impacted the feeding propensity of *S. calcitrans*. Our results showed that compared to uninfected controls, infected *S. calcitrans* took more time to consume blood. Also, these individuals were less likely to feed and when they did, they consumed a small amount of blood. For vector control purpose, reduction in feeding propensity is particularly important since pathogens they carry are transmitted during the blood meal. Pre-lethal reduction in feeding due to fungal infection has been shown in *An. stephensi* (Blanford et al., 2011, 2012), *Ae. aegyptis* (Darbro et al., 2012), and *An. gambiae* (Scholte et al., 2006). Studies indicate that this reduction in feeding is related to the fact that, individuals infected with fungi fail to locate potential blood sources owing to the reduction of the olfactory sensitivity occasioned by the infection. For example, George et al. (2011) showed that *B. bassiana* and *Metarhizium acridum* fungal spores, as well as inducing sublethal effects in *An. stephensi*, also reduce the responsiveness of its olfactory neurons. The reduction of feeding in infected *S. calcitrans* could also be due to the antifeedant activity of the secondary metabolites produced by the *M. anisopliae* blastopores, and the toxicity activity of these metabolites on the tissues of the insect midgut. For instance, Amiri et al. (1999) demonstrated that the destruxins produced by *M. anisopliae* have an antifeedant effect against larvae of *Plutella xylostella* (Lepidoptera: Plutellidae) and *Phaedon cochleariae* (Coleoptera: Chrysomelidae). Also, Skrobek and Butt (2005) showed that these molecules exhibit a cytotoxicity activity in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) cells.

Our results also revealed that gravid females *S. calcitrans* differentiated substrates treated with *M. anisopliae* ICIPE 30 from substrates without this fungus. These females laid fewer eggs on treated substrates compared to the untreated ones (**Figure 5A**ii). Using *Metarhizium brunneum*, Machtinger et al. (2016) also found this fungus-induced oviposition avoidance in *S. calcitrans*. We showed that eggs laid on substrates with fungus had lower hatchability (**Figure 5A**iii). This could explain why these substrates were avoided by gravid females *S. calcitrans*. In our previous studies, we showed that gravid females *S. calcitrans* were able to avoid substrates that could harm their progeny (Baleba et al., 2019, 2020). As the egg-laying decision of *S. calcitrans* is guided by olfactory cues (Baleba et al., 2019), we suggest that the avoidance of substrates treated with *M. anisopliae* by gravid females *S. calcitrans* could be mediated by chemical volatiles produced by *M. anisopliae*. Studies conducted on the termites *Macrotermes michaelseni* (Isoptera: Termitidae) (Mburu et al., 2011) and Coptotermes formosanus Shiraki (Isoptera: Rhinotermitidae) (Hussain et al., 2010) have already demonstrated the repellency of volatiles emitted by *M. anisopliae* conidia. Our results open new research avenues in identifying repellent odourant molecules from *M. anisopliae* that may be used to control *S. calcitrans* and reduce the spread of diseases that they transmit.

We found a reduction of eggs production in gravid females *S. calcitrans* infected with *M. anisopliae*. Also, we showed that the hatchability of eggs produced by these females was significantly low. These results suggest that once infected by *M. anisopliae*, *S. calcitrans* females are less likely to produce viable progeny for the next generation; contributing therefore to their population reduction. In *An. gambiae*, females infected with *M. anisopliae* laid fewer eggs (Scholte et al., 2006). Using *B. bassiana*, García-Munguía et al. (2011) also obtained a reduction of fecundity in infected females *Ae. aegytis*. The introduction of the entomopathogenic fungus, *Aspergillus parasiticus* (Eurotiales: Trichocomaceae) into natural populations of *An. gambiae*, *Culex fatigans*, and *Ae. aegypti* significantly reduced the fecundity and fertility of females that become infected (Nnakumusana, 1985). In our study, we hypothesize that the observed reduction of fecundity and fertility could be the immediate effect of the reduction of blood intake observed in *M. anisopliae* infected *S. calcitrans*. It has been shown that ovarian development, egg maturation and fertility heavily depend on the amount of blood taken by the insect (Gonzales and Hansen, 2016). Moreover, the reduction of fecundity and fertility in infected *S. calcitrans* could be associated with the effect of *M. anisopliae* on the maturation of *S. calcitrans* eggs. For example, Sánchez-Roblero et al. (2012) demonstrated that *B. bassiana* delay the maturation of *Anastrepha ludens* (Diptera: Tephritidae) eggs, resulting to the reduction the quantity of their mature basal oocytes and ultimately the number of eggs laid.

Also, we demonstrated that there is a fitness cost associated with the tolerance of *S. calcitrans* larvae to *M. anisopliae* infection. As Moraes et al. (2008), we found that *S. calcitrans* larvae were not susceptible to *M. anisopliae* infection. About 90% of the larvae contaminated with the 11 strains of *M. anisopliae* (**Figure 6A**i) and those contaminated with high concentrations of ICIPE 30 (**Figure 6A**ii) reached the pupal stage. Moraes et al. (2014) demonstrated the existence of antifungal activity of *S. calcitrans* larvae against entomopathogenic fungal infection. The authors found that macerated solution of *S. calcitrans* larvae reduces the growth of *B. bassiana*; suggesting that these larvae produce robust anti-fungal substances protecting them from infection. When subjected to high-performance liquid chromatography (HPLC) analysis, new peaks in the chromatogram that could represent the antifungal molecules were obtained from the solution of macerated *B. bassiana* infected larvae as compared to the control group (Moraes et al., 2014). Earlier, a peptide affecting microorganism growth (stomoxyn) was identified in the exterior midgut of *S. calcitrans* (Boulanger et al., 2002). We recommend further studies to characterize molecules secreted by fungal infected *S. calcitrans* larvae and their protective role against pathogens.

Furthermore, we found that the tolerance of *S. calcitrans* larvae to *M. anisopliae* infection compromised some of their life-history traits. For instance, even though the majority of contaminated individuals from each larval instar pupated, their pupation period was longer than that of uncontaminated individuals. Also, these contaminated larvae were smaller, and

in some contaminated larval instars such as the first and the third instars, adults had small weight and emergence percentage. Moreover, we obtained deformed adults from contaminated larvae even though their number was reduced. Vogelweith et al. (2017) explained that to optimize their response to pathogen invasion, individuals generally balance between investing in their immune system and other life-history traits. Thus, we argue that during a fungal infection in *S. calcitrans* larvae, certain fitness traits (e.g. developmental speed and weight gain) are suppressed in favor of immune reactions that will enhance the response against the infection.

In summary, our study examined the previously unexplored lethal and pre-lethal effect of *M. anisopliae* infection in *S. calcitrans*. We identified a potent and virulent *M. anisopliae* strain for *S. calcitrans* (ICIPE 30) that could be developed as biopesticide to manage the fly. We showed that the infectivity of this strain against *S. calcitrans* could be sex and age-dependent. Also, we proved that males and females of *S. calcitrans* contaminated with ICIPE 30 can horizontally transmit conidia of this fungal strain to their conspecific mates. Our results demonstrated that *M. anisopliae* ICIPE 30 infection reduces the feeding propensity, fecundity and fertility of *S. calcitrans* adults. We showed that the tolerance of *S. calcitrans* larvae to *M. anisopliae* infection has a fitness cost in these larvae. Taken together, our work provides detailed insights into the consequence of fungal infection of *S. calcitrans*, demonstrating the potentiality of the use of entomopathogenic fungi in controlling this important vector of various pathogens of human and veterinary significance. We recommend further chemical, molecular, and physiological studies that would additionally explain or elucidate our results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SB: study conceptualization, experimental design, data collection, analysis and interpretation, and first manuscript draft preparation. AA: assistance in media preparation, fungus culture, germination test, and manuscript proofreading. MG and KA: advice in experimental design and manuscript proofreading. SS: manuscript proofreading. DM: study conceptualization, fund acquisition, advise in experimental design, supervision, and manuscript proofreading. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2021.637817/full#supplementary-material

Supplementary Figure 1 | (A) Cylindrical plastic tube used as the contamination device. **(B)** Uninfected (i) and infected (ii) adults of *S. calcitrans* (Original Photo: Steve B.S. Baleba).

Supplementary Figure 2 | Individual of *S. calcitrans* before **(A)**, during **(B)** and after the blood meal **(C)** (Original Photo: Steve B. S. Baleba).

Supplementary Figure 3 | (A) Experimental setup where 10 gravid females were allowed to choose substrates with and without dried conidia of *M. anisopliae* ICIPE 30; **(B)** No choice oviposition bioassay setup using single uninfected (i) and infected (ii) female *S. calcitrans*.

REFERENCES

Abbot, W. A. (1925). Method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–267. doi: 10.1093/jee/18.2.265a

Akutse, K. S., Subramanian, S., Maniania, N. K., Dubois, T., and Ekesi, S. (2020). Biopesticide research and product development in Africa for sustainable agriculture and food security - experiences from the International Centre of Insect Physiology and Ecology (icipe). *Front. Sustain. Food Syst.* 4:563016. doi: 10.3389/fsufs.2020.563016

Alkhaibari, A. M., Carolino, A. T., Bull, J. C., Samuels, R. I., and Butt, T. M. (2017). Differential pathogenicity of Metarhizium blastospores and conidia against larvae of three mosquito species. *J. Med. Entomol.* 54, 696–704. doi: 10.1093/jme/tjw223

Amiri, B., Ibrahim, L., and Butt, T. M. (1999). Antifeedant properties of destruxins and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocontrol Sci. Technol.* 9, 487–498. doi: 10.1080/09583159929451

Baldacchino, F., Muenworn, V., Desquesnes, M., Desoli, F., Charoenviriyaphap, T., and Duvallet, G. (2013). Transmission of pathogens by Stomoxys flies (Diptera, Muscidae): a review. *Parasite* 20:26. doi: 10.1051/parasite/2013026

Baleba, S. B. S., Torto, B., Masiga, D., Getahun, M. N., and Weldon, C. W. (2020). Stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), improve offspring fitness by avoiding oviposition substrates with competitors or Parasites. *Front. Ecol. Evol.* 8:5. doi: 10.3389/fevo.2020.00005

Baleba, S. B. S., Torto, B., Masiga, D., Weldon, C. W., and Getahun, M. N. (2019). Egg-laying decisions based on olfactory cues enhance offspring

fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae). *Sci. Rep.* 9:3850. doi: 10.1038/s41598-019-40479-9

Blanford, S., Jenkins, N. E., Read, A. F., and Thomas, M. B. (2012). Evaluating the lethal and pre-lethal effects of a range of fungi against adult *Anopheles stephensi* mosquitoes. *Malar. J.* 11, 365. doi: 10.1186/1475-2875-11-365

Blanford, S., Shi, W., Christian, R., Marden, J. H., Koekemoer, L. L., Brooke, B. D., et al. (2011). Lethal and pre-lethal effects of a fungal biopesticide contribute to substantial and rapid control of malaria vectors. *PLoS One* 6:e23591. doi: 10.1371/journal.pone.0023591

Boulanger, N., Munks, R. J. L., Hamilton, J. V., Vovelle, F., Brun, R., Lehane, M. J., et al. (2002). Epithelial innate immunity: a novel antimicrobial peptide with antiparasitic activity in the blood-sucking insect *Stomoxys calcitrans*. *J. Biol. Chem.* 277, 49921–49926. doi: 10.1074/jbc.M206296200

Brunner-Mendoza, C., Reyes-Montes, M., del, R., Moonjely, S., Bidochka, M. J., and 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. doi: 10.1080/09583157.2018.1531111

Burgerjon, A. (1956). Pulvérisation de poudrage au laboratoire par des préparations pathogènes insecticides. *Ann. Epiphyties* 4, 677–688.

Carn, V. M. (1996). The role of dipterous insects in the mechanical transmission of animal viruses. *Br. Vet. J.* 152, 377–393. doi: 10.1016/S0007-1935(96)80033-9

Christensen, B. M., LaFond, M. M., and Christensen, L. A. (1986). Defense reactions of mosquitoes to filarial worms: effect of host age on the immune response to Dirofilaria immitis microfilariae. *J. Parasitol.* 72:212. doi: 10.2307/3281593

Cook, D. F., Telfer, D. V., Lindsey, J. B., and Deyl, R. A. (2018). Substrates across horticultural and livestock industries that support the development of stable fly, *Stomoxys calcitrans* (Diptera: Muscidae): substrates that support stable fly. *Aust. Entomol.* 57, 344–348. doi: 10.1111/aen.12282

Cruz-Vazquez, C., Carvajal Márquez, J., Lezama-Gutiérrez, R., Vitela-Mendoza, I., and Ramos-Parra, M. (2015). Efficacy of the entomopathogenic fungi *Metarhizium anisopliae* in the control of infestation by stable flies *Stomoxys calcitrans* (L.), under natural infestation conditions. *Vet. Parasitol.* 212, 350–355. doi: 10.1016/j.vetpar.2015.07.003

Darbro, J. M., Ritchie, S. A., Thomas, M. B., Johnson, P. H., Ryan, P. A., and Kay, B. H. (2012). Effects of *Beauveria bassiana* on survival, blood-feeding success, and fecundity of *Aedes aegypti* in laboratory and semi-field conditions. *Am. J. Trop. Med. Hyg.* 86, 656–664. doi: 10.4269/ajtmh.2012.11-0455

Dimbi, S., Maniania, N., and Ekesi, S. (2013). Horizontal transmission of *Metarhizium anisopliae* in fruit flies and effect of fungal infection on egg laying and fertility. *Insects* 4, 206–216. doi: 10.3390/insects4020206

Ezeakacha, N. F., and Yee, D. A. (2019). The role of temperature in affecting carry-over effects and larval competition in the globally invasive mosquito *Aedes albopictus*. *Parasit. Vectors* 12:123. doi: 10.1186/s13071-019-3391-1

Flores-Villegas, A. L., Cabrera-Bravo, M., Toriello, C., Bucio-Torres, M. I., Salazar-Schettino, P. M., and Córdoba-Aguilar, A. (2016). Survival and immune response of the Chagas vector Meccus pallidipennis (Hemiptera: Reduviidae) against two entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria fumosorosea*. *Parasit. Vectors* 9:176. doi: 10.1186/s13071-016-1453-1

Forlani, L., Pedrini, N., and Juarez, M. P. (2011). Contribution of the horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* to the overall performance of a fungal powder formulation against *Triatoma infestans*. *Res. Rep. Trop. Med.* 2, 135–140. doi: 10.2147/RRTM.S22961

Friesen, K., Chen, H., Zhu, J., and Taylor, D. B. (2015). External morphology of stable fly (Diptera: Muscidae) larvae. *J. Med. Entomol.* 52, 626–637. doi: 10.1093/jme/tjv052

García-Munguía, A. M., Garza-Hernández, J. A., Rebollar-Tellez, E. A., Rodríguez-Pérez, M. A., and Reyes-Villanueva, F. (2011). Transmission of *Beauveria bassiana* from male to female *Aedes aegypti* mosquitoes. *Parasit. Vectors* 4:24. doi: 10.1186/1756-3305-4-24

George, J., Blanford, S., Domingue, M. J., Thomas, M. B., Read, A. F., and Baker, T. C. (2011). Reduction in host-finding behaviour in fungus-infected mosquitoes is correlated with reduction in olfactory receptor neuron responsiveness. *Malar. J.* 10:219. doi: 10.1186/1475-2875-10-219

Gonzales, K., and Hansen, I. (2016). Artificial diets for mosquitoes. *IJERPH* 13:1267. doi: 10.3390/ijerph13121267

González-Santoyo, I., and Córdoba-Aguilar, A. (2012). Phenoloxidase: a key component of the insect immune system. *Entomol. Exp. Appl.* 142, 1–16. doi: 10.1111/j.1570-7458.2011.01187.x

Greenfield, B. P. J., Peace, A., Evans, H., Dudley, E., Ansari, M. A., and Butt, T. M. (2015). Identification of Metarhizium strains highly efficacious against Aedes, Anopheles and Culex larvae. *Biocontrol Sci. Technol.* 25, 487–502. doi: 10.1080/09583157.2014.989813

Hillyer, J. F., Schmidt, S. L., Fuchs, J. F., Boyle, J. P., and Christensen, B. M. (2005). Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with a decrease in haemocyte numbers. *Cell. Microbiol.* 7, 39–51. doi: 10.1111/j.1462-5822.2004.00430.x

Hussain, A., Tian, M.-Y., He, Y.-R., Bland, J. M., and Gu, W.-X. (2010). Behavioral and electrophysiological responses of *Coptotermes formosanus* Shiraki towards entomopathogenic fungal volatiles. *Biol. Control* 55, 166–173. doi: 10.1016/j.biocontrol.2010.08.009

Jemberie, W., Hill, S., Rännbäck, L.-M., Ondiaka, S., Raja, N., and Ignell, R. (2018). Evaluation of entomopathogenic fungi *Metarhizium anisopliae* against dengue virus mosquitoes *Aedes aegypti* (Diptera: Culicidae). *Entomology*. 43, 7–18.

Kaaya, G. P. (1989). Glossina morsitans morsitans: mortalities caused in adults by experimental infection with entomopathogenic fungi. *Acta Trop.* 46, 107–114. doi: 10.1016/0001-706X(89)90004-1

Kaaya, G. P., and Munyinyi, D. M. (1995). Biocontrol potential of the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* for tsetse flies (*Glossina* spp.) at developmental sites. *J. Inverteb. Pathol.* 66, 237–241. doi: 10.1006/jipa.1995.1095

Kingsolver, J. G., Arthur Woods, H., Buckley, L. B., Potter, K. A., MacLean, H. J., and Higgins, J. K. (2011). Complex life cycles and the responses of insects to climate change. *Integ. Comp. Biol.* 51, 719–732. doi: 10.1093/icb/icr015

Lacey, L. A. ed. (2012). *Manual of Techniques in Invertebrate Pathology*, 2nd ed. Oxford; New York, NY: Academic Press imprint of Elsevier Science.

Laveissière, C., and Grebaut, P. (1990). The trapping of tsetse flies (Diptera: Glossinidae). Improvement of a model: the Vavoua trap. *Trop. Med. Parasitol.* 41, 185–192.

Liu, H., Skinner, M., Brownbridge, M., and Parker, B. L. (2003). Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae). *J. Inverteb. Pathol.* 82, 139–147. doi: 10.1016/S0022-2011(03)00018-1

López-Sánchez, J., Cruz-Vázquez, C., Lezama-Gutiérrez, R., and Ramos-Parra, M. (2012). Effect of entomopathogenic fungi upon adults of *Stomoxys calcitrans* and *Musca domestica* (Diptera: Muscidae). *Biocontrol Sci. Technol.* 22, 969–973. doi: 10.1080/09583157.2012.699026

Machtinger, E. T., Weeks, E. N. I., and Geden, C. J. (2016). Oviposition deterrence and immature survival of filth flies (Diptera: Muscidae) when exposed to commercial fungal products. *J. Insect Sci.* 16:54. doi: 10.1093/jisesa/iew032

Maniania, N. K. (2002). A low-cost contamination device for infecting adult tsetse flies, *Glossina* spp., with the entomopathogenic fungus *Metarhizium anisopliae* in the field. *Biocontrol Sci. Technol.* 12, 59–66. doi: 10.1080/09583150120110662

Maniania, N. K., and Odulaja, A. (1998). Effect of species, age, and sex of tsetse on response to infection by *Metarhizium anisopliae*. *BioControl* 43, 311–323. doi: 10.1023/A:1009939711555

Maniania, N. K., Okech, M. A., Adino, J. O., Opere, J. O., and Ekesi, S. (2013). Transfer of inoculum of *Metarhizium anisopliae* between adult *Glossina morsitans* morsitans and effects of fungal infection on blood feeding and mating behaviors. *J. Pest Sci.* 86, 285–292. doi: 10.1007/s10340-012-0473-7

Mburu, D. M., Ndung'u, M. W., Maniania, N. K., and Hassanali, A. (2011). Comparison of volatile blends and gene sequences of two isolates of *Metarhizium anisopliae* of different virulence and repellency toward the termite *Macrotermes michaelseni*. *J. Exp. Biol.* 214, 956–962. doi: 10.1242/jeb.050419

McCormick, M. I., and Gagliano, M. (2009). "Carry-over effects-the importance of a goodstart," in *Proceedings of the 11th International Coral Reef Symposium* (Florida: Nova South Eastern University), 305–310.

Mnyone, L. L., Kirby, M. J., Lwetoijera, D. W., Mpingwa, M. W., Knols, B. G., Takken, W., et al. (2009). Infection of the malaria mosquito, *Anopheles gambiae*, with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence. *Malar. J.* 8:309. doi: 10.1186/1475-2875-8-309

Mnyone, L. L., Kirby, M. J., Mpingwa, M. W., Lwetoijera, D. W., Knols, B. G. J., Takken, W., et al. (2011). Infection of *Anopheles gambiae* mosquitoes with

entomopathogenic fungi: effect of host age and blood-feeding status. *Parasitol Res.* 108, 317–322. doi: 10.1007/s00436-010-2064-y

Mondal, S., Baksi, S., Koris, A., and Vatai, G. (2016). Journey of enzymes in entomopathogenic fungi. *Pac. Sci. Rev. A* 18, 85–99. doi: 10.1016/j.psra.2016.10.001

Moraes, A. P. R., Angelo, I., da, C., Fernandes, É. K. K., Bittencourt, V. R. E. P., and Bittencourt, A. J. (2008). Virulence of *Metarhizium anisopliae* to eggs and immature stages of *Stomoxys calcitrans*. *Ann N Y Acad Sci.* 1149, 384–387. doi: 10.1196/annals.1428.008

Moraes, A. P. R., Videira, S. S., Bittencourt, V. R. E. P., and Bittencourt, A. J. (2014). Antifungal activity of *Stenotrophomonas maltophilia* in *Stomoxys calcitrans* larvae. *Rev. Bras. Parasitol. Vet.* 23, 194–199. doi: 10.1590/S1984-29612014037

Morales Hernandez, C. E., Padilla Guerrero, I. E., Gonzalez Hernandez, G. A., Salazar Solis, E., and Torres Guzman, J. C. (2010). Catalase overexpression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*. *Appl. Microbiol. Biotechnol.* 87, 1033–1044. doi: 10.1007/s00253-010-2517-3

Mullens, B. A. (1985). Host age, sex, and pathogen exposure level as factors in the susceptibility of *Musca domestica* to *Entomophthora muscae*. *Entomol. Exp. Appl.* 37, 33–39. doi: 10.1111/j.1570-7458.1985.tb03449.x

Mweke, A., Ulrichs, C., Nana, P., Akutse, K. S., Fiaboe, K. K. M., Maniania, N. K., et al. (2018). Evaluation of the entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria* sp. for the Management of *Aphis craccivora* (Hemiptera: Aphididdae). *J. Econ. Entomol.* 111, 1587–1594. doi: 10.1093/jee/toy135

Niassy, S., Subramanian, S., Ekesi, S., Bargul, J. L., Villinger, J., and Maniania, N. K. (2013). Use of *Metarhizium anisopliae* chitinase genes for genotyping and virulence characterization. *BioMed Res. Int.* 2013:1–9. doi: 10.1155/2013/465213

Nnakumusana, E. S. (1985). Laboratory infection of mosquito larvae by entomopathogenic fungi with particular reference to Aspergillus parasiticus and its effects on fecundity and longevity of mosquitoes exposed to sporal infections in larval stages. *Curr. Sci.* 54, 1221–1228.

Ortiz-Urquiza, A., and Keyhani, N. (2013). Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects* 4, 357–374. doi: 10.3390/insects4030357

Paula, A. R., Carolino, A. T., Silva, C. P., and Samuels, R. I. (2011). Susceptibility of adult female *Aedes aegypti* (Diptera: Culicidae) to the entomopathogenic fungus *Metarhizium anisopliae* is modified following blood feeding. *Parasit. Vectors* 4:91. doi: 10.1186/1756-3305-4-91

Quesada-Moraga, E., and Vey, A. (2003). Intra-specific variation in virulence and in vitro production of macromolecular toxins active against locust among *Beauveria bassiana* strains and effects of *in vivo* and *in vitro* passage on these factors. *Biocontrol Sci. Technol.* 13, 323–340. doi: 10.1080/0958315031000110346

R Core Team. (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available online at: https://www.R-project.org/ (accessed July, 2020).

Ravindran, K., Akutse, K. S., Sivaramakrishnan, S., and Wang, L. (2016). Determination and characterization of destruxin production in *Metarhizium anisopliae* Tk6 and formulations for *Aedes aegypti* mosquitoes control at the field level. *Toxicon* 120, 89–96. doi: 10.1016/j.toxicon.2016.07.016

Samuels, R. I., Charnley, A. K., and Reynolds, S. E. (1988). The role of destruxins in the pathogenicity of 3 strains of *Metarhizium anisopliae* for the tobacco hornworm *Manduca sexta*. *Mycopathologia* 104, 51–58. doi: 10.1007/BF00437924

Sánchez-Roblero, D., Huerta-Palacios, G., Valle, J., Gómez, J., and Toledo, J. (2012). Effect of *Beauveria bassiana* on the ovarian development and reproductive potential of *Anastrepha ludens* (Diptera: Tephritidae). *Biocontrol Sci. Technol.* 22, 1075–1091. doi: 10.1080/09583157.2012.713090

Scholte, E.-J., Knols, B. G., and Takken, W. (2004). Autodissemination of the entomopathogenic fungus *Metarhizium anisopliae* amongst adults of the malaria vector *Anopheles gambiae*. *Malar. J.* 3:45. doi: 10.1186/1475-2875-3-45

Scholte, E.-J., Knols, B. G. J., and Takken, W. (2006). Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. *J. Inverteb. Pathol.*91, 43–49. doi: 10.1016/j.jip.2005.10.006

Scholte, E.-J., Njiru, B. N., Smallegange, R. C., Takken, W., and Knols, B. G. (2003). Infection of malaria (*Anopheles gambiae* s.s.) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*. *Malar. J.* 2:29. doi: 10.1186/1475-2875-2-29

Shahrestani, P., Chambers, M., Vandenberg, J., Garcia, K., Malaret, G., Chowdhury, P., et al. (2018). Sexual dimorphism in *Drosophila melanogaster* survival of *Beauveria bassiana* infection depends on core immune signaling. *Sci. Rep.* 8:12501. doi: 10.1038/s41598-018-30527-1

Skrobek, A., and Butt, T. M. (2005). Toxicity testing of destruxins and crude extracts from the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol. Lett.* 251, 23–28. doi: 10.1016/j.femsle.2005.07.029

Talaei -Hassanloui, R., Kharazi-Pakdel, A., Goettel, M., and Mozaffari, J. (2006). Variation in virulence of *Beauveria bassiana* isolates and its relatedness to some morphological characteristics. *Biocontrol Sci. Technol.* 16, 525–534. doi: 10.1080/09583150500532758

Taylor, D. B., Moon, R. D., and Mark, D. R. (2012). Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. *J. Med. Entomol.* 49, 198–209. doi: 10.1603/ME10050

Therneau, T. (2015). *A Package for Survival Analysis in S. Version 2.38*. Available online at: https://CRAN.R-project.org/package=survival (accessed July, 2020).

Unlu, I., Suman, D. S., Wang, Y., Klingler, K., Faraji, A., and Gaugler, R. (2017). Effectiveness of autodissemination stations containing pyriproxyfen in reducing immature *Aedes albopictus* populations. *Parasit. Vectors* 10:139. doi: 10.1186/s13071-017-2034-7

Valero-Jiménez, C. A., Debets, A. J., van Kan, J. A., Schoustra, S. E., Takken, W., Zwaan, B. J., et al. (2014). Natural variation in virulence of the entomopathogenic fungus *Beauveria bassiana* against malaria mosquitoes. *Malar. J.* 13:479. doi: 10.1186/1475-2875-13-479

Vogelweith, F., Körner, M., Foitzik, S., and Meunier, J. (2017). Age, pathogen exposure, but not maternal care shape offspring immunity in an insect with facultative family life. *BMC Evol. Biol.* 17:69. doi: 10.1186/s12862-017-0926-y

Walker, A. R. (1990). "Disease caused by arthropods," in *Handbook on Animal Diseases in the Tropics, 4th edn*, eds M. M. H. Sewell, and D. W. Brocklesby (London: Bailliere Tindall), 44.

Wamiti, L. G., Khamis, F. M., Abd-alla, A. M. M., Ombura, F. L. O., Akutse, K. S., Subramanian, S., et al. (2018). *Metarhizium anisopliae* infection reduces *Trypanosoma congolense* reproduction in *Glossina fuscipes* fuscipes and its ability to acquire or transmit the parasite. *BMC Microbiol.* 18:142. doi: 10.1186/s12866-018-1277-6

Zayed, A., Soliman, M. M., and El-Shazly, M. M. (2013). Infectivity *of Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) to *Phlebotomus papatasi* (Diptera: Psychodidae) under laboratory conditions. *J. Med. Entomol.* 50, 796–803. doi: 10.1603/ME12244

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Altered Expression of Chemosensory and Odorant Binding Proteins in Response to Fungal Infection in the Red Imported Fire Ant, *Solenopsis invicta*

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Social insects have evolved acute mechanisms for sensing and mitigating the spread of microbial pathogens within their communities that include complex behaviors such as grooming and sanitation. Chemical sensing involves detection and transport of olfactory and other chemicals that are mediated by at least two distinct classes of small molecular weight soluble proteins known as chemosensory- and odorant binding proteins (CSPs and OBPs, respectively) that exist as protein families in all insects. However, to date, a systematic examination of the expression of these genes involved in olfactory and other pathways to microbial infection has yet to be reported. The red imported fire ant, *Solenopsis invicta*, is one of the most successful invasive organisms on our planet. Here, we examined the temporal gene expression profiles of a suite of *S. invicta CSPs* (*SiCSPs1-22*) and *OBPs* (*SiOBPs1-16*) in response to infection by the broad host range fungal insect pathogen, *Beauveria bassiana*. Our data show that within 24 h post-infection, i.e., before the fungus has penetrated the host cuticle, the expression of *SiCSPs* and *SiOBPs* is altered (mainly increased compared to uninfected controls), followed by suppression of *SiCSP* and select *SiOBP* expression 48 h post-infection and mixed responses at 72 h post-infection. A smaller group of *SiBOPs*, however, appeared to respond to fungal infection, with expression of *SiOBP15* consistently higher during fungal infection over the time course examined. These data indicate dynamic gene expression responses of *CSPs* and *OBPs* to fungal infection that provide clues to mechanisms that might mediate detection of microbial pathogens, triggering grooming, and nest sanitation.

Keywords: odorant binding protein, chemosensory protein, *Beauveria bassiana*, red imported fire ant, *Solenopsis invicta* Buren, fungal pathogenesis

INTRODUCTION

Chemosensory and odorant binding proteins (CSPs and OBPs, respectively), represent two evolutionarily distinct protein families, that share several features (Pelosi et al., 2006, 2014; Kulmuni and Havukainen, 2013). CSPs and OBPs are small molecular weight (typically 10–18 kDa) soluble proteins capable of binding a wide range of ligands including hydrophobic and volatile compounds such as pheromones, organic and fatty acids, and other semiochemicals and environmental odors (Calvello et al., 2005; Dani et al., 2011; Pelosi et al., 2011). Transcriptome analyses continue to identify CSPs and OBPs enriched in the antennae of many insects, where they are thought to solubilize and shuttle chemical ligands to transmembrane receptors as part of olfactory and/or gustatory sensing pathways (Xue et al., 2016; Bin et al., 2017; Yuvaraj et al., 2018; Qiu et al., 2020; Yang et al., 2020). However, members of the CSP and OBP families are now recognized as functioning in a wide range of physiological processes beyond olfaction (Pelosi et al., 2018). For example, subsets of CSPs and OBPs have been found to function as reservoirs for the storage and release of semiochemicals from pheromone glands (Jacquin-Joly et al., 2001; Briand et al., 2002; Iovinella et al., 2013). Several CSPs have been shown to function in development and regeneration, and an OBP in mosquitoes has been shown to reduce host inflammation by binding leukotrienes and biogenic amines, thus blocking swelling, itching, and pain reaction in the host while feeding (Kitabayashi et al., 1998; Maleszka et al., 2007; Calvo et al., 2009). A link between CSPs and vision has been suggested by the observation of CSPs acting as carriers for β-carotene in the cotton bollworm (*Helicoverpa armigera*), and the expression of CSPs has also been linked to potential adaptive mechanisms leading to chemical insecticide resistance (Bautista et al., 2015; Zhu et al., 2016). However, to date, changes in the expression of insect CSPs and OBPs to infection by a microbial (fungal) pathogen have not been directly examined.

Red imported fire ants (*Solenopsis invicta*) are eusocial insects that live in complex societies that include communal organization, divisions of labor and reproduction, and task specialization and recruitment, all of which entail highly sophisticated social interactions and modes of communication (Wilson, 1962; Ross et al., 2007). *S. invicta* is also one of the most successful invasive species in the world, having spread from its origin in northern country-regionArgentina/southern country-regionBrazil to the United States, and from there, worldwide (Shoemaker et al., 2011). Due to the high population density of the ant nest, threats of the spread of disease-causing microbial pathogens are high. In response, these insects have evolved a number of social strategies (in addition to the innate immune system) for dealing with pathogens that include grooming and nest sanitation (Fan et al., 2012; Qiu et al., 2014). Aside from innate immune signaling (e.g., Toll-receptors), knowledge concerning mechanisms for detection and response to pathogens remains limited. The broad host range insect pathogen, *Beauveria bassiana*, is capable of parasitizing and killing *S. invicta* (Williams et al., 2003; Bextine and Thorvilson, 2004; Brinkman and Gardner, 2004). Infection begins with attachment of conidial spores to the insect integument, and subsequent growth of penetrating hyphae that enter into the insect hemolymph through the exoskeleton (Ortiz-Urquiza and Keyhani, 2013, 2015). Inside the hemolymph, the fungus undergoes a dimorphic transition, utilizing the nutrient in the hemolymph before penetrating outward to ultimately sporulate on the insect cadaver (Lewis et al., 2009; Wanchoo et al., 2009; Ortiz-Urquiza and Keyhani, 2015). Depending upon the dose, ants (and other insects) can mount successful defense strategies to counter the pathogens that include various behaviors (e.g., behavioral fever, grooming), and although infection leading to death typically progresses over a time course of 3–7 day, host (transcriptional) responses have been recorded as early as 4 h post-infection (Roy et al., 2006; Reber et al., 2011; Qiu et al., 2014; Zhang et al., 2020). These latter studies, performed in locusts, indicated potential changes in the expression of certain CSPs in response to fungal infection, although the issue was not systematically investigated.

Here, we sought to examine a time course of changes in the gene expression of 21 *S. invicta* CSPs (*SiCSPs1-4, 6-22*, note we have used the nomenclature consistent with McKenzie et al. (2014), in which there is no *SiCSP5*) and 16 *S. invicta OBPs* (*SiOBPs1-16*, no signals were seen for *SiOBP17* (Zhang et al., 2016) and this gene was not further examined) in response to *B. bassiana* infection. Expression of a large set of *SiCSPs* showed a transient stimulation 24 h post-infection, that was subsequently sharply repressed 48 h post-infection, mainly returning to uninfected levels 72 h post-infection. In contrast, changes in the expression of *SiOBPs* in response to *B. bassiana* infection were more limited, with several showing decreased expression and others increased expression during the infection course, although wave-form patterns of expression for several *SiOBPs* were noted. Of all genes examined, only *SiOBP15* showed a clear pattern of increased expression 24–72 h post-infection. These data show that CSP and OBP expression patterns can respond dynamically to pathogens during different stages of the infection process, including pre- and post-cuticle penetration, as well as during ingress and proliferation within the hemocoel.

MATERIALS AND METHODS

Insects and Fungal Isolate

Solenopsis invicta colonies were collected from the field and maintained in the plastic boxes coated with talcum powder essentially as described (Fan et al., 2012). Ants were kept at 26°C with ∼70% humidity and 16:8 dark:light photoperiod. The colony was determined to be polygyne as evidenced by the presence of multiple queens and sequencing of the *Gp-9* (*OBP3*) alleles. Sucrose (300 mM solution) and freeze-dried *Galleria mellonella* larvae were fed to colonies every 2–3 day. Randomly mixed minor and major workers were used for all bioassay and RT-qPCR analysis. *B. bassiana* (ATCC 90517) was culture on potato dextrose agar (PDA)

for 14–15 day at 26°C before conidia were collected in sterile 0.05% Tween-80 solution and mycelial debris were removed by filtration through sterile lens paper. Spores concentrations were calculated by direct counting using a Neubauer hemocytometer.

Insect Bioassays

Various concentrations of *B. bassiana* conidia were prepared via dilution to 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/mL in sterile 0.05% Tween-80. *S. invicta* workers (20/replicate × 3 replicates/experiment) were immersed in the conidial suspensions for 15 s, and the workers were removed and placed on a dry tissue paper. Control ants were treated with 0.05% Tween-80. Controls and treated workers were kept at 26°C with ∼70% humidity and 16:8 dark:light photoperiod and placed in standard Petri dishes containing an Eppendorf tubes filled with sucrose (300 mM) solution, that was replaced every 2–3 day. Mortality was recorded twice daily and dead insects were removed and placed in tubes under 70% humidity to confirm fungal outgrowth. The entire experiment was repeated with three independent batches of fungal conidia.

Gene Expression Analyses: RT-qPCR

Workers treated with suspensions of 1×10^8 *B. bassiana* conidia/mL as above were collected for quantitative RT-PCR gene expression analyses. Controls were treated with 0.05% Tween-80. After treatment, surviving workers were collected over a time course of the infection that included 12, 24, 48, and 72 h post-inoculation. Total RNAs were extracted from the whole body of workers using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and contaminating genomic DNA was removed by digestion using TURBO DNase (Invitrogen) according to the manufacturer's instructions. Agarose gel electrophoresis and NanoDrop 2000 spectrophotometric analyses were performed to determine the quality and quantity of the total RNA preparations. An aliquot of 2 μg of the purified total RNA was used to construct cDNA libraries from each sample, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Primers for RT-qPCR (**Supplementary Table 1**) were designed using Beacon Designer 8 software. The *S. invicta* elongation factor-α (EF1α) gene was used as the housekeeping (reference) gene. The amplification efficiencies of all primers were confirmed by empirical construction of standard curves and primer concentrations and annealing temperatures were determined according to the derived amplification efficiencies (Zhang et al., 2016; Wanchoo et al., 2020). For RT-qPCR experiments, the *S. invicta* cDNA libraries from various treatments were diluted 40-fold in nuclease-free ddH_2O (double-distilled H_2O), and 5 μl of diluted cDNA was used as the template in a 15 μL reaction volumes. Each reaction contained 1× Master Mix (Biotools, Houston, TX, United States), 5 μL template and 200 nM of each of gene specific primer pairs. All reactions were performed in triplicate. At least three independent RNA preparations for each sample were analyzed using the RT-qPCR protocol. The RT-qPCR reactions were performed using an Eco Real-Time qPCR System (Illumina, San Diego, CA, United States) with a thermo-profile of one cycle of 95°C 5 min, 95°C 2 min, then 45 cycles of 95°C 15 s, and 60/59°C 45 s, followed by a melting curve analysis from 55 to 95°C.

Phylogenetic Analyses

Chemosensory proteins and OBPs protein sequences from the ant species *Linepithema humile* (Argentine ant), *Camponotus floridanus* (carpenter ant), *Camponotus japonicus* (Japanese carpenter ant), *Hapergnathos saltator* (Jerdon's jumping ant), and *S. invicta* were used to build limited phylogenetic trees (**Supplementary Tables 2**, **3**, and **Supplementary Material**). For both CSP and OBP sequences, amino acid multiple sequence alignments were generated with webPRANK (Löytynoja and Goldman, 2010), and the best fitting models for amino acid substitutions were estimated with MEGA7 (Kumar et al., 2016). MEGA7 chose the models LG + G and JTT + G for the CSP and OBP sequences, respectively, and these models were used to build the phylogenetic trees with RaxML, available at the CIPRES Science Gateway (Miller et al., 2010; Stamatakis, 2014). G (gamma shape parameters) and branch length were estimated, and branch support was calculated by bootstrapping. RaxML was allowed to execute 1000 rapid bootstrap inferences and halt bootstrapping automatically after a thorough maximum likelihood search (934 bootstraps). The software MEGA7 was used to draw the tree (Kumar et al., 2016).

Data Analyses

Normalized expression data were examined by one-way ANOVAs (with *post hoc* comparisons using Bonferroni and Duncan's test) using the IBM SPSS Statistic 20 software package (SPSS, Inc., 2011). The mean lethal time (LT_{50}) and mean lethal concentration were estimated by Probit analysis.

RESULTS

Phylogenetic Analyses

The amino acid sequences for 21 SiCSPs (SiCSP1-4 and 6-22) and 16 SiOBPs (SiCSP1-16) were used to construct limited phylogenetic trees including CSPs and OBPs from the ant species *S. invicta*, *L. humile*, *C. floridanus*, *C. japonicus*, and *H. saltator* (**Figures 1**, **2**). As has been reported by others (Gotzek et al., 2011; Kulmuni and Havukainen, 2013; McKenzie et al., 2014), for the CSPs, these analyses revealed a division of the CSPs into ant-specific lineages and those shared with other insects. Within the ant-specific lineages, SiCSPs 1, 14 (somewhat related to the CSP gene expansion labeled in green), and 18 were dispersed in the tree, whereas two distinct fire ant CSP gene expansions (boxed in green and blue) were noted (**Figure 1**). These expansions consisted of SiCSPs 9, 10, 12, 13, 19, 20, and 22, in one clade (green), and SiCSPs 11, 15, 16, 17, and 21 in another (blue). SiCSPs 2, 3, 4, 6, 7, and 8 showed homology to CSPs found in order insects. Unlike CSPs, no ant specific OBPs were noted. SiOBPs 12-16 formed

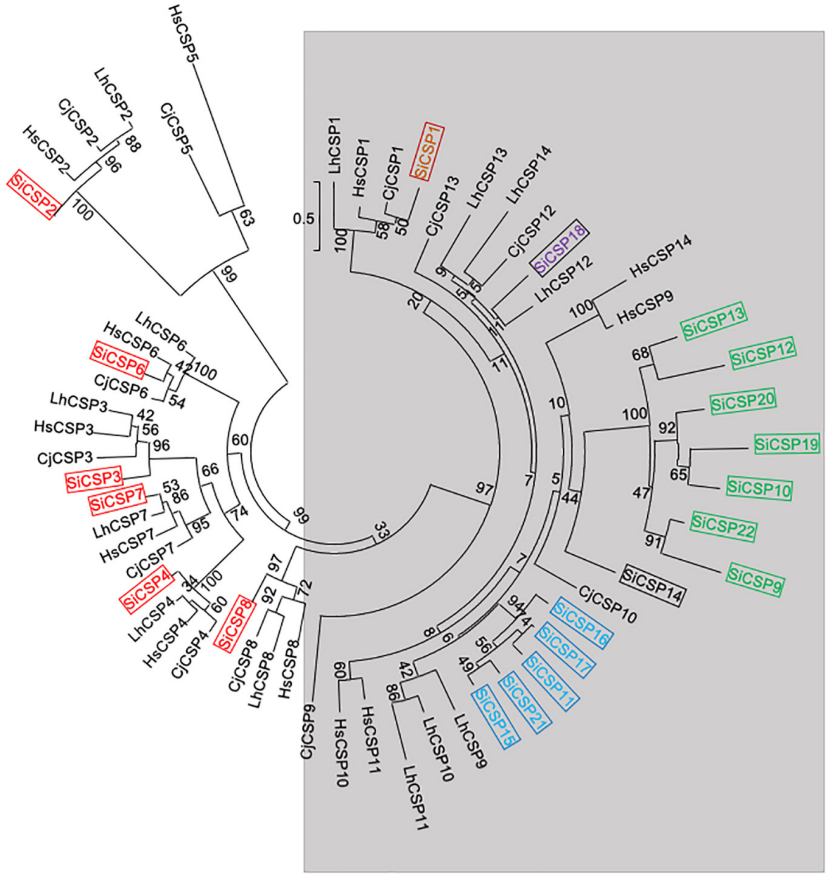

FIGURE 1 | Simplified phylogenetic tree of S. invicta CSPs. Limited maximum likelihood phylogeny of S. invicta CSPs compared to the CSP repertoires found in the ant species, C. japonicus (CjCSP), H. saltator (HsCSP), and L. humile (LhCSPs) (Accession numbers given in **Supplementary Table 2**). Numbers at nodes indicate bootstrap values. Scale shows number of substitutions per site. The tree is midpoint-rooted in the absence of a suitable out-group. More detailed phylogenetic analyses of insect CSPs can be found in Kulmuni and Havukainen (2013), McKenzie et al. (2014).

a *S. invicta*-specific OBP expansion (**Figure 2**), with the other OBPs dispersed within the tree. SiOBP3, which showed high amino acid similarity with SiOBP4 (**Figure 2**), is equivalent to GP-9 that has been linked to a "mini-chromosome" which also contains SiOBPs 4, 5, 9, 12, 13, and 15-16, that appears to show limited recombination and has been implicated as mediating important aspects of ant "social" behavior (Linksvayer et al., 2013; Nipitwattanaphon et al., 2013; Wang et al., 2013; Zhang et al., 2016).

Ant Bioassays

As a broad host range insect pathogen, *B. bassiana* is known to be able to infect ants, including *S. invicta* (Stimac et al., 1993; Oi et al., 1994), and insect bioassays indicated dose-dependent mortality of *S. invicta* after parasitism by *B. bassiana* (**Figure 3**). At low doses (10^5–10^6 conidia/mL) used in this work, infection yielded ∼25 and 60% mortality, respectively, whereas at higher doses (10^7–10^8 conidia/mL), almost all treated individuals eventually succumbed to the infection. The mean lethal time (LT_{50}) for the latter two doses (10^7–10^8 conidia/ml) was calculated as being 3.2 ± 0.63 h and 2.5 ± 0.2 days, respectively. Based on these data, an approximate mean lethal dose for 50% mortality (LD_{50}) was calculated to be 1.13×10^7 conidia/mL.

Expression of *CSPs* and *OBPs* in *B. bassiana* Infected and Non-infected *S. invicta* Workers

Changes in the expression of *SiCSPs 1-4*, *6-22*, and *OBPs 1-16* were examined by RT-qPCR in *S. invicta* workers over a time course (12–72 h) of *B. bassiana* infection using 10^8 conidia/mL as detailed in the "Materials and Methods" section (**Figures 4**, **5**). CSPs are grouped by their phylogenetic position and color

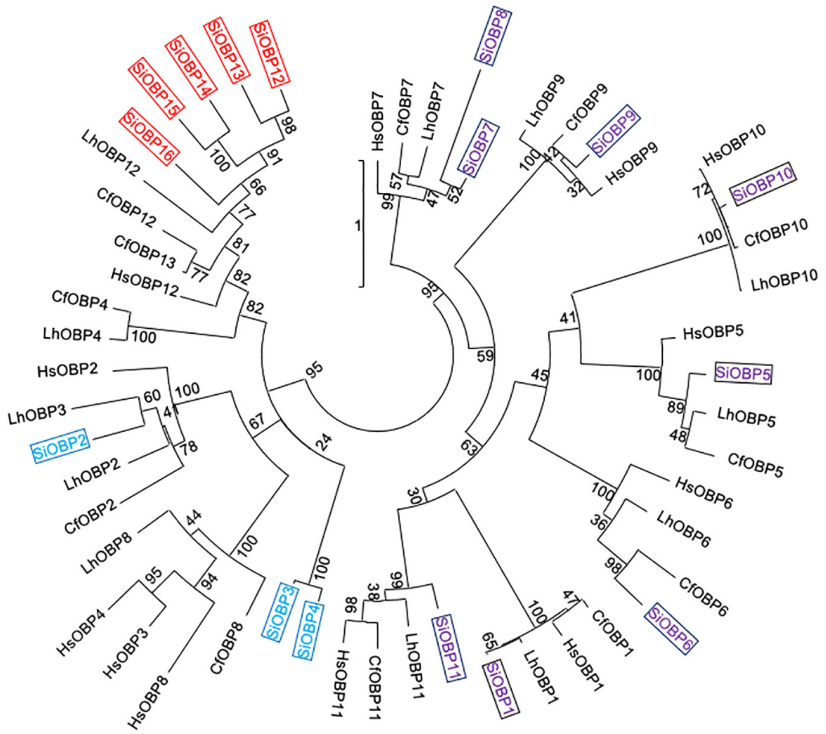

FIGURE 2 | Simplified phylogenetic tree of *S. invicta* OBPs. Limited maximum likelihood phylogeny of *S. invicta* OBPs compared to the OBP repertoires found in the ant species, *C. floridanus* (CfCSP), *H. saltator* (HsCSP), and *L. humile* (LhCSPs) (Accession numbers given in **Supplementary Table 3**). Numbers at nodes indicate bootstrap values. Scale shows number of substitutions per site. The tree is midpoint-rooted in the absence of a suitable out-group. More detailed phylogenetic analyses of insect CSPs can be found in McKenzie et al. (2014), Xue et al. (2016), Zhang et al. (2016).

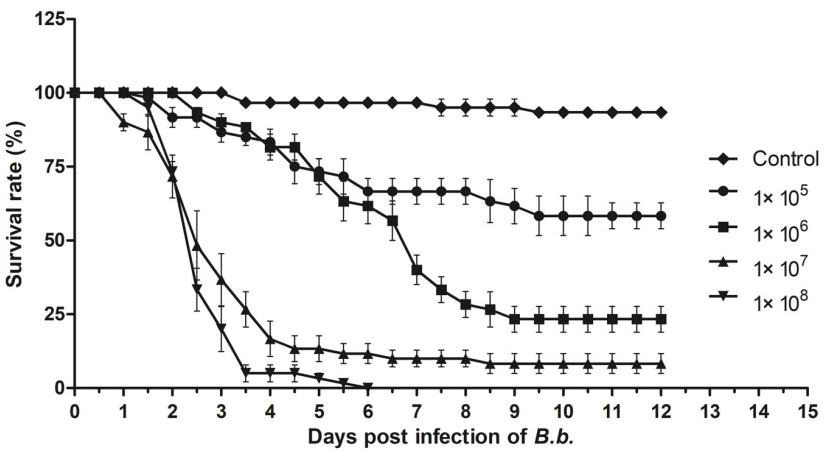

FIGURE 3 | Insect bioassays. Time course of infection of *S. invicta* workers by *B. bassiana* using indicated concentration of fungal conidia as the inoculum. All experiments were performed in triplicate. Error bars = ± SD.

coded as in **Figure 1**. No sequence for *SiCSP5* was assigned as per previously reported nomenclature (McKenzie et al., 2014). During 12 h post-infection only *SiCSP13*, within the ant specific expansion and *SiCSP2*, found in the "insect general" *CSP* clade were significantly ($P < 0.01$) downregulated as compared to untreated controls. However, by 24 h post-infection, the expression levels of a large set of *SiCSPs* showed significantly increased expression in response to *B. bassiana* infection. These included *SiCSPs 1*, *14*, *18* (ant-specific), and members of the sub-clusters, *SiCSPs 13*, *19*, *20*, and *22* (in green, fire ant *CSP* gene expansion), and *SiCSPs 11*, *15*, *16*, and *17* (in blue, representing a second fire ant *CSP* expansion), as well as the "general" *SiCSPs 3*, *4*, *6*, and *7*. With the exception of *SiCSP14*, which showed a dramatic significant increase in expression 48 h post-infection,

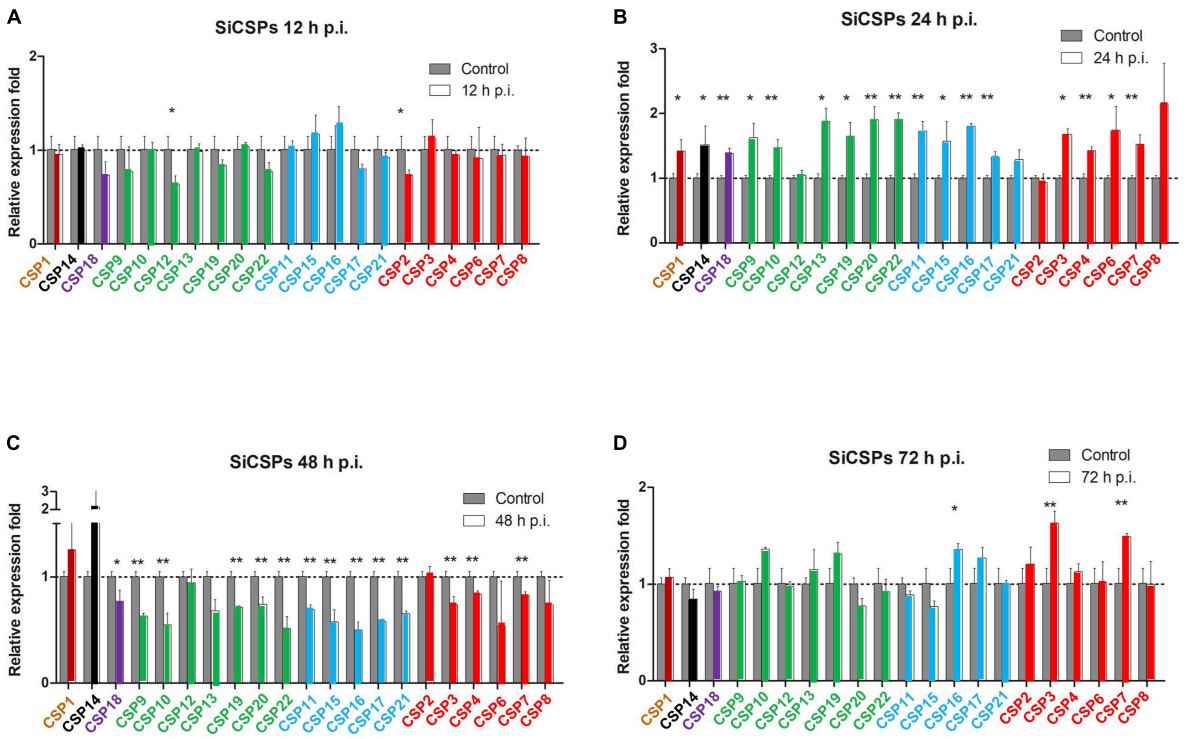

FIGURE 4 | Gene expression analyses of *S. invicta* CSPs in response to *B. bassiana* infection. *S invicta* workers were infected using 1×10^8 conidia/ml. Total RNA was isolated **(A)** 12 h, **(B)** 24 h, **(C)** 48 h, and **(D)** 72 h post-infection and RT-qPCR reactions performed as detailed in the "Materials and Methods" section. CSPs are grouped and color coded according to phylogenetic analyses shown in **Figure 1**. All experiments were performed in triplicate. Error bars = ± SD. Symbols "*" and "**" indicate statistical significances of $P < 0.05$ and $P < 0.01$, respectively.

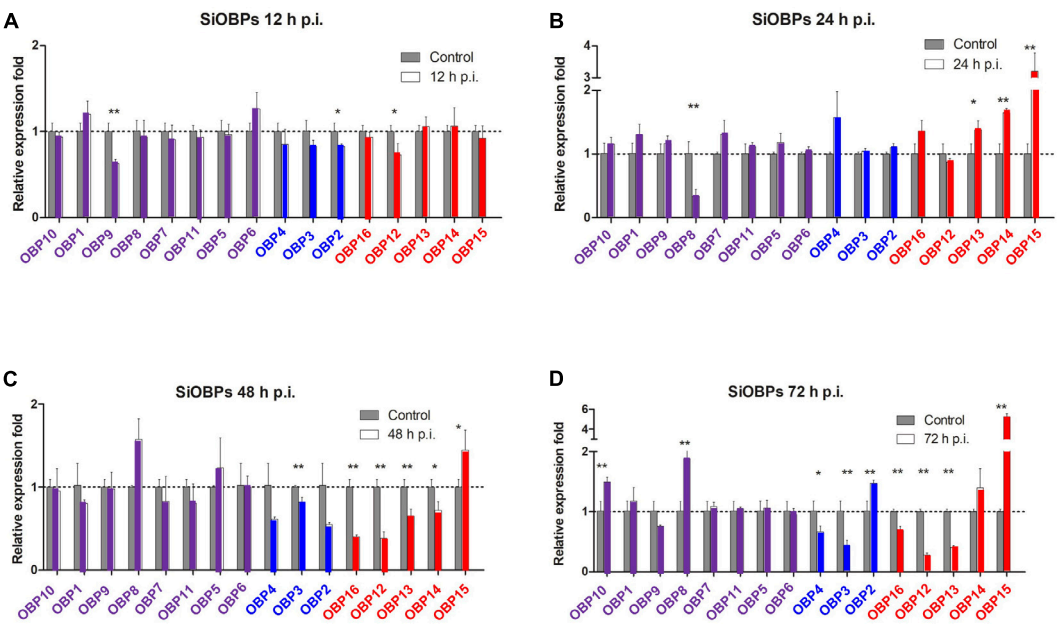

FIGURE 5 | Gene expression analyses of *S. invicta* OBPs in response to *B. bassiana* infection. *S invicta* workers were infected using 1×10^8 conidia/ml. Total RNA was isolated **(A)** 12 h, **(B)** 24 h, **(C)** 48 h, and **(D)** 72 h post-infection and RT-qPCR reactions performed as detailed in the "Materials and Methods" section. OBPs are grouped and color coded according to phylogenetic analyses shown in **Figure 2**. All experiments were performed in triplicate. Error bars = ± SD. Symbols "*" and "**"indicate statistical significances of $P < 0.05$ and $P < 0.01$, respectively.

the increased expression of the other *SiCSPs* appeared transient, and was followed by a sharp decrease in expression levels. Indeed, aside from a handful of *SiCSPs*, whose expression did not vary significantly (i.e., *SiCSPs 1*, *2*, *8*, *12,* and *13*), the expression of the other *SiCSPs* was significantly decreased in infected ants compared to controls. By 72 h post-infection, *SiCSP* expression levels were similar to controls, except for *SiCSP16*, *3*, and *7*, which were significantly upregulated (**Figure 4**).

With respect to the *SiOBPs*, at 12 h post-infection and similar to the *SiCSPs*, significant decreases in the expression of only a handful of *SiOBPs*, namely *SiOBPs 2*, *9*, and *12* were seen (**Figure 5**). By 24 h post-infection, only *SiOBP8* showed significantly decreased expression in response to *B. bassiana* infection, whereas *SiOBPs 4, 13*, and *15*, (with the latter >3–4 fold) showed significant upregulation in response to infection. At 48 and 72 h post-infection, *SiOBPs 3*, *12*, *13*, and *16* showed decreased expression, whereas *SiOBP14* and *SiOBP4* showed significantly ($P < 0.01$) decreased expression at 48 and 72 h post-infection, respectively. *SiOBP15* was the only *OBP* (and gene examined) to show a consistent pattern of increased expression throughout the 24–72 h post-infection time course. Expression of *SiOBP3/gp9* gradually decreased during *B. bassiana* infection and was at ∼50% 72 h post-infection.

DISCUSSION

Though the functions of some CSPs and OBPs in chemical perception and potential downstream behavioral regulation have been reported, any roles during microbial pathogen infection have not been systematically studied. The data presented here suggest a number of important points. First, that *SiCSP* and *SiOBP* expression is significantly more dynamic than previously considered. Second, to the best of our knowledge, these data are the first to show systematic changes in expression of the suite of *CSP* and *OBP* genes in response to microbial infection. In addition, our data show that changes in (fire ant) *CSP* and *OBP* expression occur very early in the infection process–being seen within 24 h post-infection, a time point before the fungus has penetrated the cuticle which typically occurs 24–48 h after infection. In addition, *SiCSP* and *SiOBP* gene expression responses did not follow any clear phylogenetic patterns, i.e., more closely related *SiCSPs/SiOBPs* did not appear to follow similar gene expression responses to *B. bassiana* infection than more distantly related *SiCSPs/SiOBPs*.

Upon *B. bassiana* infection, *SiCSPs* and *SiOBPs* showed some similar temporal expression dynamics, with expression of these genes downregulated, in general, at the very initial stages of the infection (i.e., likely at the initiation of germination at 12 h post-infection) and subsequently upregulated at 24 h post-infection, i.e., once most of the conidia were probably germinated and starting the processes of breaching the cuticle. At 48 h (i.e., initial hemocoel colonization), *SiCSP* and *SiOBP* expression lowered, and only a few *SiCSPs* and *SiOBPs* were upregulated at 72 h post-infection (i.e., hemocoel colonization). Two important aspects should be noted. First, many CSPs and OBPs are not involved in antennal chemosensation and instead may function as ligand carriers in other physiological functions that can include pheromone and hormone sequestration and signaling (Dani et al., 2011). Second, our experimental design does not discriminate between any self- versus allo-grooming that may occur.

Among the *SiCSPs*, the most significant increase occurred for *SiCSP14* at a time point in which expression of most other *CSPs* was sharply decreased (i.e., 48 h post-infection). Prior studies reported high and robust expression of *SiCSP14* in the worker abdomen and antennae, respectively, while showing low expression in the head and the thorax (Wanchoo et al., 2020). These gene expression analyses showed that *SiCSP3*, *7* and *17*, which were the only *CSPs* showing a change of expression in infected ants 72 h post-infection (∼1.5-fold increase), exhibited robust and high expression in the antennae and the abdomen of workers (Wanchoo et al., 2020). Of the major fire ant antennal *SiCSPs* (*SiCSPs12, 8, 19, 11, and 1*) (Wanchoo et al., 2020), *SiCSP19* and *11* showed a significant increase in expression at 24 h post-infection, followed by a decrease at 48 h post-infection. Of the three other most-expressed antennal *SiCSPs*, *SiCSP12*, and 8 showed no change in expression over time, whereas *SiCSP1* displayed a very transitory and slightly increase in expression 24 h post-infection that dropped to uninfected levels 48 and 72 h post-infection. These results again highlight the potential for dynamic changes in CSP expression patterns to environmental stimuli (including microbial infection) that has hitherto been neglected.

In *S. invicta* worker antennae, gene expression analyses indicated high expression of *SiOBPs 1*, *2*, *5*, and *6* (Zhang et al., 2016), none of which showed dramatic responses to *B. bassiana* infection. In worker head tissues, aside from high expression of *SiBOP3*, *SiOBPs 2*, *7*, *10*, *13*, and *15* were also highly expressed, and as noted *SiOBP15* was the only gene examined whose expression was consistently increased during *B. bassiana* infection. *SiOBP15* was also not very highly expressed in worker thorax and abdomen tissues (the latter showing low expression of *SiOBPs* in general) (Zhang et al., 2016). Intriguingly, *SiOBP8*, which showed the greatest increase in response to *B. bassiana* infection after *SiOBP15*, was in general poorly expressed in worker tissues (Zhang et al., 2016).

Solenopsis invicta OBP14 which was highly expressed in worker thorax, along with *SiOBP3*, (Zhang et al., 2016), showed an initial decrease in expression followed by a significant increase ($P < 0.01$) as compared to untreated controls 48 h post-infection. *SiOBP3* (*gp-9*) has been implicated as part of a control locus that mediates aspects of social behavior, notably mono- versus polygyny in fire ants (Gotzek and Ross, 2009). However, it is now recognized as being part of a significantly larger "mini-chromosome" that includes additional OBPs (i.e., *SiOBPs 4*, *5*, *9*, *12*, *13*, and *15-16*) and CSPs (i.e., *SiCSP7, 9, 12, 13, 14, and 22*) (Zhang et al., 2016), and which appears to be restricted in terms of recombination (i.e., linkage group 16) (Wang et al., 2013; Nipitwattanaphon et al., 2014). Thus, it is possible that this genomic region includes a range of genes involved in a network of regulation that ultimately impacts social organization. Expression of *SiOBP3*, together with *SiOBPs 12*, *13*, and *16*, appeared to respond to *B. bassiana* showing a consistent downward trend in expression levels 48 and 72 h post-infection.

A range of studies have indicated a clear role for soluble "olfactory" proteins in physiological processes beyond olfaction. Our data show that this can now be potentially expanded to include response to (microbial) pathogens. It remains to be determined whether these functions are within olfaction, e.g., recognition of the fungal pathogen–spores or other infectious propagules–on the insect surface, or downstream processes, e.g., inflammation, development/reproduction, regeneration and/or a combination of both. Within the olfaction context, it is known that ants engage in social behaviors that include grooming and sanitation (Reber et al., 2011; Qiu et al., 2014), and therefore CSPs and/or OBPs may be involved in the chemosensation of microbial pathogens on the insect surface. In addition, insect responses to pathogens can include changes in feeding and reproduction that may be mediated by hormones and other signaling molecules during the infection process. As CSPs/OBPs can act as carriers for these molecules, changes in their expression levels may reflect responses to the infectious agent.

As in most cases examined, changes in the expression of *CSPs*/*OBPs* were transient and appeared to follow a wave-like pattern, showing increased expression within 24 h post-infection, followed by decreased expression by 48 h post-infection. At 12 h post-infection, the fungal conidia have attached and germinated on the insect surface but have yet to penetrate the cuticle (Ortiz-Urquiza and Keyhani, 2013). Large scale transcriptomics have revealed similar changes in gene expression patterns during pre-penetration events in locusts infected by the insect fungal pathogen *Metarhizium acridum* (Zhang et al., 2015, 2020). These data indicate that insects can detect microbial pathogens early during infection and hence may attempt to quickly mobilize immune or other responses to the infection. In this context, signals (lipids and other compounds) on the insect surface are known to change as the fungus germinates and germ tubes begin to grow on the surface before penetration (Pedrini et al., 2010, 2013). At 24 h post-infection, fungal hyphae are in the process of breaching the cuticle and by 48 h can reach the hemolymph (Ortiz-Urquiza and Keyhani, 2016). It is during these stages that our data show high fluctuation (increase then decrease) in *SiCSP* and *SiOBP* expression levels, that may reflect the transition to elicitation of direct (innate) immune responses once the fungus has breached the cuticle. At 72 h post-infection, the fungus is likely proliferating within the hemocoel, forming free-floating hyphal bodies that are capable of evading immune surveillance (Lewis et al., 2009; Wanchoo et al., 2009). At this stage, changes in *SiCSP* and *SiOBP* expression levels appear to be limited. Overall, our results highlight that insect responses may be calibrated to infection dynamics and that the time course of the infection needs to be considered in any examination of such responses. Both suppression and induction of *SiCSPs* and *SiOBPs* were noted, with *SiOBP15* showing the most consistent (increased) response across the infection time course. These data allow for discrete functional hypotheses to be made concerning a number of *SiCSPs* and *SiOBPs* that may be implicated in microbial infection responses and future work examining several candidates within the context of (*B. bassiana*) infection is warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NOK and ZW initiated the project and conceived and designed the study. ZW, AO-U, and NOK performed the samples collection, library constructions, RT-qPCR, data processing, bioinformatic analyses, data interpretation, wrote the manuscript, and contributed to revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.596571/full#supplementary-material

Supplementary Table 1 | List of primers used for RT-qPCR analyses.

Supplementary Table 2 | CSPs accession numbers.

Supplementary Table 3 | OBPs accession numbers.

Supplementary Material | Amino acid sequences of CSPs and OBPs used for the phylogenetic analyses.

REFERENCES

Bautista, M. A., Bhandary, B., Wijeratne, A. J., Michel, A. P., Hoy, C. W., and Mittapalli, O. (2015). Evidence for trade-offs in detoxification and chemosensation gene signatures in *Plutella xylostella*. *Pest Manag. Sci.* 71, 423–432. doi: 10.1002/ps.3822

Bextine, B. R., and Thorvilson, H. G. (2004). Novel *Beauveria bassiana* delivery system for biological control of the red imported fire ant. *Southwest Entomol.* 29, 47–53.

Bin, S. Y., Qu, M. Q., Pu, X. H., Wu, Z. Z., and Lin, J. T. (2017). Antennal transcriptome and expression analyses of olfactory genes in the sweetpotato weevil *Cylas formicarius*. *Sci. Rep.* 7:11073.

Briand, L., Swasdipan, N., Nespoulous, C., Bezirard, V., Blon, F., Huet, J. C., et al. (2002). Characterization of a chemosensory protein (ASP3c) from honeybee (Apis mellifera L.) as a brood pheromone carrier. *Eur. J. Biochem.* 269, 4586–4596. doi: 10.1046/j.1432-1033.2002.03156.x

Brinkman, M. A., and Gardner, W. A. (2004). Red imported fire ant (Hymenoptera : Formicidae) control in nursery pots treated with *Beauveria bassiana* and bifenthrin. *J. Entomol. Sci.* 39, 175–187. doi: 10.18474/0749-8004-39.2.175

Calvello, M., Brandazza, A., Navarrini, A., Dani, F. R., Turillazzi, S., Felicioli, A., et al. (2005). Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. *Insect. Biochem. Mol. Biol.* 35, 297–307.

Calvo, E., Mans, B. J., Ribeiro, J. M., and Andersen, J. F. (2009). Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. *Proc. Natl. Acad. Sci. U S A.* 106, 3728–3733. doi: 10.1073/pnas.0813190106

Dani, F. R., Michelucci, E., Francese, S., Mastrobuoni, G., Cappellozza, S., La Marca, G., et al. (2011). Odorant-binding proteins and chemosensory proteins in pheromone detection and release in the silkmoth *Bombyx mori*. *Chem. Senses* 36, 335–344. doi: 10.1093/chemse/bjq137

Fan, Y., Pereira, R. M., Kilic, E., Casella, G., and Keyhani, N. O. (2012). Pyrokinin beta-neuropeptide affects necrophoretic behavior in fire ants (*S. invicta*), and expression of beta-NP in a mycoinsecticide increases its virulence. *PLos One* 7:e26924.doi: 10.1371/journal.pone.0026924

Gotzek, D., Robertson, H. M., Wurm, Y., and Shoemaker, D. (2011). Odorant binding proteins of the red imported fire ant, solenopsis invicta: an example of the problems facing the analysis of widely divergent proteins. *PLoS One* 6:e16289.doi: 10.1371/journal.pone.0016289

Gotzek, D., and Ross, K. G. (2009). Current status of a model system: the gene Gp-9 and its association with social organization in fire ants. *PLoS One* 4:e7713.doi: 10.1371/journal.pone.0007713

Iovinella, I., Bozza, F., Caputo, B., Della Torre, A., and Pelosi, P. (2013). Ligand-binding study of *Anopheles gambiae* chemosensory proteins. *Chem. Senses* 38, 409–419. doi: 10.1093/chemse/bjt012

Jacquin-Joly, E., Vogt, R. G., Francois, M. C., and Nagnan-Le Meillour, P. (2001). Functional and expression pattern analysis of chemosensory proteins expressed in antennae and pheromonal gland of *Mamestra brassicae*. *Chem. Senses* 26, 833–844. doi: 10.1093/chemse/26.7.833

Kitabayashi, A. N., Arai, T., Kubo, T., and Natori, S. (1998). Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (*American cockroach*). *Insect. Biochem. Mol. Biol.* 28, 785–790. doi: 10.1016/s0965-1748(98)00058-7

Kulmuni, J., and Havukainen, H. (2013). Insights into the evolution of the CSP gene family through the integration of evolutionary analysis and comparative protein modeling. *PLos One* 8:e63688.doi: 10.1371/journal.pone.0063688

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lewis, M. W., Robalino, I. V., and Keyhani, N. O. (2009). Uptake of the fluorescent probe FM4-64 by hyphae and haemolymph-derived in vivo hyphal bodies of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology-Sgm* 155, 3110–3120. doi: 10.1099/mic.0.029165-0

Linksvayer, T. A., Busch, J. W., and Smith, C. R. (2013). Social supergenes of superorganisms: do supergenes play important roles in social evolution? *Bioessays* 35, 683–689. doi: 10.1002/bies.201300038

Löytynoja, A., and Goldman, N. (2010). webPRANK: a phylogeny-aware multiple sequence aligner with interactive alignment browser. *BMC Bioinform.* 11:579. doi: 10.1186/1471-2105-11-579

Maleszka, J., Foret, S., Saint, R., and Maleszka, R. (2007). RNAi-induced phenotypes suggest a novel role for a chemosensory protein CSP5 in the development of embryonic integument in the honeybee (*Apis mellifera*). *Dev. Genes Evol.* 217, 189–196. doi: 10.1007/s00427-006-0127-y

McKenzie, S. K., Oxley, P. R., and Kronauer, D. J. C. (2014). Comparative genomics and transcriptomics in ants provide new insights into the evolution and function of odorant binding and chemosensory proteins. *BMC Genomics* 15:718.doi: 10.1186/1471-2164-15-718

Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). "Creating the CIPRES science gateway for inference of large phylogenetic trees," in *Gateway Computing Environments Workshop (GCE)*, (New Orleans, LA).

Nipitwattanaphon, M., Wang, J., Dijkstra, M. B., and Keller, L. (2013). A simple genetic basis for complex social behaviour mediates widespread gene expression differences. *Mol. Ecol.* 22, 3797–3813. doi: 10.1111/mec.12346

Nipitwattanaphon, M., Wang, J., Ross, K. G., Riba-Grognuz, O., Wurm, Y., Khurewathanakul, C., et al. (2014). Effects of ploidy and sex-locus genotype on gene expression patterns in the fire ant *Solenopsis invicta*. *Proc. R. Soc. B-Biol. Sci.* 281:20141776. doi: 10.1098/rspb.2014.1776

Oi, D. H., Pereira, R. M., Stimac, J. L., and Wood, L. A. (1994). Field applications of *Beauveria bassiana* for control of the red imported fire ant (Hymenoptera. Formicidae). *J. Econ. Entomol.* 87, 623–630. doi: 10.1093/jee/87.3.623

Ortiz-Urquiza, A., and Keyhani, N. O. (2013). Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects* 4, 357–374. doi: 10.3390/insects4030357

Ortiz-Urquiza, A., and Keyhani, N. O. (2015). Stress response signaling and virulence: insights from entomopathogenic fungi. *Curr. Genet.* 61, 239–249. doi: 10.1007/s00294-014-0439-9

Ortiz-Urquiza, A., and Keyhani, N. O. (2016). "Chapter Six - molecular genetics of *Beauveria bassiana* infection of insects," in *Advances in Genetics*, eds B. Lovett and R. J. St Leger (Cambridge, MA: Academic Press), 165–249. doi: 10.1016/bs.adgen.2015.11.003

Pedrini, N., Ortiz-Urquiza, A., Zhang, S., and Keyhani, N. (2013). Targeting of insect epicuticular lipids by the entomopathogenic fungus *Beauveria bassiana*: hydrocarbon oxidation within the context of a host-pathogen interaction. *Front. Microbiol.* 4:24. doi: 10.3389/fmicb.2013.00024

Pedrini, N., Zhang, S., Juárez, M. P., and Keyhani, N. O. (2010). Molecular characterization and expression analysis of a suite of cytochrome P450 enzymes implicated in insect hydrocarbon degradation in the entomopathogenic fungus *Beauveria bassiana*. *Microbiology* 156, 2549–2557. doi: 10.1099/mic.0.039735-0

Pelosi, P., Calvello, M., and Ban, L. P. (2011). Diversity of odorant-binding proteins and chemosensory proteins in insects. *Chem. Senses* 30, I291–I292.

Pelosi, P., Iovinella, I., Felicioli, A., and Dani, F. R. (2014). Soluble proteins of chemical communication: an overview across arthropods. *Front. Physiol.* 5:320. doi: 10.3389/fphys.2014.00320

Pelosi, P., Iovinella, I., Zhu, J., Wang, G., and Dani, F. R. (2018). Beyond chemoreception: diverse tasks of soluble olfactory proteins in insects. *Biol. Rev. Camb. Philos. Soc.* 93, 184–200. doi: 10.1111/brv.12339

Pelosi, P., Zhou, J. J., Ban, L. P., and Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cell Mol. Life Sci.* 63, 1658–1676. doi: 10.1007/s00018-005-5607-0

Qiu, H.-L., Lu, L., Shi, Q.-X., and He, Y.-R. (2014). Fungus exposed Solenopsis invicta ants benefit from grooming. *J. Insect Behav.* 27, 678–691. doi: 10.1007/s10905-014-9459-z

Qiu, L., He, L., Tan, X., Zhang, Z., Wang, Y., Li, X., et al. (2020). Identification and phylogenetics of *Spodoptera frugiperda* chemosensory proteins based on antennal transcriptome data. *Comp. Biochem. Physiol. Part D Genom. Proteom.* 34:100680. doi: 10.1016/j.cbd.2020.100680

Reber, A., Purcell, J., Buechel, S. D., Buri, P., and Chapuisat, M. (2011). The expression and impact of antifungal grooming in ants. *J. Evol. Biol.* 24, 954–964. doi: 10.1111/j.1420-9101.2011.02230.x

Ross, K. G., Krieger, M. J. B., Keller, L., and Shoemaker, D. D. (2007). Genetic variation and structure in native populations of the fire ant Solenopsis invicta: evolutionary and demographic implications. *Biol. J. Linn. Soc.* 92, 541–560. doi: 10.1111/j.1095-8312.2007.00853.x

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., and Pell, J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annu. Rev. Entomol.* 51, 331–357. doi: 10.1146/annurev.ento.51.110104.150941

Shoemaker, D., Ascunce, M. S., Yang, C. C., Oakey, J., Calcaterra, L., Wu, W. J., et al. (2011). Global invasion history of the fire ant *Solenopsis invicta*. *Science* 331, 1066–1068.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Stimac, J. L., Pereira, R. M., Alves, S. B., and Wood, L. A. (1993). Mortality in laboratory colonies of *Solenopsis invicta* (Hymenoptera, Formicidae) treated with *Beauveria bassiana* (Deuteromycetes). *J. Econ. Entomol.* 86, 1083–1087. doi: 10.1093/jee/86.4.1083

Wanchoo, A., Lewis, M. W., and Keyhani, N. O. (2009). Lectin mapping reveals stage-specific display of surface carbohydrates in in vitro and haemolymph-derived cells of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology-Sgm* 155, 3121–3133. doi: 10.1099/mic.0.029157-0

Wanchoo, A. Z. W., Ortiz-Urquiza, A., Xia, Y., and Keyhani, N. O. (2020). Red imported fire ant (*Solenopsis invicta*) chemosensory proteins are expressed in tissue, developmental, and caste-specific patterns. *Front. Physiol.* 11:585883. doi: 10.3389/fphys.2020.585883

Wang, J., Wurm, Y., Nipitwattanaphon, M., Riba-Grognuz, O., Huang, Y. C., Shoemaker, D., et al. (2013). A Y-like social chromosome causes alternative colony organization in fire ants. *Nature* 493, 664–668. doi: 10.1038/nature11832

Williams, D. F., Oi, D. H., Porter, S. D., Pereira, R. M., and Briano, J. A. (2003). Biological control of imported fire ants. *Am. Entomol.* 49, 150–163.

Wilson, E. O. (1962). Chemcial communication among workers of the fire ant *Solenopsis saevissima* (Fr. Smith) Parts 1, 2, and 3. *Anim. Behav.* 10, 131–164.

Xue, W., Fan, J., Zhang, Y., Xu, Q., Han, Z., Sun, J., et al. (2016). Identification and expression analysis of candidate odorant-binding protein and chemosensory protein genes by antennal transcriptome of *Sitobion avenae*. *PLoS One* 11:e0161839.doi: 10.1371/journal.pone.0161839

Yang, H., Dong, J., Sun, Y., Hu, Z., Lv, Q., and Li, D. (2020). Antennal transcriptome analysis and expression profiles of putative chemosensory soluble proteins in *Histia rhodope* cramer (Lepidoptera: Zygaenidae). *Comp. Biochem. Physiol. Part D Genom. Proteom.* 33:100654. doi: 10.1016/j.cbd.2020.100654

Yuvaraj, J. K., Andersson, M. N., Zhang, D. D., and Lofstedt, C. (2018). Antennal transcriptome analysis of the chemosensory gene families from trichoptera and basal lepidoptera. *Front. Physiol.* 9:1365. doi: 10.3389/fphys.2018.01365

Zhang, W., Chen, J., Keyhani, N. O., Zhang, Z., Li, S., and Xia, Y. (2015). Comparative transcriptomic analysis of immune responses of the migratory locust, *Locusta migratoria*, to challenge by the fungal insect pathogen, *Metarhizium acridum*. *BMC Genom.* 16:867. doi: 10.1186/s12864-015-2089-9

Zhang, W., Wanchoo, A., Ortiz-Urquiza, A., Xia, Y., and Keyhani, N. O. (2016). Tissue, developmental, and caste-specific expression of odorant binding proteins in a eusocial insect, the red imported fire ant. *Solenopsis invicta*. *Sci. Rep.* 6:35452.

Zhang, W., Zheng, X., Chen, J., Keyhani, N. O., Cai, K., and Xia, Y. (2020). Spatial and temporal transcriptomic analyses reveal locust initiation of immune responses to *Metarhizium acridum* at the pre-penetration stage. *Dev. Comp. Immunol.* 104:103524. doi: 10.1016/j.dci.2019.103524

Zhu, J., Iovinella, I., Dani, F. R., Liu, Y. L., Huang, L. Q., Liu, Y., et al. (2016). Conserved chemosensory proteins in the proboscis and eyes of *Lepidoptera*. *Int. J. Biol. Sci.* 12, 1394–1404. doi: 10.7150/ijbs.16517

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Synergy in Efficacy of *Artemisia sieversiana* Crude Extract and *Metarhizium anisopliae* on Resistant *Oedaleus asiaticus*

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In order to explore the synergistic control effect of crude extracts of *Artemisia sieversiana* and *Metarhizium anisopliae* on *Oedaleus asiaticus*, we used different doses of *M. anisopliae* and crude extracts of *A. sieversiana* singly and in combination, to determine their toxicities to fourth instar *O. asiaticus*. The results showed that the combination of 10% crude extract of *A. sieversiana* with 10^7 and 10^8 spores/g *M. anisopliae* concentrations and the combination of 20% crude extract of *A. sieversiana* with 10^7 and 10^8 spores/g *M. anisopliae* concentrations had significant effects on the mortality, body weight gain, body length gain, growth rate, and overall performance of *O. asiaticus* than those of the crude extract of *A. sieversiana* and *M. anisopliae* alone. Among them, the 20% *A. sieversiana* crude extract mixed with 10^8 spores/g *M. anisopliae* and 10% *A. sieversiana* crude extract combined with 10^7 spores/g *M. anisopliae*, had the best control efficacy. In order to clarify the biochemical mechanism underlying the immune responses of *O. asiaticus* to the pesticide treatments, we monitored the activities of four enzymes: superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO). The results showed that the activities of three enzymes (SOD, CAT, and PPO) were significantly increased from the treatment with the combination of *M. anisopliae* mixed with crude extract of *A. sieversiana*. Interestingly, compared to the crude extract, the combination treatment did not significantly induce the expression of POD enzyme activity, which may be a biochemical factor for increasing the control effect of the combination treatment. Our results showed that the combination treatment had synergistic and antagonistic effects on host mortality, growth, development, and enzyme activities in *O. asiaticus*.

Keywords: *Artemisia*, *Metarhizium anisopliae*, *Oedaleus asiaticus*, synergetic control, biopesticide

INTRODUCTION

As important biological control agents, botanical pesticides have received extensive research attention due to characteristics such as their low toxicities, easy degradation, and miscibility with other pesticides and against which insects are yet to develop resistance (Dubey et al., 2010). Crude plant extracts, the most notable of botanical pesticides, contain secondary metabolites such as alkaloids, terpenoids, phenols (mainly including flavonoids), tannins, lignin, nitrogenous organic compounds, etc., which have shown to be effective against insects (Wink, 1988). They have been extracted from plants species such as *Melilotus suavcolen*, *Peganum harmala* (zygophylllaceae), *Ajuga iva* (labiateae), *Aristolochia baetica* (Aristolochiaceae), *Raphanus raphanistrum* (Brassicaceae), etc. (Jbilou et al., 2006). These secondary metabolites are chemical compounds which have evolved in plants, to have specialized functions such as adaptation, survival of competition, defense against herbivorous insects, etc. They deter herbivorous insects from feeding on plants by poisoning them or interfering with digestion and the absorption of nutrients in herbivorous insects (Rattan, 2010). For instance, flavonoids can inhibit many insect species from feeding and oviposition (Simmonds, 2001). In addition, these substances also induce a rise in the detoxification mechanism of foreign compounds in insects (Bi and Felton, 1995). They can also stimulate and induce insects to feed or to produce sex hormones. Thus, they have mutually beneficial and co-evolutionary relationships (Kergoat et al., 2017).

China is rich in plant resources. There are more than 220 plant species that can be used as botanical insecticides. Compositae, one of the plant families which accounts for more than 20% of these species, is characterized by the most complex and diverse chemical compositions (Shang et al., 1997). *Artemisia*, a dominant genus in grasslands in Inner Mongolia, has shown promising insecticidal activities. In recent years, it has increasingly become one of the common plants, with the intensification of degradation of the grasslands in Inner Mongolia. The main chemical components of *Artemisia* are flavonoids, terpenoids, sesquiterpene lactones, cyanogenic glycides, flavonal, coumarins, etc. (Vander et al., 2008). Extensive studies have been conducted on the insecticidal activity of *Artemisia* plants (da Silva et al., 2005). For example, the crude extract of Anthemideae was shown to have strong anti-feedant properties against *Tribolium castaneum* Herbst, *Tetranychus cinnabarinus*, and *Maruca testulalis*. Also, *Artemisia mongolica*, *Artemisia lavandulaefolia*, and *Acacia vestita* showed excellent insecticidal effects against *Sitophilus zeamais* (Wu, 2015).

Artemisia sieversiana, a typical Artemisia plant, also known as Artemisia, *A. alba*, *A. scoparia*, etc., is one of the dominant species in grasslands in China. It is widely distributed in India, Pakistan, Afghanistan, Russia, Mongolia, and China's Heilongjiang, Shanxi, Hebei, Ningxia, Gansu, Xinjiang, Inner Mongolia, and other regions (Cui et al., 2020). *A. sieversiana* contains a variety of insecticidal and antibacterial chemical components, such as flavonoids, lignins, sesquiterpenes, and volatile oils (Liu et al., 2010; Cui et al., 2020). In addition, its essential oil was shown to have fumigation and killing activity on *Callosobruchus chinensis*, grubs, and other insects (Ayub et al., 2019). Further, the ethanol extract of *A. sieversiana* was shown to have significantly inhibited and killed the growth of human colon cancer cells (HT-29, HCT-15, and Colo-205; Tang et al., 2015).

Entomopathogenic fungi are another group of important biological control agents with low environmental risk, used in pest management. Insect pests are not easily resistant to entomopathogenic fungi. They have great potential for pest control in integrated pest management programs (Blanford et al., 2005; Sabbour and Sahab, 2005; Zimmermann, 2007). *Metarhizium anisopliae*, one of such entomopathogenic fungal species, is widely used globally, and has been studied for more than 100 years in China. It has been used on more than 200 insect species and has shown significant control effects against the larvae of Curculio chinensis Chevrolat, *Monochamus alternatus*, Polyphylla laticollis Lewis, Locusta migratoria manilensis, *Plutella xylostella*, *O. asiaticus*, etc. (Peng et al., 2008; Kaaya et al., 2011).

M. anisopliae infects insects through the insect cuticle or when insects feed (stomach poison; Mannino et al. 2019). *M. anisopliae* directly enters the midgut through the feeding of insects, and the mycelium propagates rapidly in the midgut cells, resulting in the loss of cellular functions, directly affecting cellular metabolism, and ultimately destroying the midgut tissue. These result in the death of insects. Mortality rates of insects from midgut infections are significantly higher than that from cuticle infections. *M. anisopliae* infections are very easy to spread in their colonies. However, the long lethal time of fungal insecticides lowers their efficacy (MeCoy, 1981; Dimbi et al., 2009).

All groups of insecticides, including botanical insecticides, fungicides, and chemical insecticides, etc., have advantages and disadvantages (Marican and Durán-Lara, 2018). For example, botanical insecticides are environment friendly, but also have unstable formulations, easy photodegradation and their active ingredients are difficult to extract (Pavela, 2016). Insects do not easily develop resistance against fungal insecticides, but their lethal times are longer, and their prevention and control of pest outbreaks is slower. Chemical insecticides show rapid effectiveness and good control effects, but their excessive usage generates residues which pollute the environment and also have negative impact on food safety for humans and animals (Sabarwal et al., 2018). However, the use of a combination of these insecticides can effectively solve these challenges by producing a synergistic efficacy to achieve better control of insect pest. It can also solve the challenge of excessive use of chemical agents (Gibson et al., 2014). For example, the use of a combination of *M. anisopliae* V275 and imidacloprid or fipronil had a significantly higher control effect on *Otiorhynchus sulcatus* than that from each insecticide alone (Shah et al., 2007). Also, neem oil effectively maintained the toxicity of the entomopathogenic fungus *M. anisopliae*, protected it from UV radiation, and increased the control effect on *Aedes aegypti* larvae (Paula et al., 2019). The use of a combination of the insecticide chlorantraniliprole and *M. anisopliae* showed excellent

synergistic control on locusts, scarabaeidea, Empoasca pirisuga Matumura, rice planthoppers, and other agricultural pest (Farenhorst et al., 2010; Jia et al., 2016).

Oedaleus decorus asiaticus (Bey-Bienko), is a major pest in the grasslands of northern China. In recent years, its frequent large-scale outbreaks are partly responsible for the degradation and desertification of grasslands in Inner Mongolia, in China (Zhang et al., 2013). At present, the main control of *O. asiaticus* is based on biological control methods such as the use of poxvirus, *M. anisopliae*, *Beauveria bassiana*, and the introduction of insect natural enemies. Among them, *M. anisopliae* has shown a very significant control effect on *O. asiaticus*. It has the advantage of being green, safe, and has long duration, but has a slow effect on insects. Quercetin (a kind of flavonoids) was shown to reduce the survival rate, inhibit the growth and development of *O. asiaticus* (Cui et al., 2019). The crude extract of *A. sieversiana* contains flavonoids, which has been shown to have a toxic effect on insects, but its efficacy lasted for a short period of time. For practical applications, the simultaneous use of fungi and chemical agents for insect pest control, may result in higher mortality than from the use of each alone (Duarte et al., 2016; Jia et al., 2016). Therefore, we evaluated the synergetic control effect of the crude extract of *A. sieversiana* and *M. anisopliae* on *O. asiaticus* in this study, to provide a new direction for the research and development of new biological agents for the effective control of locust or grasshopper pest.

MATERIALS AND METHODS

Experimental Materials

Test Insects

O. asiaticus were caught from the wild, in the grasslands of Xilinhot City, Inner Mongolia, China. They were reared on their host plant, *Stipa krylovii*, in a cage (1 m × 1 m × 1 m) at room temperature (about 27°C) for 1 week.

Test Plant

A. sieversiana was collected from the Scientific Observing and Experimental Station of Pests in Xilingol Rangeland, Ministry of Agriculture, P.R. China (E116°36", N43.57'12"), located in the Xilingol League, Inner Mongolia, northeast China.

Test Strain

M. anisopliae IMI330-189 was provided by the Grassland Pest Group, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPPCAAS), which was introduced from the International Centre for Biological Control, United Kingdom, and preserved in the IPPCAAS.

Test Instruments

Insect net (mesh diameter: 5 mm, mesh bag diameter: 40 cm, and mesh length: 80–130 cm), insect cage (1 m × 1 m × 1 m), insect rearing frame (26 × 13 × 7 cm), glass cover plate (30 × 50 × 0.3 cm), conical flask (100 ml), test tube (10 ml), petri dish, writing brush, stirring rod, glass beads (dm = 3 mm), hemocytometer (25 × 16 mm), microscope (OLYMPUS-SZX16), oscillator MX-F, oven DHG-9123A, autoclave YXQ-LS-SII, and electronic balance PL-403.

Test Agent

Liquid nitrogen, distilled water, soil temperature 80 (dosage was 0.001), vegetable oil, and carrier (wheat bran).

Method

Preparation of Crude Plant Extract

The crude plant extract was extracted with water and two concentrations (10 and 20%) of the extracts were prepared (**Table 1**). Briefly, the fresh plant materials of *A. sieversiana* were cut into small pieces of about 1 cm and put into a conical flask. Distilled water was added and then autoclaved at 120°C for 90 min. They were taken out after heating and cooled to room temperature to filter plant residues.

Toxicological Tests

Separate Treatments With *A. sieversiana* Extract or *M. anisopliae*

Healthy 4th-instar *O. asiaticus* were selected from the insect cage in the laboratory, and raised in a 26 × 13 × 7 cm^3 breeding frame with a glass cover (30 × 50 × 0.3 cm). Each cage contained 10 females and 10 males (20 heads in each frame). One frame served as a replicate, and five repetitions were treated as a group. In the *A. sieversiana* extract treatment, the 10 and 20% *A. sieversiana* extracts were spread separately on leaves of *S. krylovii* with a brush and fed to *O. asiaticus* in the cages. Leaves on which distilled water was spread, served as the control. The experiment lasted for 7 days and data on mortality were recorded.

In the *M. anisopliae* treatment, 1 g each of the three concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) was weighed and separately mixed with a carrier (10 g vegetable oil and 100 g wheat bran) as poison bait. The mixture was divided into five petri dishes (dm = 7 cm) and placed in the insect rearing frame containing 20 healthy 4th instar *O. asiaticus* which had been prior fed with fresh S. kirnii for 24 h. The experiment lasted for 7 days and data on mortality were recorded.

Treatment With the Combination of *A. sieversiana* Extract + *M. anisopliae*

In this treatment, mixtures which were made up of each concentration of *A. sieversiana* extract and each concentration of *M. anisopliae* were prepared (**Table 2**). These combinations were then added to a mixture made up of 10 g vegetable oil

TABLE 1 | Proportion of components in the different concentrations of the prepared crude extract from *Artemisia sieversiana*.

Concentration (%)	Amount of distilled water (g)	Amount of *A. sieversiana* (g)
10	500	50 g
20	500	100 g

TABLE 2 | Mixed formula of crude extract of *A. sieversiana* and *Metarhizium anisopliae*.

Order number	Concentration of crude extract of *A. sieversiana* (%)	*M. anisopliae* concentration (spores/g)
1	10	10^6
2	10	10^7
3	10	10^8
4	20	10^6
5	20	10^7
6	20	10^8

mixed into 100 g wheat bran. They were mixed evenly, and then dispensed into glass culture dishes to feed *O. asiaticus* in the cages. After feeding for 24 h, the diet was changed to fresh *S. kirnii* for continuous feeding for 7 days. Data on mortality were recorded during this period.

Determination of Body Weight and Body Length of *O. asiaticus*

Female and male 4th instar *O. asiaticus* caught from the Xilinguole grassland were used to determine the effects of the treatments on body weight. We randomly selected 30 healthy 4th females and 4th males, respectively, from the captured 4th instar *O. asiaticus* to detect their initial body weight and body length. We use a vernier caliper to measure these selected females and males, and then put them in the oven at 90°C for 24 h after which the dry weights were measured using an electronic balance.

We also selected enough healthy 4th instar females and 4th instar males from the captured *O. asiaticus* for experiments. We conducted the experiment twice, each with five biological replicates, each cage contained 10 females and 10 males (20 heads in each frame). The survival and developmental time of *O. asiatica* in the cages were recorded every day to ensure that there were enough males and females for body weight and body length measurement at the end of the experiment. Dead individuals were removed in time when we change fresh *S. krylovii*. At the end of 7 days, the body lengths of 30 locusts were measured using the vernier caliper. They were then dried in the oven at 90°C for 24 h after which the dry weights were measured using an electronic balance.

Determination of Activities of Antioxidant Enzymes in *O. asiaticus*

The effect of the pesticide treatments on activities of four antioxidant enzymes [superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO)] in *O. asiaticus* were measured. To extract crude enzyme solutions, about 0.1 g of insect tissue was weighed, to which 1 ml of an extraction solution was added (purchased from Suzhou Keming Biotechnology Co., Ltd.). They were then placed in an ice bath for homogenization. The supernatant was then centrifuged at 8000 *g* at 4°C for 10 min and placed on ice for the measurement of enzyme activities with detection kits of each antioxidant enzymes (Suzhou Keming Biotechnology Co., Ltd.). Preparation of the test samples followed the manufacturer's instructions. The absorbance wavelength of each enzyme was measured on a microplate reader (DNM-9602G).

For POD, the wavelength was adjusted to 470 nm, and that of distilled water adjusted to zero. The initial absorbance value, A1, was recorded at 470 nm at 1 min and that of A2 at 2 min. The final absorbance value was given by ΔA = A2−A1. For CAT, the wavelength was adjusted to 240 nm, and that of distilled water was adjusted to zero. The initial absorbance value A1 was recorded at 240 nm and the 2nd absorbance value A2, was recorded after 1 min. The final absorbance value was given by ΔA = A1−A2. For PPO, the wavelength was adjusted to 525 nm, and that of distilled water was adjusted to zero. The absorbance of the determination tube and reference tube was detected at 525 nm. The final absorbance value was given by ΔA = A measured-A control. For SOD, the absorbance was measured at 560 nm. The final absorbance value was given by the formula, Inhibition percentage = (A control tube-control tube) ÷ A control tube × 100%.

Data Analysis

(1) The mortality rate (%) was calculated as the ratio of the number of *O. asiaticus* deaths in each frame to the number of *O. asiaticus* in the initial 4th instar (N = 20). (2) Weight gain (increased body dry mass, mg) = dry weight of *O. asiaticus* after 7 days (mg) − 4th instar *O. asiaticus* weight (mg). (3) Increase in body length (mm) = 7 days later *O. asiaticus* length (mm) − the length of the first 4th instar *O. asiaticus* (mm). (4) The developmental period was the experimental period of 7 days. (5) Growth rate (mg/day) = weight gain ÷ developmental duration. (6) Overall performance = survival rate × growth rate (Cease et al., 2012; Cui et al., 2019).

The activities of the enzymes were determined using the following formulae;Paragraph text.

$$\begin{aligned}\mathrm{POD}\left(\mathrm{U}/\mathrm{g}_{\text{fresh weight}}\right) &= \Delta A \times \mathrm{V}_{\text{total reaction}} / \\ &\quad \left(500 \times \mathrm{V}_{\text{sample}} / \mathrm{V}_{\text{sample total}}\right) / 0.005 / \mathrm{T} \\ &= 4000 \times \Delta A / \mathrm{W} \end{aligned} \quad (1)$$

$$\begin{aligned}\mathrm{CAT}\left(\mathrm{U}/\mathrm{g}_{\text{fresh weight}}\right) &= \left[\Delta A \times \mathrm{V}_{\text{total reaction}} / \left(\mu \times \mathrm{d}\right) \times 109\right] / \\ &\quad \left(\mathrm{W} \times \mathrm{V}_{\text{sample}} / \mathrm{V}_{\text{sample total}}\right) / \mathrm{T} \\ &= 918 \times \text{”}\ \mathrm{A} / \mathrm{W} \end{aligned} \quad (2)$$

$$\begin{aligned}\mathrm{PPO}\left(\mathrm{U}/\mathrm{g}_{\text{fresh weight}}\right) &= \Delta A \times \mathrm{V}_{\text{total reaction}} / \\ &\quad \left(\mathrm{W} \times \mathrm{V}_{\text{sample}} / \mathrm{V}_{\text{sample total}}\right) / 0.005 / \mathrm{T} \\ &= 120 \times \Delta A / \mathrm{W} \end{aligned} \quad (3)$$

$$\begin{aligned}&\text{SOD activity} \\ &\left(\mathrm{U}/\mathrm{g}_{\text{fresh weight}}\right) = \begin{bmatrix}\text{inhibition percentage} \div \\ \left(1 - \text{inhibition percentage}\right) \times \\ \mathrm{V}_{\text{total}}\end{bmatrix} \\ &\quad \div \left(\mathrm{W} \times \mathrm{V}_{\text{sample}} \div \mathrm{V}_{\text{sample total}}\right) \\ &\quad = 11.11 \times \text{inhibition percentage} \div \\ &\quad \left(1 - \text{inhibition percentage}\right) \div \mathrm{W} \end{aligned} \quad (4)$$

($V_{total\ reaction}$: total volume of reaction; V_{sample}: volume of added sample; $V_{sample\ total}$: volume of added extract; T: reaction time; W: sample mass; ε: molar extinction coefficient of H_2O_2, 4.36 × 104 L/mol/cm)

The experimental data were all analyzed using Excel 2010, the means were evaluated *via* the one-way ANOVAs at the 5% significance level, and differences among means were compared by using the Turkey at $p < 0.05$ (Jia et al., 2016). And Prism6 was used for making the figure, and the error bars is the SE.

RESULTS

Effects of the 10% *A. Sieversiana* (10% AS) Extract, Different Concentrations of *M. anisopliae* and Their Combinations on the Growth and Development of *O. asiatica*

Effect on Weight

The 10% crude extract of *A. sieversiana* had significant inhibitory effect on the body weight of female (**Figure 1A**) and male 4th instar (**Figure 1B**) *O. asiatica*. However, the different concentrations (10^6, 10^7, and 10^8 spores/g) of *M. anisopliae* promoted weight gain in female (**Figure 1A**) and male 4th instar (**Figure 1B**) *O. asiatica*. Among them, the 10^7 and 10^8 spores/g *M. anisopliae* concentrations had the strongest effect on the weight gain of female (29.5 ± 1.63 g) and male *O. asiatica* (26.9 ± 2.00 g), respectively. The combined treatment of 10% crude extract of *A. sieversiana* and each of the different concentration of *M. anisopliae*, had a significant inhibitory effect on the weight gain of *O. asiatica*. Among them, the combination of 10% *A. sieversiana* crude extract + 10^7 spores/g of *M. anisopliae* had the most significant inhibitory effect on the weight gain of the female (**Figure 1A**) and male 4th instar (**Figure 1B**) *O. asiatica* ($F = 233.829$, DF = 7, $p = 0$), with the weight gain of 129.73 ± 1.97 g and −7.2 ± 1.07, respectively.

Effects on Body Length

The 10% crude extract of *A. sieversiana* and *M. anisopliae* alone or in combination had effects on the body length of female (**Figure 2A**) and male (**Figure 2B**) *O. asiatica*. The 10% crude extract of *A. sieversiana* had an inhibitory effect on the growth of the body length of the *O. asiatica*. The different concentration (10^6, 10^7, and 10^8 spores/g) of *M. anisopliae* all increased the body length of the *O. asiatica*, of which the 10^8 spores/g *M. anisopliae* concentration had the most obvious effect on male (10.78 ± 0.36 mm) and female (9.28 ± 0.31 mm) *O. asiatica*. The combined treatment of 10% crude extract of *A. sieversiana* and the different concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) had an inhibitory effect on the body length of *O. asiatica*. The combined treatment of 10% of *A. sieversiana* crude extract + 10^8 spores/g of *M. anisopliae*, had the most significant inhibitory effect on the increase in body length of *O. asiatica* (Female: 3.66 ± 0.23 mm; male: 2.87 ± 0.18 mm).

Effects on Growth Rate

The 10% crude extract of *A. sieversiana* and *M. anisopliae* alone or in combination had effects on the growth rate of female (**Figure 3A**) and male (**Figure 3B**) *O. asiatica*. The growth rate of *O. asiatica* was significantly inhibited by the 10% crude extract of *A. sieversiana*, However, all the concentrations of *M. anisopliae* (10^6,10^7, and 10^8 spores/g) promoted the growth rate of female and male grasshoppers. For female, the 10^7 spores/g concentration had the highest effect on growth rate. For male, the 10^8 spores/g concentration had the highest effect. The results showed that the combination of the 10% crude extract of *A. sieversiana* and the different concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) had significant inhibitory effect on the growth rate of *O. asiatica*. The combination of 10% crude extract + 10^8 spores/g *M. anisopliae* and 10% crude extract + 10^7 spores/g *M. anisopliae* had the most significant inhibitory effect on the growth rate of female and male *O. asiatica*, respectively.

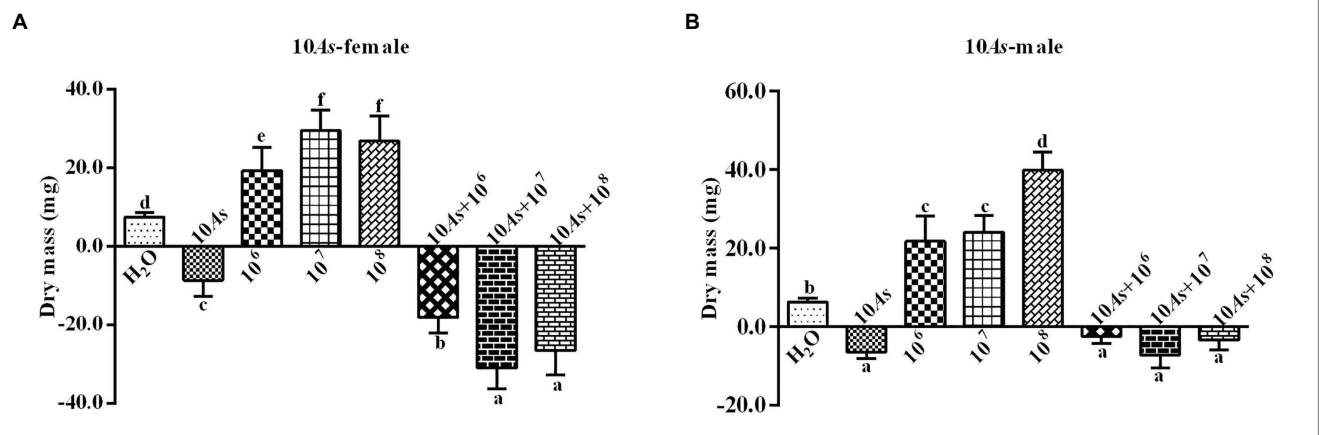

FIGURE 1 | Body weight gain and loss of **(A)** female and **(B)** male 4th instar *Oedaleus asiatica* from treatments with 10% *A. sieversiana* (10% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the growth rate of the *O. asiatica* in the same year.

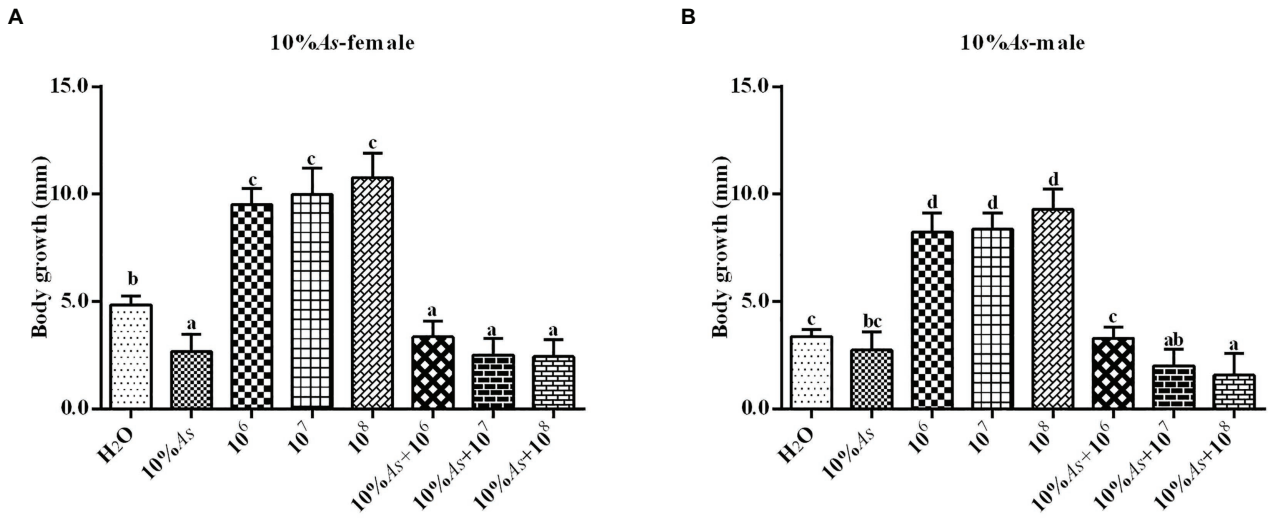

FIGURE 2 | Increase in body length of the **(A)** female and **(B)** male 4th instar *O. asiatica* from the treatment with 10% *A. sieversiana* (10% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the growth rate of the *O. asiatica* in the same year.

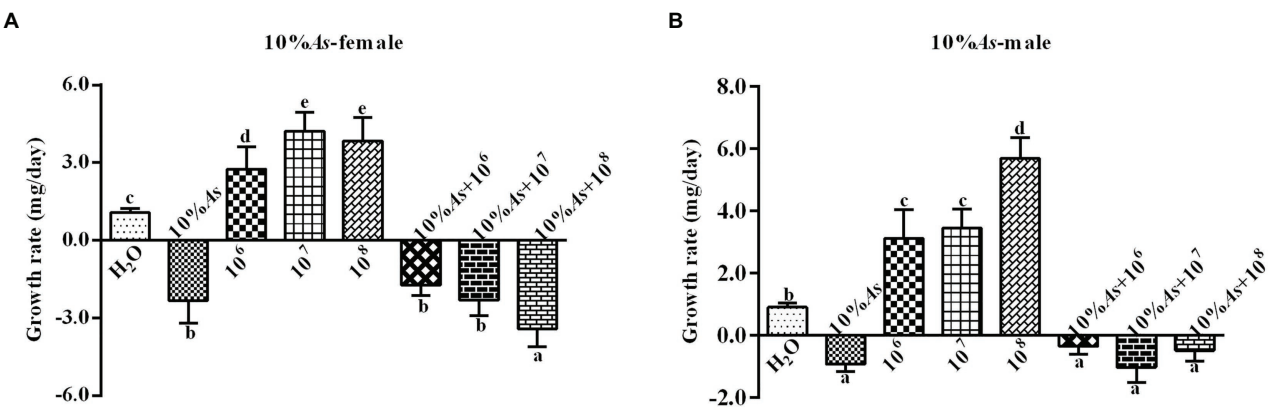

FIGURE 3 | Growth rates of **(A)** female and **(B)** male 4th instar *O. asiatica* from the treatments with 10% *A. sieversiana* (10% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the growth rate of the *O. asiatica* in the same year.

Effects on Overall Performance

The 10% *A. sieversiana* crude extract and *M. anisopliae* alone or in combination had effect on the overall performance of female (**Figure 4A**) and male (**Figure 4B**) *O. asiatica*. The 10% crude extract of *A. sieversiana* had an extremely significant effect on the overall performance. All concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) promoted the growth and development of *O. asiatica*. Among them, the 10^7 spores/g *M. anisopliae* concentration had the most significant effect on the growth and development of male (1.13 ± 0.37) and female (0.96 ± 0.08) *O. asiatica*. The combined treatment of 10% crude extract of *A. sieversiana* + each of the concentration of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g), had an effection on the growth of *O. asiatica*. The combined treatment of 10% *A. sieversiana* crude extract + 10^6 spores/g *M. anisopliae* and 10% crude extract of *A. sieversiana* + 10^7 spores/g *M. anisopliae* had the best inhibition on the overall performance of female *O. asiatica* (−0.95 ± 0.15) and male *O. asiatica* (−0.21 ± 0.07), respectively.

Effects of the 20% *A. sieversiana* (20% AS), Different Concentrations of *M. anisopliae* and Their Combinations on the Growth and Development of *O. asiatica*

Effects on Body Weight

The 20% crude extract of *A. sieversiana* had a significant inhibitory effect on the body weight of female (**Figure 5A**) and male (**Figure 5B**) 4th instar *O. asiatica*. However, the different concentrations of *M. anisopliae* (10^6, 10^7, and

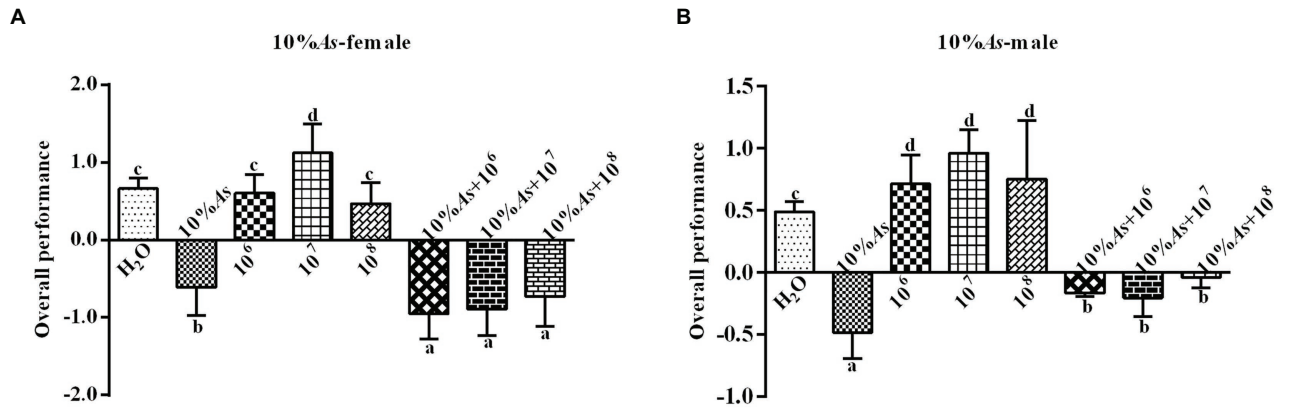

FIGURE 4 | Overall performance of **(A)** female and **(B)** male 4th instar *O. asiatica* from the treatments with 10% *A. sieversiana* (20% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the overall performance of the *O. asiatica* in the same year.

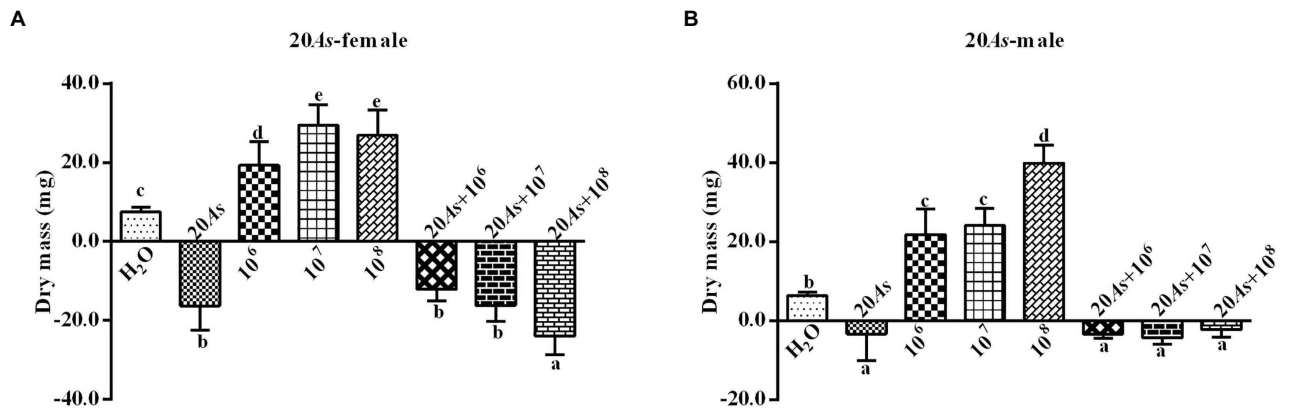

FIGURE 5 | Weight gain and loss of the **(A)** female and **(B)** male 4th instar *O. asiatica* from the treatments with 20% *A. sieversiana* (20% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the weight gain of the *O. asiatica* in the same year.

10^8 spores/g) promoted weight gain in the female and male *O. asiatica*. Among them, the 10^7 spores/g *M. anisopliae* concentration had the strongest effect on the weight gain of female *O. asiatica* (19.3 ± 1.88 mg). The combined treatment of 20% *A. sieversiana* crude extract and the different concentrations of *M. anisopliae* (10^6, 10^7, 10^8 spores/g) had a significant inhibitory effect on the weight gain of the *O. asiatica*. Among them, the combined treatment of 20% crude extract of *A. sieversiana* + 10^8 spores/g of *M. anisopliae* and 20% *A. sieversiana* crude extract + 10^7 spores/g of *M. anisopliae* had the strongest inhibitory effect on the weight gain of the male (−23.9 ± 1.05 mg) and female (−4.3 ± 0.52 mg) *O. asiatica*, respectively.

Effects on Body Length

The 20% crude extract of *A. sieversiana* and *M. anisopliae* alone or in combination had effect on the body length of the female (**Figure 6A**) and male (**Figure 6B**) *O. asiatica*. The 20% crude extract of *A. sieversiana* had a relatively inhibitory effect on the body length of the *O. asiatica*. The different concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) increased the body length of *O. asiatica*. Among them, the 10^8 spores/g *M. anisopliae* concentration had the most significant effect on the body length. The combined treatment of 20% crude extract of *A. sieversiana* and different concentrations of *M. anisopliae* (10^6, 10^7, and 10^8 spores/g) had an inhibitory effect on the body length of female and male *O. asiatica*. The combined treatment of 20% crude extract of *A. sieversiana* + 10^8 spores/g *M. anisopliae* had the most significant inhibitory effect on the body length of *O. asiatica* (female 2.44 ± 2.52 mm; male: 1.58 ± 0.32 mm).

Effects on Growth Rate

The 20% *A. sieversiana* crude extract and *M. anisopliae* alone or in combination had effects on the growth rate of female

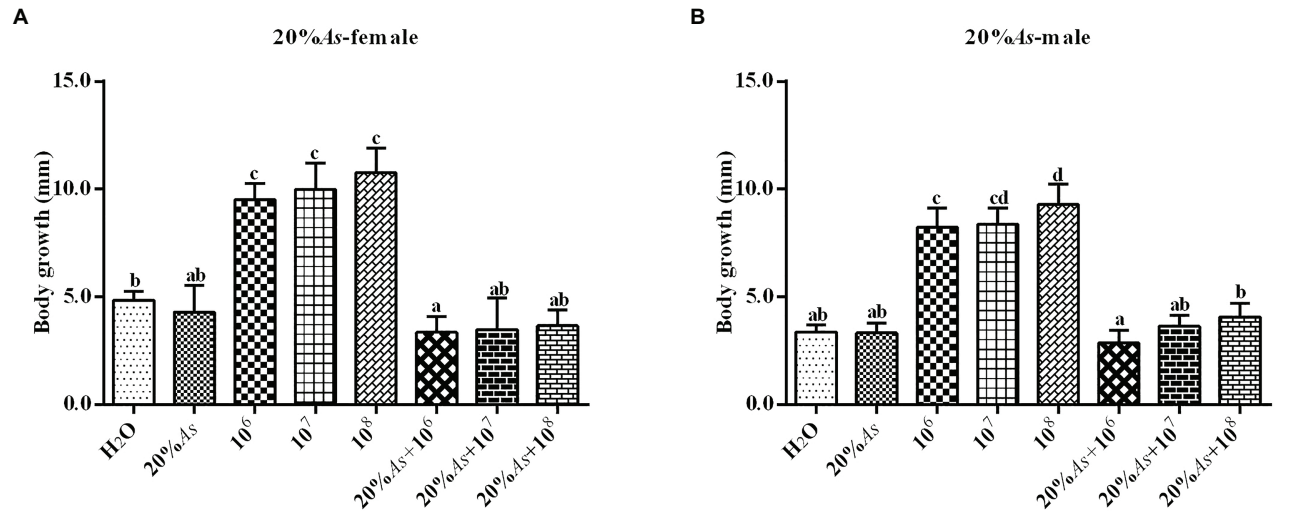

FIGURE 6 | Increase in body length of **(A)** female and **(B)** male 4th instar *O. asiatica* from treatments with 20% *A. sieversiana* (20% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the body length of the *O. asiatica* in the same year.

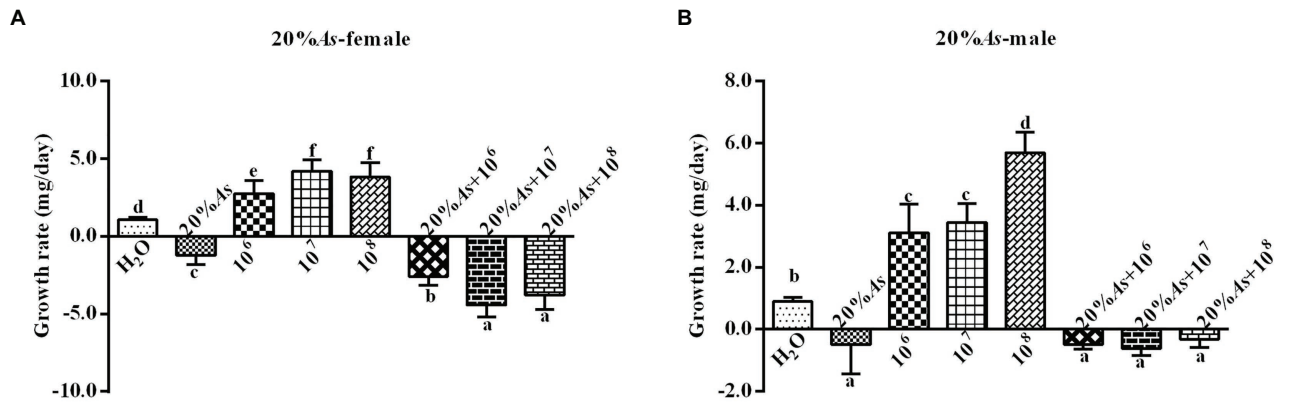

FIGURE 7 | Growth rates of **(A)** female and **(B)** male 4th instar *O. asiatica*, from treatments with 20% *A. sieversiana* (20% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the growth rate of the *O. asiatica* in the same year.

(**Figure 7A**) and male (**Figure 7B**) *O. asiatica*. The 20% crude extract of *A. sieversiana* had inhibitory effects on the growth rates of *O. asiatica*. The results also showed that the concentration of 10^7 spores/g *M. anisopliae* and 10^8 spores/g of *M. anisopliae* had a significant effect on the growth rate of female ($F = 185.11$, DF = 7, $p = 0$), and male ($F = 161.59$, DF = 7, $p = 0$) *O. asiatica*. The combination treatment of 20% crude extract + 10^8 spores/g *M. anisopliae* had the most significant inhibitory effect on the growth rate of female (−3.41 ± 0.22 mg/d) and combination treatment of 20% crude extract + 10^7 spores/g *M. anisopliae* had the most significant inhibitory effect on the growth rate of male (−0.61 ± −0.78 mg/d) *O. asiatica*.

Effects on Overall Performance

The 20% *A. sieversiana* crude extract and *M. anisopliae* alone or in combination had effects on the overall performance of female (**Figure 8A**) and male (**Figure 8B**) *O. asiatica*. The 20% crude extract of *A. sieversiana* had an inhibition on the overall performance of female (−0.93 ± 0.10) and male (−0.44 ± 0.03). The 10^7 spores/g *M. anisopliae* concentration had a promoting effect on the growth and development of female (1.13 ± 0.16) and male (0.96 ± 0.08) *O. asiatica* compared to the control. The combination of the 20% *A. sieversiana* crude extract and different concentration of *M. anisopliae* had a significant inhibitory effect on the overall performance of *O. asiatica*.

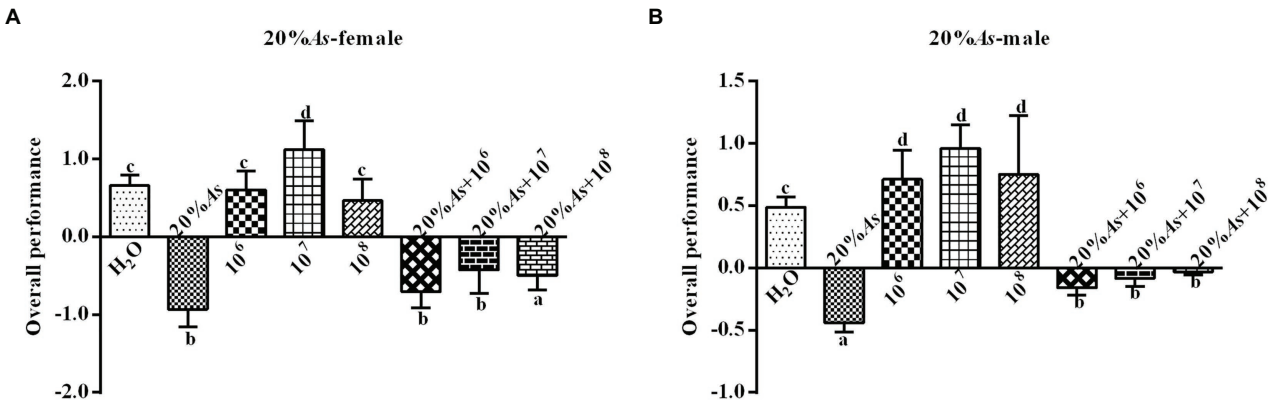

FIGURE 8 | Overall performance of **(A)** female and **(B)** male 4th instar *O. asiatica* from treatments with 20% *A. sieversiana* (20% As) crude extract, different concentrations of *M. anisopliae* and their combinations. Different lowercase letters indicate significant differences in the overall performance of the *O. asiatica* in the same year.

Among them, the combination of 20% *A. sieversiana* crude extract + 10^6 spores/g had the strongest inhibition on the overall performance of female (−0.71 ± 0.09; **Figure 8A**) and male (−0.16 ± 0.03) *O. asiatica* (**Figure 8B**).

Effect of Crude Extracts of *A. sieversiana*, Different Concentrations of *M. anisopliae* and Their Combinations on the Mortality of *O. asiatica*

When *A. sieversiana* was used alone, the lethal effect of 20% crude extract was greater than that of 10% crude extract. The lethal effect of the different concentrations of *M. anisopliae* on *O. asiatica* followed a decreasing order as follows: 10^8 spores/g > 10^6 spores/g > 10^7 spores/g, but there was no significant difference between the 10^6 and 10^7 spores/g concentration. The lethal effect of combined treatment on *O. asiatica* also followed a decreasing order as follows: 20% As + 10^8 spores/g *M. anisopliae* > 20% As + 10^7 spores/g *M. anisopliae* > 10% As + 10^7 spores/g *M. anisopliae* > 10% As + 10^6 spores/g *M. anisopliae* > 20% As + 10^6 spores/g *M. anisopliae*. Among them, 10^8 spores/g *M. anisopliae* had the best efficacy, resulting into a mortality rate of 85 ± 5.08%. The results showed that the combination of 10% crude extract of *A. sieversiana* + 10^7 and 10^8 spores/g *M. anisopliae*, the combination of 20% crude extract + 10^7 and 10^8 spores/g *M. anisopliae* had significantly better control effect on *O. asiatica* than that of using only crude extract of *A. sieversiana* ($F = 14.23$, DF = 11, $p = 0$; **Figure 9**).

Effect of Crude Extracts of *A. sieversiana*, Different Concentrations of *M. anisopliae* and Their Combinations on the Activities of Antioxidant Enzymes in *O. asiatica*

There was no significant difference in the lethal effect of the 20% As + 10^7 spores/g *M. anisopliae* and 10% As + 10^7 spores/g *M. anisopliae* on *O. asiatica* (**Figure 9**). Likewise the effect of

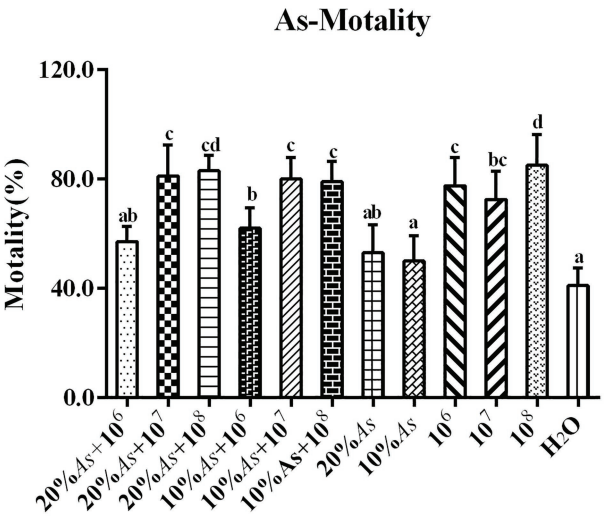

FIGURE 9 | Mortalities of *O. asiatica* from treatments with 10 and 20% crude extract *A. sieversiana* and *M. anisopliae* alone or in combination. Different lowercase letters indicate significant differences in the mortality of the *O. asiatica* in the same year.

the increase in concentration of *A. sieversiana* extracts, on the mortality of *O. asiatica* was not significantly different. Therefore, in this experiment, we tested the activities of antioxidant enzymes in *O. asiatica* from the following treatments: 10% *A. sieversiana* extract + 10^7 spores/g *M. anisopliae*, 10% *A. sieversiana* extract alone, 10^7 spores/g *M. anisopliae* alone, and distilled water (control). The activities of catalase (CAT; 414.94 ± 16.94 U/g) and polyphenol oxidase (PPO; 88.20 ± 5.49 U/g) were significantly increased by the 10% *A. sieversiana* extract + 10^7 spores/g *M. anisopliae* (**Figure 10**). The activities of SOD and POD were also activated by this treatment. The highest activity of SOD (2564.10 ± 218.08 U/g) was recorded under the 10^7 spores/g *M. anisopliae* treatment,

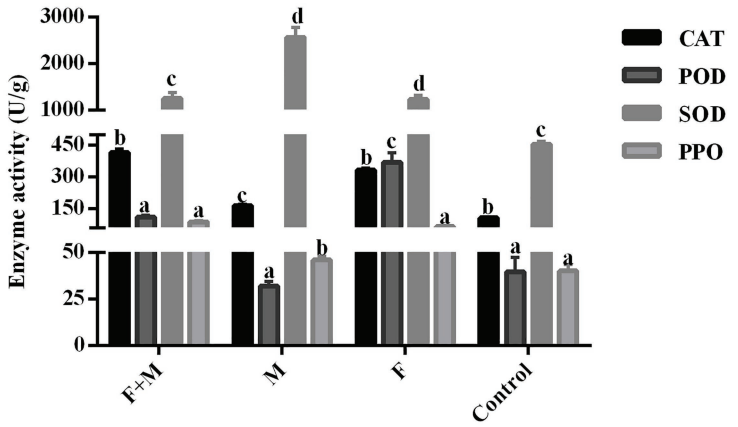

FIGURE 10 | Activities of antioxidant enzymes in *O. asiatica* from the treatment with 10% crude extract *A. sieversiana* and *M. anisopliae* alone or their combined use. 1. F, *M. anisopliae* alone; M, crude extract of 10% *A. sieversiana* alone; F+M, combination of *M. anisopliae* and the 10% crude extract of *A. sieversiana*; Control, distilled water; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; PPO, polyphenol oxidase.

and that of POD (368.40 ± 47.45 U/g) was recorded under the 10% *A. sieversiana* extract treatment (**Figure 10**).

DISCUSSION

The frequent large-scale outbreaks of *O. asiatica* in the grasslands of northern China, are partly responsible for the recent degradation of grasslands and desertification in Inner Mongolia in China. The entomopathogenic fungus *M. anisopliae*, a recognized non-polluting and non-toxic pesticide, has been widely studied for pest control and has shown potential as a chemical substitute for locust and grasshopper control (Gillespie et al., 2000; Peng et al., 2008). However, insect pathogenic fungi have shortcomings such as a longer time of attacking pest and unstable control effect (Pilz et al., 2009). In addition, external conditions such as temperature, humidity, density, and height of vegetation also affect the control efficacy of *M. anisopliae* on locust. In dense vegetation areas, the reduced infectivity of *M. anisopliae* conidia on the vegetation surface also reduces its efficacy (Hunter et al., 2001).

Therefore, the use of appropriate adjuvants can increase the persistence of fungi. Many entomopathogenic fungi are compatible with a range of chemical and natural insecticides (Santos et al., 2007; Pelizza et al., 2015). One possible candidate is crude plant extracts. For example, the combination of neem oil and *M. anisopliae* significantly increased the mortalities of *A. aegypti* and locusts. By this, the efficacy of this biological control agent was increased (Haroona et al., 2011; Paula et al., 2019). However, neem oil inhibited the vegetative growth of Beauveria bassiana and reduced the yield and viability of conidia (Depieri et al., 2005).

The secondary metabolites of Artemisia plants include terpenoids, flavonoids, coumarins, glycosides, steroids, and polyacetylenes (Tan et al., 1998). The crude extract of *A. sieversiana* was shown to have insecticidal activity (Liu et al., 2010). In this regard, we also found that the treatment with *A. sieversiana* alone significantly reduced the mortality of *O. asiatica*. Correspondingly, it caused a series of immune and physiological responses in the host insect after *M. anisopliae* infected the host (Wang et al., 2013; Ling et al., 2016). Therefore, the high concentration of conidia may enhance host fungal infections and cause high mortality in a relatively short time (Kirkland et al., 2004; Shrestha et al., 2015). This study also indirectly confirmed that a higher concentration of *M. anisopliae* enhances its lethality.

Fungal insecticides have good potential for pest control, as there is currently no record of development of insect resistance against them (Behie and Bidochka, 2013; Erler and Ates, 2015). In order to reduce the risk of resistance, the use of fungal agents should be minimized. The efficacy of a combination of fungi and chemicals was shown to be higher under laboratory or field conditions, and has been reported in several studies involving *Anomala cuprea* Hope and *Locusta migratoria* (Hiromori and Nishigaki, 1998; Jia et al., 2016). Although there was no significant difference between the mortality of *O. asiatica* treated with 20% crude extract of *A. sieversiana* combined with 10^8 spores/g *M. anisopliae* and 10^8 spores/g *M. anisopliae* alone in this study, the overall performance of *O. asiatica* was decreased. This indicates that the efficacy of the crude extract of *A. sieversiana* was significantly improved.

The death time of pest is an important factor affecting the control efficacy of insecticides for pest management (Asi et al., 2008; Gupta et al., 2009). The results of a field application of 6×10^7 spore *M. anisopliae* bait on locusts, showed that the peak infection of the locust population was reached on the 15th day after infection, with a mortality rate of 6.5%, and the mortality rate increased with time (Dong et al., 2011). Compared to this experiment, the mortality rate was much lower than that in the treatment with crude extract of *A. sieversiana* and *M. anisopliae*, and the peak time of infection was three times longer than that under the mixture of crude extract of *A. sieversiana* and *M. anisopliae*. The results showed that treatment with the combination of the crude extract of *A. sieversiana* and *M. anisopliae* significantly shortened the

lethal time, and its efficacy was significantly better than that of other groups and any single agent. This shows that the synergetic control effect from the combination treatment was faster and better than the control effect of each pesticide alone. The effect of 10% *A. sieversiana* crude extract mixed with 10^7 spores/g *M. anisopliae* on the mortality of *O. asiatica* reached the effect of 10^8 spores/g *M. anisopliae* alone in this study; therefore, the dosage of *M. anisopliae* was reduced, and may largely prevent the development of resistance in insects against it.

Although it has not been reported that insects resistance to *M. anisopliae* (Dubovskiy et al., 2013), the risk of resistance to *M. anisopliae* can be reduced by observing the effects of *A. sieversiana* and *M. anisopliae* on the protective enzyme activities of *O. asiatica*. We found that the enzymatic activities of catalase and polyphenol oxidase of *O. asiatica* were over-expressed under the mixed treatment of the combination of the crude extract of *A. sieversiana* and *M. anisopliae*. And some studies have found that the high expression of catalase could shorten the germination time and increase the pathogenicity. of *M. anisopliae* (Hernandez et al., 2010). Therefore, we found that the crude extract of *A. sieversiana* can be used as a synergist of *M. anisopliae* to improve its pathogenicity. Under the conditions of external factors, such as plants and biological pesticides, the slower growth and development of insects are often associated with the expression of enzyme activity in their bodies (Serebrov et al., 2006). Our results also confirmed the conclusion that the mortality, body weight, growth rate, and overall performance of *O. asiatica* were significantly reduced under the treatment with crude extract of *A. sieversiana* and *M. anisopliae*, while the activity of its protective enzymes was significantly increased.

The use of *M. anisopliae* in combination with chemical agents have caused varied mortality rates of locusts, and even showed antagonistic effect (Jia et al., 2016). In our research, similar results of different concentration ratios causing varied mortality of *O. asiatica* were recorded. Therefore, in the considerations for the use of a combination of new biopesticides and fungal preparations for pest control, the compatibility of the two should be carefully evaluated. A number of studies have comprehensively evaluated the compatibility of fungi and chemicals agents under laboratory and field conditions (Cuthbertson et al., 2005; Asi et al., 2010).

We found an interesting phenomenon in this study. *Metarhizium anisopliae* can promote the increase of body weight and body length of *O. asiatica*. However, when *M. anisopliae* was mixed with the crude extract of *A. sieversiana*, it inhibited the growth of body weight and length, especially the growth of body weight, and the effect of the mixture of *M. anisopliae* and the crude extract of *A. sieversiana* on the development of female was greater than that of male. However, the reasons for this phenomenon need to be further studied. Our work was carried out under laboratory conditions. We, therefore, propose that further studies under field conditions characterized by many complicated and uncontrollable factors, be carried out to determine the best compound formula which shows promising efficacy. Additionally, other parameters such as the toxicity of fungi, the influence of the mixture of crude extract of *A. sieversiana* and *M. anisopliae* on natural enemies and non-target organisms, etc. should be considered in the evaluation of the combination of fungi and new biopesticides.

In summary, our research showed that *M. anisopliae* was a good biological control agent for on *O. asiatica*. The crude extract of *A. sieversiana* increased the virulence of *M. anisopliae* and shortened the mortality time of the pest. These results indicate that the crude extract of *A. sieversiana* could be used as a synergist for *M. anisopliae*, and this compound formulation can become an alternative biological control agent to control insect pest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

ZZ and XT designed the experiments. SL and XT performed the experiments. SL and ZZ wrote the manuscript. SL, CX, and GD collected the data and analyzed it. ZZ and GW provided technical and material support. All authors contributed to the article and approved the submitted version.

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REFERENCES

Asi, M. R., Afzal, M., Anwar, S. A., and Bashir, M. H. (2008). Comparative efficacy of insecticides against sucking insect pests of cotton. *Pakistan J. Life Soc. Sci.* 15, 631–638.

Asi, M. R., Bashir, M. H., Afzal, M., Ashfaq, M., and Sahi, S. T. (2010). Compatibility of entomopathogenic fungi, *Metarhizium anisopliae* and Paecilomyces fumosoroseus with selective insecticides. *Pak. J. Bot.* 42, 4207–4214.

Ayub, M., Hussain, S., Rasool, A., Hussain, I., Khan, F., and Sultan, A. (2019). Insecticidal activity of *Artemisia sieversiana* Ehrh. plant extract against white grub (Scarabaeidae: Coleoptera). *J. Entomo. Zool. Studies* 7, 304–308.

Behie, S. W., and Bidochka, M. J. (2013). Potential agricultural benefits through biotechnological manipulation of plant fungal associations. *Bioessays* 35, 328–331. doi: 10.1002/bies.201200147

Bi, J. L., and Felton, G. W. (1995). Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as

components of induced resistance. *J. Chem. Ecol.* 21, 1511–1530. doi: 10.1007/BF02035149

Blanford, S., Chan, B. H. K., Jenkins, N., Sim, D., Turner, R. J., Read, A. F., et al. (2005). Fungal pathogen reduces potential for malaria transmission. *Science* 308, 1638–1641. doi: 10.1126/science.1108423

Cease, A. J., Elser, J. J., Ford, C. F., Hao, S. G., Kang, L., and Harrison, J. F. (2012). Heavy livestock grazing promotes locust outbreaks by lowering plant nitrogen content. *Science* 335, 467–469. doi: 10.1126/science.1214433

Cui, B. Y., Huang, X. b., Li, S., Hao, K., Chang, B. H., Tu, X. B., et al. (2019). Quercetin affects the growth and development of the grasshopper *Oedaleus asiaticus* (Orthoptera: Acrididae). *J. Econ. Entomol.* 112, 1175–1182. doi: 10.1093/jee/toz050

Cui, J., Nuermaimaiti, M., Zou, G., and Xin, X. (2020). Chemical composition of *Artemisia sieversiana*. *Chem. Nat. Compd.* 56, 1120–1121. doi: 10.1007/s10600-020-03241-6

Cuthbertson, A. G., Walters, K. F., and Deppe, C. (2005). Compatibility of the entomopathogenic fungus *Lecanicillium muscarium* and insecticides for eradication of sweetpotato whitefly, *Bemisia tabaci*. *Mycopathologia* 160, 35–41. doi: 10.1007/s11046-005-6835-4

da Silva, J. A. T., Yonekura, L., Kaganda, J., Mookdasanit, J., Nhut, D. T., and Afach, G. (2005). Important secondary metabolites and essential oils of species within the anthemideae (Asteraceae). *Int. J. Geogr. Inf. Syst.* 11, 1–46. doi: 10.1300/J044v11n01_01

Depieri, R. A., Martinez, S. S., and Menezes, J. (2005). Compatibility of the fungus Beauveria bassiana (Bals.) Vuill. (Deuteromycetes) with extracts of neem seeds and leaves and the emulsible oil. *Neotrop. Entomol.* 34, 601–606. doi: 10.1590/S1519-566X2005000400010

Dimbi, S., Maniania, N. K., and Ekesi, S. (2009). Effect of *Metarhizium anisopliae* inoculation on the mating behavior of three species of African Tephritid fruit flies, *Ceratitis capitata*, *Ceratitis cosyra* and *Ceratitis fasciventris*. *Biol. Control* 50, 111–116. doi: 10.1016/j.biocontrol.2009.04.006

Dong, H., Gao, S., Nong, X. Q., Cong, B., Zhang, Z. H., and Sun, D. Y. (2011). The prevailing of *Metarhizium anisopliae* in rangeland and its interaction with parasitic flies for biological control of grasshoppers. *Chin. J. Biol. Control* 2011, 52–56.

Duarte, R. T., Goncalves, K. C., Espinosa, D. J., Moreira, L. F., De Bortoli, S. A., Humber, R. A., et al. (2016). Potential of entomopathogenic fungi as biological control agents of Diamondback Moth (Lepidoptera: Plutellidae) and compatibility with chemical insecticides. *J. Econ. Entomol.* 109, 594–601. doi: 10.1093/jee/tow008

Dubey, N. K., Shukla, R., Kumar, A., Singh, P., and Prakash, B. (2010). Prospects of botanical pesticides in sustainable agriculture. *Curr. Sci.* 98, 479–480. doi: 10.1126/science.1183962

Dubovskiy, I. M., Whitten, M. M. A., Yaroslavtseva, O. N., Greig, C., Kryukov, V. Y., Grizanova, E. V., et al. (2013). Can insects develop resistance to insect pathogenic fungi? *PLoS One* 8:e60248. doi: 10.1371/journal.pone.0060248

Erler, F., and Ates, A. O. (2015). Potential of two entomopathogenic fungi, Beauveria bassiana and *Metarhizium anisopliae* (Coleoptera: scarabaeidae), as biological control agents against the June beetle. *J. Insect Sci.* 15:44. doi: 10.1093/jisesa/iev029

Farenhorst, M., Knols, B. G. J., Thomas, M. B., Howard, A. F. V., Takken, W., Rowland, M., et al. (2010). Synergy in efficacy of fungal entomopathogens and permethrin against west african insecticide-resistant anopheles gambiae mosquitoes. *PLoS One* 5:e12081. doi: 10.1371/journal.pone.0012081

Gibson, D. M., Donzelli, B. G., Krasnoff, S. B., and Keyhani, N. O. (2014). Discovering the secondary metabolite potential encoded within entomopathogenic fungi. *Nat. Prod. Rep.* 31, 1287–1305. doi: 10.1039/C4NP00054D

Gillespie, J. P., Burnett, C., and Charnley, A. K. (2000). The immune response of the desert locust Schistocerca gregaria during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var acridum. *J. Insect Physiol.* 46, 429–437. doi: 10.1016/S0022-1910(99)00128-6

Gupta, S., Sharma, R. K., Gupta, R. K., Sinha, S. R., Singh, R., and Gajbhiye, V. T. (2009). Persistence of new insecticides and their efficacy against insect pests of okra. *Bull. Environ. Contam. Toxicol.* 82, 243–247. doi: 10.1007/s00128-008-9581-8

Haroona, W. M., Pagesb, C., Vassalb, J. M., Abdallaa, A. M., Luong-Skovmandb, M., and Lecoq, M. (2011). Laboratory and field investigation of a mixture of Metarhizium acridum and neem seed oil against the tree locust Anacridium melanorhodon melanorhodon (Orthoptera: Acrididae). *Biocontrol Sci. Tech.* 21, 353–366. doi: 10.1080/09583157.2010.550678

Hernandez, C. E. M., Guerrero, I. E. P., Hernandez, G. A. G., Solis, E. S., and Guzman, J. C. T. (2010). Catalase overexpression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*. *Appl. Microbiol. Biotechnol.* 87, 1033–1044. doi: 10.1007/s00253-010-2517-3

Hiromori, H., and Nishigaki, J. (1998). Joint action of an entomopathogenic fungus (Metarhizium anisopliae) with synthetic insecticides against the scarab beetle, Anomala cuprea (Coleoptera: Scarabaeidae) Iarvae. *Appl. Entomol. Zool.* 33, 77–84. doi: 10.1303/aez.33.77

Hunter, D. M., Milner, R. J., and Spurgin, P. A. (2001). Aerial treatment of the Australian plague locust, *Chortoicetes terinifera* (Orthoptera: Acrididae) with *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Bull. Entomol. Res.* 91, 93–99.

Jbilou, R., Ennabili, A., and Sayah, F. (2006). Insecticidal activity of four medicinal plant extracts against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Afr. J. Biotechnol.* 5, 936–940.

Jia, M., Cao, G., Li, Y., Tu, X. B., Wang, G. J., Nong, X. Q., et al. (2016). Biochemical basis of synergism between pathogenic fungus *Metarhizium anisopliae* and insecticide chlorantraniliprole in Locusta migratoria (Meyen). *Sci. Rep.* 6:28424. doi: 10.1038/srep28424

Kaaya, G. P., Samish, M., Hedimbi, M., Gindin, G., and Glazer, I. (2011). Control of tick populations by spraying *Metarhizium anisopliae* conidia on cattle under field conditions. *Exp. Appl. Acarol.* 55, 273–281. doi: 10.1007/s10493-011-9471-3

Kergoat, G. J., Meseguer, A. S., and Jousselin, E. (2017). Evolution of plant–insect interactions: insights from macroevolutionary approaches in plants and herbivorous insects. *Adv. Bot. Res.* 81, 25–53. doi: 10.1016/bs.abr.2016.09.005

Kirkland, B. H., Cho, E. M., and Keyhani, N. O. (2004). Differential susceptibility of *Amblyomma maculatum* and *Amblyomma americanum* (Acari: ixodidea) to the entomopathogenic fungi Beauveria bassiana and *Metarhizium anisopliae*. *Biol. Control* 31, 414–421. doi: 10.1016/j.biocontrol.2004.07.007

Ling, E., Cao, G. C., Jia, M., Zhao, X., Wang, L., Tu, X. B., et al. (2016). Different effects of *Metarhizium anisopliae* strains IMI330189 and IBC200614 on enzymes activities and hemocytes of Locusta migratoria. *PLoS One* 11:e0155257. doi: 10.1371/journal.pone.0167914

Liu, Z. L., Liu, Q. R., Chu, S. S., and Jiang, G. H. (2010). Insecticidal activity and chemical composition of the essential oils of *Artemisia lavandulaefolia* and *Artemisia sieversiana* from China. *Chem. Biodivers.* 7, 2040–2045. doi: 10.1002/cbdv.200900410

Mannino, M. C., Huarte-Bonnet, C., Davyt-Colo, B., and Nicolás, P. (2019). Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. *J. Fungi* 5:33. doi: 10.3390/jof5020033

Marican, A., and Durán-Lara, E. F. (2018). A review on pesticide removal through different processes. *Environ. Sci. Pollut. Res.* 25, 2051–2064. doi: 10.1007/s11356-017-0796-2

MeCoy, C. W. (1981). "Pest control by the fungus Hirsutell thompsonii//Burges" in *Microbial control of pests and plant disease 1970–1980*. eds. H. D. Burges and N. W. Hussey (London: Academic Press), 499–512.

Paula, A. R., Ribeiro, A., Lemos, F. J. A., Silva, C. P., and Samuels, R. I. (2019). Neem oil increases the persistence of the entomopathogenic fungus *Metarhizium anisopliae* for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. *Parasit. Vectors* 12:163. doi: 10.1186/s13071-019-3415-x

Pavela, R. (2016). History, presence and perspective of using plant extracts as commercial botanical insecticides and farm products for protection against insects–a review. *Plant Prot. Sci.* 52, 229–241. doi: 10.17221/31/2016-pps

Pelizza, S. A., Scorsetti, A. C., Fogel, M. N., Pacheco-Marino, S. G., Stenglein, S. A., Cabello, M. N., et al. (2015). Compatibility between entomopathogenic fungi and biorational insecticides in toxicity against *Ronderosia bergi* under laboratory conditions. *BioControl* 60, 81–91. doi: 10.1007/s10526-014-9606-7

Peng, G., Wang, Z., Yin, Y., Zeng, D. Y., and Xia, Y. X. (2008). Field trials of *Metarhizium anisopliae* var. acridum (Ascomycota: Hypocreales) against oriental migratory locusts, Locusta migratoria manilensis (Meyen) in Northern China. *Crop Prot.* 27, 1244–1250. doi: 10.1016/j.cropro.2008.03.007

Pilz, C., Keller, S., Kuhlmann, U., and Toepfer, S. (2009). Comparative efficacy assessment of fungi, nematodes and insecticides to control western corn rootworm larvae in maize. *BioControl* 54, 671–684. doi: 10.1007/s10526-009-9209-x

Rattan, R. S. (2010). Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Prot.* 29, 913–920. doi: 10.1016/j.cropro.2010.05.008

Sabarwal, A., Kumar, K., and Singh, R. P. (2018). Hazardous effects of chemical pesticides on human health-Cancer and other associated disorders. *Environ. Toxicol. Pharmacol.* 63, 103–114. doi: 10.1016/j.etap.2018.08.018

Sabbour, M. M., and Sahab, A. F. (2005). Efficacy of some microbial control agents against cabbage pests in Egypt. *Pak. J. Biol. Sci.* 8, 1351–1356. doi: 10.3923/pjbs.2005.1351.1356

Santos, A., Oliveira, B. L., and Samuels, R. I. (2007). Selection of entomopathogenic fungi for use in combination with sub-lethal doses of imidacloprid: perspectives for the control of the leaf-cutting ant *Atta sexdens* rubropilosa Forel (Hymenoptera: Formicidae). *Mycopathology* 163, 233–240. doi: 10.1007/s11046-007-9009-8

Serebrov, V. V., Gerber, O. N., Malyarchuk, A. A., Martemyanova, V. V., Alekseevc, A. A., and Glupova, V. V. (2006). Effect of Entomopathogenic fungi on detoxification enzyme activity in greater wax moth galleria mellonella L. (Lepidoptera, Pyralidae) and role of detoxification enzymes in development of insect resistance to Entomopathogenic Fungi. *Biol. Bull.* 33, 581–586. doi: 10.1134/S1062359006060082

Shah, F. A., Ansari, M. A., Prasad, M., and Butta, T. M. (2007). Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. *Biol. Control* 40, 246–252. doi: 10.1016/j.biocontrol.2006.10.005

Shang, F. J., Du, X. Q., and Liu, G. J. (1997). Resources and utilization of medicinal plants of Eupatorium in Northeast China. *For. By-Prod. Spec. China* 3:42.

Shrestha, G., Enkegaard, A., and Steenberg, T. (2015). Laboratory and semi-field evaluation of Beauveria bassiana (Ascomycota: Hypocreales) against the lettuce aphid, *Nasonovia ribisnigri* (Hemiptera: aphididae). *Biol. Control* 85, 37–45. doi: 10.1016/j.biocontrol.2015.03.005

Simmonds, M. S. J. (2001). Importance of flavonoids in insect-plant interactions: feeding and oviposition. *Phytochemistry* 56, 245–252. doi: 10.1016/S0031-9422(00)00453-2

Tan, R. X., Zheng, W. F., and Tang, H. Q. (1998). Biologically active substances from the genus *Artemisia*. *Planta Med.* 64, 295–302. doi: 10.1055/s-2006-957438

Tang, J., Zhao, J. J., and Li, Z. H. (2015). Ethanol extract of *Artemisia sieversiana* exhibits anticancer effects and induces apoptosis through a mitochondrial pathway involving DNA damage in COLO-205 colon carcinoma cells. *Bangladesh J. Pharmacol.* 10:518. doi: 10.3329/bjp.v10i3.23196

Vander, K. F., Verpoorte, R., and Meyer, J. J. M. (2008). Metabolomic quality control of claimed anti-malarial *Artemisia afra* herbal remedy and *A. afra* and *A. annua* plant extracts. *S. Afr. J. Bot.* 74, 186–189. doi: 10.1016/j.sajb.2007.10.004

Wang, Y., Yang, P., Cui, F., and Kang, L. (2013). Altered immunity in crowded locust reduced fungal (*Metarhizium anisopliae*) pathogenesis. *PLoS Pathog.* 9:e1003102. doi: 10.1371/journal.ppat.1003102

Wink, M. (1988). Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75, 225–233. doi: 10.1007/BF00303957

Wu, H. Q. (2015). Research progress on insecticidal effect of *Artemisia* in Compositae. *Bulletin Agric. Sci. Technol.* 5, 221–223.

Zhang, W. Z., He, B., Cao, G. C., Zhang, Z. H., Wu, Y. H., Liu, S. C., et al. (2013). Quantitative analysis of the effects of Stipa krylovii and *Leymus chinensis* on the factors of vitiality of Oedaleus decorus asiaticus. *Acta Pratacul. Sin.* 22, 302–309. doi: 10.13268/j.cnki.fbsic.1997.03.037

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Tech.* 17, 879–920. doi: 10.1080/09583150701593963

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Transcriptional Responses of *Beauveria bassiana* Blastospores Cultured Under Varying Glucose Concentrations

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Culturing the entomopathogenic fungus, *Beauveria bassiana*, under high glucose concentrations coupled with high aeration results in a fungal developmental shift from hyphal growth to mostly blastospores (yeast-like cells). The underlying molecular mechanisms involved in this shift remain elusive. A systematic transcriptome analysis of the differential gene expression was preformed to uncover the fungal transcriptomic response to osmotic and oxidative stresses associated with the resulting high blastospore yield. Differential gene expression was compared under moderate (10% w/v) and high (20% w/v) glucose concentrations daily for three days. The RNAseq-based transcriptomic results depicted a higher proportion of downregulated genes when the fungus was grown under 20% glucose than 10%. Additional experiments explored a broader glucose range (4, 8, 12, 16, 20% w/v) with phenotype assessment and qRT-PCR transcript abundance measurements of selected genes. Antioxidant, calcium transport, conidiation, and osmosensor-related genes were highly upregulated in higher glucose titers (16-20%) compared to growth in lower glucose (4-6%) concentrations. The class 1 hydrophobin gene (*Hyd1*) was highly expressed throughout the culturing. *Hyd1* is known to be involved in spore coat rodlet layer assembly, and indicates that blastospores or another cell type containing hydrophobin 1 is expressed in the haemocoel during the infection process. Furthermore, we found implications of the HOG signaling pathway with upregulation of homologous genes *Ssk2* and *Hog1* for all fermentation time points under hyperosmotic medium (20% glucose). These findings expand our knowledge of the molecular mechanisms behind blastospore development and may help facilitate large-scale industrial production of *B. bassiana* blastospores for pest control applications.

Keywords: biocontrol, dimorphic growth, morphogenesis, Cordycipitaceae, liquid fermentation

INTRODUCTION

The arthropod-pathogenic fungus *Beauveria bassiana* (Ascomycota: Cordycipitaceae) is globally used as a biological control agent against many crop pests and vectors of human diseases. Moreover, this fungus has been recently identified as an endophyte associated with various benefits including plant growth, drought resistance, and induced disease resistance (Mascarin et al., 2016; Tomilova et al., 2020). The current use of *B. bassiana*, as well as other filamentous (anamorphic phase) entomopathogenic fungi, as biopesticides have been primarily focused on the inundative biocontrol approach, *i.e.* large quantities of fungal inoculum are produced then deployed in the field to deliver a lethal dose to the target pest, aiming to achieve quick and effective pest suppression (Jaronski, 2010). Therefore, to meet the growing demands of high-quality large-scale production of fungal propagules for the biopesticide industry, liquid culture may be the preferred method over solid-state fermentation. Liquid culture production technology provides various advantages in terms of easiness, automated operation, scalability, quality control, batch-to-batch uniformity, and cost-effective, large-scale production afforded by deep-tank stirred bioreactors for some entomopathogenic fungi like *B. bassiana* (Jaronski, 2010; Mascarin et al., 2015a; Mascarin et al., 2015b; Mascarin et al., 2016).

As many Hypocrealean insect-pathogenic fungi, dimorphic growth in *B. bassiana* is ubiquitous. It renders unicellular vegetative, thin-walled, yeast-like cells termed blastospores, which can be easily mass-produced *in vitro* by submerged liquid fermentation (Pham et al., 2009; Mascarin et al., 2015a; Mascarin et al., 2015b). These *in vitro* yeast-like cells possess distinct biochemical properties and surface structures from hemolymph-derived hyphal bodies (Wanchoo et al., 2009; Yang et al., 2017). This cellular phenotype evolved to facilitate fungal infection in arthropod hosts by successfully allowing the fungus to bypass the host immune system while simultaneously colonizing and exploiting host tissues and resources leading to nutrient depletion, tissue degradation by enzymes, and synthesis of secondary metabolites (Humber, 2008). For pest control purposes, blastospores can be formulated as sprayable products and outperform in virulence to the traditional solid-grown conidia against various target pests because this cell type can germinate quicker and infect the host sooner (Cliquet and Jackson, 1999; Kirkland et al., 2004a; Kirkland et al., 2004b; Mascarin et al., 2015a; Mascarin et al., 2015b; Alkhaibari et al., 2016; Alkhaibari et al., 2018; Bernardo et al., 2018; Bernardo et al., 2020). Besides that, blastospores' virulence can be markedly affected by culture conditions and media composition against arthropod targets (Kirkland et al., 2004b; Mascarin et al., 2015a). Based on our previous studies, we have found a cost-effective technology for rapidly producing high yields of blastospores with low mycelium content by submerged liquid fermentation under high glucose titers and aeration rates (Mascarin et al., 2015a; Mascarin et al., 2015b; Mascarin et al., 2016). This phenomenon has intrigued us and has prompted investigation on the molecular mechanisms governing this improved phenotypic response during dimorphic growth, resulting in high production yields of blastospores in *B. bassiana* induced by greater glucose titer gradient coupled with high aeration supply.

Growth media for entomopathogenic fungi generally use up to 4% glucose as the carbon source to support conidia or blastospore production in solid or liquid media. A low carbon rate usually renders high mycelial growth and low blastospore yields (Mascarin et al., 2015a). However, our previous studies have shown proper C:N ratio coupled with high aeration rate and glucose titers induce higher blastospore yield with low hyphal growth in liquid culture of *B. bassiana* (Mascarin et al., 2015a; Mascarin et al., 2016). The mechanism behind this phenotype remains elusive but a plausible scenario falls under the hypothesis that oxidative and osmotic stress could be involved as part of the fungal response to such growth conditions.

Therefore, this study aimed at understanding the transcriptional signatures involved in blastospore formation and multiplication in response to the hyperosmolarity caused by a high gradient of glucose titer. This study also provides novel insights on osmotic and oxidative stress genes involved in the regulatory system of blastospore development in *B. bassiana* during submerged fermentation. In this regard, expression profiles of key genes were characterized across glucose gradients from 4% to 20%. This study reveals high osmotic pressure (> 0.5 MPa) induces improved blastospores' production in *B. bassiana* and other related insect-pathogenic fungi. Thus, this study may help improve the scale-up industrial production of blastospores in other filamentous entomopathogenic fungi, including those recalcitrant isolates which often provide low yields.

METHODS

Fungal Isolate and Culture Conditions

The *B. bassiana* strain GHA (ARSEF 201) selected for RNA sequencing was initially isolated from infected *Diabrotica undecimpunctata* in a greenhouse in Corvallis, Oregon, USA, in October of 1977. This strain is the active ingredient in several commercial mycoinsecticides. The isolate was cultured on potato dextrose agar (PDA) and preserved as a glycerol stock (10%) at -80 °C at the USDA, Peoria, IL. The culture conditions used for RNA-seq were selected because of high blastospore yields (Mascarin et al., 2015a; Mascarin et al., 2015b). The experiment consisted of seven treatments represented by an increased glucose gradient of 0, 4, 8, 10, 12, 16, and 20% glucose (w/v). Blastospore concentrations were recorded 24, 48, and 72 h post-inoculation of the fermentation course. Media composition consisted of 2.5% cottonseed flour (Pharmamedia®, ADM™, USA) as the nitrogen source, amended with basal salts and vitamin mix (Mascarin et al., 2015a). The cultures were placed on a rotary shaker incubator (New Brunswick™, Innova 4000®, NJ, U.S.A.) with rotating orbit diameter of 19 mm and agitation speed of 350 rpm, at 28°C, and a filling volume of 50 mL liquid medium in 250-mL baffled Erlenmeyer flasks to provide high aeration, as previously reported by (Mascarin et al., 2015a). Liquid media were inoculated with a pre-culture to deliver a final concentration of 5×10^6 blastospores/mL. The

experiment consisted of three biological replicates and was repeated twice or thrice on different dates using new fungal inoculum. The fungal growth parameters evaluated during the course of fermentation were: a) growth kinetics to determine the blastospore concentration from 24 h to 72 h post-inoculation; b) maximum specific growth rate determined during the log-growth phase of blastospore production curve at different glucose titers using the following formula: $\mu\ (h^{-1}) = \ln(N_i) - \ln(N_0)/(t_i - t_0)$, where N_i = blastospore population recorded in log-growth at time t_i, N_0 = initial blastospore population at time zero t_0 (i.e., 5×10^6 blastospores/mL); and c) glucose utilization by subtracting the difference between initial glucose titer and the remaining glucose measured in the spent medium at the end of the fermentation period (day 3).

Blastospore concentration data were fitted to a generalized linear model with gamma distribution and inverse link function, including glucose titer, fermentation day, and interaction term as fixed effects in the linear predictor. Specific growth rate and glucose consumption datasets were separately fitted to general linear models with and without a random effect for replicate flasks, and normal distribution for errors including glucose titer as the fixed effect. Multiple pairwise post-hoc comparisons using the Tukey HSD method (p-value < 0.05) were used to separate significant means between glucose titers within each fermentation day. This analysis was performed in the statistical environment R (version 4.0.2, R Core Team, 2020) and plots created with the "ggplot2" package (Wickham, 2016).

RNA Isolation and Sequencing

Three independent biological samples, corresponding to 1 mL of the whole culture in shake flasks, were collected for each fermentation time (*i.e.*, days 1, 2, and 3). Samples were centrifuged at 16,000*g* for five minutes to pellet the cells, the supernatant was decanted, and the pellet was flash-frozen in liquid nitrogen. The pellet was homogenized with a mortar and pestle under liquid nitrogen. RNA was extracted with a Qiagen RNAeasy Kit (Germantown, MD, USA). RNA quality was confirmed using an Agilent 2200 tapestation. RNA concentration was measured using the Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). RNA integrity number (RIN) was considered acceptable if greater than 6. The library preparation and sequencing were performed by the Brigham Young University DNA sequencing center. The mRNA was selected with an oligo poly dT selection, and the library was prepared with an Illumina TruSeq RNA library kit. The final library was sequenced with an Illumina HiSeq 2500 with 50 cycle single read sequencing V4. The transcriptome data are available on NCBI-GEO under the GEO accession GSE163673 and can be accessed through the link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163673.

RNA-Seq Data Analysis

Raw sequencing reads were checked for adapters and quality trimmed to Q30 using Genomics Workbench (version 10.0.1) (Qiagen, Inc, Germantown, MD, USA). The reads were mapped to the *B. bassiana* strain D1-5 reference genome (GenBank accession no. ANFO01000000) using the 85% length alignment and 85% similarity. Expression values were normalized using RPKM and a separate General Linear Model with a negative binomial distribution under Genomics Workbench. Statistical comparisons of differential gene expression between treatments were conducted pairwise with Wald tests and Bonferroni corrected p-values determined under Genomics Workbench. Differentially expressed genes with False Discovery Rate (FDR) adjusted p-value (< 0.005) and log2(fold change, FC) > 2 or < -2 were used for the final data set.

Gene-Set Enrichment Analysis

The Gene Set Enrichment Analysis (GSEA) software determines whether *a priori* defined set of genes is statistically significant between two biological states (Subramanian et al., 2005). GSEA rank gene sets by enrichment magnitude and indicates classes of over-represented genes in the gene set. 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: the $\log_2$ fold change, adjusted p-value, or inverse p-value, and a list of gene sets. GSEAPreranked calculates an enrichment score by matching genes from gene sets to those in the user ranked index. Next, the gene set enrichment score shows how often members of that gene set occur at the top or bottom of the ranked data set. This study used GSEAPreranked mode with gene sets categorized by Gene Ontology (GO) annotation. The metric used in the GSEA input file was the sign of fold change multiplied by its inverse p-value (Iwanicki et al., 2020a). The p-value provided was used as an output of differential gene expression analysis. When the p-value output was "0", the "0" value was replaced by artificially high or low values "+1E+308" or "-1E+308" for up and down-regulated genes according to the sign of fold change. The parameter adopted for running the GSEAPreranked for GO were: minlengh 10 and maxlengh 500, enrichment statistic: "classic" and FDR-correction for multiple testing < 0.25 for enriched gene sets (Iwanicki et al., 2020a).

Expression of Heat Shock Protein and Key Genes Associated With Response to Stress Conditions and High Osmolarity Glycerol Pathway

Heat shock proteins (HSPs) are a large superfamily of intracellular, molecular chaperones with molecular sizes ranging from less than 30 to ~100 kD and are involved in maintaining protein homeostasis in several physiological processes including response to stress conditions. A recent study has investigated the role heat shock proteins (Hsp70) have in virulence, cell wall integrity, antioxidant activity, and stress tolerance of *B. bassiana* strain ARSEF 2860 (Wang et al., 2020). This study explored gene expression of 14 Hsp70 homologs identified by Wang et al. (2020) in blastospores cultivated with high (20%) and moderate (10%) glucose amended liquid media with focus on those related to antioxidant activity and stress tolerance. In addition, gene

expression of four Mitogen-activated protein kinases (MAPK) homologs: *Ste11* (BBAD15_g9298), *Ssk2* (BBAD15_g1898), *Pbs2* (BBAD15_g4699), *Hog1* (BBAD15_g6467) associated with HOG pathway in *B. bassiana* ARSEF 2860, and one homolog associated to multiple stresses were also explored (Wsc1, BBAD15_g1986) obtained from Liu et al. (2017) and Tong et al., (2019). In addition, we examined the expression profile of another set of select *B. bassiana* homologous genes involved in osmotic stress, including *CnA1* (BBAD15_g8778), *Ecm33* (BBAD15_g9622), *Ras1* (BBAD15_g11654), *Mkk1* (BBAD15_g4332), *pmr1* (BBAD15_g2615), *Gpcr* (BBAD15_g8874), *Cdk1* (BBAD15_g6748), *Mtd* (BBAD15_g10510), *Ssk1* (BBAD15_g8860), *Bmh1* (BBAD15_g2365), *Ohmm* (BBAD15_g7953), *Ktr4* (BBAD15_g4067), and *Mbf1* (BBAD15_g10650) (Ortiz-Urquiza and Keyhani, 2016). Sequences were blasted against the genome of *B. bassiana* D1-5 to obtain homologous genes (*e*-value < 0.0001 and sequence similarity > 80%). An interactive heatmap was drawn to illustrate the selected genes on days 1, 2, and 3. This analysis was performed in the statistical environment R (version 4.0.2, R Core Team, 2020) and heatmap created with the "ggplot2" package (Wickham, 2016) using the mean log2-FC values of gene expression patterns at 1, 2, and 3 days post-inoculation across the increased glucose gradient. Four selected genes (*Pbs2*, *Ste11*, *Hog1*, and *Ssk2*), known to be associated with response to osmotic stress were mapped against MAPK-signaling pathways by searching against the Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) database (https://www.kegg.jp/kegg/tool/map_pathway.html). The mapping was performed through the K numbers of genes, while the pathways were shown as a schematic illustration.

qRT-PCR-Based Gene Expression Analysis of Key Blastospore-Related Genes

In this experiment, *B. bassiana* GHA was cultivated with different glucose concentrations ranging from 4% to 20% at incremental intervals of 4%. The blastospore concentration, glucose consumption, and specific growth rate of blastospores were recorded daily for three days under the same set of fermentation conditions described previously. This bioassay's primary purpose was to evaluate the expression of key genes involved in blastospore development across a greater glucose titer gradient. Samples stored at -80°C were used for RNA extraction *via* TRIzol reagent (Invitrogen®), and the concentration and quality of total RNA were evaluated *via* Nanodrop (Thermo Scientific). Synthesis of cDNA was conducted with 1ug of total RNA from each sample using the QuantiTec reverse transcription kit with DNA Wipeout (Qiagen). Measures of gene expression were performed in a 10 μL reaction using one microliter of cDNA and *B. bassiana* GHA gene-specific primers (**Supplemental Table S1**). Primers were designed on loci from *B. bassiana* strain D1-5 reference genome (GenBank accession no. ANFO01000000), which were updated with read mapping of *B. bassiana* GHA reads and a new consensus extracted. The PowerUp SYBR green Master mix qPCR kit (Qiagen) was used in all reactions using the qPCR cycling condition recommended by the manufacturer (holding at 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C). The qPCR assays included a Melt Curve analysis at the end of the reaction, and was carried out on an Applied Biosystems QuantStudio 6 Flex Real-time PCR system (ThermoFisher Scientific™). The gene expression assays evaluated at least three biological replicates, and each sample was assessed in duplicate. The gene expression levels were normalized against the fungal gene actin previously validated in Zhou et al. (2012) (**Supplemental Table S1**) and were analyzed post-run using the ΔΔCt method (Livak and Schmittgen, 2001). The temporal mean expression levels in fold change of selected *B. bassiana* genes during different blastospore growth stages under liquid fermentation were described in line plots, and an asterisk indicates significant differences between glucose titers at 5% probability level by ANOVA within each growth interval. The heatmap was created with the "ggplot2" package using the mean $\log_2$ fold change values of gene expression patterns at 1, 2, and 3 days post-inoculation across the increased glucose gradient.

RESULTS

We have previously argued blastospore production of *B. bassiana* requires a combination of high aeration and high osmotic pressure imposed by glucose to achieve high yields in shorter fermentation times. In our model system designed in this study, we set out transcriptome analysis to scrutinize the molecular and genetic factors driving blastospore formation in high glucose cultures (20% w/v) under high aeration rates against moderate glucose cultures (10% w/v) during three days of culture.

Blastospore Yield and Kinetics Under Different Glucose Concentrations

Liquid cultures of *B. bassiana* amended with varying glucose titers started with an initial density of 5×10^6 blastospores/mL and dramatically increased over time with greater glucose gradient (Interaction day × glucose titer: $\chi^2_{12;79} = 53.15$, $p < 0.0001$), indicating blastospore concentration is strongly dependent on fermentation time and glucose titer in the liquid medium (**Figure 1A**). Regardless of glucose titers tested, blastospore concentration at day 1 post-inoculation suggests fungal culture is under lag-phase adaptation to medium conditions. The lack of glucose in the medium resulted in steady and low blastospore yields over time. Notably, these fungal cultures exhibited more mycelium development than blastospores, suggesting this carbon source is indispensable to sustain the fungus yeast-like growth. From day 2 to day 3 post-inoculation, blastospore yields were always below 1×10^9 blastospores/mL when grown in the range of 4-10% glucose with no significant blastospore concentration changes between titers. Blastospore production increased with increasing glucose concentration and peaked when grown with 20% glucose, yielding higher than 1×10^9 blastospores/mL at 2 days post-inoculation.

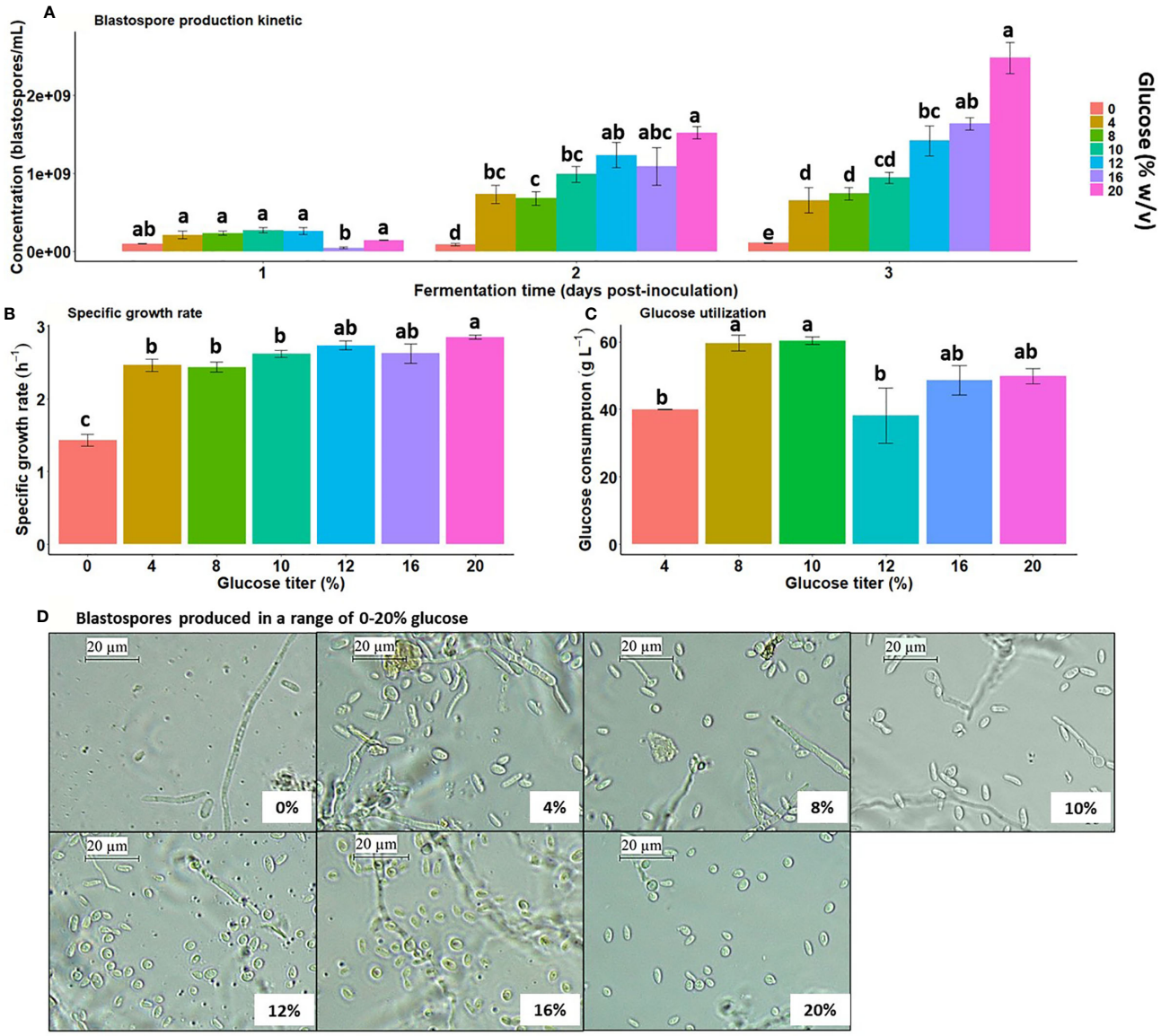

FIGURE 1 | Blastospore yield and growth kinetics are significantly boosted by greater glucose gradient (0 to 20% w/v) under high aeration during submerged liquid culture conditions. **(A)** *B. bassiana* (strain GHA) blastospore concentration in liquid culture with a greater glucose titer gradient at 28 °C, 350 rpm, and 50 mL of filling volume. **(B)** Maximum specific growth rate during log-phase growth across glucose titer gradient. **(C)** Glucose consumption at day 3 post-inoculation across glucose titer gradient. Bar heights represent means, and error bars are standard error of means (± S.E.). **(D)** Blastospore morphotypes induced by different glucose titers (photographs taken at day 3 of fermentation). Vertical bars within each fermentation time are significantly different when followed by distinct letters (Tukey HSD, $p < 0.05$).

The specific growth rate during the log-growth phase within the interval 0 and 2 days post-inoculation, was significantly increased with greater glucose titers ($F_{6;36} = 22.76$, $p < 0.0001$), reaching a plateau at 12% glucose and remained constant at 16% and 20% glucose (2.63 – 2.85 h^{-1}). The specific growth rate at 20% glucose was greater than 0-10% glucose (**Figure 1B**). Glucose utilization by GHA cultures was significantly affected by the initial glucose titer ($F_{5;28} = 4.66$, $p = 0.003$), but the consumption trend did not increase with the glucose gradient. The maximum glucose uptake was around 60 g L^{-1} when cultures were grown under 8-10% initial glucose titers (**Figure 1C**). Moreover, the fungus's glucose uptake remained lower than 50 g L^{-1} (= 5% w/v) when cultured with 12-20% glucose, indicating a surplus of spent glucose in the medium. Morphological examination of blastospores grown under greater glucose titers from 12% to 20% exhibited more ovoid shape and a smaller size than those produced with a range of 0-10% glucose (**Figure 1D**).

Considering the main objective to study blastospore growth, the glucose concentrations used in the transcriptome analysis

(20% *versus* 10%) were chosen to avoid the interference of hyphal growth. Although there were no statistical differences among specific growth rates in media ranging from 4% to 10% glucose, fungal cultures in 10% glucose presented reduced hyphal growth compared to lower glucose concentrations (Mascarin et al., 2015a). Hence, 10% glucose was selected as the lower glucose titer to compare with the higher, 20% glucose, in the transcriptome study.

Summary of RNAseq Data

A total of 108.56 million clean and mapped RNA-Seq reads were generated. Of these, 103.7 million reads could be aligned to the *B. bassiana* strain D1-5 reference genome (concordant unique pairs, see details in **Table 1**). A total of 54.3 and 53.8 million unique match reads were obtained for blastospores grown in medium amended with either 10% or 20% glucose. The average rate of reads alignment against the reference genome were 95.1% and 96.3% for 10% and 20% glucose treatments. **Table 1** summarizes the RNA-Seq experiment results, and transcriptomics data are available on NCBI-GEO under the GEO accession GSE163673.

The principal component analysis showed the highest variation in the gene expression profile is from biological samples from days 1, 2 and 3 (75%), regardless of glucose concentration. According to the second principal component (accounted for 9% total variance), genes were differently expressed in blastospores grown with moderate (10%) glucose in relation to blastospores grown high glucose cultures (20%) at days 2 and 3 of fermentation. In general, biological samples were grouped within treatments indicating high similarity (**Figure 2**).

Comparisons Between 20% and 10% Glucose-Amended Medium Within Days of Cultivation

Gene-Set Enrichment Analysis

To characterize the set of genes significantly upregulated in blastospores cultivated in 20% and 10% glucose-amended media, GO-term gene set enrichment analysis using GSEA was utilized (Subramanian et al., 2005). A clear difference was found between GO-terms enriched in blastospores grown in 20% glucose medium compared to fungus grown in 10% glucose across three days of cultivation (**Figure 3**, **Supplemental Table S2**). On day 1 of culture, 53 GO terms were significantly enriched (FDR adjusted $p < 0.25$) in blastospores grown in 20% glucose medium. Of these 53 terms, 21 were assigned to biological process (Fig 3A), 8 to cellular components, and 24 to molecular function (**Supplemental Table S2**). Also on day 1, blastospores grown in 10% glucose medium had 12 GO-terms enriched, 2 in biological process category: response to oxidative stress (GO:0006979) and metabolic process (GO:0008152), and 8 in molecular functions category (**Figure 3A**, **Supplemental Table S2**). On day 2 of culture, 33 enriched GO-terms were

TABLE 1 | Summary of the read-mapping statistics on the transcriptomic features of *B. bassiana* liquid cultures grown over time.

Glucose level	10%			20%		
Fermentation time	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Total mapped reads (%)	18,266,154 (95.88%)	23,776,977 (94.95%)	12,471,404 (94.44%)	21,119,505 (96.19%)	17,892,329 (95.89%)	15,029,519 (96.96%)
Total unmapped reads (%)	785,841 (4.12%)	1,265,577 (5.05%)	734,891 (5.56%)	836,200 (3.81%)	766,802 (4.11%)	471,220 (3.04%)
Unique match (%)	18,212,828 (95.59%)	23,699,923 (94.64%)	12,433,499 (94.15%)	21,071,725 (95.97%)	17,837,883 (95.60%)	14,9860,077 (96.68%)

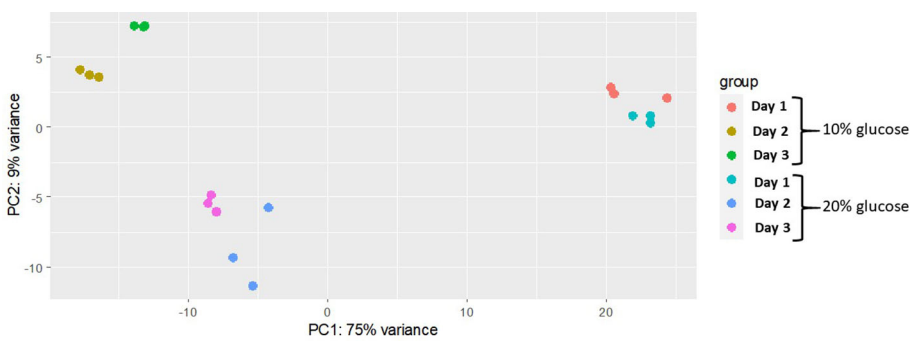

FIGURE 2 | Multivariate analysis separating glucose treatments across different fermentation time points. Principal component analysis of regularized-logarithmic (rlog) transformed gene counts of *B. bassiana* (strain GHA) blastospores cultivated in medium with 10% or 20% glucose during three days of growth. Biological replicates are represented by each dot (n = 3 per treatment).

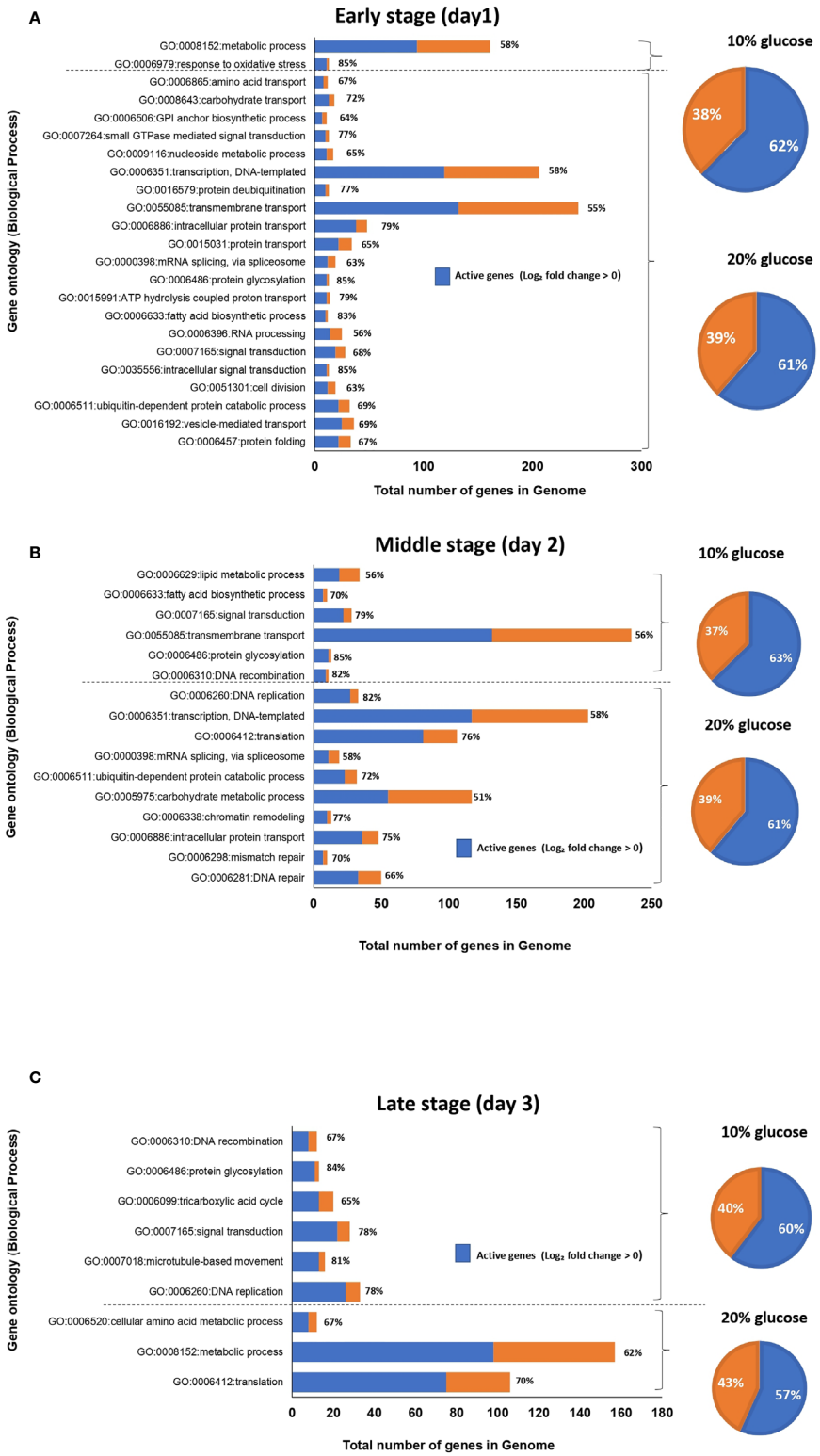

FIGURE 3 | Graphical representation of enriched gene ontology (GO) terms for the biological process of *B. bassiana* blastospores produced in liquid medium amended with 20% glucose compared to 10% glucose after one **(A)**, two **(B)**, and three **(C)** days of culture (FDR $p < 0.25$). The value in front of each bar represents the percentage of active genes (log2-FC > 0), in the set of genes associated with each GO-term. The pie chart illustrates the percentage of active genes (in blue) in 20% or 10% glucose treatment considering all enriched GO-terms for each day of fermentation (see also **Supplemental Table S2**).

found for blastospores grown in 20% glucose medium. Of these 33 terms, 11 were assigned to biological process, 6 to cellular components, and 16 to molecular functions and division (GO:0006310, GO:0006260, GO:0006412, GO:0006351) (**Figure 3**, **Supplemental Table S2**). Likewise, on day 2 for blastospores grown in 10% glucose medium 14 enriched GO-terms were found; 5 were assigned to biological processes, 3 to cellular components, and 6 to molecular functions (**Figure 3B; Supplemental Table S2**). These results agree with the laboratory observations of peak blastospore production on day 2 in both glucose-amended media. However, this peak is significantly higher for fungus grown under 20% glucose (**Figure 1A**). On day 3, blastospores grown in 20% glucose medium 22 enriched GO-terms were found; 3 were assigned to biological process (**Figure 3C**), 3 to cellular component, and 16 to molecular function (**Supplemental Table S2**). For blastospores grown in 10% glucose medium 6 GO-terms enriched among the biological process (**Figure 3C**) associated mainly with cellular growth/germination (GO:0006260, GO:0006310) were found. Additionally, 2 GO-terms assigned to cellular components and 9 to molecular function (**Supplemental Table S2**) were also identified.

The majority of GO-terms assigned to biological process: intracellular protein transport (GO:0006886), transcription, DNA-templated (GO:0006351), ubiquitin-dependent protein catabolic process (GO:0006511) were all enriched in blastospores grown in the high glucose medium at days 1 and 2, as well as for GO-term translation (GO:0006412) at days 2 and 3 compared to 10% glucose-amended medium (**Figure 3C**). These results indicate higher cell activity related to signal transduction, transmembrane transport, cell division, and transcription in blastospores grown with 20% glucose medium compared to those produced in the 10% glucose–amended medium.

Differentially Expressed Genes

From 11,861 genes annotated in the *B. bassiana* reference genome, 198 (1.66% of genome) genes were differentially expressed (FDR adjusted $p < 0.005$, log2-FC > 2 or < -2) between blastospores grown in medium amended with 10% and 20% glucose during three cultivation days. Most of the differentially expressed genes (DEGs) were downregulated in blastospores grown under 20% glucose representing a total of 161 genes (81% of the DEG) over 3 days of cultivation. Of these, 11 common genes were downregulated in all three days (**Figure 4**), with 7 encoding for "hypothetical proteins", 1 for "trypsin" (BBAD15_g10782), 1 for "putative endo-1,3(4)-beta-glucanase" (BBAD15_g10674), and 1 for "Kinesin light chain" 4 (BBAD15_g12537) (**Supplemental Table S3**). Day 2 of cultivation corresponded to the highest number of downregulated genes (n=94), whereas day 1 showed the lowest number (n=50). Analyzing all time points of fermentation, a total of 37 genes (19% of the DEGs) were found to be upregulated in blastospores grown in the 20% glucose medium at least in one culture day. Day 2 of cultivation exhibited the highest number of upregulated genes (n=20), whereas, on day 1, there were only 7 upregulated genes (**Supplemental Table S3**). Moreover, a total of 37 genes (19% of the DEGs) were found to be upregulated in blastospores grown in the 20% glucose medium at least in one culture day.

The 37 genes upregulated in blastospores cultivated in the medium with 20% glucose were plotted in a heatmap to investigate these genes' dynamism during the three days of cultivation (**Figure 5**, **Supplemental Table S3**). This process

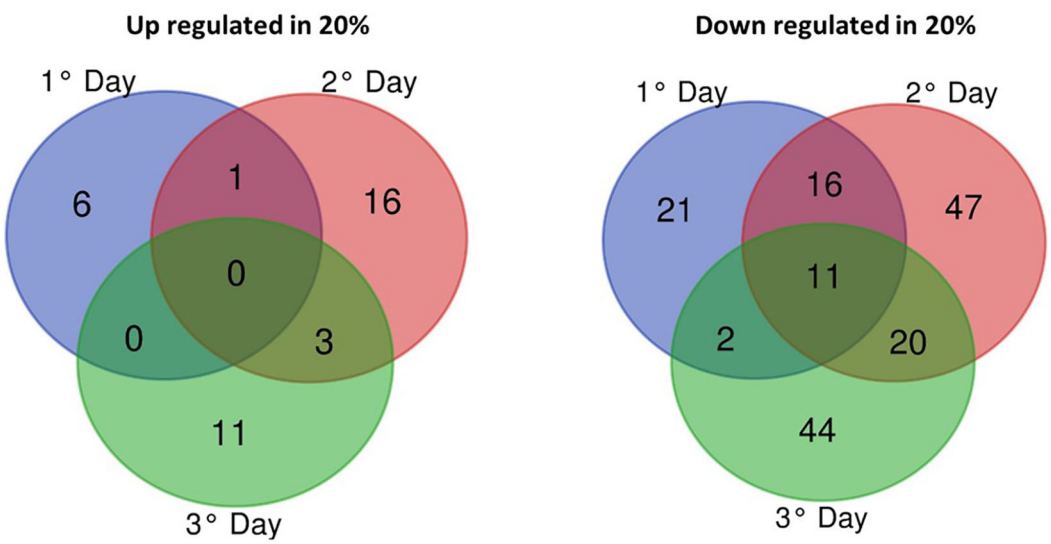

FIGURE 4 | Venn diagram of differentially-expressed genes (log2-fold > 2 (upregulated) or < -2 (downregulated) of *B. bassiana* grown in liquid cultures supplemented with 20% vs. 10% glucose on days 1, 2, and 3 of fermentation. The cutoff used was false-discovery rate adjusted *p*-value less than 0.005 (FDR $p < 0.005$). DEGs, statistically significantly differentially-expressed genes (see also **Supplemental Table S3**).

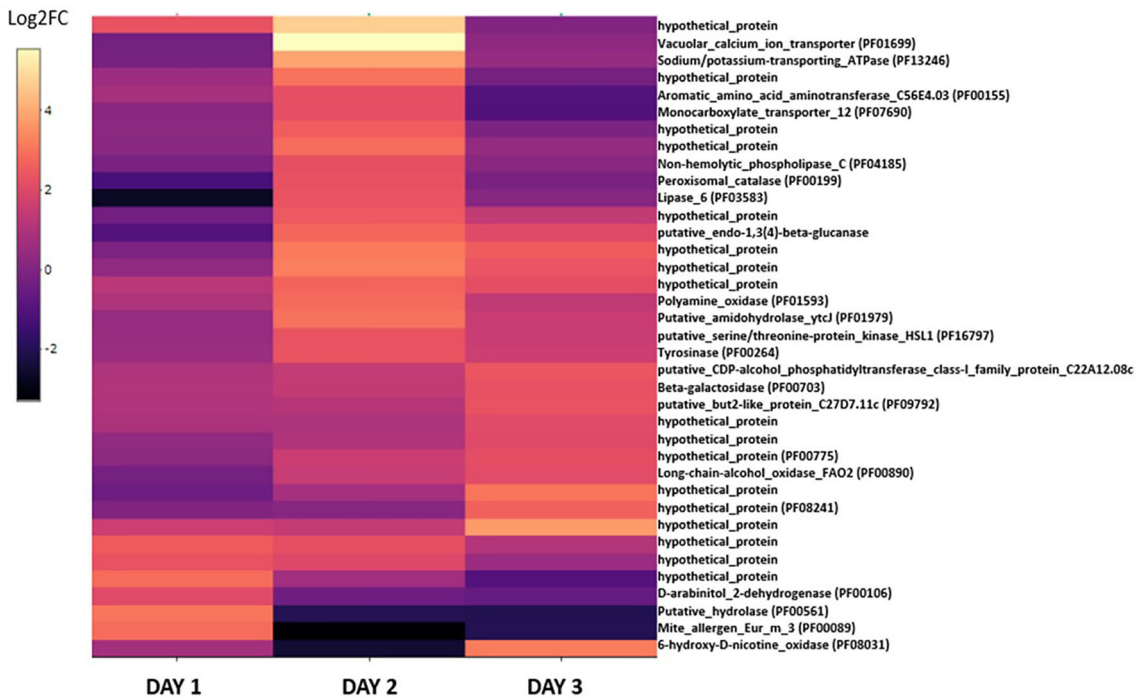

FIGURE 5 | The heatmap displays the $\log_2$-fold changes of DEGs in the *B. bassiana* cultures grown under 20% glucose compared to 10% glucose-amended cultures. DEGs for at least one culture day (log2-FC > 2 (upregulated), FDR $p < 0.005$). In parentheses, the protein family code (pfam) provided when properly identified. Unknown proteins are assigned to "hypothetical proteins" (see also **Supplemental Table S3**).

identified 17 genes assigned to hypothetical proteins with little or none annotated information available. On day 1, few genes were found upregulated and were represented mainly by enzymes such as hydrolase (PF00561), trypsins (PF00089), dehydrogenase (PF00106), and an oxidase (PF08031) (**Figure 5**, **Supplemental Table S3**). However, on day 2, a more significant change in the expression of genes related to nutrient transport (PF07690), oxidoreductase (PF01593), catalase (PF00199), and other enzymes such as lipase (PF03583) and tyrosinase (PF00264) were observed. Many upregulated genes found on day 2 were then down-regulated on day 3, and new groups of genes related to oxidase (PF00890), beta-galactosidase (PF00703), and hypothetical proteins were upregulated. Interestingly, the vacuolar calcium ion transporter (PF01699) and the sodium/potassium transporting ATPase (PF13246) were both strongly upregulated on day 2 when fungal cultures were grown with 20% glucose. This suggests blastospores on day 1 of fermentation relied on these proteins to maintain cation homeostasis through these cations' active transport.

Comparisons Between Culture Days Within 20% or 10% Glucose Medium

To identify the primary biological processes involved in the rapid blastopores growth in 20% glucose medium, GO terms enriched on day 2 in relation to day 1 and day were contrasted with those found in blastospores population grown in 10%-glucose medium.

Gene-Set Enrichment Analysis

Comparing day 2 with day 1, 4 and 5 exclusive GO-terms assigned to biological processes enriched in blastospores grown in 10% and 20% glucose medium, respectively, and 5 common enriched GO-terms (**Figure 6A**, **Supplemental Table S2**). On day 2, 53 GO-terms were significantly (FDR $p < 0.25$) enriched in blastospores grown in 10% glucose medium; 9 biological processes, 4 cellular components, and 22 molecular functions (**Figure 6A**, **Supplemental Table S2**). Likewise, 32 GO-terms significantly (FDR $p < 0.25$) enriched were found in blastospores grown in 20% glucose medium, 5 biological processes, 1 cellular component, and 26 molecular functions (**Figure 6A**, **Supplemental Table S2**). The common enriched GO-terms on day 2 were assigned to biological processes such as lipidic metabolic process (GO:0006629), lipidic biosynthetic process (GO:0008610), mycotoxin biosynthetic process (GO:0043386), ATP hydrolysis coupled proton transport (GO:0015991), and response to oxidative stress (GO:0006096) (**Figure 6A**). These results suggest 5 biological processes occur in the exponential growth phase (day 2), regardless of the initial glucose concentration supplied in liquid cultures of *B. bassiana*.

Comparing day 3 with day 2, 10 exclusive GO-terms assigned to biological processes enriched in blastospores grown in 10%, 1 exclusive GO-term enriched in blastospores cultivated under 20% glucose, and 2 common enriched GO-terms (**Figure 6B**) were found. On day 3, 35 GO-terms were significantly (FDR $p < 0.25$) enriched in blastospores grown in 10% glucose medium; 12 biological processes, 4 cellular components, and 19 molecular

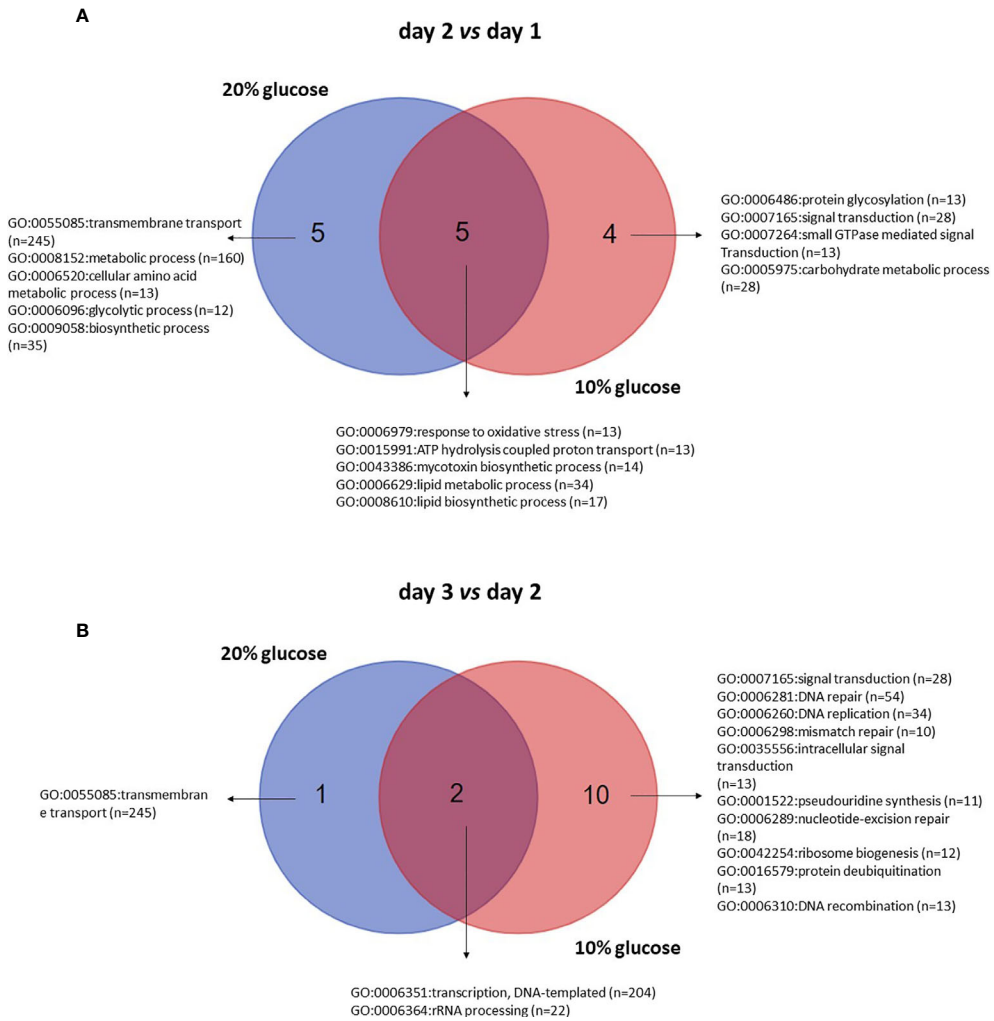

FIGURE 6 | Venn diagram of enriched gene ontology (GO) terms for biological processes of blastospores of *B. bassiana* produced in medium amended with 20% or 10% glucose comparing day 2 with day 1 **(A)** and day 3 with day 2 **(B)** within each glucose concentration tested. FDR $p < 0.25$ (see also **Supplemental Table S4**).

functions (**Figure 6B**, **Supplemental Table S2**). Additionally, 13 GO-terms enriched in blastospores grown in the 20% glucose medium; 3 biological processes (**Figure 6B**), 2 cellular components, and 8 molecular functions (**Supplemental Table S2**). The common enriched GO-terms at day 3 were assigned to biological processes such as transcription, DNA-templated (GO:0006351), and rRNA processing (GO:0006364). The only exclusive GO-term assigned to biological processes enriched in blastospores cultivated with 20% glucose was transmembrane transport (GO:0055085). The most significant difference ($p < 0.05$) in blastospore yield between 10% and 20% glucose media was observed in the *in vivo* experiment on day 3 (**Figure 1A**). On day 3 of cultivation, the blastospore concentration was twice greater in 20% glucose than when grown in 10% glucose.

Differentially Expressed Genes

The most considerable difference in gene expression of blastospores grown in different glucose concentrations (10% or 20%) was observed on day 2 compared to day 1, regardless of the initial glucose titer. A total of 507 down-regulated genes and 195 upregulated genes were observed on day 2 compared to day 1 for fungus grown with 20% glucose, and 560 upregulated and 259 down-regulated on day 2 compared to day 1 for fungus grown with 10% glucose (**Supplemental Table S3**). Additionally, 371 common upregulated genes on day 2 compared to day 1 when blastospores were produced with either 10% or 20% glucose medium with a significant number (n=181) classified as "hypothetical proteins". Also, 189 and 137 genes were upregulated at day 2 in 10% and 20% glucose medium, respectively (**Supplemental Table S3**). Comparing day 2 with day 3, 49 DEGs were found in 10% glucose medium, of which 37 were upregulated and 12 were down-regulated. Conversely, 73 DEGs were found in 20% glucose medium, of which 24 were upregulated and 49 downregulated. Only 4 genes were commonly upregulated on day 3 for both 10% and 20% glucose enriched cultures; 3 of them were classified as

"hypothetical proteins" (BBAD15_g2461, BBAD15_g7457, BBAD15_g5885), and 1 (BBAD15_g4395) was an isoflavone reductase (**Supplemental Table S3**), which is involved in the pathway pterocarpan phytoalexin biosynthesis.

Expression of Heat Shock Protein Genes and Key Genes Associated With Response to Stress Conditions and High Osmolarity Glycerol Pathway

In the *B. bassiana* GHA, 13 homologs HSP were identified out of the 14 HSP involved in stress responses, virulence, and cell wall integrity identified by Wang et al. (2020) in the *B. bassiana* ARSEF2860 genome. The expression of the 13 HSP70s homologous genes did not differ statistically between blastospores produced in 20% and 10% glucose, within each culture day (log2-FC > 2 < -2, FDR $p < 0.005$) (**Supplemental Table S4**). However, when fixing the glucose concentration and comparing the gene expression between culture days, 2 HSP70 homologs were found and are located in the cell cytoplasm: heat shock protein Ssb1 (BBAD15_g2252) and heat shock protein Ssz1 (BBAD15_g4682). Both were upregulated at day 1 compared to day 2 in blastospores grown in 20% and 10% glucose amended medium. Considering MAPK genes associated with the HOG pathway characterized by Liu et al. (2017), no statistical differences in the expression of homologs of *B. bassiana* D1-5 genes: *Ste11* (BBAD15_g9298), *Ssk2* (BBAD15_g1898), *Pbs2* (BBAD15_g4699), and *Hog1* (BBAD15_g6467), between blastospores produced in 20% and 10% glucose, within each culture day, nor between culture days when fixing glucose concentration (log2-FC > 2 < -2, FDR $p < 0.005$) (**Supplemental Tables S3** and **S4**). Although, the genes *Ssk2* and *Hog1* were found to be active (log2 -FC > 0) in 20% glucose medium in all culture days compared to 10% glucose (**Figure 8**). However, the homologs of the sensing protein Wsc1 (BBAD15_g1986), localized in the vacuoles and cell wall/membrane in *B. bassiana*, were upregulated on day 2 compared to day 1 in blastospores produced with both 20% and 10% glucose (**Supplemental Table S3**).

Because the media conditions suggest osmotic stress tolerance responses may be an essential factor in promoting this morphology's development, 40 genes were reviewed involved in osmotic stress in *B. bassiana*, summarized in Ortiz-Urquiza and Keyhani (2016). The identification of 9 homologous genes were found in *B. bassiana* GHA involved in osmotic stress response in blastospores grown with 20% vs. 10% glucose on days 1, 2, and 3 of fermentation (log2-FC >1 or < -1 in at least one of culture days) (**Figure 7**, **Supplemental Table S4**). During the early phase of culture growth, at day 1 with 20% glucose, *B. bassiana* oxidative and osmotic related genes upregulated included the class I hydrophobin, *Hyd1* (BBAD15_g6903), and the mitochondrial membrane protein, Ohmn (BBAD15_g7953). These results inspired a closer look at the expression of these genes. *Hyd1* was highly expressed under all time points and both glucose rates, so a compiled list of the most expressed genes were made (**Supplemental Table S5**). All 6 genes were highly expressed, and represented the most expressed genes under all time points and both glucose rates. The genes with known function represent proteins associated with the cell wall, which is consistent with these proteins being the basic cell building blocks for these propagules.

In summary, by mapping key genes (*Ssk1*, *Pbs2*, *Ste11*, *Hog1*, and *Ssk2*) involved in the osmotic stress response in *B. bassiana* growing under 20% glucose (hyperosmotic medium) in relation to 10% glucose medium, we found implications of the HOG signaling pathway with upregulation of homologous genes *Ssk2* and *Hog1* for all time points of cultivation (**Figure 8**). These two genes are associated with cell osmoregulation leading to osmolyte synthesis and enhanced blastospore yield.

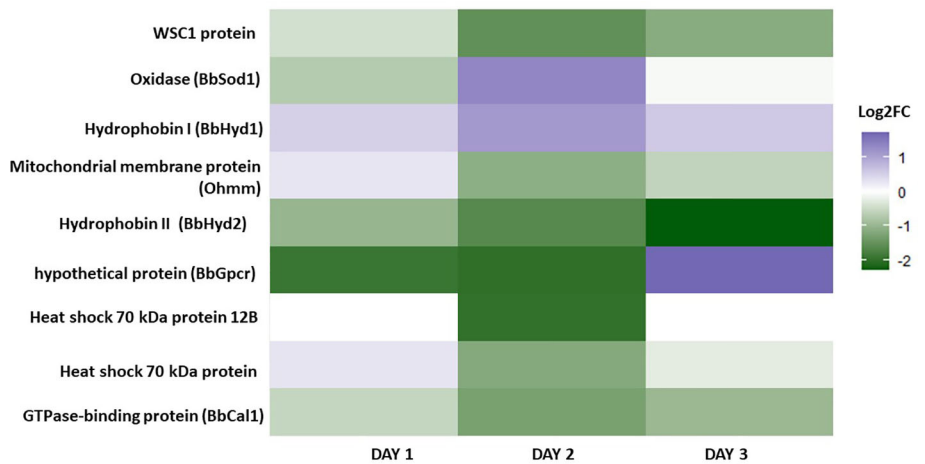

FIGURE 7 | Heatmap depicting the gene expression of nine genes associated with osmotic stress in *B. bassiana* blastospores grown in liquid cultures supplemented with 20% vs. 10% glucose on days 1, 2, and 3 of fermentation (log2-FC >1 or < -1 in at least one of culture days) (see also **Supplemental Table S4**).

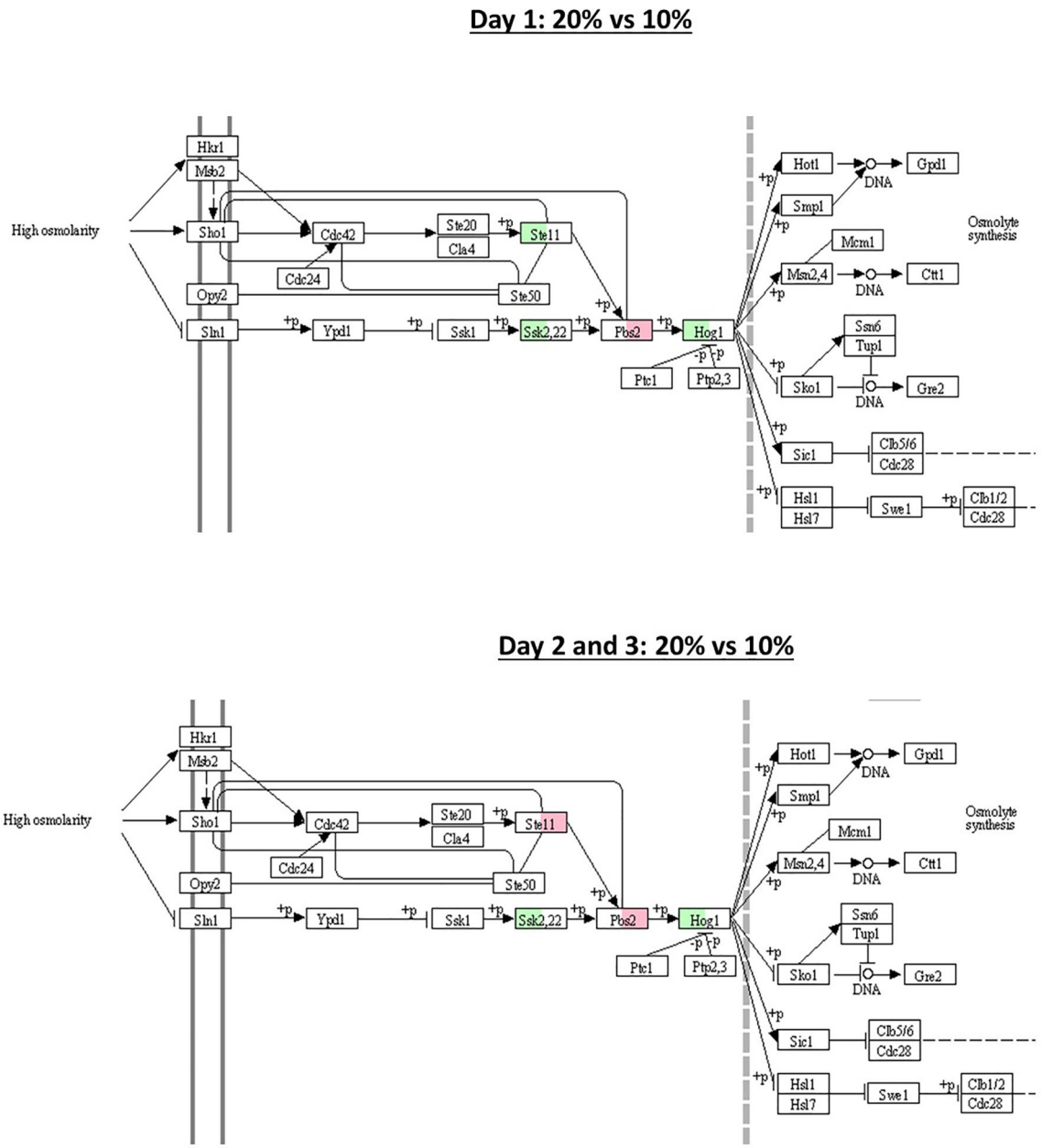

FIGURE 8 | Schematic illustration of MAPK-signaling pathway with 5 selected genes (*Ssk1*, *Pbs2*, *Ste11*, *Hog1*, and *Ssk2*), known to be associated with response to osmotic stress. Green and red boxes indicate the gene is active (log2-FC > 0) in 20% and 10% glucose (log2-FC < 0) treatment, respectively (see also **Supplemental Table S4**). The pathway was obtained from the Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) database.

Gene Expression of Key Blastospores-Related Genes *via* qRT-PCR

The expression of key genes involved in oxidative/respiration, carbon catabolite, cell wall synthesis, and osmoregulation pathways were tested via qRT-PCR. This was used to identify the essential fungal genes needed during blastospore growth under increased glucose rates on different fermentation days (**Figure 9**). The most pronounced gene expression, down- or upregulated, was seen at day 2 of cultivation, which coincided with the intense metabolic activity (catabolism and anabolism) during log-growth phase of *B. bassiana* blastospores. Notably, the expression level peak of all genes analyzed through qRT-PCR occurred with the growth peak of blastospores grown in 20% glucose (ANOVA, $p < 0.05$), which was the highest osmotic pressure simulated in this study (**Figure 9A**). Of particular interest, the gene expression profile showed strongly upregulated genes related to cell growth and cellular multiplication from day 2 to day 3 of development, compared

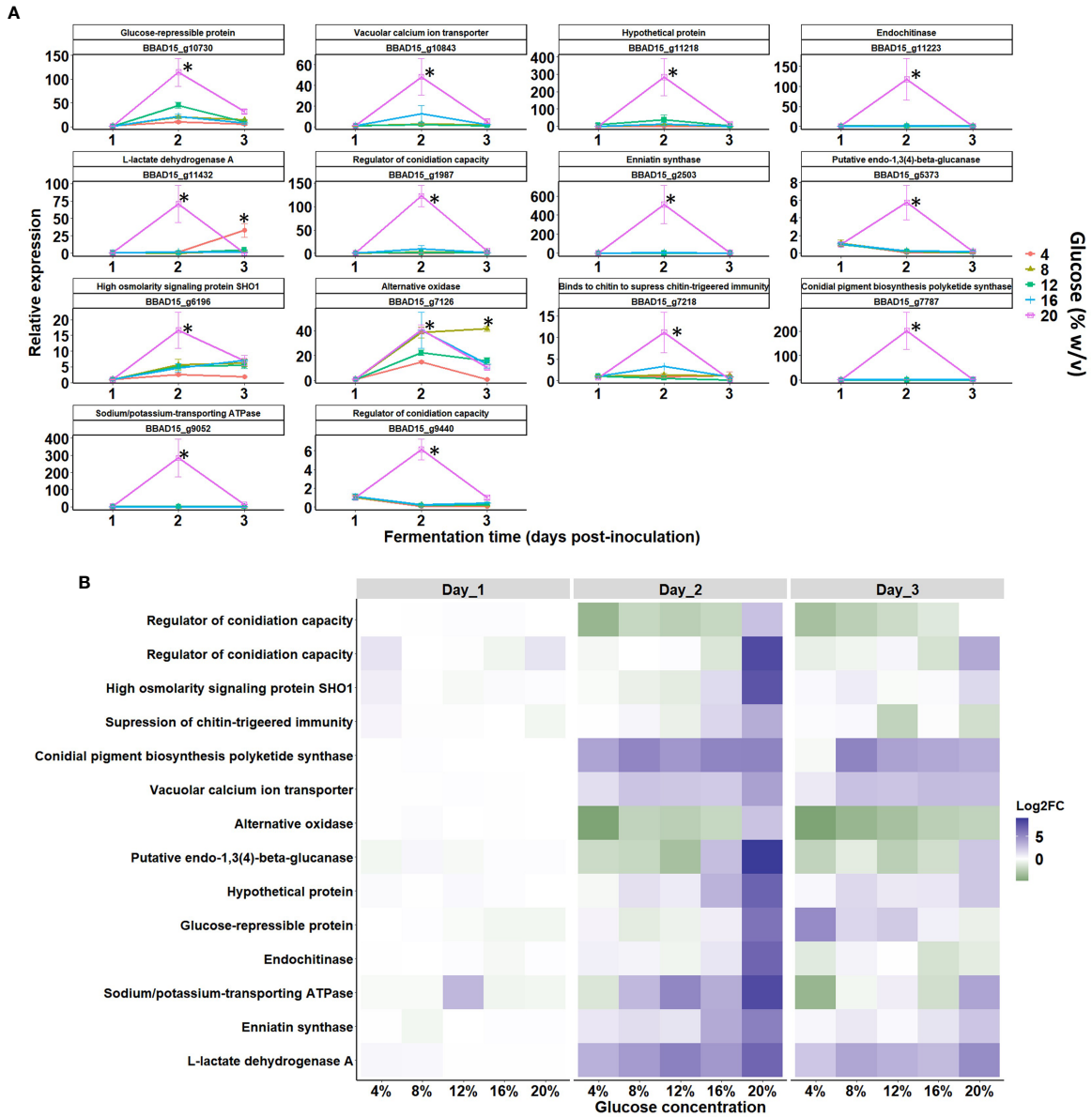

FIGURE 9 | Gene expression of key blastospore-related genes *via* qPCR during growth stages of *B. bassiana* in response to an increased glucose gradient. **(A)** Temporal elicitation of selected *B. bassiana* genes (expressed in fold change values normalized by the reference actin gene *via* ΔΔCt method) during the blastospore growth stages: early (day 1), middle (day 2), and late (day 3) responses under liquid fermentation (mean ± standard error). Significant differences between glucose concentrations within each growth phase are indicated by asterisks ($p < 0.05$*) according to ANOVA. **(B)** Heatmap shows gene expression patterns at 1, 2, and 3 days post-inoculation and mean $\log_2$ fold change values from three biological replicates and two technical replicates. The log2-FC values and the color scale are shown at the right of the heat map, where blue indicates upregulation, while green indicates downregulation.

to day 1, where genes were weakly expressed (**Figure 9B**). In this sense, all genes were highly upregulated after 2 days of cultivation in 20% glucose medium. More interestingly, the transcript levels of an osmosensor gene (BBAD15_g6196) were significantly upregulated at 20% glucose-enriched medium with a peak at day 2 and was maintained until day 3 of cultivation. Osmosensor gene expression levels initiated with an 8% glucose-amended medium and then continued to increase with the glucose gradient up to 20% (**Figure 9B**).

Furthermore, a high expression of two genes involved in regulating conidiation capacity was recorded in cultures grown with 20% glucose on day 2 compared to cultures grown at lower glucose levels (≤ 16% glucose) (**Figure 9B**). On day 3, the activity of endochitinase, beta-glucanase, and regulatory conidiation encoding genes decreased. Finally, analysis of gene BBAD15_g7126, which encodes for alternative oxidase (AOX), revealed a strong upregulation at all five glucose titers at day 2 and 3 of cultivation. Interestingly, gene regulation was slight or

null on day 1 of cultivation, but on day 2, all genes were mostly upregulated with pronounced induction of all genes in blastospores grown under 20% glucose on day 2. During day 3, expression levels declined for most genes, irrespective of glucose concentration (**Figure 9B**).

DISCUSSION

Dimorphic growth characterized by the hyphae-blastospores transition in filamentous (anamorphic) entomopathogenic fungi is intriguing, primarily on how environmental and nutritional cues under certain liquid culture conditions trigger this transitional development into blastospores. This dimorphism of hyphae-blastospores in the host hemolymph is also critical for pathogen virulence (Wang et al., 2008; Ortiz-Urquiza and Keyhani, 2013). Previous studies have asserted that the coupling of high oxygenation and high glucose concentrations remarkably boost *in vitro* submerged multiplication of blastospores of *B. bassiana*, *Cordyceps javanica* and *Metarhizium robertsii* compared to cultures grown with low to moderate levels of glucose and aeration (Mascarin et al., 2015a; Mascarin et al., 2015b; Mascarin et al., 2016; Jaronski and Mascarin, 2017; Iwanicki et al., 2019; Iwanicki et al., 2020b). As a result, this combination of high osmotic pressure and oxygenation in the medium leads to an increased cell weight yield of a culture primarily composed of blastospores in 2 to 3 days of cultivation. In this regard, our results sheds light on the metabolic pathways linked to blastospore formation in highly aerated, glucose-rich cultures induced by oxidative and osmotic stresses that result in increased blastospore yield of *B. bassiana*. A high specific growth rate accompanied this improved blastospore yield, revealing an essential role of hyperosmolarity imposed by high glucose rate on the accelerated growth kinetics of blastospores, mainly represented by smaller (shrinkage) cells compared to those produced with low titers of glucose (≤10%), as noted by Mascarin et al. (2015b). The underlying molecular mechanisms involved with intense transmembrane transport, intracellular carbohydrate metabolism, intense antioxidant activity due to oxidative stress, enriched HOG-signaling pathway in response to osmotic stress, and DNA replication/transcription are boosted in day 2 with 20% glucose. This is the period with a distinct log-growth phase reflected in higher numbers of blastospores compared to cultures grown in 10% glucose. Future efforts to identify the underlying molecular mechanisms of cell shrinkage, osmoregulation, and cell cycle machinery should be related to kinetic growth dynamics and the multiplication of blastospores.

Our results indicate that the culture condition responses on day 1 were more pronounced in blastopores grown in a hyperosmotic medium amended with 20% than with 10% glucose. Although no differences in blastospore yield were observed on day 1 cultivated in 10% and 20% glucose media, our results showed a high number of enriched biological processes in blastopores grown in 20% glucose. The processes are associated with sensing and signal transduction, transport of protons against an electrochemical gradient using energy from ATP hydrolysis, proteins, amino acids, carbohydrate transport, and fatty acids biosynthesis. To maintain the shape and redox balance in the cytosol for optimal functioning of biochemical reactions in hyperosmotic conditions, yeast and other filamentous fungi use signal transduction process to respond rapidly by increasing intracellular solute concentrations, such as free amino acids, sugars, and fatty acids (Cliquet and Jackson, 1999; Westfall et al., 2004; Ding et al., 2019). Consequently, the rapid increase in solute concentration in the cytosol requires high ATP consumption by cells, which does not contribute to biomass synthesis (Varela et al., 2004). In hypersaline conditions, the halophilic fungus *Aspergillus montevidensis* accumulate fatty acids, amino acids, and soluble sugars to keep osmotic balance (Ding et al., 2019). Therefore, our findings indicate the 20% glucose medium triggers a rapid metabolic response of blastospores to deal with the imbalance in redox state by exchanging ions and accumulating solutes as observed by (Westfall et al. (2004) and Salmerón-Santiago et al. (2011) regarding fungal stresses.

Although we determined blastospores cultivated on high glucose (20%) concentration activate several more metabolic responses than those grown on 10% glucose on the first day of culture, we did not observe statistically significant gene expression differences (log2-FC > 2 < -2, FDR $p < 0.005$) among several MAPK homologous to *B. bassiana* ARSEF 2860 (Liu et al., 2017) associated with the HOG pathway. However, some individual genes were upregulated when using lower thresholds in the 20% glucose medium, as identified in **Figure 8**. In this sense, we hypothesized blastospores grown in 10% and 20% glucose medium are both under osmotic stress and activate genes associated with the HOG pathway at similar intensity.

Besides MAPK proteins associated with the HOG pathway, other important groups of proteins involved in response to stress conditions are the heat shock proteins (Hsps). Here, we identified two Hsps homologs to *Ssb1* (BBAD15_g2252) and *Ssz1* (BBAD15_g4682) upregulated at day 1 compared to day 2 in blastospores grown in 20% and 10% glucose. In *B. bassiana* ARSEF 2860, *Ssz1* and *Ssb1* homologous play similar roles as *FgSsb* and *FgSsz* in *Fusarium graminearum* (Liu et al., 2017) to tolerate high osmolarity and heavy metal cations (Wang et al., 2020), while in *Magnaporthe oryzae*, homologous to HSP *Ssb1* (*MoSsb1*) and HSP *Ssz1* (*MoSsz1*) were shown to be crucial for cell wall integrity, pathogenicity, growth, and conidiation (Yang et al., 2018). Altogether, these results indicate *Hsp, Ssb1* and *Ssz1* may be associated with sensing osmotic stress in blastospores, especially on day 1, regardless of the two glucose concentrations tested. These *Hsp* were not differently expressed on day 2, regardless of glucose concentration, indicating blastospores successfully respond to external stimuli. However, on day 2, blastospores had upregulated a gene homologous to *Wsc1* (BBAD15_g1986) protein, regardless of glucose concentration. Tong et al. (2019) characterized the functionally of WSC domain-containing protein Wsc1 in *B. bassiana* describing it as responsible for sensing multiple stress cues upstream of the Hog1 signaling pathway. These findings indicate blastospores

might require genes homologous to the Wsc1 protein gene to deal with osmotic stress.

Our results from RNAseq analyses showed a remarkable variation in gene expression along the timeline of the experiment. Time accounted for 75% of the data variation, according to PCA-plot. Higher differences in gene expressions were observed on day 2 compared to day 1 of growth, regardless of glucose concentration. Enriched biological processes associated with DNA activities were observed in blastospores cultivated in 20% glucose on day 2. This can be related to intense cell growth and multiplication, as evidenced by *in vitro* experiments; statistically higher blastospore yield was confirmed in 20% glucose than in 10% glucose. This increase in blastospore yield in 20% glucose medium was also coupled with high upregulation of vacuolar calcium ion transporter (PF01699) and the sodium/potassium transporting ATPase (PF13246), as evidenced by the transcriptome and qRT-PCR results. Those genes are involved in the maintenance of cell homeostasis and were among the top 10 upregulated genes at day 2 in 20% glucose compared to 10% glucose.

We found blastospores cultivated in 10% and 20% glucose increased the expression of genes associated with oxidative stress responses more at day 2 than day 1. Corroborating this, a homologous gene to the sensing protein Wsc1 (BBAD15_g1986), localized in *B. bassiana* vacuoles and cell wall/membrane, was strongly upregulated at day 2 compared to day 1 in blastospores grown in both glucose concentrations. The deletion of this gene in a *B. bassiana* mutant led to a significant elevation in cell sensitivity to high oxidation, osmotic stress, and metal cations (Tong et al., 2019). At a moderate level, oxidative stress is an essential prerequisite for the growth and metabolism of several microorganisms including cell wall biosynthesis, cell proliferation, and morphogenesis (Nunes et al., 2005; Song et al., 2013; Iwanicki et al., 2020a). However, at high levels, free radicals' accumulation with high reactivity such as superoxide, oxygen radicals, and hydroxyl, becomes harmful to the cells' components. To cope with oxidative stress, blastospores activate enzymatic defense mechanisms by expressing enzymes such as superoxide dismutase, catalase, peroxidases, and oxidases. Several of these enzymes were upregulated at day 2. Moreover, we associated the response to oxidative stress as an essential component to boost blastospore production regardless of glucose concentration (Mascarin et al., 2015b; Iwanicki et al., 2020a). To some extent, the medium's osmotic stress mimics insect hemolymph, the natural environment conducive for blastospores formation, and mediates the HOG signaling pathway enabling cell osmoregulation and water homeostasis (Wang et al., 2008; Liu et al., 2017).

Filamentous fungal insect pathogens like *B. bassiana* usually grow by hyphal extension outside the host and propagate by yeast-like budding only after entry into the host hemocoel subsequent to cuticular penetration. This allows the fungus to rapidly colonize the host hemocoel and evade the host's immune responses. This dramatic change in morphology (phenotype) requires a dimorphic switch to regulate the hundreds of genes required for the transition. Since both aerial conidiation and dimorphic transition were closely linked to the transcriptional expression of the developmental activator genes (Zhang et al., 2019), it was hypothesized like aerial conidiation, the submerged dimorphic transition required for fungal virulence could be a process of asexual development to be primarily governed by the central pathway in *B. bassiana* and other filamentous fungal insect pathogens. These same authors unveiled *BrlA* and *AbaA* genes served as master regulators of both aerial conidiation and submerged blastospore production (dimorphic transition) (Zhang et al., 2019). In agreement with these findings, our qRT-PCR data revealed an increased transcript expression of genes responsible for the regulation of conidiation capacity (BBAD15_g9440 and BBAD15_g1987) in *B. bassiana* grown in liquid culture. This was mainly induced in blastospores produced in 20% glucose on day 2, thus hinting at their involvement in blastospores and conidia propagation. Further studies correlating the *BrlA* and *AbaA* genes with *B. bassiana* blastospore *in vitro* growth under high glucose titers should be addressed.

Carbon Catabolite Repression (CCR) is an important mechanism allowing preferential utilization of an energy-efficient and available carbon source over relatively less accessible carbon sources. This mechanism helps fungi to obtain the maximum amount of glucose for the energy invested. Fungi assimilate glucose and highly favorable sugars before switching to less suitable carbon sources such as organic acids and alcohols (Adnan et al., 2017). A glucose-repressible protein (BBAD15_g10730) examined in our qRT-PCR study was expressed in all glucose-amended *B. bassiana* cultures, but it was highly upregulated across all time points when the fungus was grown with 20% glucose. This result implies the catabolite repression pathway is strongly activated under high glucose titers required for increased and rapid production yields of *B. bassiana* blastospores in submerged liquid culture. Catabolite repression occurs at high glucose concentrations and is seen as the repression of synthesis of mitochondrial and other enzymes. Under these conditions, glucose is metabolized rapidly by glycolysis and considerable fermentation (anaerobic energy metabolism) occurs even under aerobic conditions. In other words, the NADH generated during glycolysis is oxidized by fermentation rather than by respiration, even though sufficient oxygen may be present. In this sense, the gene encoding for L-lactate dehydrogenase (BBAD15_g11432) analyzed by qRT-PCR was remarkably upregulated in blastospores cultured with 20% glucose by day 2, indicating the production of NAD+ (oxidized state), utilization of carbon sources, and maintenance of redox homeostasis during blastospores multiplication. The high metabolic activity of fungal blastospores cultivated with higher titers of glucose, in the range of 8-20%, increased up to day 3 and reflected the upregulation of alternative oxidase (AOX). The alternative oxidase (AOX) is a protein localized on the matrix side of the inner mitochondrial membrane. It plays a vital role in controlling reactive oxygen species (ROS), metabolic homeostasis, cellular energy demand, redox state, and stress response in fungal cells (Tian et al., 2020). Thus, AOX

expression, mainly for high metabolic activity in cultures with higher glucose titers, helps the fungus cope with oxidative and osmotic stresses during fungal growth in liquid culture. AOX genes have been closely associated with fungal pathogenesis, morphogenesis, stress signaling, and drug resistance (Tian et al., 2020).

One interesting observation was hydrophobin *Hyd1* as one of the highest expressed genes during the propagule production. Hydrophobins are involved in fungal spore coat rodlet layer assembly, consistent with it being a key member of the cell wall. Previous studies have suggested *Hyd1* is not found in blastospores based on protein extraction (Holder and Keyhani, 2005) and immunostaining (Zhang et al., 2011), which raises the possibility that these might be mischaracterized as blastospores and are actually "submerged conidia" (Bidochka et al., 1987). It is tempting to conclude these are "submerged conidia" based on the amount of hydrophobin present (*Hyd2* is also relatively highly expressed, although much less than *Hyd1* [data not shown]), and this rodlet layer is a hallmark of conidiogenic propagules (Zhang et al., 2011). In addition to the rodlet layer, the main distinguishing features of submerged conidia are size and shape (spherical 3-4 um), and germination speed (Bidochka et al., 1987). Blastospores are typically ellipsoidal and larger (~6 µm), germinate faster, and lack a rodlet layer (Bidochka et al., 1987). In this study, the cells produced in 10% glucose appear more similar to the blastospore description (**Figure 1D**). In comparison, the cells grown in 20% glucose are smaller and more spherical (**Figure 1D**), as previously documented by Mascarin et al. (2015b). It is important to note *Hyd1* was one of the top five genes expressed under all time points and treatments during this experiment. Additionally, the 10% and 20% glucose-grown cells likely contain significant and similar amounts of hydrophobin 1. One possibility is hydrophobin 1 was not accessible during the previous attempts to observe it in blastospores (Holder and Keyhani, 2005; Zhang et al., 2011). If hydrophobin 1 is found in blastospores, it should be expressed in the haemocoel during the infection process, which it has been observed in previous transcriptomic studies (Cho et al., 2007; Dong et al., 2017). The *Hyd1* promoter has even been used to deliver an insecticidal toxin during the infection process (Wang et al., 2013). Interestingly, a *Hyd1* strain knockout had similar virulence when injected directly into the haemocoel (Zhang et al., 2011). Alternatively, if these are concluded to be a form of blastospore, it suggests size and shape are not useful to cell types indicators. It would also indicates that blastospores or another cell type containing hydrophobin 1 is expressed in the haemocoel during the infection process. Additional studies are needed to determine if these propagules should be considered blastospores, submerged conidia, or a different morphology. It also highlights the utility of transcriptomics in understanding the differences in morphologies when applied to a culture pushed to one predominant morphology.

Finally, by mapping key genes involved in the osmotic stress response in *B. bassiana* growing under a hyperosmotic environment (**Figure 8**), we found implications of the HOG signaling pathway with upregulation of homologous genes *Ssk2* and *Hog1*. We hypothesize these two genes are associated with cell osmoregulation, leading to osmolyte synthesis and enhanced blastospore yield. The *Hog1* has also been linked to regulating environmental stresses in *B. bassiana* (Zhang et al., 2009). Deletion of the *Hog1* gene also reduced *Hyd1* expression by 46x, suggesting strong regulation of *Hyd1 via Hog1* (Zhang et al., 2009).

CONCLUSIONS

Our findings pave the way towards improving the fermentation process for *B. bassiana* and generates much-needed new knowledge on the primary metabolic processes and gene expression signatures involved in *B. bassiana* grown under osmotic and oxidative conditions with improved blastospore yields. Considering other hypocrealean entomopathogenic fungi, such as *Cordyceps* (formerly *Isaria*) and *Metarhizium*, have their blastospore production enhanced when grown with highly aerated and hyperosmotic liquid cultures, future comparative transcriptome studies should confirm the universal genetic regulations underlying this intriguing phenomenon. We envision the transcriptome tool allied with metabolomics will help improve the overall industrial production of blastospores and their metabolites for agricultural, pharmaceutical, and biotechnological purposes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163673.

AUTHOR CONTRIBUTIONS

CD, GM, and JLR conceived the experiments. GM, JLR, and CD conducted the experiments. All authors analyzed the results, prepared a manuscript draft, and edited the manuscript. All authors revised the manuscript for technical and scientific accuracy. JLR and CD acquired funding. CD and GM supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.644372/full#supplementary-material

Supplementary Table 1 | Primer sequences used for qRT-PCR gene expression analyses.

Supplementary Table 2 | Enriched Gene Ontology (GO), terms in *Beauveria bassiana* blastospores (between 20% and 10% within each culture day, and between culture days within each glucose concentration) resulted from Gene set enrichment analysis.

Supplementary Table 3 | Dataset with significantly differentially expressed genes between glucose concentrations within each culture day, and between culture days within each glucose concentration, based on log2-FC > 2 (upregulated) or < -2 (downregulated) with FDR at $p < 0.005$.

Supplementary Table 4 | Homologous (*e*-value < 0.0001 and sequence similarity > 80%) to 14 Hsp70 sequences, four MAPK sequences, and one sequence (Wsc1) obtained from Liu et al. (2017); Wang et al. (2020), and Tong et al. (2019), respectively, and 40 related-genes to osmotic stress in *B. bassiana* (Ortiz-Urquiza and Keyhani, 2016).

Supplementary Table 5 | The six highest expressed genes over all treatments based on total summed daily percentages.

REFERENCES

Adnan, M., Zheng, W., Islam, W., Arif, M., Abubakar, Y. S., Wang, Z., et al. (2017). Carbon Catabolite Repression in Filamentous Fungi. *Int. J. Mol. Sci.* 19 (1), 48. doi: 10.3390/ijms19010048

Alkhaibari, A. M., Carolino, A. T., Yavasoglu, S. I., Maffeis, T., Mattoso, T. C., Bull, J. C., et al. (2016). *Metarhizium brunneum* Blastospore Pathogenesis in *Aedes aegypti* Larvae: Attack on Several Fronts Accelerates Mortality. *PloS Pathog.* 12, e1005715. doi: 10.1371/journal.ppat.1005715

Alkhaibari, A. M., Lord, A. M., Maffeis, T., Bull, J. C., Olivares, F. L., Samuels, R. I., et al. (2018). Highly specific host-pathogen interactions influence *Metarhizium brunneum* blastospore virulence against *Culex quinquefasciatus* larvae. *Virulence* 9, 1449–1467. doi: 10.1080/21505594.2018.1509665

Bernardo, C. C., Barreto, L. P., Csr, E. S., Luz, C., Arruda, W., and Fernandes, E. K. K. (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*. *Ticks Tick Borne Dis.* 9, 1334–1342. doi: 10.1016/j.ttbdis.2018.06.001

Bernardo, C. D. C., Pereira-Junior, R. A., Luz, C., Mascarin, G. M., and Kamp Fernandes É, K. (2020). Differential susceptibility of blastospores and aerial conidia of entomopathogenic fungi to heat and UV-B stresses. *Fungal Biol.* 124, 714–722. doi: 10.1016/j.funbio.2020.04.003

Bidochka, M. J., Pfeifer, T. A., and Khachatourians, G. G. (1987). Development of the entomopathogenic fungus *Beauveria bassiana* in liquid cultures. *Mycopathologia* 99, 77–83. doi: 10.1007/BF00436909

Cho, E. M., Kirkland, B. H., Holder, D. J., and Keyhani, N. O. (2007). Phage display cDNA cloning and expression analysis of hydrophobins from the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. *Microbiol. (Reading)* 153, 3438–3447. doi: 10.1099/mic.0.2007/008532-0

Cliquet, S., and Jackson, M. (1999). Influence of culture conditions on production and freeze-drying tolerance of *Paecilomyces fumosoroseus* blastospores. *J. Ind. Microbiol. Biotechnol.* 23, 97–102. doi: 10.1038/sj.jim.2900698

Ding, X., Liu, K., Lu, Y., and Gong, G. (2019). Morphological, transcriptional, and metabolic analyses of osmotic-adapted mechanisms of the halophilic *Aspergillus montevidensis* ZYD4 under hypersaline conditions. *Appl. Microbiol. Biotechnol.* 103, 3829–3846. doi: 10.1007/s00253-019-09705-2

Dong, W. X., Ding, J. L., Gao, Y., Peng, Y. J., Feng, M. G., and Ying, S. H. (2017). Transcriptomic insights into the alternative splicing-mediated adaptation of the entomopathogenic fungus *Beauveria bassiana to* host niches: autophagy-related gene 8 as an example. *Environ. Microbiol.* 19, 4126–4139. doi: 10.1111/1462-2920.13862

Holder, D. J., and Keyhani, N. O. (2005). Adhesion of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana to* substrata. *Appl. Environ. Microbiol.* 71, 5260–5266. doi: 10.1128/AEM.71.9.5260-5266.2005

Humber, R. A. (2008). Evolution of entomopathogenicity in fungi. *J. Invertebr. Pathol.* 98, 262–266. doi: 10.1016/j.jip.2008.02.017

Iwanicki, N. S. A., Pereira, A. A., Botelho, A., Rezende, J. M., Moral, R. A., Zucchi, M. I., et al. (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. doi: 10.1038/s41598-019-38594-8

Iwanicki, N. S., Júnior, I. D., Eilenberg, J., and De Fine Licht, H. H. (2020a). Comparative RNAseq Analysis of the Insect-Pathogenic Fungus *Metarhizium anisopliae* Reveals Specific Transcriptome Signatures of Filamentous and Yeast-Like Development. *G3 (Bethesda)* 10, 2141–2157. doi: 10.1534/g3.120.401040

Iwanicki, N. S. A., Mascarin, G. M., Moreno, S. G., Eilenberg, J., and Delalibera Junior, I. (2020b). Growth kinetic and nitrogen source optimization for liquid culture fermentation of *Metarhizium robertsii* blastospores and bioefficacy against the corn leafhopper *Dalbulus maidis*. *World J. Microbiol. Biotechnol.* 36, 71. doi: 10.1007/s11274-020-02844-z

Jaronski, S. T., and Mascarin, G. M. (2016). "Mass Production of Fungal Entomopathogens," in *Microbial Control of Insect and Mite Pests*. ed. L.A. Lacey (Cambridge, MA: Academic Press), 141–155. doi: 10.1016/B978-0-12-803527-6.00009-3

Jaronski, S. T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl* 55, 159–185. doi: 10.1007/s10526-009-9248-3

Kirkland, B. H., Cho, E.-M., and Keyhani, N. O. (2004a). Differential susceptibility of *Amblyomma maculatum* and *Amblyomma americanum* (Acari:Ixodidea) to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Biol. Control.* 31, 414–421. doi: 10.1016/j.biocontrol.2004.07.007

Kirkland, B. H., Westwood, G. S., and Keyhani, N. O. (2004b). Pathogenicity of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to Ixodidae tick species *Dermacentor variabilis, Rhipicephalus sanguineus*, and *Ixodes scapularis*. *J. Med. Entomol.* 41, 705–711. doi: 10.1603/0022-2585-41.4.705

Liu, J., Wang, Z. K., Sun, H. H., Ying, S. H., and Feng, M. G. (2017). Characterization of the Hog1 MAPK pathway in the entomopathogenic fungus *Beauveria bassiana*. *Environ. Microbiol.* 19, 1808–1821. doi: 10.1111/1462-2920.13671

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mascarin, G. M., Jackson, M. A., Kobori, N. N., Behle, R. W., and Delalibera Junior, I. (2015a). Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. *J. Invertebr. Pathol.* 127, 11–20. doi: 10.1016/j.jip.2014.12.001

Mascarin, G. M., Jackson, M. A., Kobori, N. N., Behle, R. W., Dunlap, C. A., and Delalibera Junior, I. (2015b). 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. doi: 10.1007/s00253-015-6620-3

Mascarin, G. M., Jackson, M. A., Behle, R. W., Kobori, N. N., and Junior, I. D. (2016). Improved shelf life of dried *Beauveria bassiana* blastospores using convective drying and active packaging processes. *Appl. Microbiol. Biotechnol.* 100, 8359–8370. doi: 10.1007/s00253-016-7597-2

Nunes, L. R., Costa De Oliveira, R., Leite, D. B., Da Silva, V. S., Dos Reis Marques, E., Da Silva Ferreira, M. E., et al. (2005). Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. *Eukaryot. Cell* 4, 2115–2128. doi: 10.1128/ec.4.12.2115-2128.2005

Ortiz-Urquiza, A., and Keyhani, N. O. (2013). Action on the Surface: Entomopathogenic Fungi versus the Insect Cuticle. *Insects* 4, 357–374. doi: 10.3390/insects4030357

Ortiz-Urquiza, A., and Keyhani, N. O. (2016). "Molecular Genetics of Beauveria bassiana Infection of Insects," in *Advances in Genetics*. Eds. B. Lovett and R. J. S. Leger (Cambridge, MA: Academic Press), 165–249. 2016/05/01 ed.

Pham, T. A., Kim, J. J., Kim, S. G., and Kim, K. (2009). Production of Blastospore of Entomopathogenic *Beauveria bassiana* in a Submerged Batch Culture. *Mycobiology* 37, 218–224. doi: 10.4489/MYCO.2009.37.3.218

R Core Team (2020). R: A language and environment for statistical computing. In: *R Foundation for Statistical Computing* (Vienna, Austria). Available at: https://www.R-project.org/ (Accessed 2020).

Salmerón-Santiago, K. G., Pardo, J. P., Flores-Herrera, O., Mendoza-Hernández, G., Miranda-Arango, M., and Guerra-Sánchez, G. (2011). Response to osmotic stress and temperature of the fungus *Ustilago maydis*. *Arch. Microbiol.* 193, 701–709. doi: 10.1007/s00203-011-0706-9

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

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., 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, 15545–15550. doi: 10.1073/pnas.0506580102

Tian, F., Lee, S. Y., Woo, S. Y., and Chun, H. S. (2020). Alternative Oxidase: A Potential Target for Controlling Aflatoxin Contamination and Propagation of *Aspergillus flavus*. *Front. Microbiol.* 11, 419. doi: 10.3389/fmicb.2020.00419

Tomilova, O. G., Shaldyaeva, E. M., Kryukova, N. A., Pilipova, Y. V., Schmidt, N. S., Danilov, V. P., et al. (2020). Entomopathogenic fungi decrease *Rhizoctonia* disease in potato in field conditions. *PeerJ* 8, e9895. doi: 10.7717/peerj.9895

Tong, S. M., Wang, D. Y., Gao, B. J., Ying, S. H., and Feng, M. G. (2019). The DUF1996 and WSC domain-containing protein Wsc1I acts as a novel sensor of multiple stress cues in *Beauveria bassiana*. *Cell Microbiol.* 21, e13100. doi: 10.1111/cmi.13100

Varela, C. A., Baez, M. E., and Agosin, E. (2004). Osmotic stress response: quantification of cell maintenance and metabolic fluxes in a lysine-overproducing strain of *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 70, 4222–4229. doi: 10.1128/aem.70.7.4222-4229.2004

Wanchoo, A., Lewis, M. W., and Keyhani, N. O. (2009). Lectin mapping reveals stage-specific display of surface carbohydrates in in vitro and haemolymph-derived cells of the entomopathogenic fungus *Beauveria bassiana*. *Microbiol. (Reading)* 155, 3121–3133. doi: 10.1099/mic.0.029157-0

Wang, C., Duan, Z., and St Leger, R. J. (2008). MOS1 osmosensor of *Metarhizium anisopliae* is required for adaptation to insect host hemolymph. *Eukaryot. Cell* 7, 302–309. doi: 10.1128/ec.00310-07

Wang, Z. L., Ying, S. H., and Feng, M. G. (2013). Recognition of a core fragment of *Beauveria bassiana* hydrophobin gene promoter (P hyd1) and its special use in improving fungal biocontrol potential. *Microb. Biotechnol.* 6, 27–35. doi: 10.1111/j.1751-7915.2012.00351.x

Wang, J., Chen, J., Hu, Y., Ying, S. H., and Feng, M. G. (2020). Roles of six Hsp70 genes in virulence, cell wall integrity, antioxidant activity and multiple stress tolerance of *Beauveria bassiana*. *Fungal Genet. Biol.* 144, 103437. doi: 10.1016/j.fgb.2020.103437

Westfall, P. J., Ballon, D. R., and Thorner, J. (2004). When the stress of your environment makes you go HOG wild. *Science* 306, 1511–1512. doi: 10.1126/science.1104879

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

Yang, Z., Jiang, H., Zhao, X., Lu, Z., Luo, Z., Li, X., et al. (2017). Correlation of cell surface proteins of distinct *Beauveria bassiana* cell types and adaption to varied environment and interaction with the host insect. *Fungal Genet. Biol.* 99, 13–25. doi: 10.1016/j.fgb.2016.12.009

Yang, J., Liu, M., Liu, X., Yin, Z., Sun, Y., Zhang, H., et al. (2018). Heat-Shock Proteins MoSsb1, MoSsz1, and MoZuo1 Attenuate MoMkk1-Mediated Cell-Wall Integrity Signaling and Are Important for Growth and Pathogenicity of *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* 31, 1211–1221. doi: 10.1094/mpmi-02-18-0052-r

Zhang, Y., Zhao, J., Fang, W., Zhang, J., Luo, Z., Zhang, M., et al. (2009). Mitogen-activated protein kinase hog1 in the entomopathogenic fungus *Beauveria bassiana* regulates environmental stress responses and virulence to insects. *Appl. Environ. Microbiol.* 75, 3787–3795. doi: 10.1128/aem.01913-08

Zhang, S., Xia, Y. X., Kim, B., and Keyhani, N. O. (2011). Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. *Mol. Microbiol.* 80, 811–826. doi: 10.1111/j.1365-2958.2011.07613.x

Zhang, A.-X., Mouhoumed, A.-Z., Tong, S.-M., Ying, S.-H., and Feng, M.-G. (2019). BrlA and AbaA Govern Virulence-Required Dimorphic Switch, Conidiation, and Pathogenicity in a Fungal Insect Pathogen. *mSystems* 4, e00140–e00119. doi: 10.1128/mSystems.00140-19

Zhou, Y. H., Zhang, Y. J., Luo, Z. B., Fan, Y. H., Tang, G. R., Liu, L. J., et al. (2012). Selection of optimal reference genes for expression analysis in the entomopathogenic fungus *Beauveria bassiana* during development, under changing nutrient conditions, and after exposure to abiotic stresses. *Appl. Microbiol. Biotechnol.* 93, 679–685. doi: 10.1007/s00253-011-3561-3

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Survival of the Sawfly *Athalia rosae* Upon Infection by an Entomopathogenic Fungus and in Relation to Clerodanoid Uptake

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Larvae of the turnip sawfly *Athalia rosae* are a pest of Brassicacae plants, as their feeding can cause defoliation of various crops of economic importance. The larvae and the adults of this sawfly species are known to take up different classes of chemical compounds from their respective host plants, with potentially deterrent functions against predators. In addition, compounds taken up by the adults, the clerodanoids, are known for their antimicrobial activity. These features could be a challenge to biocontrol strategies. Several natural enemies of *A. rosae* have been identified, targeting larval and pupal stages of *A. rosae*, which could potentially be used as biocontrol agents. However, targeting the adult stage of a larval pest in addition to targeting the juvenile stages may improve population control. In this study, we ask whether a strain of the entomopathogenic fungus *Beauveria bassiana* shows biological activity against *A. rosae* adults. We also investigate whether the behavior of clerodanoid uptake by the adults, which is commonly found, affects their survival in response to a *B. bassiana* exposure. We found a clear dose-response relationship, i.e., with increasing fungal conidia concentrations survival of *A. rosae* decreased. However, there was only a low incidence of mycelial growth and sporulation from *A. rosae* cadavers, indicating that either the fungus is not successfully developing inside this host, or it is not able to re-emerge from it. Clerodanoid uptake decreased the survival of healthy adults; however, it did not increase their survival to *B. bassiana*. Our results revealed that this strain of *B. bassiana* if applied alone is probably not suitable for biocontrol of this sawfly species, because *A. rosae* showed a high baseline resistance against this fungus. The behavior of clerodanoid uptake is unlikely to have evolved as a defense against this entomopathogenic fungus.

Keywords: *Athalia rosae*, *Beauveria bassiana*, *Ajuga reptans*, clerodanoids, pharmacophagy, phytochemicals, chemical ecology

INTRODUCTION

Insect herbivores can cause tremendous losses in agriculture, when feeding as larvae or adults on diverse plant parts of crops (Zalucki et al., 2012; Sharma et al., 2017). Natural enemies of insect pests are used as effective control agents against adult as well as juvenile stages of their herbivorous hosts (Kuhar et al., 2000; Laznik et al., 2010; Boivin et al., 2012). However, most examples of biological pest control involve targeting juvenile stages of insect pests. For example, the effectiveness of the most widely used biopesticide to date, *Bacillus thuringiensis*, decreases with increasing developmental stage of the insect host (Glare and O'Callaghan, 2000). Likewise, baculoviruses, applied mainly against lepidopteran pest species, target larval hosts (Inceoglu et al., 2001). In many cases, juvenile stages might be more accessible to a biocontrol agent, since they are the stages which aggregate in high densities in crop fields, and have a high feeding rate (Ohnesorge, 1979; Ferro et al., 1983). Manipulating the adult population has been shown to be an effective method of reducing the damage to crops (Hunt and Vernon, 2001; Cotes et al., 2018). However, adults are often the dispersing stage and might be harder to target (Vallat and Dorn, 2005; Lombaert et al., 2006). Another obstacle to the use of parasites and pathogens against adult insect pests is that several microbial control agents, such as *B. thuringiensis* and baculoviruses, need to be taken up orally by species which might not feed in the adult stage or feed on a food source which might not be a suitable vector for a biocontrol agent (Glare and O'Callaghan, 2000), preventing the uptake of oral pathogens.

This setback is circumvented by entomopathogenic fungi, which infect their host by penetrating through its cuticle. In the case of *Beauveria bassiana* (Ascomycota: Hypocreales), the conidia attach, germinate and penetrate the cuticle of a broad range of insect hosts (Ortiz-Urquiza and Keyhani, 2013). When the hemolymph of the host is reached, the fungus differentiates into yeast-like cells called blastospores, which proliferate in the insect's body cavity and use its nutrients. After host death, hyphae germinate, colonize host tissues, catabolize their remaining nutrients and breach the cuticle from the inside. After colonizing the insect surface and if the environmental conditions allow it, conidia will sporulate and complete the life-cycle of the fungus (Ortiz-Urquiza and Keyhani, 2013; Pedrini et al., 2013). The ease of production of *B. bassiana* on an industrial scale (Santa et al., 2005) as well as the fact that this is a necrotrophic parasite which needs to kill its host to complete its life-cycle made it a microorganism of interest in the context of biological control and integrated pest management (Lacey et al., 2015). However, its application in the field faces several challenges: Its speed of killing is sometimes considered as too slow to prevent significant damage to the crops by feeding insects (Inglis et al., 2001), and its lethality might also decrease as a result of environmental conditions (Rangel et al., 2008) and host defenses (Maistrou et al., 2018). Insect behavior can be an important line of defense against entomopathogenic fungi (Bonadies et al., 2019) including *B. bassiana* (Meyling and Pell, 2006), which might further jeopardize the success of biocontrol strategies.

The turnip sawfly, *Athalia rosae* (Hymenoptera: Tenthredinidae), is a feeding specialist on plants of Brassicaceae. The larvae feed on leaves and flowers (Bandeili and Müller, 2010) and can cause serious losses on crops of economic importance (Sawa et al., 1989), as they often occur in high densities, leading to complete defoliation (Riggert, 1939). The control of this pest includes the use of insecticides for coating seeds of Brassicacae, as well as for later pulverization in the field (Daniel and Ion, 2019). As an alternative or complement to chemical pesticides, several natural enemies of *A. rosae* have been described, including nematodes and insect parasitoids (ichneumonids, tachinids, and chalcidids), which exclusively infest the larval stages (Riggert, 1939). The adults of *A. rosae* feed on nectar of various plants, mostly belonging to the Apiaceae. In addition, adult males and females visit different plants of the Lamiales, on which they do not cause any visible damage but take up *neo*-cleradane diterpenoids (in the following called clerodanoids; Nishida et al., 2004). These compounds act as feeding stimulants (Opitz et al., 2012), but also affect the mating behavior, making particularly females more successful once they have had access to clerodanoids (Amano et al., 1999). Moreover, adults that have incorporated these compounds are better protected against diverse predators and seem to be bitter tasting (Nishida and Fukami, 1990). Thus, adults are pharmacophagous, as they take up these compounds for purposes other than nutrition, a behavior also called self-medication (Abbott, 2014). Clerodanoids isolated from different Lamiaceae are known to have antimicrobial activity *in vitro* (Bozov et al., 2015). However, it is to our knowledge unknown yet whether exposure to clerodanoids *via* uptake from leaves can also act antimicrobial *in vivo*.

Efforts to control the populations of *A. rosae* focus on egg and larval stages, probably because of their ease of collection in the field (Sáringer et al., 1996; Kamangar et al., 2010). However, controlling adults while they aggregate on plants for clerodanoid uptake and reproduction may reduce the number of ovipositing adult females on crop fields. Moreover, it is yet unknown whether entomopathogenic fungi could be an option for the control of *A. rosae* populations. In this study, we investigated whether *B. bassiana* could be a prospective biocontrol agent against adult *A. rosae*. Additionally, we investigated whether the lethality of the fungus is influenced by the behavior of clerodanoid uptake by the sawflies.

MATERIALS AND METHODS

Insect Maintenance

Adults of the sawfly *A. rosae* were collected from Apiaceae in the surroundings of Bielefeld, Germany, and reared in the laboratory for several generations. Adults were provided with potted host plants of *Sinapis alba* for oviposition. Larvae were offered *S. alba* and *Brassica rapa* var. *pekinensis* for feeding. The host plants were grown in the greenhouse (about 20°C, 70% r.h., 16 h:8 h light:dark) and offered to the insects at a pre-flowering stage, when about 5–6 weeks old. The insects were kept on their host plants in large mesh cages

(60 × 60 × 60 cm) at room temperature and 16 h:8 h light:dark until the last larval instar, the eonymphs, crawled into the soil. Pots with soil and eonymphs were removed to containers with a gauze-lid. Once adults emerged, they were collected and kept in groups of up to 10 adults in Petri dishes (9 cm diameter) lined with filter paper and a source of honey-water mixture (1:50) in a refrigerator until use.

Clerodanoid Exposure

Within 2 days after adult hatching, adult males and females were individually placed in small Petri dishes (5 cm diameter) lined with slightly moistened filter paper and placed at room temperature. Throughout the experiment, we did not further consider the sex of the animals but randomly distributed males and females into all treatments. They were provided with the honey-water mixture and half of the individuals were offered a small piece (1 × 1 cm) of a leaf of *Ajuga reptans* (Lamiaceae). Plants used for this experiment were kept outside in pots and were flowering. Adults usually start within less than 20 min to voraciously "nibble" on the leaves (Müller, personal observation) and take up clerodanoid compounds in that way (Nishida et al., 2004; Paul et al., 2021). We refer to this experimental manipulation as "exposure treatment." After 48 h, the leaf was removed from the Petri dish and the bioassay was initiated (see below). Even though we do not have a direct quantification of the amount of compounds taken up after nibbling on the leaves, we know that nibbling on *A. reptans* leaves for a short time leads to qualitative changes in the chemical profiles of adults. After such exposure, adults become attractive to their conspecifics, which exhibit a nibbling behavior only on exposed but not on non-exposed conspecifics (Paul et al., 2021). We observed this behavior toward insects exposed to clerodanoids in our experiment, but not toward insects of the non-exposed treatment.

Culture of *Beauveria bassiana* and Condidia Suspension

A *Beauveria bassiana* strain (KVL 03-122) was collected from an agroecosystem in Denmark (Meyling et al., 2009) isolated from *Leptoterna dolobrata* (Homoptera: Miridae). It was kept at −80°C in a culture collection at University of Copenhagen before cultivation. Isolates were cultivated on quarter-strength Sabouraud Dextrose Agar + Yeast 10% (SDAY) and incubated for 10 days at 23°C to allow for sporulation. Conidia were collected by scraping the surface of the culture with a sterile loop and transferred in sterile 1 ml phosphate buffered saline (PBS) with 0.05% of Triton-X. The resulting solution was centrifuged twice at 23°C, 4000 rpm for 3.5 min and the supernatant discarded in order to remove agar and hyphae. The pellet was resuspended in 1 ml PBS/Triton-X 0.05%, and the conidia concentration assessed with a hemocytometer (Neubauer Improved). The concentration of the inoculum was adjusted through serial dilutions before exposing the adults of *A. rosae*.

We chose to expose the sawflies to 2.10^7, 2.10^8, and 2.10^9 conidia/ml. In a preliminary assay, 2.10^6 conidia/ml did not cause any mortality significantly different from control insects (**Supplementary Figure S1**). We, therefore, chose to focus our sampling efforts on the three aforementioned inoculum concentrations.

After each infection bout (replicate), the germination rate of the inoculum was assessed by plating 100 µl of a 10^5 conidia/ml solution on quarter strength SDAY 10% plates. After incubation for 24 h at 23°C, the germination of 3 × 100 conidia was counted to ensure that the germination rate or the inoculum was higher than 90%.

We chose the strain KVL 03-122 based on previous bioassays performed on *Tenebrio molitor* (Coleoptera: Tenebrionidae), which showed that KVL 03-122 was one of the most virulent strains we had access to, maybe because of its fast germination on the insect cuticle (unpublished data). Its host range is unknown but is likely to be broad, since it infects species of different orders, including beetles and mirids. Whether it has been in contact with populations of *A. rosae* in the field is unknown, but the ecology of *B. bassiana* makes the existence of an adaptation to the latter unlikely (Ortiz-Urquiza and Keyhani, 2013). Another strain, KVL 03-144, showing an intermediate virulence to *T. molitor*, did not cause a mortality significantly different from control animals in a preliminary experiment on *A. rosae* despite the relatively high concentration of the inoculum (2.10^8 conidia/ml, **Supplementary Figure S2**).

Bioassay

Adults with or without clerodanoid exposure were transferred individually from their Petri dishes to medicine cups (Carl Roth, Karlsruhe, Germany) containing 2 × 2 cm filter paper dipped in a filter-sterilized solution of honey in distilled water (1:50 v:v).

One microliter of conidia suspension of a given concentration was applied with a micropipette laterally where the legs are connected to the thorax, in order to facilitate the adsorption of the droplet during the movement of the adults. As a control, individuals received an application of PBS/Triton-X 0.05% only (referred to as "Triton" treatment). Care was taken that the whole droplet was adsorbed on the cuticle before including each individual in the experiment and closing the lid of the medicine cup. The medicine cups of a whole replicate were then kept in an incubator at 20°C and with a natural photoperiod. Every 2nd day, we transferred the individuals into a clean medicine cup with a fresh filter paper soaked in honey water. The experiments were carried out between April and May 2020. The day of inoculation was counted as day 0, and we refer to this experimental manipulation as "inoculation treatment." Per treatment combination, between 17 and 34 individuals were set up (**Table 1**).

Individual sawflies were checked daily for movement, in which case, they were scored as alive. On the contrary, individuals lying at the bottom of the cup and showing no leg movement, or individuals showing a paralyzed and dehydrated look (characteristic of the death by *B. bassiana*) were scored as dead. Dead individuals were transferred into a new sterile medicine up and kept with a 3 × 3 cm piece of filter paper dipped in filter-sterilized distilled water, and kept in similar conditions. We checked daily for the re-emergence of the mycelium from the cadavers for 20 days.

TABLE 1 | Number of adult sawflies, which where either exposed or not to the plant *Ajuga reptans*, for uptake of clerodanoids, and then inoculated with different concentrations of *B. bassiana* conidia.

Inoculation treatment in number of conidia per ml	Exposure treatment	
	Non clerodanoids	Clerodanoids
Triton	32	34
2.10^7	20	19
2.10^8	17	22
2.10^9	19	25

Each fungal inoculation treatment concentration was performed twice in two separate inoculation sessions (replicates). Due to the number of insects we could obtain for each inoculation session, we could test only two concentrations simultaneously, alongside the corresponding Triton control. Therefore, the number of insects is higher in the Triton inoculation treatment.

Statistics

All statistical analyses and graphical representations were performed with the R software (R Core Team, 2013).

The survival of *A. rosae* when inoculated with different concentrations of conidia showed non-proportional hazards. We analyzed the survival data with an accelerated failure time model fitted for a Weibull distribution with the "survreg" function of the "survival" package (Therneau and Grambsch, 2000; Therneau, 2015). Between-group comparisons were performed using the method of contrasts (Crawley, 2007).

Regarding the proportion of sawfly individuals from which we could see mycelium germinating on the external surface of the cadaver, we analyzed presence or absence of mycelium on the cadaver with a generalized linear model (GLM) fitted for a binomial distribution, with presence or absence of mycelium being the response variable, and the clerodanoid exposure (or no exposure) and concentration of the inoculum of conidia as explanatory variables. Since the adults inoculated with Triton never showed any mycelial growth on their surface after death, we excluded them from the analysis.

For both analyses, we started with the most complex models including two-way interactions between the inoculation and the exposure treatments as well as the inoculation session as a frailty term in the survival analysis, and as a random factor. We performed model selection by comparing Akaike's information criterion (AIC) of the full models and all possible nested models, including the null one. We kept as optimal models the ones with the lowest AICs (Akaike, 1976). The figures were made with the "ggplot2" and "survminer" packages (Wickham, 2016; Kassambara and Kosinski, 2018).

RESULTS

Survival of *Athalia rosae* Adults to *Beauveria bassiana* KVL 03-122 and in Depedence of Clerodanoid Exposure

The mortality of the experimental sawflies population showed a clear dose response to the concentration of conidia present in the inoculum, with the longevity of the sawflies decreasing with increasing concentrations of the inoculum between the treatment groups (**Figure 1**; **Supplementary Table S3** for *post hoc* comparisons).

The effect of clerodanoid exposure on the survival of *A. rosae* adults over 14 days differed depending on the inoculation with *B. bassiana* conidia (inoculation treatment*exposure treatment: deviance = 10.13; $p = 0.018$; df = 9,182). Individuals that were exposed to clerodanoids prior to inoculation with the non-infectious control (0.05% Triton X-PBS) died after a shorter time compared to those not exposed to clerodanoids. In contrast, when the individuals were inoculated with conidia of *B. bassiana*, there was no difference between individuals exposed to clerodanoids or not (**Figure 1**; **Supplementary Table S3** for *post hoc* comparisons).

Sporulation of *Beauveria bassiana* From Adult Sawfly Cadavers

We found a very low incidence of mycelial growth and sporulation from cadavers of *A. rosae* that had been inoculated with different concentrations of conidia. The proportion of cadavers from which fungal sporulation occurred was between 10 and 26% in the different experimental groups (**Figure 2**). Among the adult sawflies inoculated with conidia, neither the clerodanoid exposure nor the concentration of conidia of the inoculum explained the incidence of mycelial growth on the cadavers (inoculation treatment*exposure treatment: deviance = 2.51; $p = 0.28$; df = 5,119; inoculation treatment: deviance = 0.4; $p = 0.82$; df = 2,122; exposure treatment: deviance = 0.43; $p = 0.51$; df = 1,123). As expected, no mycelium germinated on the external surface of adults not exposed to conidia.

DISCUSSION

This study aimed at assessing the virulence of a *B. bassiana* strain against adult sawflies of *A. rosae*, which are known to expose themselves to clerodanoids in their natural environment. Manipulating both the amount of conidia the sawfly individuals received and whether or not they were allowed to take up clerodanoids enabled us to gather data on both the sawfly-fungus interaction and the potential benefit of clerodanoid uptake.

The inoculum concentrations of *B. bassiana* we had to use to induce mortality significantly higher than in control individuals of *A. rosae* were relatively high compared to lethal concentrations used in other insect-*B. bassiana* interactions (Maistrou et al., 2018, 2020). This indicates that adults of *A. rosae* have a rather high baseline resistance against this fungus. This species is indeed often confronted with entomopathogenic fungi, as the larvae bury themselves in the soil, where they pupate. Thus, also the emergence of the young adults happens in the soil, where entomopathogenic fungi are omnipresent (Clifton et al., 2015). This constant pressure could explain the evolution of a high level of constitutive resistance against such pathogens in *A. rosae*.

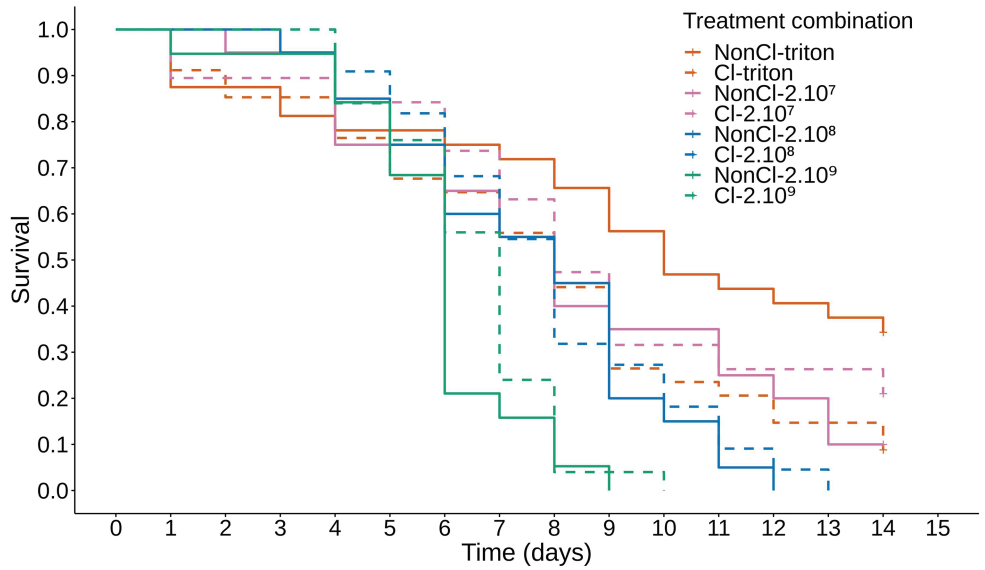

FIGURE 1 | Survival of *Athalia rosae* adults inoculated with *Beauveria bassiana* and depending on clerodanoid exposure. Kaplan-Meier curve showing the proportion of live individuals over 14 days of *A. rosae* adults inoculated with several concentrations of conidia of *B. bassiana* strain KVL 03-122. Orange lines: 0.05% Triton X-PBS (triton); pink lines: 2.10^7; blue lines: 2.10^8; green lines: 2.19^9 conidial/ml. Dashed lines represent adults allowed to take up clerodanoids (Cl) from a leaf of *Ajuga reptans* prior to inoculation (NonCl); survival of adults treated with Cl-triton was significantly different from those of the NonCl-triton treatment. There was no difference between survival of Cl and NonCl individuals in the other inoculation treatments with different concentrations of *B. bassiana*. Survival decreased with increasing concentrations of the inoculation treatment. See **Supplementary Table S3** for *post hoc* comparisons.

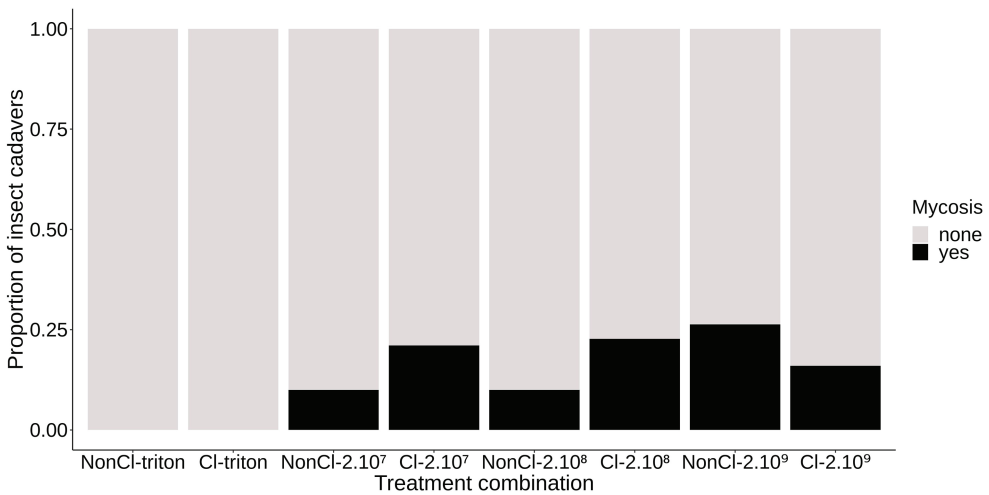

FIGURE 2 | Mycelial colonization of the cadavers of *A. rosae*. Stacked bar graph representing the proportion of *A. rosae* cadavers showing mycelial colonization, i.e., mycosis (yes) or not (none) by *B. bassiana* KVL 03-122 depending on the inoculation and exposure treatments: Non-Cl, no clerodanoids exposure, Cl, clerodanoids exposure; triton, 2.10^7, 2.10^8, and 2.10^9 represent the inoculation control and the inoculum concentration in conidial/ml. Neither the inoculation nor the clerodanoids exposure treatments significantly affected the proportion of cadavers showing mycelial colonization.

Another surprising result is that very few cadavers supported the re-emergence of our strain of *B. bassiana*. The mycosis developing at the surface of the insect cadaver is often used by invertebrate pathologists as a means of confirming that the insect died of the inoculated fungus instead of dying of a co-occuring opportunistic infection (Goettel and Inglis, 1997). In this context, it may seem that very few sawflies directly died of our strain of *B. bassiana*. However, this is unlikely, because their survival showed a clear dose-response to the concentration of conidia in the inoculum. Combined with the fact that we needed to inoculate the sawflies with a high dose of conidia, this confirms, as proposed by Maistrou et al. (2020),

that the ability of one strain of *B. bassiana* to develop mycosis on the cadaver of the host is a fitness trait, which is correlated with other virulence traits, such as its ability to kill the host. It has been proposed that the ability to cause mycosis on the insect cadaver relies strongly on the ability to outcompete bacterial growth after host death (Fan et al., 2017). More experiments are required to explore this hypothesis in *A. rosae* adults in order to know whether this can explain a significant reduction in lifespan of the individuals compared to control with a low occurrence of mycosis on cadavers.

Interestingly, we observed no protective effect of the prior exposure of individual sawflies to clerodanoids, neither in the presence, nor absence of the fungus. This is an unexpected result considering that some clerodanoids show antifungal activity (reviewed by Li et al., 2016; Qing et al., 2017). The lack of a protective effect in this interaction suggests that the *in vitro* antimicrobial activity reported for some clerodanoids does not necessarily translate into a better protection against a potential pathogen *in vivo*. The activity may thus depend on the specific structure of the clerodanoids or other factors that modulate the insect's physiology when incorporating compounds from *A. reptans*. There was moreover a slightly negative effect of clerodanoid uptake on survival of sawflies in the absence of fungal inoculation, as these insects showed a lower survival than the control. This corroborates results found by Kubo et al. (1982) who found insecticidal activities of two ajugarins, which are clerodanoids from *Ajuga* plants. Clerodanoids have been shown to affect insect physiology both by cuticular contact and feeding (Abbaszadeh et al., 2012). Thus, clerodanoid uptake may also come with some costs.

In contrast to the slightly negative effect of clerodanoids on adult survival, clerodanoid uptake has been shown to increase the mating success of female *A. rosae* (Amano et al., 1999), indicating a fitness benefit. A trade-off between reproduction and longevity has been observed in several insect species (Ellers, 1995; Hunt et al., 2006), and pharmacophagous uptake of plant compounds may have both costs and benefits (Erb and Robert, 2016). Moreover, in our study, the sawflies were not allowed to mate and had no opportunity to lay any eggs, as no host plant was provided. This may have affected their longevity as well. The longevity of the control animals treated with 0.05% Triton-X only was highly similar to the life-span observed for *A. rosae* in other studies, in which adult longevity had been investigated in dependence of the larval host plant quality (Müller and Sieling, 2006: on average 14 days; Bandeili and Müller, 2010: ranging from 9 to 17 days). Considering this relatively short adult lifespan of this species, the ability to reproduce during this time window is crucial for the individuals. Further studies should assess the effects of clerodanoid uptake on other life history traits such as fecundity and its relationship to longevity.

A negative effect of clerodanoids on the survival was not visible when the insects were inoculated with conidia, indicating that the deleterious effect of *B. bassiana* overwrites a potential negative effect of clerodanoids on *A. rosae*. In our system, we could not control for the amount of clerodanoids taken up by the adult sawflies during exposure. However, as *A. rosae* start within a very short time to nibble on leaves and as they had access to a leaf for 48 h, we can ensure that all insects had sufficient chance to take up clerodanoids. This was confirmed by the characteristic nibbling behavior conspecifics exhibited toward insects of the clerodanoids-exposed treatment. Adult sawflies could have either incorporated plant compounds and/or spread some over the surface of their cuticle when contacting the leaves or while cleaning themselves (Paul et al., 2021). Further experiments are needed to determine the exact location of clerodanoids in and/or on the individual sawflies.

Overall, our data suggest that the behavior of clerodanoid uptake did not evolve as a means to increase survival to an exposure to entomopathogenic fungi, at least not toward this *B. bassiana* strain. Since its deleterious effect on control individuals represents a cost which might be non-negligible in natural populations, we can assume that other benefits selected for the evolution and persistence of this trait in *A. rosae*. The above-mentioned benefit in mating and a reduction in the predation by birds and reptiles in insects which had taken up clerodanoids are some of these benefits (Nishida and Fukami, 1990).

To conclude, our study did not reveal our strain of *B. bassiana* to be a good candidate as a biocontrol agent of *A. rosae*, since it was only able to cause significant mortality at high doses. More importantly, our strain was not able to cause mycosis on the cadavers. This means that in the eventuality of a massive release of conidia of this strain on *A. rosae* populations, the fitness of the fungus would be almost zero, since the persistence of *B. bassiana* in the environment has been shown to rely mainly on conidia production (Vänninen, 1995). In case of a massive release of conidia for biocontrol, the lack of their persistence in the environment would prevent the infection of non-target species, as well as prevent the disruption of the microbial community of the soil, or select for resistance in pest populations. A suitable strategy may be to test other strains of *B. bassiana* in order to identify more virulent ones or some in which virulence may be decoupled from the ability to cause mycosis. Another lead would be to combine *B. bassiana* with cuticle-disrupting agents, like entomopathogenic nematodes or diatomaceous earth, which are known to have a synergistic effect with *B. bassiana* on host mortality (Barberchek and Kaya, 1990; Riasat and Wakil, 2011). These agents might increase host death without enabling the fungus to compete against bacteria after host death. Finally, the pharmacophagous uptake of clerodanoids did not reveal to improve resistance of *A. rosae* individuals against this fungus but may be an effective self-medication against other microorganisms and pathogens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is provided in the **Supplementary Table S4**.

AUTHOR CONTRIBUTIONS

CZ, JK, and CM conceived the experiments. CZ, LL, RR, and IM performed the experiments. CZ analyzed the data. CZ and CM wrote the manuscript, with inputs from LL, RR, and JK. CM and JK acquired funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.637617/full#supplementary-material

REFERENCES

Abbaszadeh, G., Srivastava, C., and Walia, S. (2012). Insect growth inhibitory activity of clerodane diterpenoids isolated from *Clerodendron infortunatum* L. on the cotton bollworm, *Helicoverpa armigera* (Hubner). *Natl. Acad. Sci. Lett.* 35, 457–464. doi: 10.1007/s40009-012-0077-z

Abbott, J. (2014). Self-medication in insects: current evidence and future perspectives. *Ecol. Entomol.* 39, 273–280. doi: 10.1111/een.12110

Akaike, H. (1976). "Canonical correlation analysis of time series and the use of an information criterion" in *System identification advances and case studies*. eds. R. K. Mehra and D. G. Lainiotis (New York, NY, USA: Academic Press Inc.).

Amano, T., Nishida, R., Kuwahara, Y., and Fukami, H. (1999). Pharmacophagous acquisition of clerodendrins by the turnip sawfly (*Athalia rosae ruficornis*) and their role in the mating behaviour. *Chemoecology* 9, 145–150. doi: 10.1007/s000490050046

Bandeili, B., and Müller, C. (2010). Folivory versus florivory—adaptiveness of flower-feeding. *Naturwissenschaften* 97, 79–88. doi: 10.1007/s00114-009-0615-9

Barberchek, M. E., and Kaya, H. K. (1990). Interactions between Beauveria bassiana and the entomogenous nematodes, Steinernema feltiae and Heterorhabditis heliothidis. *J. Invertebr. Pathol.* 55, 225–234. doi: 10.1016/0022-2011(90)90058-E

Boivin, G., Hance, T., and Brodeur, J. (2012). Aphid parasitoids in biological control. *Can. J. Plant Sci.* 92, 1–12. doi: 10.4141/cjps2011-045

Bonadies, E., Wcislo, W. T., Gálvez, D., Hughes, W., and Fernández-Marín, H. (2019). Hygiene defense behaviors used by a fungus-growing ant depend on the fungal pathogen stages. *Insects* 10:130. doi: 10.3390/insects10050130

Bozov, P., Girova, T., Prisadova, N., Hristova, Y., and Gochev, V. (2015). Antimicrobial activity of neo-clerodane diterpenoids isolated from Lamiaceae species against pathogenic and food spoilage microorganisms. *Nat. Prod. Commun.* 10, 1797–1800. doi: 10.1177/1934578X1501001101

Clifton, E. H., Jaronski, S. T., Hodgson, E. W., and Gassmann, A. J. (2015). Abundance of soil-borne entomopathogenic fungi in organic and conventional fields in the midwestern USA with an emphasis on the effect of herbicides and fungicides on fungal persistence. *PLoS One* 10:e0133613. doi: 10.1371/journal.pone.0133613

Cotes, B., Rämert, B., and Nilsson, U. (2018). A first approach to pest management strategies using trap crops in organic carrot fields. *J. Crop Prot.* 112, 141–148. doi: 10.1016/j.cropro.2018.05.025

Crawley, M. J. (2007). *The R book*. Chichester, England: John Wiley & Sons.

Daniel, R. A., and Ion, M. (2019). The protection of the rapeseed crop against the attack of *Athalia rosae* in the S-E of Boianului plain. Annals of the University of Craiova, Agriculture, Montanology, Cadastre Series Vol. XLIX.

Ellers, J. (1995). Fat and eggs: an alternative method to measure the trade-off between survival and reproduction in insect parasitoids. *Neth. J. Zool.* 46, 227–235. doi: 10.1163/156854295X00186

Erb, M., and Robert, C. A. M. (2016). Sequestration of plant secondary metabolites by insect herbivores: molecular mechanisms and ecological consequences. *Curr. Opin. Plant. Sci.* 14, 8–11. doi: 10.1016/j.cois.2015.11.005

Fan, Y., Liu, X., Keyhani, N. O., Tang, G., Pei, Y., Zhang, W., et al. (2017). Regulatory cascade and biological activity of *Beauveria bassiana* oosporein that limits bacterial growth after host death. *Proc. Natl. Acad. Sci. U. S. A.* 114, E1578–E1586. doi: 10.1073/pnas.1616543114

Ferro, D. N., Morzuch, B. J., and Margolies, D. (1983). Crop loss assessment of the Colorado potato beetle (Coleoptera: Chrysomelidae) on potatoes in Western Massachusetts. *J. Econ. Entomol.* 76, 349–356. doi: 10.1093/jee/76.2.349

Glare, T. R., and O'Callaghan, M. (2000). *Bacillus thuringiensis: Biology, ecology and safety*. *1st Edn.* New York, NY, USA: Wiley.

Goettel, M. S., and Inglis, G. D. (1997). "Chapter V-3—Fungi: hyphomycetes" in *Manual of techniques in insect pathology*. ed. L. A. Lacey (San Diego, CA: Academic Press), 213–249.

Hunt, J., Jennions, M. D., Spyrou, N., and Brooks, R. (2006). Artificial selection on male longevity influences age dependent reproductive effort in the black field cricket *Teleogryllus commodus*. *Am. Nat.* 168, E72–E86. doi: 10.1086/506918

Hunt, D. W. A., and Vernon, R. S. (2001). Portable trench barrier for protecting edges of tomato fields from Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 94, 204–207. doi: 10.1603/0022-0493-94.1.204

Inceoglu, A. B., Kamita, S. G., Hinton, A. C., Huang, Q., Severson, T. F., Kang, K. D., et al. (2001). Recombinant baculoviruses for insect control. *Pest Manag. Sci.* 57, 981–987. doi: 10.1002/ps.393

Inglis, G. D., Goettel, M. S., Butt, T. M., and Strasser, H. (2001). "Use of hyphomycete fungi for managing insect pests" in *Fungi as biological agents: Progress, problems and potential*. eds. T. M. Butt, C. W. Jackson and N. Magan (Wallingford, UK: CABI Publishing), 23–69.

Kamangar, S., Gharali, B., Kaihanian, A. A., and Ebrahimi, E. (2010). Identification of natural enemies of *Athalia rosae* and investigation on effects of these agents on the control of the pest in Kurdistan province.

Kassambara, A., and Kosinski, M. (2018). Survminer: Drawing Survival Curves using "ggplot2." R Package Version 0.4.2. 2018. Available at: https://CRAN.R-project.org/package=survminer (Accessed July 20, 2020).

Kubo, I., Klocke, J. A., Miura, l., and Fukuyama, Y. (1982). Structure of Ajugarin IV. *J. Chem. Soc. Chem. Commun.* 11, 618–619.

Kuhar, T. P., Youngman, R. R., and Laub, C. A. (2000). Alfalfa weevil (Coleoptera: Curculionidae) population dynamics and mortality factors in Virginia. *Environ. Entomol.* 29, 1295–1304. doi: 10.1603/0046-225X-29.6.1295

Lacey, L. A., Grzywacz, D., Sapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi: 10.1016/j.jip.2015.07.009

Laznik, Ž., Tóth, T., Lakatos, T., Vidrih, M., and Trdan, S. (2010). Control of the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) on potato under field conditions: a comparison of the efficacy of foliar application of two strains of *Steinernema feltiae* (Filipjev) and spraying with thiametoxam. *J. Plant. Dis. Prot.* 117, 129–135. doi: 10.1007/BF03356348

Li, R., Morris-Natschke, S. L., and Lee, K. H. (2016). Clerodane diterpenes: sources, structures, and biological activities. *Nat. Prod. Rep.* 33, 1166–1226. doi: 10.1039/c5np00137d

Lombaert, E., Boll, R., and Lapchin, L. (2006). Dispersal strategies of phytophagous insects at a local scale: adaptive potential of aphids in an agricultural environment. *BMC Evol. Biol.* 6:75. doi: 10.1186/1471-2148-6-75

Maistrou, S., Natsopoulou, M. E., Jensen, A. B., and Meyling, N. V. (2020). Virulence traits within a community of the fungal entomopathogen Beauveria: associations with abundance and distribution. *Fungal Ecol.* 48:100992. doi: 10.1016/j.funeco.2020.100992

Maistrou, S., Paris, V., Jensen, A. B., Rolff, J., Meyling, N. V., and Zanchi, C. (2018). A constitutively expressed antifungal peptide protects *Tenebrio molitor* during a natural infection by the entomopathogenic fungus *Beauveria bassiana*. *Dev. Comp. Immunol.* 86, 26–33. doi: 10.1016/j.dci.2018.04.015

Meyling, N. V., Lübeck, M., Buckley, E. P., Eilenberg, J., and Rehner, S. A. (2009). Community composition, host-range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and semi-natural habitats. *Mol. Ecol.* 18, 1282–1293. doi: 10.1111/j.1365-294X.2009.04095.x

Meyling, N. V., and Pell, J. K. (2006). Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecol. Entomol.* 31, 162–171. doi: 10.1111/j.0307-6946.2006.00781.x

Müller, C., and Sieling, N. (2006). Effects of glucosinolate and myrosinase levels in *Brassica juncea* on a glucosinolate-sequestering herbivore – and vice versa. *Chemoecology* 16, 191–201. doi: 10.1007/s00049-006-0347-7

Nishida, R., and Fukami, H. (1990). Sequestration of distasteful compounds by some pharmacophagous insects. *J. Chem. Ecol.* 16, 151–164. doi: 10.1007/BF01021276

Nishida, R., Kawai, K., Amano, T., and Kuwahara, Y. (2004). Pharmacophagous feeding stimulant activity of neo-clerodane diterpenoids for the turnip sawfly, *Athalia rosae ruficornis*. *Biochem. Syst. Ecol.* 32, 15–25. doi: 10.1016/S0305-1978(03)00160-1

Ohnesorge, B. (1979). Beobachtungen zur Biologie der Rubsenblattwespe *Athalia rosae* L. (Hym., Tenthredinidae). *Anzeiger fur Schadlingskunde Pflanzenschutz Umweltschutz* 52, 70–73. doi: 10.1007/BF01903535

Opitz, S. E. W., Boevé, J. -L., Nagy, Z. T., Sonet, G., Koch, F., and Müller, C. (2012). Host shifts from Lamiales to Brassicaceae in the sawfly genus *Athalia*. *PLoS One* 7:e33649. doi: 10.1371/journal.pone.0033649

Ortiz-Urquiza, A., and Keyhani, N. O. (2013). Action on the surface: entomopathogenic fungi versus the insect cuticle. *Insects* 4, 357–374. doi: 10.3390/insects4030357

Paul, S. C., Dennis, A. B., Tewes, L. -J., Freidrichs, J., and Müller, C. (2021). Consequences of pharmacophagous uptake from plants and conspecifics in a sawfly elucidated using chemical and molecular techniques. bioRxiv [preprint] doi: 10.1101/2021.02.09.430406

Pedrini, N., Ortiz-Urquiza, A., Huarte-Bonnet, C., Zhang, S., and Keyhani, N. O. (2013). Targeting of insect epicuticular lipids by the entomopathogenic fungus *Beauveria bassiana*: hydrocarbon oxidation within the context of a host-pathogen interaction. *Front. Microbiol.* 15, 4–24. doi: 10.3389/fmicb.2013.00024

Qing, X., Yan, H., Ni, Z. Y., Vavricka, C., Zhang, M., Shi, Q., et al. (2017). Chemical and pharmacological research on the plants from genus *Ajuga*. *Heterocycl. Commun.* 23, 245–268. doi: 10.1515/hc-2017-0064

Rangel, D. E., Anderson, A. J., and Roberts, D. W. (2008). Evaluating physical and nutritional stress during mycelial growth as inducers of tolerance to heat and UV-B radiation in *Metarhizium anisopliae* conidia. *Mycol. Res.* 112, 1362–1372. doi: 10.1016/j.mycres.2008.04.013

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/ (Accessed March 15, 2018).

Riasat, T., and Wakil, W. (2011). Effect of Beauveria bassiana mixed with diatomaceous earth on mortality, mycosis and sporulation of *Rhyzopertha dominica* on stored wheat. *Phytoparasitica* 39, 225–231. doi: 10.1007/s12600-011-0164-6

Riggert, E. (1939). Untersuchungen über die Rübenblattwespe *Athalia colobri* Christ (*A. spinarum* F.). *Z. Angew. Entomol.* 26, 462–516. doi: 10.1111/j.1439-0418.1939.tb01575.x

Santa, H. S., Santa, O. R., Brand, D., Vandenberghe, L. P., and Soccol, C. R. (2005). Spore production of Beauveria bassiana from agro-industrial residues. *Braz. Arch. Biol. Technol.* 48, 51–60. doi: 10.1590/S1516-89132005000400007

Sáringer, G., Fodor, A., Nadasy, M., Lucskai, A., and Georgis, R. (1996). Possibilities of biological control using entomopathogenic nematodes against *Leptinotarsa decemlineata* L. and *Athalia rosae* L. larvae. Mededelingen-Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent (Belgium).

Sawa, M., Fukunaga, A., Naito, T., and Oishi, K. (1989). Studies on the sawfly, *Athalia rosae* (Insecta, Hymenoptera, Tenthredinidae) I. general biology. *Zool. Sci.* 6, 541–547.

Sharma, S., Kooner, R., and Arora, R. (2017). "Insect pests and crop losses" in *Breeding insect resistant crops for sustainable agriculture*. eds. R. Arora and S. Sandhu (Singapore: Springer).

Therneau, T. M. A. (2015). Package for Survival Analysis in S. Package Version 2.38. Available at: https://CRAN.R-project.org/package=survival (Accessed April 10, 2020).

Therneau, T. M., and Grambsch, P. M. (2000). *Modeling Survival Data: Extending the Cox Model*. New York, NY: Springer.

Vallat, A., and Dorn, S. (2005). Changes in volatile emissions from apple trees and associated response of adult female codling moths over the fruit-growing season. *J. Agric. Food Chem.* 53, 4083–4090. doi: 10.1021/jf048499u

Vänninen, I. (1995). Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. *Mycol. Res.* 100, 93–101. doi: 10.1016/S0953-7562(96)80106-7

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. New York, NY, USA: Springer.

Zalucki, M. P., Shabbir, A., Silva, R., Adamson, D., Shu-Sheng, L., and Furlong, M. J. (2012). Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): just how long is a piece of string? *J. Econ. Entomol.* 105, 1115–1129. doi: 10.1603/EC12107

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Estimating Contact Rates Between *Metarhizium anisopliae*–Exposed Males With Female *Aedes aegypti*

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Introduction: Effective control of *Aedes aegypti* will reduce the frequency and severity of outbreaks of dengue, chikungunya, and Zika; however, control programs are increasingly threatened by the rapid development of insecticide resistance. Thus, there is an urgent need for novel vector control tools, such as auto-dissemination of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana.* The aim of this study was to estimate contact rates of *M. anisopliae*-exposed males with wild female *Ae. aegypti*. As a control the contact rates of untreated males with wild females was contrasted.

Methods: The study was conducted in Reynosa, Mexico. The treatment and control households (n = 15 per group) were geographically separated by an arid and hot area that naturally prevented the flight of males between arms. In each control household, 40 *M. anisopliae*-exposed male *Ae. aegypti* were released per week for 8 weeks (specimens were exposed to a concentration of 5.96×10^7 conidia/cm^2 for 24 h; n = 4,800 males). In each control household, 40 untreated males were released per week for 8 weeks (n = 4,800 males). All specimens were dust-marked prior to release. Mosquito abundance was monitored with human landing collections, and captured *Ae. aegypti* were examined for any dust-marking.

Results: In the treatment households, the contact rate of *Ae. aegypti* females with marked, fungus-treated males was 14% (n = 29 females marked from 197). Where in the control households, the contact rate of females with marked, untreated males was only 6% (n = 22 marked from 365). In the treatment households the recapture rate of released males was at 5% and higher than that for the control households (which was 2%). Auto-dissemination of *M. anisopliae* from infected males to female *Ae. aegypti* was demonstrated through the recovery of an infected female from the floor of a household.

Conclusions: Overall, the contact rate between *M. anisopliae*-infected males with the natural female population was 60% higher than for the control group of healthy males. The results provide further support to the release of fungus-exposed males as a potentially useful strategy against *Ae. aegypti*, though further research is required.

Keywords: males' releases, *Metarhizium anisopliae*, auto-dissemination, biological control, dengue, *Aedes aegypti*

Abbreviations: FEMs, fungus-exposed males; L:D, light: dark.

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INTRODUCTION

The global expansion of *Aedes aegypti* and its vectored arboviruses is the widest ever recorded (Kraemer et al., 2015). Vector control is the primary tool used in the fight against arbovirus transmission, but is hampered by inadequate program management, limited human, financial and infrastructural capacity, community apathy to eliminate water-storage containers, plus increased travel and uncontrolled urbanization (Gould et al., 2017). Of serious concern, is the large-scale and intensive use of insecticides for *Aedes* control, which increases the selection pressure on vector populations to develop insecticide resistance. In fact, resistance to all four classes of insecticide commonly used in vector control has been recorded for *Ae. aegypti* (Moyes et al., 2017). In response, novel methods of *Aedes* control are under development and the most promising options are: 1) transinfection with *Wolbachia* (Moreira et al., 2009), 2) incompatible insect treatment (Marris, 2017; Ritchie, 2018), 3) sterile insect treatment (Lees et al., 2015), 4) the use of adult mosquitoes to transfer insecticides (Devine et al., 2009; Mains et al., 2015; Brelsfoard et al., 2019), biological control using *Bacillus thuringiensis* and oomyceto *Leptolegnia chapmanii* (Rodríguez-Pérez et al., 2012), and entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana* (Scholte et al., 2007; Paula et al., 2008; Reyes-Villanueva et al., 2011; García-Munguía et al., 2011).

Aedes control with entomopathogenic fungi has demonstrated promise (Reyes-Villanueva et al., 2011; García-Munguía et al., 2011; Garza-Hernández et al., 2013; Garza-Hernández et al., 2015). Previous laboratory studies have shown that *M. anisopliae* is auto-disseminated from fungus-exposed *Ae. aegypti* males (FEMs) to unexposed females, and can kill 85% of females infected with DENV-2 in less than 10 days (Reyes-Villanueva et al., 2011; Garza-Hernández et al., 2013; Garza-Hernández et al., 2015). In order to gain regulatory acceptance and effectively implement bio-control with the Ma-CBG-2 strain fungus of high virulence at 6×10^8 conidia/mL (LT_{50} of 7.5 ± 0.4 days; which reduced fecundity by up to 99% (Reyes-Villanueva et al., 2011), the lab-to-field development process has an end goal of demonstrating effectiveness and feasibility in the field (Vontas et al., 2014). Although control of *Ae. aegypti* using entomopathogenic fungi has been widely studied in laboratory conditions (*e.g.* stages of infection, formulations, fungal susceptibility against different stages of mosquito development, virulence of different fungal propagules), our method here assessed, *i.e.* the *M. anisopliae* conidia transfer from FEMs to females provided an additional tool for integrated dengue vector control programs through intra-domicile releases of FEMs. Under semi-field conditions, FEMs made over twice mating attempts without insemination than the uninfected males and during both attempts and successful matings, the FEMs were able to transfer the amount of fungus to females, even after the 5th mating (about 10% of male's conidia load) which was sufficient to kill 50% of females within 3 days (Garza-Hernández et al., 2015). Thus, indicating that there is potential for auto-dissemination of *M. anisopliae* from males to females as a dengue control tool.

The overall aim of this study was to evaluate the contact rates of FEMs to unexposed females in a small-scale field trial. This was measured by releasing either FEMs or unexposed males in the treatment and control arms (*i.e.* a parallel arm experiment), respectively, and examining the ratio of recaptured, dust-marked FEMs and unexposed males to the number of dust-marked females. Thus, we tested the hypothesis whether the contact rates between released males and the natural female population differed between the treatment and control arms.

MATERIALS AND METHODS

Study Site and Period

The study was conducted in the neighborhood "Nuevo Amanecer" at Reynosa, Mexico (19° 14' 39.91"W and 26° 3' 16.2"N), located 33 meters above sea level and with 700,000 inhabitants. The climate is hot-dry with an annual mean temperature of 22°C; the dry season lasts around 40 days in July-August with daily temperatures reaching 40°C to 42°C; conversely, the winter encompasses at least 20 days in December to January with minimum temperatures of 0°C to 5°C (INEGI, 2014). The *Ae. aegypti* local seasonality is bimodal with activity in March-June and September-November (Rodriguez-Perez et al., 2020). This study was conducted in October and November of 2016, in a cluster of 120 households arranged in two arms (control and treatment) each separated clearly by an arid and hot field (500 m length, 200 m wide) with only a 10% grass coverage. This field acted as a natural barrier to prevent the dispersal of released males between treated and control arm (**Figure 1**). Preliminary human landing collections were conducted to select 30 households (15 per group) with similar abundances of *Ae. aegypti* to be designated as the experimental households. The experimental households had an average of five female mosquitoes recorded through 2015 (Rodriguez-Perez et al., 2020). The households were distributed across 101,000 m^2, and were occupied by an average of five residents (**Figure 1**). Southward, the blocks were surrounded by non-experimental households, while eastward and westward, there was a 300-m-wide open grassland field. Northward, there is an 80-m-wide water canal, and beyond there are 4 km of grassland, with *Prosopis* spp. and *Acacia* spp. bushes and few dispersed non-experimental households until the USA boundary wall. Daily temperature and relative humidity in field, measured by waterproof digital thermometer, varied from 18°C to 28°C, from 70% to 85%, respectively, during the survey interval.

Experimental Design

Ten days before the survey, the low but similar *Ae. aegypti* abundance was verified by human landing collections conducted in the 30 experimental households. Human landing catch was conducted for 20 min per household, and 10 households were covered during 17:00 to 20:30 h. This was repeated across three consecutive days to examine all 30 households. Here, one and zero female *Ae. aegypti* were found in the 15 treated and in the 15 control households, respectively, during the preliminary surveys.

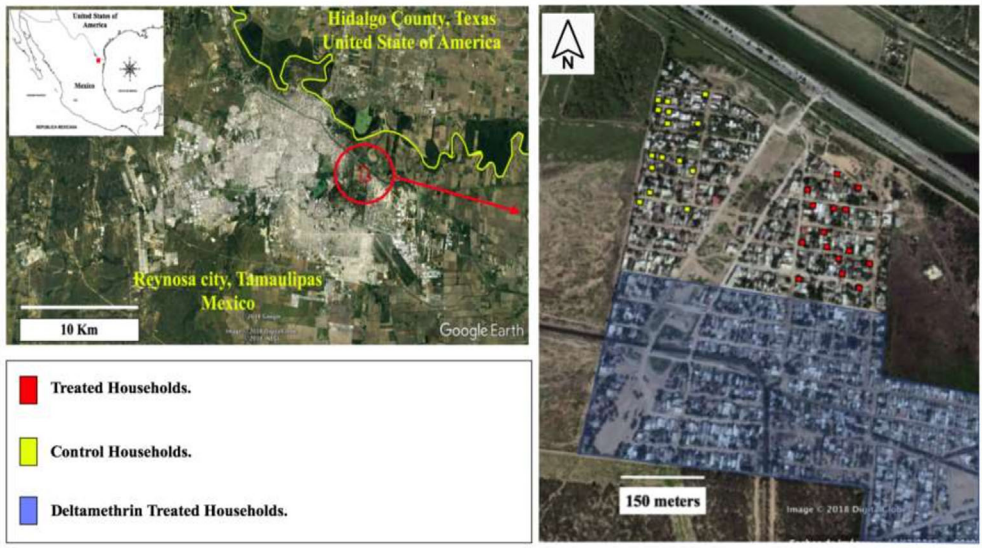

FIGURE 1 | Map including the 30 experimental households at Reynosa, Mexico: Households with releases of *Metarhizium anisopliae* - exposed male *Aedes aegypti* in red-filled circles; Households treated with uninfected males in yellow-filled circles. The 12 blocks with experimental households (with colored circles), and the non-experimental households (in blue) located southward were treated with deltamethrin and cleaned of man-made containers. North, east and west-side of surveyed area are feral/inhabited areas.

Next, the experimental releases of male *Ae. aegypti* commenced. Each individual release event involved releasing 40 males (either FEMs or unexposed males) per household. Specifically, half (n=20 red-marked males) were released inside the living room of the household, while the other half (n=20 yellow-marked males) were released in the front yard at 3 m from the main entrance of each household. FEMs and unexposed males were marked with the same powder colors (red and yellow). Males were released between 17:00 and 20:30 h and all 30 households were treated in three consecutive days, releasing 200 FEMs in five households and 200 unexposed males in five control households per day per week, during the 8 weeks.

Insectary Maintenance and Preparation of *Ae. aegypti*

The released males were 4 to 6 days old, unmated, sugar-fed *Ae. aegypti* taken from a colony set up in 2006 with larvae from Monterrey, Mexico and reared following published protocols (Reyes-Villanueva et al., 2011). Briefly, larvae were held at a density of 200 per liter of deionized water in an enamel pan; pupae were confined in a screened cage; females were blood fed on the arm of only one voluntary person.

Origin and Maintenance of *M. anisopliae* and Production of Conidia

The Ma- CBG-2 strain of *M. anisopliae sensu lato* was isolated from a *Galleria mellonella* exposed, in a plastic cup, to a sample of soil collected at rural habitat around the city of Arteaga, Coahuila, México; then it was cultured on potato-dextrose- agar (PDA) and incubated at 25°C for 20 days to allow sporulation. The Ma-CBG-2 strain was tested at an exposure concentration of *circa* 5.96×10^7 conidia/cm^2 on a filter paper prepared as previously reported (Reyes-Villanueva et al., 2011). Briefly, the fungus was cultured on potato-dextrose-agar plates incubated at 25 ± 2°C for 20 days in the dark. The conidia yield was estimated by using a mixture of 0.5% Tween-20 and 0.5% Triton-X in 0.85% saline solution. The spore suspension was centrifuged at 3,500 rpm for 10 min and then diluted to 1.6×10^8 conida/mL based on hemocytometer. To facilitate the following experiments, 5 to 7 mL (depending on the conidia harvested using 20 standard Petri dishes) of the final suspension was applied to 8 cm diameter filter papers (2.5 µm pore).

Exposure of Adult Males to the Fungi

Seven mL of the mix of the conidia suspension was poured onto a sterile Whatman filter paper that was placed on the bottom half of a Petri dish and then dried at room temperature (about 24°C) for 24 h. After drying, a second half-dish was placed over the first one to create an exposure chamber as depicted in **Figure 2** (Reyes-Villanueva et al., 2011). Both treated with dry conidia and untreated (clean) filters were placed in chambers (**Figure 2**) also described elsewhere (Garza-Hernández et al., 2015), where 20 males were confined per chamber for 24-h; the confinement was from 11:00 to 11:00 h, then each group of 20 males was transferred to 1-L meshed–cardboard cup where mosquitoes had a "resting" time of 3 h and then were marked with yellow or red dust by the procedure described previously (Garza-Hernández et al., 2015) after 3 h post-treatment. The mosquitoes were kept during and after treatment under insectary conditions which were maintained at 25 ± 1°C, relative humidity of 80 ± 5%, and a photoperiod of 14:10-h L:

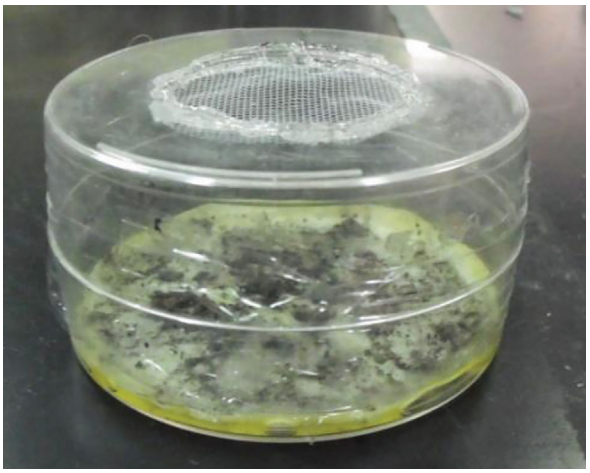

FIGURE 2 | Chamber for exposure of *Aedes aegypti* to *Metarhizium anisopliae* containing a filter at the bottom impregnated with a concentration of 5.96×10^7 conidia cm^2 of the fungus. Mosquitoes were fed with 5%-sucrose in a cotton ball placed on the hole at top half. Another hole at the lateral dish served for transferring the mosquitoes into the chamber.

D. Around the 15:00 h the cups were placed in dry-ice boxes and transported to the field to be released.

Release of Exposed and Untreated Males, Mosquito Sampling, and Processing

A parallel arm study was used. In the treatment arm, 40 FEMs were released in each experimental household (n = 15) each week; while in the control arm, 40 unexposed males were released weekly in each household (n = 15). The household was the experimental unit and the whole experiment encompassed 8 weeks (October to November). In order to avoid the presence of *Ae. aegypti* larvae in or around the households and to have a similar and low population of mosquitoes at the beginning of the experiment, a month prior to the survey the *Ae. aegypti* population was placed under control pressure. First, in all indoor walls (cement-blocks), closets and bathrooms of each household, deltamethrin (Deltametrina 25, Química del Golfo, Monterrey, México) wettable granules 0.25 mg (with a residual effect of ~ 30 days) was applied at Ultra Low Volume (equipment Cifarelli® model Nuvola 5 horse power, Voghera, Italy) adjusted to 800 mL per minute to delivering an average of 2.4 L (3 min. of 800 mL per min. per household). Then, 3 weeks prior the male releases, backyards of all households were cleaned by removing any water-storage container (this activity took 5 days to complete). The vector control was conducted in each experimental household, as well as all other households of the neighborhood block.

The mosquito abundance in each household was sampled with human landing collections. The human landing collections were conducted in the living rooms of the households and were carried out by a two-person team: one person exposed their upper body, while the other person collected the mosquitoes that had landed on the partner's exposed skin with a mouth aspirator. Human landing catches were conducted using the same schedule as the mosquito releases, described above, and occurred about 15 min after the mosquitoes were released in each household.

Immediately after each human landing collection, the mosquitoes were examined in the aspirator. When conducting human landing collection for *Ae. aegypti*, it is common to capture both host-seeking females and swarming males (Roth, 1948), thus all individuals were morphologically identified to both species and sex (Darsie and Ward, 2005). Marked mosquitoes were then detected with the aid of an ultra-violet light lamp. The marked males and female mosquitoes captured were counted and released immediately in order not to modify the experimental cohort of *Ae. aegypti* through removal trapping. After completing human landing collection, the floor of each households was examined for the presence of any dead mosquitoes through exhaustive searching. Mosquito cadavers were placed into sterile 5-mL tubes and transported to the lab where they were immersed in 1% chloride for 1 min, dried for 10 min, and then placed into Petri dishes containing PDA for sporulation.

Prior ethical approval was received, see Ethics approval and consent to participate section below.

Statistical Analysis

A dataset was constructed with numbers of total and marked females, unmarked (wild) males, marked males (FEMs and unexposed males) in red and yellow per household/week/treatment. Marked females and males were compared between treatments by 2 × 2 contingency tables based on the chi-square statistic. The means of females, FEMs, unexposed males and wild males as response variables were compared between treated and control households (arms) by Student's t *post-hoc* tests conducted with a negative binomial regression model with treatment as class variable with proc glimmix in SAS 9.4 (SAS Institute Inc, 2013).

RESULTS

Contact Rate With Females

Overall, there were 197 and 365 wild females collected in treated and control households, respectively (**Table 1**). In the treatment households, the contact rate of *Ae. aegypti* with marked, fungus-exposed males was 14.7% (n = 29). In contrast, the contact rate of females with marked, untreated males was only 6% (n = 22) in the control houses. These percentages represent the proportion of the total females that were dust-marked through auto-dissemination from the released males. Therefore, the capture of marked females was 60% higher in households where FEMs were released (15% = 29/197) than in control households treated with unexposed males (6% = 22/365; $\chi 2 = 11.71$, df=1, p =0.0006). Note that the fungus incidence of sampled mosquitoes was not recorded due to specimens being released immediately after capture and identification.

TABLE 1 | *Aedes aegypti* documented in human-landing counts conducted in 15 households/week during 8 weeks in treated and control site.

Week	Total females		Marked females[1]		Recaptured males[2,3]		Wild males	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
1	3	2	1	1	21 (9,12)	36 (17,19)	12	27
2	13	7	2	2	16 (7,9)	33 (17,16)	8	21
3	20	9	2	4	9 (5,4)	42 (19,23)	9	20
4	47	29	6	2	10 (5,5)	26 (14,12)	2	5
5	43	20	4	2	7 (3,4)	24 (11,13)	4	9
6	115	60	1	4	12 (6,6)	41 (17,24)	1	4
7	121	65	5	13	8 (5,3)	19 (10,9)	2	4
8	3	5	1	1	13 (6,7)	22 (12,10)	2	1
Total	365	197	22	29	96 (46,50)	243(117,126)	40	91

[1]*The proportion of marked females was 24% higher in treated households where fungus-exposed males were released than in control ones where unexposed marked males were released. ($\chi 2 = 11.71$, df =1, $p < 0.05$).*

[2]*Each column comprises data for 8 weeks and three numbers per week: The number of total recaptured males, then red and yellow males in parenthesis.*

[3]*The proportion of recaptured males was 60% higher in treated than in control households ($\chi 2 = 66.07$, df =1, $p < 0.001$).*

In treated, forty males previously exposed for 24 h to a filter with a dose of 5.2×10^6 conidia per cm^2 of Metarhizium anisopliae were released per household/week; in control, 40 uninfected males exposed for 24 h to a clean filter, were released per household/week. Total female mosquitoes including marked females, recaptured males containing red and yellow males (in parentheses), and wild males (with no mark), are shown.

Abundance of Marked Males

A total of 339 dust-marked males (FEMs and unexposed males) were recaptured out of the 9,600 released being an overall recapture rate of 3.5%. In the treated households, the recapture rate was 5% (n = 243/4,800), with a mean/household of 2.02 ± 0.11. In the control households, the recapture rate was significantly less at 2% (96/4,800), with a mean of 0.80 ± 0.22 ($t = 4.86$, df =378, $p<0.001$). The males that were released indoors versus outdoors were marked with different color dust. At both treated and control households, relatively equal ratios of males released indoor: outdoor were recaptured, with the ratios being 1:1.07 and 1:1.08 from treated and control households respectively ($\chi 2 = 0.0015$, df=1, p =0.96).

Abundance of Wild Males

A total of 131 unmarked male *Ae. aegypti* were captured. These wild males were 2.2 times more abundant in the treated households with a mean of 0.75 ± 0.11 (n= 91), against a mean of 0.33 ± 0.07 (n = 40) in control households ($t = 3.30$, df = 238, $p <0.001$). Note that the abundance of released males far outweighed the wild population, with wild males representing only 27.9% of all captured males (n = 131/470).

Retrieval of Cadavers

During searches for dead mosquito on the household floors, a total of 17 cadavers were found (16 marked males and one female). Of these, from the red-marked female the fungal surveyed strain was successfully re-isolated; that female was mated.

DISCUSSION

Conidia of *M. anisopliae* germinate in less than 20 h (Hywel-Jones and Gillespie, 1990) and all FEMs released here, putatively, already had a 27-h infection by direct exposure to the fungus in the chamber (**Figure 2**), which is relevant because an earlier study conducted in a greenhouse reported that FEMs marked with red powder seized the double of female *Ae. aegypti* (7) than the unexposed males marked with yellow powder (3) (Garza-Hernández et al., 2015). Within approximately 4 days post-exposure, 50% of FEMs die (Reyes-Villanueva et al., 2011); so, it seems that once FEMs start to be impacted by fungal infection the response is to prioritize mating over flight for dispersal, which is in line to the predominance of FEMs (60%) in treated households than unexposed males in control households reported here. The increase in mating activity in males challenged by pathogens has been documented in other insects, such as in *Schistocerca gregaria* infected by *M. anisopliae* (Clancy et al., 2017) and in the cricket *Gryllus texensis* threatened by iridovirus (Adamo et al., 2014). During the current experiment it was observed that FEMs, unexposed males, and wild males were all captured in the same swarms formed on a volunteer (bait) during human landing collection. It is known that when some males of *Ae. aegypti* start hovering on a human head, males and females are attracted to the swarm at an intensity contingent on the number of males present in the swarm (Fawaz et al., ; Roth, 1948); this is linked with the higher capture (2x) of wild males in treated households than in control households.

The effect of fungal infection on the flight of male *Ae. aegypti* remains yet unknown. The low recapture (40%) of marked males in control households is possibly related to higher dispersal of the healthy males after they were released. It is worthy to mention that the ratio of indoor: outdoor marked males was similar for FEMs (1:1.08) and unexposed males (1:1.07), which suggests a similar dispersal of those released indoors or outdoors (Verdonschot and Besse-Lototskaya, 2014). In a prior mark-release-recapture study of untreated male *Ae. aegypti* in Mexico, the maximum recapture rate and distance recorded (by backpack aspirator) were 6.55% (138/2,107) and 166 m (Valerio et al., 2012); nevertheless, they also found that more than 50% of recaptured males were found in the three houses nearest to the release point. In this study, non-experimental households were inter-dispersed between the experimental households, and then possibly the unexposed males migrated to adjacent non-experimental households, which were not surveyed by human

landing collections and therefore must be examined in future studies. Last, in a recent study conducted approximately at 15 km (in USA, across the border) from this study site, examined larvae marked with isotopes in tires that produced males collected in BG Sentinel traps at 220 m (Juarez et al., 2010).

CONCLUSIONS

This is the first report about contact rates recorded by mark-release-recapture of *M. anisopliae*-exposed males with female *Ae. aegypti*, in field. Overall, the contact rate between *M. anisopliae*-infected males with the natural female population was 60% higher than for the control group of healthy males. This pilot data provides strong evidence in support of the potential of entomopathogenic fungi to control *Ae. aegypti* through auto-dissemination. The next step in the evaluation process of this tool is to investigate the effectiveness in a large-scale field trial.

DATA AVAILABILITY STATEMENT

The data set analyzed in the present study is available from the corresponding author upon reasonable request. Requests to access these data sets should be directed to FR-V, frv65@hotmail.es.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethics Committee of the Escuela Nacional de Medicina y Homeopatia of the Instituto Politecnico Nacional (Mexico City) under reference ENMH-CB-061-2013. 202. Written informed consent was obtained from all human volunteers to collect mosquitoes (HLC collectors).

AUTHOR CONTRIBUTIONS

FR-V and MR-P conceived and designed the experiment. MR-P conducted field-work. FR-V and TR analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Adamo, S. A., Kovalko, I., Easy, R. H., and Stoltz, D. (2014). A Viral Aphrodisiac in the Cricket *Gryllus Texensis*. *J. Exp. Biol.* 217, 1970–1976. doi: 10.1242/jeb.103408

Brelsfoard, C. L., Mains, J. W., Mulligan, S., Cornel, A., Holeman, J., Kluh, S., et al. (2019). *Aedes aegypti* Males as Vehicles for Insecticide Delivery. *Insects* 10, 230. doi: 10.3390/insects10080230

Clancy, L. M., Copper, A. L., Griffith, G. W., and Santer, R. D. (2017). Increased Male-Male Mounting Behavior in Desert Locusts During Infection With an Entomopathogenic Fungus. *Sci. Rep.* 7, 56–59. doi: 10.1038/s41598-017-05800-4

Darsie, R. F.Jr., and Ward, R. A. (2005). *Identification and Geographical Distribution of the Mosquitoes of North America* Vol. 383 (North of Mexico: University Press of Florida).

Devine, G. J., Zamora-Perea, E., Kileen, G. F., Stancil, J. D., Clark, S. J., and Morrison, A. C. (2009). Using Adult Mosquitoes to Transfer Insecticides to *Aedes aegypti* Larval Habitats. *PNAS* 106, 11530–11534. doi: 10.1073/pnas.0901369106

Fawaz, E. Y., Allan, S. A., Bernier, U. R., Obenauer, P. J., and Diclaro, J. W. (2014). Swarming Mechanisms in the Yellow Fever Mosquito: Aggregation Pheromones are Involved in the Mating Behavior of *Aedes aegypti*. *Vector Ecol.* 39, 347–354. doi: 10.1111/jvec.12110

Garza-Hernández, J. A., Reyes-Villanueva, F., Russell, T. L., Braks, M. A. H., Garcia-Munguia, A. M., and Rodríguez-Pérez, M. A. (2015). Copulation Activity, Sperm Production and Conidia Transfer in *Aedes aegypti* Males Contaminated by *Metarhizium anisopliae*: A Biological Control Prospect. *PLoS Negl. Trop. Dis.* 10, e0004144. doi: 10.1371/journal.pntd.0004144

Garza-Hernández, J. A., Rodríguez-Pérez, M. A., Salazar, M. I., Russell, T. L., Adeleke, M. A., de Luna-Santillana, E. J., et al. (2013). Vectorial Capacity of for Dengue Virus Type2 is Reduced With Co-Infection of *Aedes aegypti Metarhizium anisopliae*. *PLoS Negl. Trop. Dis.* 7, e2013. doi: 10.1371/journal.pntd.0002013

García-Munguía, A., Garza-Hernández, J., Rebollar-Tellez, E., Rodríguez-Pérez, M., and Reyes-Villanueva, F. (2011). Transmission of *Beauveria bassiana* From Male to Female *Aedes aegypti* Mosquitoes. *Parasit. Vectors* 4, 24. doi: 10.1186/1756-3305-4-24PMID:21352560

Gould, E., Pettersson, J., Higgs, S., Charrel, R., and Lamballerie, X. (2017). Emerging Arboviruses: Why Today? *One Health* 4, 1–13. doi: 10.1016/j.onehlt.2017.06.001

Hywel-Jones, N. L., and Gillespie, A. T. (1990). Effect of Temperature on Spore Germination *in Metarhizium anisopliae* and *Beauveria bassiana*. *Mycol. Res.* 94, 389–392. doi: 10.1016/S0953-7562(09)80363-8

INEGI (2014). "Anuario Estadístico Y Geográfico De Tamaulipas 2014," in *Instituto Nacional De Estadística, Geografía E Informática* (México: Gobierno Federal).

Juarez, J. G., Garcia-Luna, S., Chaves, L. F., Carbajal, E., Valdez, E., Avila, C., et al. (2010). Dispersal of Female and Male *Aedes aegypti* From Discarded Container Habitats Using a Stable Isotope Mark-Capture Study Design in South Texas. *Sci. Rep.* 10, 6803. doi: 10.1038/s41598-020-63670-9

Kraemer, M. U. G., Sinka, M. E., Duda, K. A., Mylne, A. Q. N., Shearer, F. M., Barker, C. M., et al. (2015). The Global Distribution of the Arbovirus Vectors *Aedes aegypti* and *Ae. albopictus*. *ELife* 4, e08347. doi: 10.7554/eLife.0834

Lees, R. S., Gilles, J. R. I., Hendrichs, J., Vreysen, M. J. B., and Bourtzis, K. (2015). Back to the Future: The Sterile Insect Technique Against Mosquito Disease Vectors. *Curr. Opin. Insect Sci.* 10, 156–162. doi: 10.1016/j.cois.2015.05.011

Mains, J. W., Brelsfoard, C. L., and Dobson, S. L. (2015). Male Mosquitoes as Vehicles for Insecticide. *PLoS Negl. Trop. Dis.* 9, e0003406. doi: 10.1371/journal.pntd.0003406

Marris, E. (2017). Bacteria Could be Key to Freeing South Pacific of Mosquitoes. *Nature* 548, 17–18. doi: 10.1038/548017a

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., van den Hurk, A. F., Ryan, P. A., and O´Neill, S. L. (2009). A *Wolbachia* Symbiont in *Aedes aegypti* Limits Infection With Dengue, Chikungunya, and *Plasmodium*. *Cell* 139, 1268–1278. doi: 10.1016/j.cell.2009.11.042

Moyes, C. L., Vontas, J., Martins, A. J., Ching-Ng, L., Ying-Koou, S., Dusfour, I., et al. (2017). Contemporary Status of Insecticide Resistance in the Major *Aedes* Vectors of Arboviruses Infecting Humans. *PLoS Negl. Trop. Dis.* 11, e0005625. doi: 10.1371/journal.pntd.0005625

Paula, A. R., Brito, E. S., Pereira, C. R., Carrera, M. P., and Samuels, R. I. (2008). Susceptibility of Adult *Aedes aegypti* (Diptera: Culicidae) to Infection by *Metarhizium anisopliae* and *Beauveria bassiana*: Prospects for Dengue Vector Control. *Biocontrol Sci. Technol.* 18, 1017–1025. doi: 10.1080/09583150802509199

Reyes-Villanueva, F., Garza-Hernández, J., Garcia-Munguia, A., Tamez-Guerra, P., Howard, A. F., and Rodriguez-Perez, M. A. (2011). Dissemination of *Metarhizium anisopliae* of Low and High Virulence by Mating Behavior in *Aedes aegypti*. *Parasit. Vectors* 4, 171. doi: 10.1186/1756-3305-4-171

Ritchie, S. A. (2018). *Wolbachia* and the Near Cessation of Dengue Outbreaks in Northern Australia Despite Continued Dengue Importations Via Travelers. *J. Travel Med.* 25, 1–3. doi: 10.1093/jtm/tay084

Rodríguez-Pérez, M. A., Howard, A. F. V., and Reyes-Villanueva, F. (2012). "Chapter 11: Biological Control of Dengue Vectors, 241-270 Pp," in *Integrated Pest Management and Pest Control*, vol. 668 . Eds. M. L. Larramendy and S. Soloneski (Rijeka, Croatia: Intech Open Access Publisher).

Rodriguez-Perez, M. A., Russell, T. L., Olguin-Rodriguez, O., Laredo-Tiscareño, S. V., Garza-Hernandez, J. A., and Reyes-Villanueva, F. (2020). Dengue Serotypes Circulating in *Aedes aegypti* and Humans in a Poor or Peripheral Neighborhood At Reynosa, Mexico. *Southwest. Entomol.* 45 (4), 1025–1038. doi: 10.3958/059.045.0420

Roth, L. M. (1948). A Study of Mosquito Behavior. An Experimental Laboratory Study of the Sexual Behavior of *Aedes aegypti* (Linneus). *Am. Midl. Nat.* 40, 265–352. doi: 10.2307/2421604

SAS Institute Inc (2013). *What's New in SAS 9.4* (Cary, NC: SAS Institute).

Scholte, E. J., Takken, W., and Knols, B. G. J. (2007). Infection of Adult *Aedes aegypti* and *Ae. albopictus* Mosquitoes With the Entomopathogenic Fungus *Metarhizium anisopliae*. *Acta Trop.* 102, 151–158. doi: 10.1016/j.actatropica.2007.04.011

Valerio, L., Facchinelli, L., Ramsey, J. M., and Scott, T. W. (2012). Dispersal of Male *Aedes aegypti* in a Coastal Village in Southern Mexico. *Am. J. Trop. Med. Hyg.* 86, 665–676. doi: 10.4269/ajtmh.2012.11-0513

Verdonschot, P. F. M., and Besse-Lototskaya, A. A. (2014). Flight Distance of Mosquitoes (Culicidae): A Metadata Analysis to Support the Management of Barrier Zones Around Rewetted and Newly Constructed Wetlands. *Limnologica* 45, 69–79. doi: 10.1016/j.limno.2013.11.002

Vontas, J., Moore, S., Kleinschmidt, I., Ranson, H., Lindsay, S., Lengeler, C., et al. (2014). Framework for Rapid Assessment and Adoption of New Vector Control Tools. *Trends Parasitol.* 30, 191–204. doi: 10.1016/j.pt.2014.02.005

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REVIEW
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Entomopathogenic Fungi for Tick Control in Cattle Livestock From Mexico

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Ticks are one of the main economic threats to the cattle industry worldwide affecting productivity, health and welfare. The need for alternative methods to control tick populations is prompted by the high prevalence of multiresistant tick strains to the main chemical acaricides and their ecological consequences. Biological control using entomopathogenic fungi (EPF) is one of the most promising alternative options. The objective of this paper is to review the use of EPF as an alternative control method against cattle ticks in Mexico. *Metarhizium anisopliae* sensu lato (s.l.) and *Beauveria bassiana* s.l. are the most studied EPF for the biological control of ticks in the laboratory and in the field, mainly against *Rhipicephalus microplus*; however, evaluations against other important cattle ticks such as *Amblyomma mixtum* and *R. annulatus*, are needed. A transdisciplinary approach is required to incorporate different types of tools, such as genomics, transcriptomics and proteomics in order to better understand the pathogenicity/virulence mechanism in EPF against ticks. Laboratory tests have demonstrated the EPF efficacy to control susceptible and resistant/multiresistant tick populations; whereas, field tests have shown satisfactory control efficiency of *M. anisopliae* s.l. against different stages of *R. microplus* when applied both on pasture and on cattle. Epidemiological aspects of ticks and environmental factors are considered as components that influence the acaricidal behavior of the EPF. Finally, considering all these aspects, some recommendations are proposed for the use of EPF in integrated control schemes for livestock ticks.

Keywords: ***Rhipicephalus microplus*, *Metarhizium*, *Beauveria*, bovines, biological control, ticks**

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INTRODUCTION

Food security is one of the main concerns worldwide, where cattle play a fundamental role in the supply of milk and meat (Falvey, 2015). Cattle production in Mexico is an activity of social and economic importance that is carried out throughout the national territory, occupying more than 110 million hectares, with 1.1 million registered livestock farms (SIAP (Servicio de Información Agroalimentaria y Pesquera), 2020). This large area of the national territory dedicated to livestock has an impact on the use of natural resources and can affect the quality and preservation of ecosystems (González-Padilla et al., 2019). Bovine livestock in the country has an inventory of 35.2 million cattle heads (SIAP (Servicio de Información Agroalimentaria y Pesquera), 2018), and is based mainly on direct grazing in extensive production systems (Castillo-Gallegos et al., 2005);

where one of the main threats are ticks and the pathogens they transmit, affecting productivity, health and well-being. It has been estimated that more than 80% of cattle population worldwide is exposed to tick infestations (Snelson, 1975; Giles et al., 2014), where the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), *R.* (*B.*) *annulatus* (Say), and *Amblyomma mixtum* (Koch) are considered the most important livestock ticks in Mexico. Previously, tick control has been based on therapeutic interventions using chemical treatments (acaricides and endectocides). These methodologies have definitely contributed to improving productivity and welfare; however, the intensive and frequent use, and inappropriate use as well, of these chemicals has resulted in the development of acaricidal resistance in ticks (Fernández-Salas et al., 2012a,b,c; Alonso-Díaz et al., 2013a). Tick resistance has been reported for almost all the main chemical acaricides (Alonso-Díaz et al., 2013a; Rodríguez-Vivas et al., 2014a) and this phenomenon, added to an exacerbated chemical control problem, has had other consequences such as environmental and food contamination by secondary chemical metabolites, spread of ticks into free zones, restrictions on cattle export and increase in diseases transmitted by these parasites (De Castro, 1997; Domínguez-García et al., 2016; Rodríguez-Vivas et al., 2017). Results have shown that dependence on these chemical products, as the only form of control, is neither economically nor ecologically sustainable. Sustainable cattle production needs strong changes, such as considering both agroecology-oriented and novel tick-control approaches (Alonso-Díaz et al., 2014). This latter has motivated the exploration of alternative methods for tick control (Samish et al., 2004), such as the use of entomopathogenic fungi (EPF). Biological control by EPF is one of the most promising options for tick control (Polar et al., 2005). The most widely used EPF species against cattle ticks are *Metharizium anisopliae* s.l., *Beauveria bassiana* and *Akanthomyces lecanii* (formerly, *Lecanicillium lecanii*) (Fernandes et al., 2012; Romo-Martínez et al., 2013). EPF show clear advantages, such as being environmentally safe, can be mass-produced, and have the ability to infect their hosts through the cuticle rather than wait for ingestion in order to cause infection (Rajula et al., 2020). It has also been reported that EPF may affect the entire tick cycle (free-living and parasitic stages) (Fernández-Salas et al., 2017, 2018, 2019), a characteristic that allows broadening the spectrum of use in a tick control strategy. The research requires a transdisciplinary approach in order to be able to integrate the necessary knowledge on the use of EPF in the control of ticks. Through this integration, it will be possible to identify links between the studies carried out, generate research hypotheses to improve biological control and design viable EPF application schemes based on experiences that help guide future studies in the use of these fungi against livestock ticks. The objective of this paper is to review the use of EPF as an alternative control method against cattle ticks in Mexico.

Cattle Ticks

Ticks are obligate blood-feeding ectoparasites that infest 80% of the cattle worldwide (Giles et al., 2014; Grisi et al., 2014). These ectoparasites are one of the most important health problems for the livestock industry and are responsible for high economic losses around the world, putting food safety at risk (Rodríguez-Vivas et al., 2017). In addition to having direct effects on their hosts, ticks are also the most important group of parasitic arthropods as vectors of pathogens that affect domestic animals and wildlife (Pérez de León et al., 2020). Tick-borne pathogens are the main cause of transboundary livestock diseases (e.g., bovine babesiosis, anaplasmosis, theileriosis, and heartwater disease), which are among the diseases listed as notifiable by the World Organization for Animal Health (Esteve-Gasent et al., 2020). Estimated annual global costs associated with ticks and the pathogens transmitted by them range between US$ 13.9 billion and US$ 18.7 billion (De Castro, 1997). Ticks that affect cattle around the world belong to two families: Ixodidae and Argasidae. The first, also known as hard ticks, includes all species from *Amblyomma*, *Dermacentor*, *Haemophysalis*, *Hyalomma*, *Ixodes,* and *Rhipicephalus*; while the second family or soft ticks, includes the *Ornithodoros* and *Otobius* ticks (**Figure 1**).

Cattle Ticks in Mexico

In Mexico there are 82 species of ticks that parasitize domestic and wild animals (Higa et al., 2020). The main ticks of domesticated cattle belong to the Ixodidae family. Among these, *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), *R.* (*B.*) *annulatus* (Say), and *A. mixtum* (Koch 1844) have been reported with a high prevalence in cattle farms across the country. However, there are other ticks such as *Dermacentor albipictus*, *R. sanguineus*, *Anocentor nitens* and *Otobius megnini* that also have a considerable livestock impact (Martínez et al., 2019). In Mexico, the economic losses caused only by *R. microplus* were US$ 573.61 million per year (Rodríguez-Vivas et al., 2017). Although, some other ticks such as *Otobius megnini* which is present throughout the country, are also very important in livestock inspections for the export market (Martínez et al., 2019).

Cattle Fever Ticks *Rhipicephalus microplus* and *Rhipicephalus annulatus*

Cattle fever ticks (CFT) *R. microplus* and *R. annulatus* remain endemic in Mexico (Esteve-Gasent et al., 2020). Both ticks have similar biological processes and morphology; however, their geographic distribution is different (Estrada-Peña and Venzal, 2006; SENASICA, 2013). While *R. microplus* is present in tropical and subtropical regions, *R. annulatus* is endemic to arid and semiarid regions (Northern Mexico) (SENASICA, 2013) (**Figure 2**). CFT are present in 65% of the national territory and have the capacity to infest mainly cattle, but they have also been reported to infest equines, deer and other wild animals (CFSPH (The Center For Food Security and Public Health), 2007; Rodríguez-Vivas et al., 2013a). These ticks have the ability to transmit notifiable animal diseases in cattle, such as anaplasmosis and babesiosis (Klafke et al., 2020). For this reason, CFT have special attention and constant vigilance in the border area between Mexico and the United States, in order to prevent their spread in free-tick areas (Lohmeyer et al., 2011), where there has already been an increase in infestations or outbreaks (Pound et al., 2010; Araya-Anchetta et al., 2015). The biological cycle of *R. microplus* is shown in **Figure 3**.

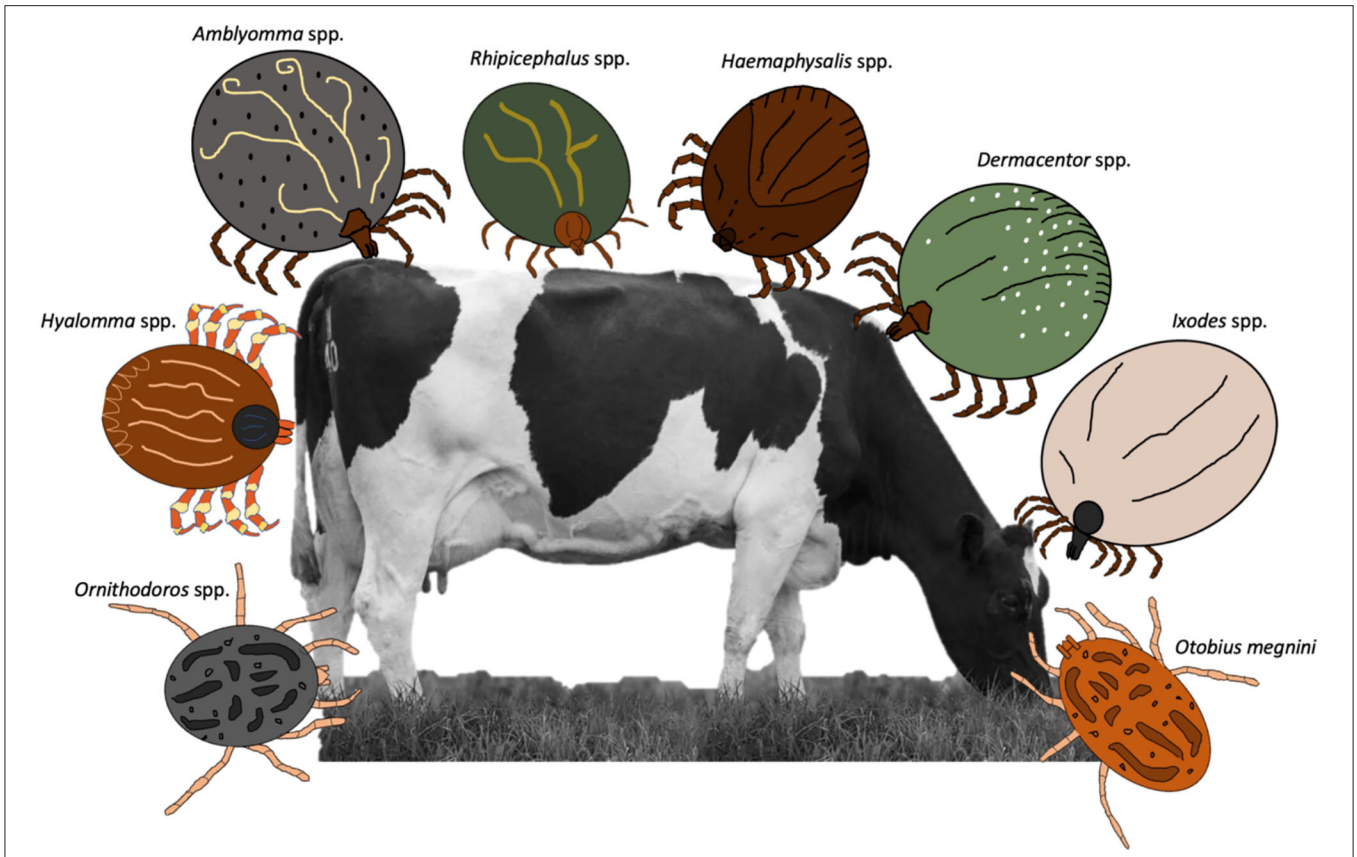

FIGURE 1 | Ticks infesting cattle around the world.

Amblyomma mixtum

A. mixtum has a similar distribution to that of *R. microplus* in Mexico (**Figure 2**) (SENASICA, 2013); where, concomitant infestations are common in ∼86% of farms (Alonso-Díaz et al., 2013a). Currently, it seems that *A. mixtum* has a greater distribution, since this species has been able to adapt to various ecological niches, including semi-arid grasslands and subtropical secondary forests (Estrada-Peña et al., 2004); in addition to its great capacity to occupy the ecological niches of other ticks (i.e., *R. microplus* under high pressure from acaricides) (Alonso-Díaz et al., 2013b). This ectoparasite has a heteroxenous life cycle and is a generalist species that infests livestock, humans and, wildlife in Mexico (Aguilar-Domínguez et al., 2019; Higa et al., 2020). It causes economic losses due to the large amount of blood taken from its hosts and the transmission of infectious diseases to domestic/wild animals (*Anaplasma marginale*) and humans (*Rickettsia ricketsii*) (Alonso-Díaz et al., 2013b; Aguilar-Domínguez et al., 2019). Additionally, other potentially zoonotic species such as *Rickettsia amblyommatis* have been detected in *A. mixtum* from Mexico (Sánchez-Montes et al., 2016; Merino et al., 2020), making this parasite one of the most important tick species in veterinary medicine and public health in the country (Pérez de León et al., 2020). The biological cycle of *A. mixtum* is shown in **Figure 4**.

Table 1 shows the temperature, rainfall and relative humidity per climate zone in Mexico to observe the characteristics of each ecological niche where the main ticks are distributed.

Impact of Climate Change on the Epidemiology of Ticks

Climate change is viewed as a long-term change in average weather patterns that have come to define Earth's local, regional and global climates (NASA-GCC, 2019). Perhaps, some of the most important alterations caused by climate change are warmer temperatures in temperate zones, altered precipitation patterns, increased frequency and severity of extreme weather events (hurricanes or droughts), and sea level rise (Kutz et al., 2009; Polley and Thompson, 2009). These last changes have affected, directly or indirectly, the biology and ecology of a great number of organisms on the planet; therefore, these climate variations have impacted on the habits and biological cycles of ectoparasites (Cumming and Van Vuuren, 2006; Kutz et al., 2009), including ticks (Pérez de León et al., 2012). In this regard, some authors in Mexico have mentioned that *R. microplus* can present between four to five successful generations per year in tropical and subtropical areas (Rodríguez-Vivas et al., 2005). Ticks have had the ability to evolve, adapt and spread within the changing climatic conditions, which, for the most part,

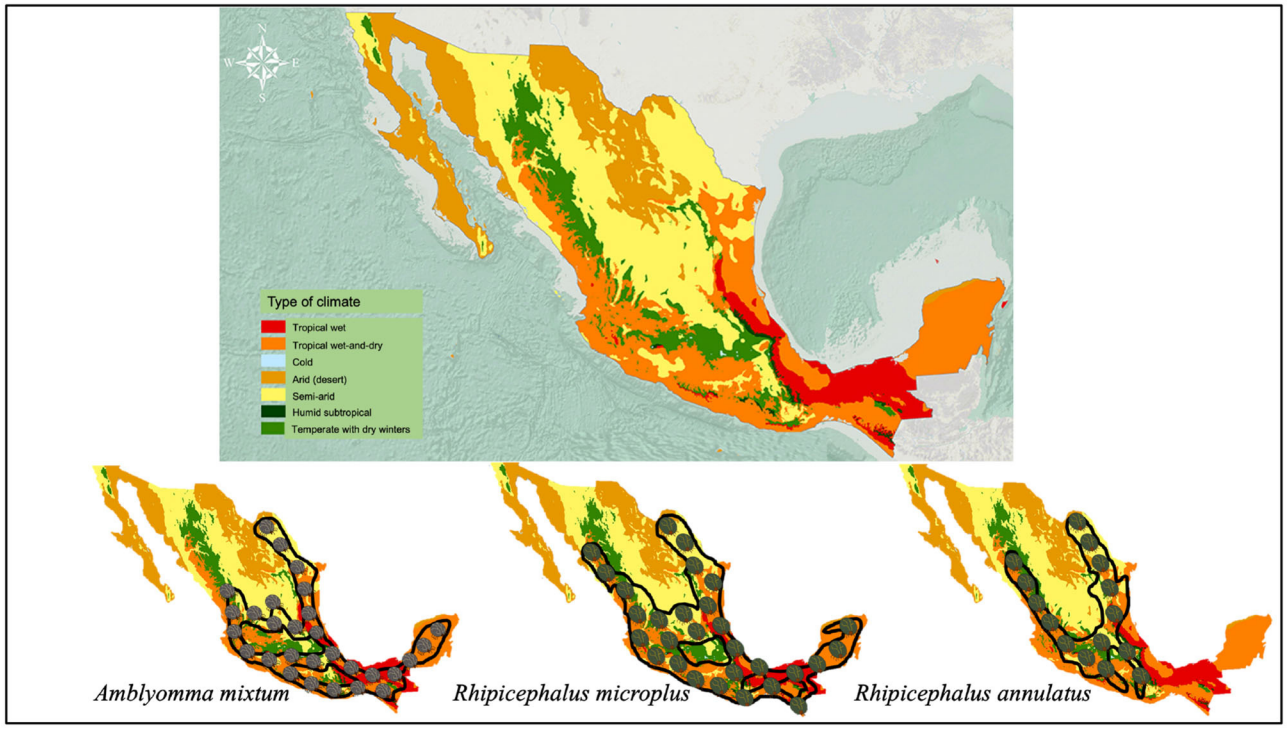

FIGURE 2 | Climate zones of Mexico and their relationship with the distribution of the main ticks that affect cattle in Mexico. Imaged edited according to information from SEMARNAT (Secretaría de Medio Ambiente y Recursos Naturales) (2003) and SENASICA-SAGARPA (2015).

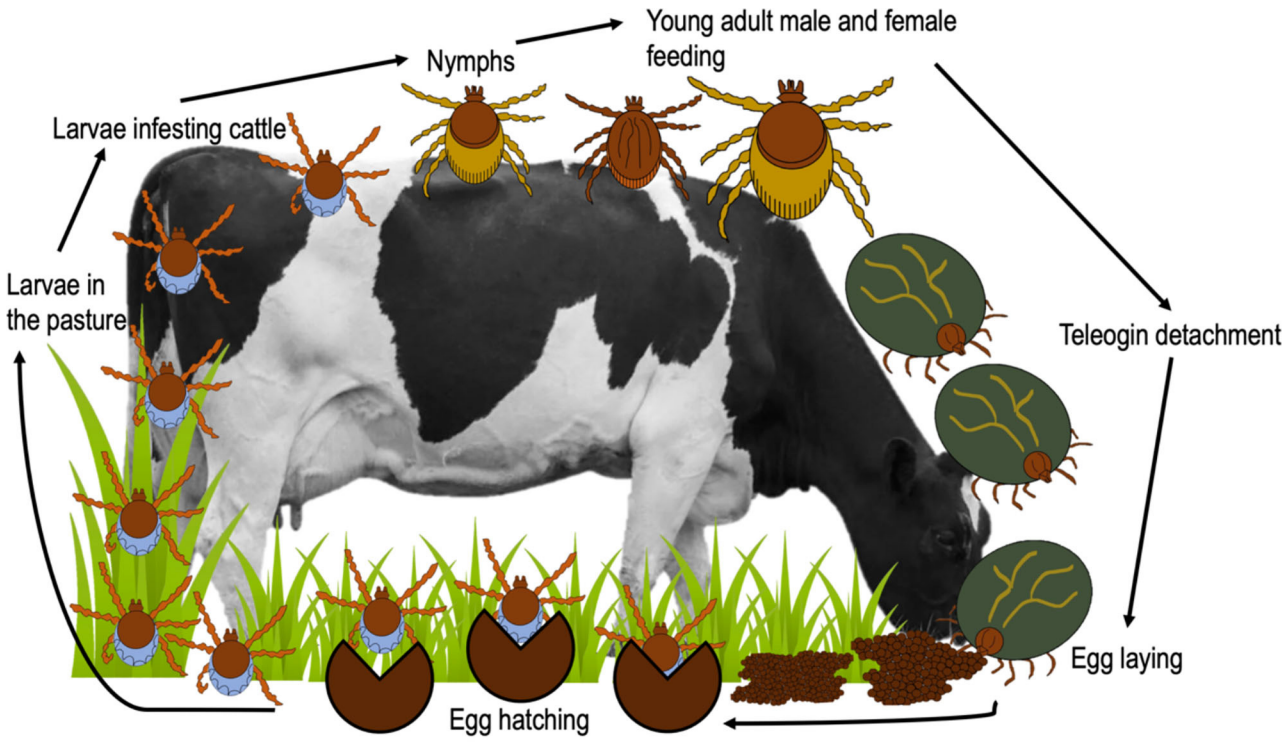

FIGURE 3 | Life cycle of *R. microplus*.

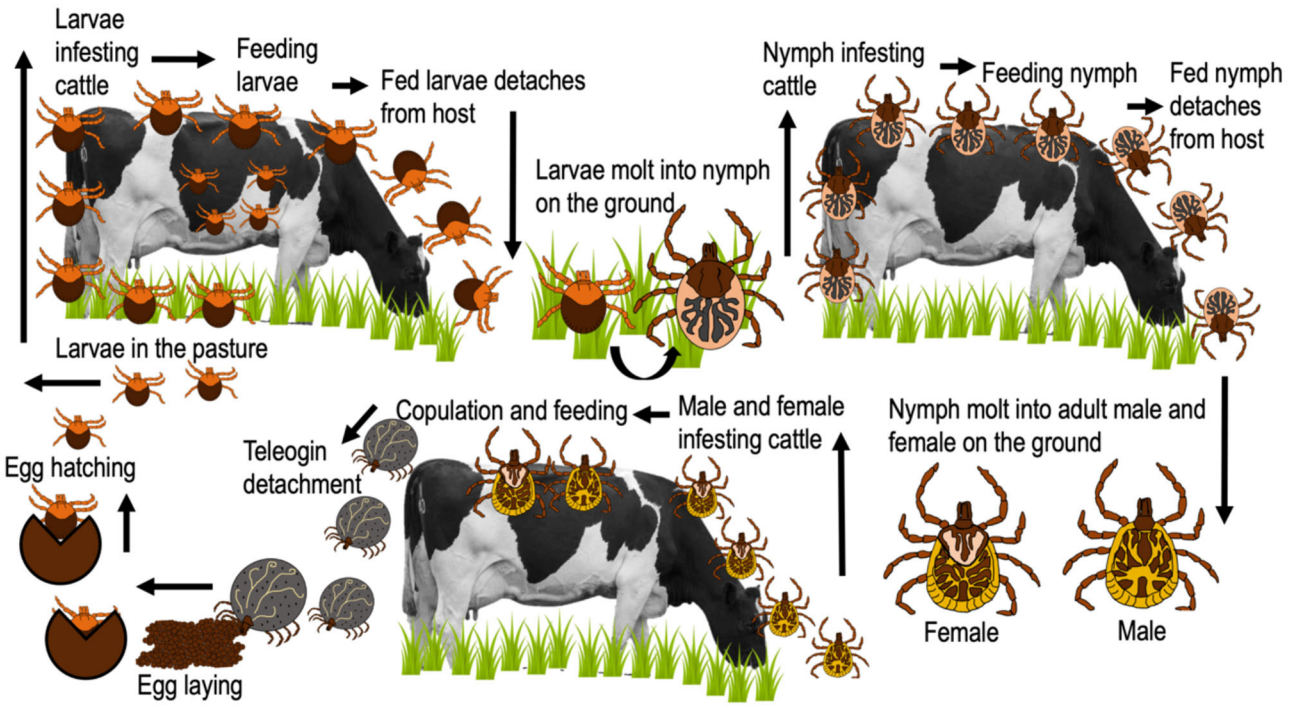

FIGURE 4 | Life cycle of *A. mixtum*.

TABLE 1 | Annual mean temperature, rainfall and relative humidity per climate zone in Mexico.

Climatic zone	Mean temperature (°C)	Mean rainfall (mm)	Relative humidity (%) Min–Max
Tropical wet	22–26	2000–4000	50.0–100
Tropical wet and dry	22–26 (>26 in some areas)	1000–2000	40.0–91.4
Cold	10–15	500–850	25.7–77.7
Arid (desert)	18–22	100–300	25.0–82.0
Semi-arid	18–26	300–600	25.7–77.7
Humid subtropical	18–22	2000–4000	40.0–91.4
Temperate with dry winters	10–22	600–1000	20.0–80.0

Information obtaining from Prieto (2005) and Morillón et al. (2018).

have favored the dynamics and population movement of these arachnids in different geographical areas (Barré and Uilenberg, 2010). This situation has led to the presentation of relatively new infestations in some livestock areas, or the diagnosis of diseases transmitted by these vectors, which were not common for certain latitudes in the past (Estrada-Peña, 2008; Montero et al., 2016). Climate change can also affect domestic or wild hosts (Barré and Uilenberg, 2010; Rodríguez-Vivas et al., 2013a), which influences the geographical distribution of ticks, their infestations and the diseases they transmit in non-endemic areas (Giles et al., 2014). The presence of CFT has been frequently reported in tick-free zones or quarantine zones in the US. The risk of introducing ticks into or outside the quarantine zone is mainly high due to the movement of tick host species, such as the white-tailed deer (Pound et al., 2010; Webb et al., 2010), the nilgai antelope (Cárdenas-Canales et al., 2011), stray cattle and interactions between *R. microplus* and exotic weeds along the transboundary region with Mexico (Racelis et al., 2012; Esteve-Gassent et al., 2014). Likewise, the red deer (*Cervus elaphus*) has been reported as a wild host for the *R. microplus* tick (Rodríguez-Vivas et al., 2013a), helping it to spread within the Mexican territory. Obviously, the movement of these hosts is also closely related to human activities, the temperature increases in some areas, and the scarcity of water. All these characteristics can participate in a possible complex change in the ecology of ticks, since their biological cycles can be affected by these conditions.

Tick Control

Tick control is mainly based on the use of chemical acaricides, which in recent decades have played a crucial role in the

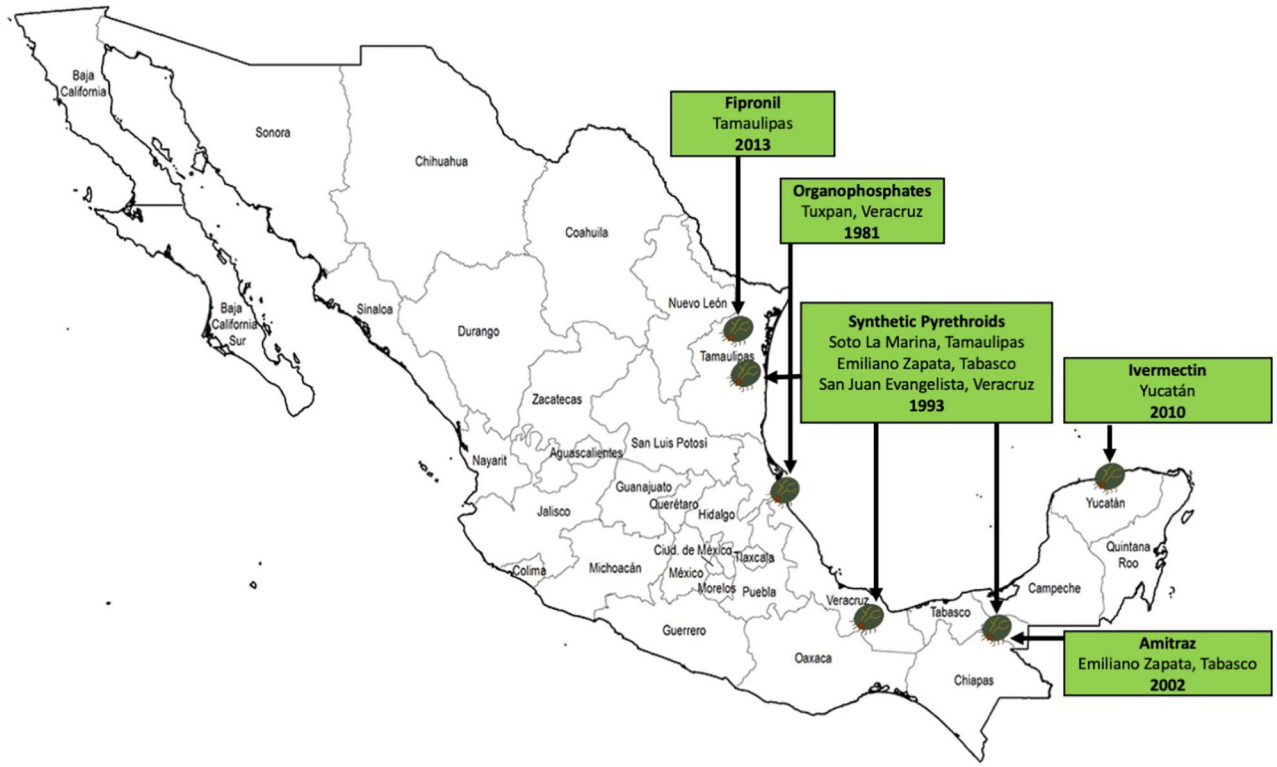

FIGURE 5 | First reports of *R. microplus* tick populations resistant to acaricides in Mexico (Ortiz et al., 1995; Soberanes et al., 2002; Perez-Cogollo et al., 2010a; Miller et al., 2013).

sustainability of the livestock production. However, since the development of the first broad-spectrum parasiticides, they have been used extensively by farmers in order to control or eliminate parasites. When ectoparasiticides are administered correctly (dosed and targeted), they are effective and have wide safety margins for both the animals and the people who apply them. However, there are factors such as resistant or multiresistant parasites and/or incorrect ways of applying the medications, which decrease their effectiveness (Alonso-Díaz et al., 2014). Currently, global results reveal that parasite control schemes based on a rigorous and exclusive use of chemical applications are not sustainable. The continuing propagation of these serious problems on a large scale involves many people in the pharmaceutical industries, professionals, farmers and in public health. It should be noted that chemical acaricides are and will be the fundamental basis of tick control, that is why they should be considered as a precious resource for cattle farming, since the cost of having an acaricide on the market implies expenses of more than 250 million dollars and between 8 and 12 years of research (De Alva, 1995; Omkar, 2016).

Acaricide Resistance of Livestock Ticks in Mexico

One of the biggest concerns that has arisen on cattle farms across the country is the ability of ticks to resist the deadly effects of the chemicals used for their control. Tick resistance to acaricides is defined as "the specific heritable trait or traits in a tick population, selected as a result of the population's contact with an acaricide. This translates into a significant increase in the percentage of the population that survives after exposure to a certain concentration of this acaricide" (Rodriguez-Vivas et al., 2018). In Mexico, several investigations have been conducted to identify and monitor populations of resistant and multiresistant ticks to acaricides, and to know the risk factors associated with the presence of this growing problem (Fernández-Salas et al., 2012a,b,c; Alonso-Díaz et al., 2013a; Higa et al., 2020). **Figure 5** shows the first cases of ticks resistant to acaricides in Mexico. **Table 2** shows a summary of the epidemiological studies of resistant or multiresistant ticks by state over time in the country, highlighting *R. microplus*, which has developed resistance to all the main types of acaricides. Multiple acaricide resistance is an alarming phenomenon in Mexico, considering that there are no new synthetic compounds on the market with a novel mode of action to control multidrug resistant ticks (Esteve-Gasent et al., 2020).

This type of resistance has been reported in different regions of Mexico and the most common in *R. microplus* are: coumaphos, flumethrin, and amitraz; chlorfenvinphos, flumethrin, and amitraz; diazinon, deltamethrin, and amitraz (Rodriguez-Vivas et al., 2007); permethrin, coumaphos, and fipronil; permethrin, coumaphos, fipronil, and amitraz (Miller

TABLE 2 | Main reports of resistance of *R. microplus* to acaricides by state in Mexico.

State	Chemical Family	Acaricide/endectocide	References
Yucatán	OP	Diazinon, Coumaphos, Chlorfenvinphos	Rodriguez-Vivas et al., 2006a
	SP	Flumethrin, Deltamethrin, Cypermethrin	Rodriguez-Vivas et al., 2006a, 2012; Rodríguez-Vivas et al., 2013b; Cabrera-Jimenez et al., 2008; Rosario-Cruz et al., 2009
	Am	Amitraz	Rodriguez-Vivas et al., 2006b; Rosado-Aguilar et al., 2008
	ML	Ivermectin	Perez-Cogollo et al., 2010a,b; Alegría-López et al., 2015
Veracruz	OP	Chlorpyrifos, Diazinon	Fernández-Salas et al., 2012c
	SP	Flumethrin, Deltamethrin, Cypermethrin	Fernández-Salas et al., 2012a,c
	Am	Amitraz	Fernández-Salas et al., 2012a
	ML	Ivermectin	Fernández-Salas et al., 2012b,c
Tamaulipas	OP	Diazinon, Coumaphos, Chlorfenvinphos, Lindane	Armendáriz-González, 2003
	SP	Flumethrin, Deltamethrin, Cypermethrin	
	PP	Fipronil	Miller et al., 2013
Tabasco	Am	Amitraz	Soberanes et al., 2002
Campeche	OP	Diazinon, Coumaphos	Li et al., 2003
	Am	Amitraz	Li et al., 2004
Nuevo León	OP	Diazinon	Miller et al., 2008
Coahuila	P	Permethrin	Miller et al., 2007
Chiapas	Am	Amitraz	Aguilar-Tipacamú et al., 2009

OP, organophosphates; SP, synthetic pyrethroids; P, pyrethroids; Am, amidines; ML, macrocyclic lactones; PP, phenylpyrazoles.

TABLE 3 | Reports of *A. mixtum* resistant to acaricides in Mexico.

State	Chemical family	Acaricide	References
Veracruz	OP	Diazinon, Coumaphos, Chlorpyrifos	Alonso-Díaz et al., 2013a
	Am	Amitraz	Alonso-Díaz et al., 2013a; Higa et al., 2020

OP, organophosphates; Am, amidines.

et al., 2013); amitraz, cypermethrin, and ivermectin (Fernández-Salas et al., 2012a,b); and coumaphos, cypermethrin, amitraz, ivermectin and fipronil (Rodríguez-Vivas et al., 2014a). Although less studied, multiresistant strains of *A. mixtum* to acaricides have also been detected (**Table 3**). As for *R. annulatus*, there is insufficient evidence to know the resistance degree of this tick to chemical acaricides in Mexico; however, some studies suggest that it may be underdiagnosed as in some other countries (Klafke et al., 2020). Recently, the first evidence of permethrin resistance in *R. annulatus* strains was reported near the US-Mexico border, in Maverick County, Texas (Klafke et al., 2020). It is important to consider that populations of *R. annulatus* resistant to pyrethroids (Ziapour et al., 2017; Aboelhadid et al., 2018) and ivermectin have already been reported in other countries.

Since acaricides will continue to be the basis of tick control, their lifespan and effectiveness need to be extended. To achieve this, it is suggested to know, evaluate and adopt other alternative control strategies in order to design an adequate integrated control scheme for ticks. It has been mentioned that the best way to control ticks in cattle farms is to combat them simultaneously in different ways (Alonso-Díaz et al., 2014; Pérez de León et al., 2020). By doing this, the parasites have less ability to defend themselves and develop resistance.

Entomopathogenic Fungi

EPF are a species of fungal pathogens for arthropods (Rajula et al., 2020). They are considered cosmopolitan saprophytic organisms that live in diverse ecosystems and climates (e.g., tropical, temperate, arid and artic), where they interact with arthropods in many terrestrial and aquatic habitats (Skinner et al., 2014). It is estimated that there are between 750 and 1,000 EPF placed in more than 100 genera (Mantzoukas and Eliopoulos, 2020; Rajula et al., 2020), which play an important role in the dynamics of arthropod populations in natural ecosystems (Maina et al., 2018). EPF comprise a wide range of genera and species with high morphological, phylogenetic and ecological diversity (Araújo and Hughes, 2016), and their interactions with arthropods are of great interest for environmental microbiology, determination of the balance of ecosystems, biodiversity, evolution of eukaryotic organisms and insect pest control (Semenova et al., 2020). These fungi have the ability to evolve to be more successful in their biological cycle, and they are also capable of colonizing and using arthropods as a substrate for their reproduction (Humber, 2008). Among the arthropods to which they have adapted throughout this evolutionary transition are ticks, which

is why they have been studied as a biological control for these pest arachnids. Biological control using EPF represents one of the most promising approaches for sustainable tick control schemes and can therefore be included as a component in an integrated pest management strategy for tick control. In addition, EPF have some advantages over conventional acaricides, such as: cost-benefit ratio, absence of harmful or secondary effects to non-target organisms, reduction of chemical residues in the environment and foods of animal origin, and short time between fungal generations (high production) (Porfirio and Schwentesius, 2016). On the other hand, EPF can protect biodiversity in the natural ecosystem and can be used in combination with synthetic chemical products, since their residues have no known adverse effects on the environment, and are self-perpetuating under ideal environmental conditions (Maina et al., 2018). For example, Webster et al. (2105) reported that the combination of *M. anisopliae* with commercial acaricides (cypermethrin and chlorpyriphos) enhance the tick control against *R. microplus* (97.9% of efficacy). EPF have also been shown to play additional roles in nature, including endophytism, antagonism of plant diseases, promoting plant growth, and rhizosphere colonization (Jaber and Ownley, 2018). The most studied EPF worldwide as biological control for ticks are *Metarhizium anisopliae* s.l., *B. bassiana* and *A. lecanii* (formerly, *Lecanicillium lecanii*) (Fernandes et al., 2012; Romo-Martínez et al., 2013). This is consistent with the reports that exist in Mexico (Ojeda-Chi et al., 2011; Fernández-Salas et al., 2018); however, some other EPF have been reported in the country, such as *Isaria* (*Paecilomyces*) *fumosorosea* (*fumosoroseus*), which has also stood out for its effectiveness (Ángel-Sahagún et al., 2010).

Currently, the taxonomic identification and reporting of *M. anisopliae* and *B. bassiana* strains are based on the studies proposed by Bischoff et al. (2009) and Rehner et al. (2011), respectively. These proposals are supported by various studies of molecular phylogeny of multiple loci and taxonomic classification, where various monophyletic lineages have been identified concluding that both species of fungi actually comprise a complex of species, which, in many cases, they are difficult to delimit without molecular tools and analysis. After these studies, where the taxonomy of the species is clarified, the strains of these EPF that have not been reidentified according to this current taxonomy should be reported as sensu lato (s.l.), and those that have been reidentified and delimited with the taxonomic techniques and proposed molecular phylogenetic studies will be reported as sensu stricto (s. str). The EPF strains used in Mexico for the control of cattle ticks have been identified through morphological analysis of their reproductive structures, and some through molecular analysis. However, in the case of *M. anisopliae* and *B. bassiana*, some of the molecular identification techniques used were not sufficient for the delimitation of the monophyletic lineages and, for other strains, the information from the molecular analyzes is not available. Therefore, in the present review, the strains of these fungi used in Mexico will be considered as *M. anisopliae* sensu lato (s.l.) and *B. bassiana* sensu lato (s.l.) as well.

Infection Mechanism of EPF

The basic advantages related to the infection mode of EPF, compared to commercial acaricides, correspond to their ability to use different mechanisms to colonize and kill ticks. Fungi use enzymatic, toxicological and mechanical invasion systems, which suggest a difficulty for ticks to develop resistance to EPF. Furthermore, it is known that they can target almost all stages of the arthropod life cycle, which means another great advantage as a member of pest control schemes (Srinivasan et al., 2019). According to Beys-da-Silva et al. (2020), the infection mode of EPF in ticks occurs as follows: (1) recognition of the susceptible host; (2) adhesion of conidia and germination on host cuticle; (3) development of specific structures (germ tube and appressorium); (4) penetration through the host's cuticle; (5) intense fungal growth and death of the host; and (6) production of conidia after hyphae emergence through the host cuticle. **Figure 6** schematizes the infection mode of EPF in ticks.

Recognition of Susceptible Host, Adhesion of Conidia and Germination on the Host Cuticle

Aerial fungal conidia adhere to the host's cuticle through hydrophobic mechanisms (Ortiz-Urquiza and Keyhani, 2013), which are mainly mediated by proteins on the surface of the conidia named hydrophobins (St. Leger et al., 1992; Skinner et al., 2014) and adhesins (i.e., Mad1 and Mad2 identified in *M. anisopliae*) (Wang and St Leger, 2007; Valero-Jiménez et al., 2016). It is important to mention that homologous proteins of these adhesins have been reported in *B. bassiana* (Gao et al., 2020), but it has been suggested that some genes that encode these proteins between the two main EPF (*M. anisopliae* and *B. bassiana*) could be different (Chen et al., 2018). Beys da Silva et al. (2010a) reported that lipolytic activity due to the action of enzymes such as lipase and esterase in ticks, could also contribute to the recognition and adhesion of conidia during the infection of *R. microplus* by *M. anisopliae*.

Penetration Through the Host's Cuticle

Once the conidia are attached, they will germinate under optimal humidity and temperature conditions, producing a germination tube followed by a peg or appressorium for penetration into the host's cuticle (Skinner et al., 2014; Brunner-Mendoza et al., 2019). The penetration process begins and is aided by the production of several hydrolytic cuticular enzymes such as lipases, proteases and chitinases, and the mechanical pressure exerted by the appressorium (Brunner-Mendoza et al., 2019). Lipolytic enzymes including lipases, act primarily on the epicuticle, followed by proteases and chitinases, according to the presence of polymeric substrates in the different portions of the cuticle (Beys da Silva et al., 2010a,b). Among the proteases that can act at this level are subtilisins, trypsins, chymotrypsins, metallopeptidases, aspartyl peptidases, and exopeptidases (Semenova et al., 2020); where their expression from fungi such as *M. anisopliae* will depend specifically on the composition of the cuticle and hemolymph (Freimoser et al., 2005). EPF such as *M. anisopliae* and *B. bassiana* can express up to 11 different subtilisins, one of the

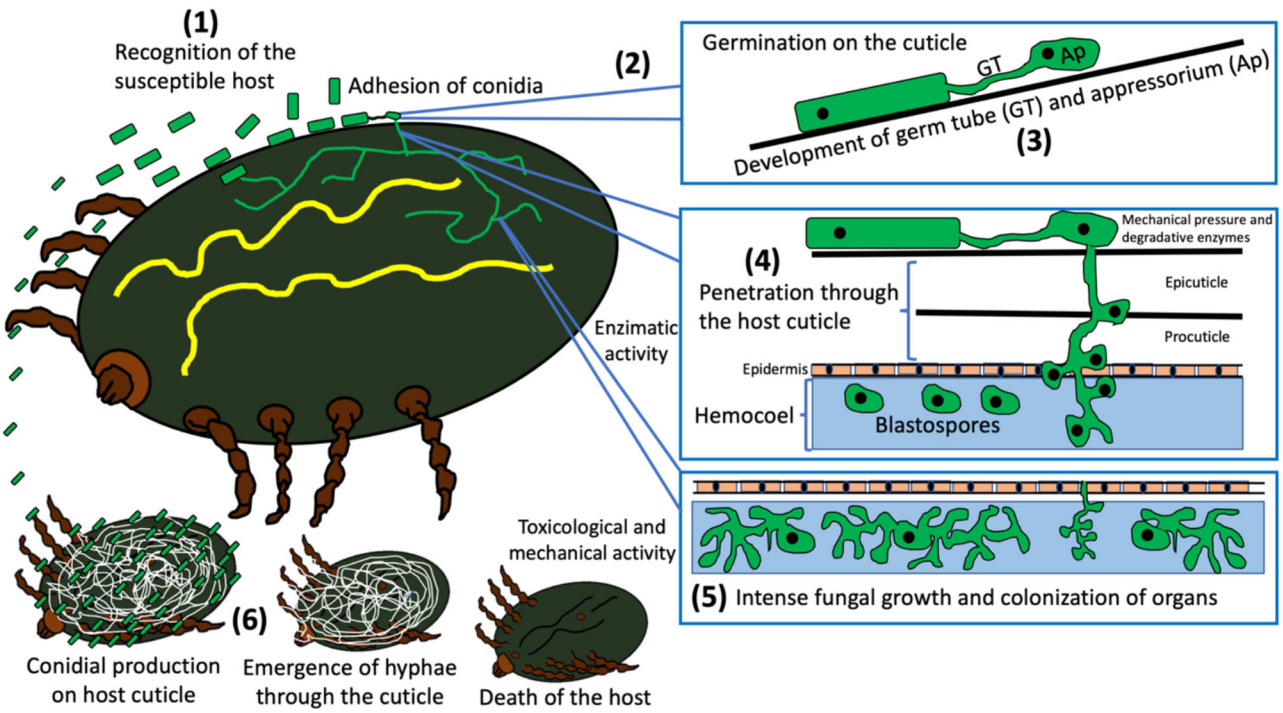

FIGURE 6 | General infection process of entomopathogenic fungi in ticks and their biological mechanisms.

most important being the Pr1 subtilisin-like peptidases, which intervene in the arthropod pathogenesis, causing hydrolysis of the cuticle and providing nutrients to the fungus (Gao et al., 2020; Semenova et al., 2020). To our knowledge, there are few reports elucidating the participation of these proteases during the infection process of cattle ticks (*R. microplus*, *R. annulatus* and *A. mixtum*) by EPF. In this regard, Golo et al. (2015) reported that spores of *M. anisopliae* s.l. expressed the Pr1 gene and that there was an increase in the specific activity of Pr1, when the fungus was cultured in *R. microplus* larvae; however, these authors concluded that increased Pr1 activity in conidia and its expression levels were not associated with significant changes (up or down) in larval mortality. It was recently reported that five of the 11 members of the Pr1 family are essential for the maintenance of the total extracellular activity of Pr1, which is necessary for the degradation of the host cuticle during hyphal invasion of EPF (such as *B. bassiana*), providing capacity for a broadest host spectrum (Gao et al., 2020). We suggest that it is necessary to continue evaluating the participation of subtilisins produced by EPF during the infection process against cattle ticks. A full understanding of the pathogenicity and/or virulence mechanism is essential for the development of an effective biological control scheme.

Fungal Growth and Death of the Host

After penetration and once inside the host, the EPF develop hyphal bodies and blastopores that multiply and disseminate through the hemolymph to invade different tissues (Maina et al., 2018; Beys-da-Silva et al., 2020) using circulation as a vehicle for colonization and for nutrient absorption (Valero-Jiménez et al., 2016; Brunner-Mendoza et al., 2019). During this event, different virulence factors act on host colonization in order to spread inside the arthropod's body, causing its death. Among the most important factors are mycotoxins such as Beauvericin, Beauverolides, Bassiannolide (by *B. bassiana*, *V. lecanii,* and *Paecilomyces* spp.) and Dextruxins A, B, C, D, E, F (by *M. anisopliae*), which act as poisons for the host (Maina et al., 2018). These fungal toxins might cause flaccid paralysis, cellular alterations and inhibit the normal functioning of muscle tissues, the middle intestine and the Malpighian tubes (Mora et al., 2017). After the dead of the host and when the nutrients within it are depleted, the fungus open the integument, forms aerial mycelia and carries out sporulation on the corpse, initiating the dispersal of its conidia (Valero-Jiménez et al., 2016). As we can see, EPF can have a wide variety of toxins that could affect the biology of livestock ticks. Although the general mechanisms of infection have been described for most of the useful EPF against arthropods, including ticks, the scientific community needs to understand the complexity of the molecular mechanisms in each infection phase, which is not completely elucidated. A transdisciplinary approach is required to incorporate different tools, such as genomics, transcriptomics, proteomics and metabolomics in order to better comprehend the mechanism of pathogenicity in EPF against ticks. It is important to note that not all EPF populations have the same capacity to produce all the enzymes or toxins reported in the scientific literature (Schrank and Vainstein, 2010), neither in variety nor in quantity. For example, in *R. microplus*, the immersion or

inoculation of dextruxin A from *M. anisopliae* on engorged ticks neither affected their biological parameters nor caused paralysis (Golo et al., 2011); but other study reported an acaricidal effect of distinct destruxins from Beauveria feline against *R. microplus* (Morais-Urano et al., 2012). In addition, different environmental and nutritional factors have been identified as the main triggers of the genetic expression of these components (Campos et al., 2005; Fang et al., 2005). The production capacity of these toxins and enzymes is reflected in the time of death of the tick, which varies according to the EPF strain, the type of fungus (genus and species), the treated tick genus, the method of application and the number of infectious spores (Fernández-Salas et al., 2017; Mantzoukas and Eliopoulos, 2020). Pathogenicity or virulence factors give EPF the ability to be specific to one or other pest arthropod, a characteristic that helps control them, without affecting other organisms beneficial to nature (Kirkland et al., 2004). It should be noted that EPF are considered "non-specialized" mite pathogens, and it has not been conclusively demonstrated that they develop specificity against ticks to the extent of causing epizootics (Fernandes et al., 2012), as has happened with some insects such as *Lymantria dispar* (Lepidoptera: Erebidae), *Diprion pini* (Hymenoptera: Diprionidae), *Dendrolimus pini* (Lepidoptera: Lasiocampidae), *D. punctatus* (Lepidoptera: Lasiocampidae), *Malacosoma disstria* (Lepidoptera: Lasiocampidae), *Fiorinia externa* (Hemiptera: Diaspididae) (Augustyniuk-Kram and Kram, 2012). However, several authors have mentioned the high capacity of these organisms to become specialists for certain arthropods, without losing their ability to be general pathogens (Beys-da-Silva et al., 2020). In this regard, a recent study has found an increase in mortality caused by Mexican strains of *M. anisopliae* s.l. on *R. microplus* and *A. mixtum*, after submitting them to four passages using these ticks as substrate (Romero-Pérez, 2020). Furthermore, Adames et al. (2011) reported in Mexico that four to seven passages of *M. anisopliae* s.l. on *R. microplus* increase its virulence against this tick. Although more studies are needed in this regard (e.g., what are the molecular or metabolic mechanisms that are triggered to increase this virulence?), the mortality behavior that these EPF develop in their evaluation against ticks is interesting. This possibility shows a promising outlook to maintain or improve virulence in those Mexican EPF strains that show favorable characteristics such as high sporulation, resistance or tolerance to UV rays, thermotolerance and/or probable specificity against ticks.

ENTOMOPATHOGENIC FUNGI AS LIVESTOCK TICK PATHOGENS

Laboratory Tests

The evaluations of *in vitro* studies regarding the acaricidal effect of EPF against livestock ticks (including resistant populations) and their reproduction in Mexico are shown in **Tables 4**–**6**. Most laboratory studies have demonstrated the effectiveness of EPF and, in some cases, their potential to control susceptible and resistant/multiresistant ticks; however, the following details can also be observed and summarized.

TABLE 4 | Laboratory evaluations of the EPF effectiveness (dosage: 1×10^8 conidia/ml) against engorged female of *R. microplus* (including resistant populations) in Mexico.

EPF	Strain	Mortality %(evaluation days)	References
M. anisopliae	ESC1	100 (20)*	Fernández-Ruvalcaba et al., 2005
	M379	37.78–53.33 (15)**	Adames et al., 2011
	Ma14	100 (20)	Ojeda-Chi et al., 2010
	Ma34	100 (20)	Ojeda-Chi et al., 2010
	Ma14+Ma34	100 (20)	Ojeda-Chi et al., 2010
	5 strains	87–100 (20)	Alcalá-Gómez et al., 2017
	55 strains	3.3–100 (20)***	Fernández-Salas et al., 2017
B. bassiana	3 strains	84–100 (20)	Alcalá-Gómez et al., 2017
	6 strains	3.3–86.7 (20)***	Fernández-Salas et al., 2018

**Including populations resistant to OP (organophosphates); **including populations resistant to OP and SP (synthetic pyrethroids); ***including populations resistant to OP, SP, Am (amidines) and Iv (ivermectin).*

Most Studies Have Been Using *R. microplus* as a Model

There is only one report on the acaricidal effect of EPF against *A. mixtum* larvae and to our knowledge, there are not reports on the acaricidal effect on *R. annulatus* in Mexico. *R. microplus*, *R. annulatus* and *A. mixtum* are the most prevalent ticks on cattle farms across the country, causing great economic losses. Therefore, it is important to evaluate the acaricidal effect of fungi on a higher diversity of ticks in order to identify strains that have a wide or reduced action spectrum, since this information could help design tick control schemes in the field (i.e., 86% of cattle farms have cohabiting *R. microplus* and *A. mixtum* in Mexico), explore the EPF infection mechanisms in each tick genus, and elucidate some biological aspects of the fungal-host immune system interaction. In recent studies, several strains of *M. anisopliae* s.l. were highly effective against larvae of *R. microplus*, but not against *A. mixtum* larvae (Jiménez-Ruíz, 2015; Fernández-Salas et al., 2017). In other countries, a high variability in the virulence of EPF against different genera of ticks and different tick populations of the same genus has been reported (Fernandes et al., 2012; Perinotto et al., 2012). Webster et al. (2017) also reported that field populations of *R. microplus* show high variation in their susceptibility to *M. anisopliae*. It is necessary to understand the factors that are involved in this differentiated response, and especially to be able to find a strain of EPF with a broad acaricidal effect against different genera of ticks and different field populations.

Acaricidal Activity of EPF Against Resistant and Multiresistant Populations of *R. microplus*

An essential goal of implementing EPF in tick control programs is the mitigation of the economic and sanitary impact of the resistance that these arthropods have developed. When a tick population is resistant to chemical acaricides, it is inappropriate

TABLE 5 | Laboratory evaluations of the EPF effectiveness (dosage: 1 × 10^8 conidia/ml) against larvae of *R. microplus* and *A. mixtum* (including resistant populations) in Mexico.

EPF	Strain	Ticks	Mortality %(evaluation days)	References
M. anisopliae	33 strains	*R. microplus*	2–100 (NS)	Ángel-Sahagún et al., 2010
	Ma14	*R. microplus*	45–62 (20)	Ojeda-Chi et al., 2010
	Ma34	*R. microplus*	34–57 (20)	Ojeda-Chi et al., 2010
	Ma14+Ma34	*R. microplus*	90 (20)	Ojeda-Chi et al., 2010
	5 strains	*R. microplus*	64.6–100 (16)	Cruz-Avalos et al., 2015
	3 strains	*R. microplus*	69.2–78.5 (4)	Díaz et al., 2014
	54 strains	*R. microplus*	1.7–100 (20)*	Fernández-Salas et al., 2019
B. bassiana	4 strains	*R. microplus*	2.5–42.9 (16)	Cruz-Avalos et al., 2015
	6 strains	*R. microplus*	3.2–53.2 (20)*	Fernández-Salas et al., 2019
I. fumosorosea	20 strains	*R. microplus*	5–94 (NS)	Ángel-Sahagún et al., 2010
	Ifr22	*R. microplus*	28.6 (16)	Cruz-Avalos et al., 2015
P. lilacinum	PlV1	*R. microplus*	92.3–94.9 (20)*	Fernández-Salas et al., 2019
M. anisopliae	23 strains	*A. mixtum*	0–32.7 (20)	Jiménez-Ruíz, 2015
B. bassiana	2 strains	*A. mixtum*	0–1.9 (20)	Jiménez-Ruíz, 2015

*Including populations resistant to OP, SP, Am and Iv; NS, Not specified.

TABLE 6 | Effect of EPF at laboratory level (dosage 1 × 10^8 conidia/ml) on the reproductive parameters of *R. microplus* in Mexico.

EPF	Strain	Inhibition of oviposition %(evaluation days)	Inhibition of egg hatching (%)	References
M. anisopliae	ESC1	74.6–75.2 (10)	Without effect	Fernández-Ruvalcaba et al., 2005
	M379	72.48–83.94 (15)	Not evaluated	Adames et al., 2011
	Ma14	12.5 (10)	Not evaluated	Ojeda-Chi et al., 2010
	Ma34	55.5 (10)	Not evaluated	Ojeda-Chi et al., 2010
	Ma14 + Ma34	39.1 (10)	Not evaluated	Ojeda-Chi et al., 2010
	5 strains	14–73 (20)	20–86	Alcalá-Gómez et al., 2017
	55 strains	8.24–55.68 (12)	Without effect	Fernández-Salas et al., 2017
B. bassiana	6 strains	0–38.2 (12)	Without effect	Fernández-Salas et al., 2018
	3 strains	12.4–98 (20)	36.7–98	Alcalá-Gómez et al., 2017

to neglect the possible interference of tick resistance mechanisms in the EPF performance (Perinotto et al., 2012). In Mexico, several strains of *M. anisopliae* s.l. induced a mortality of 90–100% in populations of *R. microplus* multiresistant to acaricides (OP, SP, Am) and ivermectin (Fernández-Salas et al., 2017, 2018, 2019). In fact, some studies reported a higher susceptibility to the EPF effect in *R. microplus* resistant to OP and SP than in susceptible strains (Adames et al., 2011). Fernández-Ruvalcaba et al. (2005) reported a similar mortality caused by *M. anisopliae* s.l. in *R. microplus* populations susceptible and resistant to OP. In general, *M. anisopliae* strains showed high acaricidal effects against resistant or acaricide-susceptible *R. microplus* and no differences in effects were observed between tick populations (**Table 4**). This is important because the resistance mechanism used by ticks in order to avoid the toxic effects of chemical acaricides did not affect the action mechanism of the EPF strains. This supports the opinion of some authors, who state that resistance to biological agents as EPF is less likely to occur compared to resistance to chemical acaricides (Polar et al., 2005).

Most Studies Have Been Targeting on a Single Stage of the Tick Life Cycle

A great advantage of EPF, compared to chemical acaricides, is that they can attack almost all stages of the arthropod life cycle, making them a unique component in integrated pest management approaches (Rajula et al., 2020). The few reports that exist on *A. mixtum* in Mexico were made in larvae, but the effect of EPF on adult ticks remains unknown. Regarding *R. microplus*, only a few EPF strains have been evaluated in engorged adult ticks and in larvae. A greater susceptibility of tick larvae to the lethal effect of EPF has been reported compared to engorged females (Fernandes and Bittencourt, 2008; Fernandes et al., 2012); however, it seems that in the bioassays carried out in Mexico there is a possible tendency of susceptibility in engorged adult ticks than in the larval stage. It would be interesting to evaluate the factors that could intervene within this possible tendency with Mexican EPF strains, considering different factors such as the nutritional, genetic, origin and biological conditions of both fungi and ticks. Regarding tick reproduction, EPF have

TABLE 7 | Field evaluations (on cattle and pastures) of the EPF effectiveness against various biological stages of *R. microplus* ticks in Mexico.

EPF	Strain	Biological cycle stage	Control %(evaluation days)	References
M. anisopliae	Ma14	Larvae on pastures	58.3–94.2 (14–28)	Ángel-Sahagún et al., 2010
	Ma14+Ma34	Larvae on pastures	40.3–100 (28–35)	Ojeda-Chi et al., 2010
	Ma379	Larvae on cattle	99.5 (14)	Romo-Martínez et al., 2013
	Ma379	Nymphs on cattle	99.7 (14)	Romo-Martínez et al., 2013
	Ma14+Ma34	Larvae and nymphs on cattle	36.5–72 (until from 4 treatment) (42)	Rodríguez-Alcocer et al., 2014
	Ma34	Adults on cattle	45.7 – 91.2 (until from 4 treatment) (42)	Alonso-Díaz et al., 2007
	Ma198	All stages on cattle	88.5 (7)	Rivera-Cervantes et al., 2017
	Ma379	Adults and teleogins on cattle	95.4–98.17 (14)	Romo-Martínez et al., 2013
	MM01	Adults on cattle	47.7 (10) 37.7 (44.5)	Bautista-Gálvez et al., 2017
	Ma14+Ma34	Adults on cattle	60.6–84.0 (until from 4 treatment) (42)	Rodríguez-Alcocer et al., 2014
B. bassiana	*B. bassiana*	Adults on cattle	76.6 (37.3)	Bautista-Gálvez et al., 2017

shown a significant inhibition of oviposition (most strains cause more than 50% effectiveness in a short time) (**Table 6**). However, the fungal effect on egg hatching inhibition percentages is not reported or has not been evaluated. This could be different in the field, since in the laboratory, the egg mass is generally removed from the engorged female between 10 to 15 days from the beginning of oviposition in order to evaluate hatching, which avoids future contact of these eggs with the spores produced on the surface of teleogin corpses, a situation that would not occur in the field. Therefore, it is recommended to include in the evaluations the direct application of EPF in the egg mass to determine its effect on its viability. In addition, it is also advisable to carry out simultaneous studies that include all stages of the tick life cycle.

Most Studies Have Been Based in the Use of *M. anisopliae* s.l. and *B. bassiana* s.l. Strains

Most of the studies carried out in Mexico have used *M. anisopliae* s.l. as the main EPF against ticks and, to a lesser extent, *B. bassiana* s.l. and *I. fumosorosea*. However, according to genetic diversity studies, other fungi have been identified that could cause high mortality effects in ticks, such as *Purpureocillium lilacinum* (Fernández-Salas et al., 2019). Therefore, it is highly recommended to continue research that identifies and evaluates the effect of different genera and species of EPF against ticks in livestock. Also, it is important to highlight that the main states where these fungi have been isolated and evaluated are located in the tropical and subtropical areas of Mexico, which stand out for their extensive livestock activity and reports of tick resistance to conventional chemical acaricides. On the other hand, these studies have allowed not only to have different EPF strains effective against ticks, but also to isolate strains from different sources and the standardization of techniques through various bioassays in distinct laboratories in the country. Taking all these aspects into account for future research, it will help to improve the EPF's effect against ticks, and include them as an important tool in tick control programs. It should be noted that the main EPF used in the studies have been isolated from different orders of insects, ticks and culture soils. In recent studies, EPF isolates have been made directly from grassland soils (Bautista-Gálvez et al., 2017; Fernandez-Salas et al., 2020), investigating whether this native characteristic may influence the tickcide effect. So far, the results have been promising (Fernández-Salas et al., 2017, 2019), attributing them to coexistence with ticks and the evolutionary adaptation of EPF to various nutritional substrates. In Mexico, studies in which EPF are isolated directly from tick corpses are scarce, although it would be interesting to carry out investigations in search of naturally colonized ticks to determine their virulence and effectiveness under controlled conditions.

Field Tests

Field studies evaluating the acaricidal effect of EPF against livestock ticks in Mexico are presented in **Table 7**. These studies have shown satisfactory control efficiency of *M. anisopliae* s.l. against different stages of *R. microplus* when applied both in pastures as in cattle; however, the following details can also be observed and summarized.

Some Field Studies Have Demonstrated the Effectiveness of *M. anisopliae* on *R. microplus* Free-Living Stage (Larvae)

Worldwide, tick control has been based on therapeutic treatments mainly directed at parasitic stages of *R. microplus*. However, this tick spends between 80 and 90% of its life cycle outside the host in pastures (Needham and Teel, 1991); therefore, a good strategy to reduce the negative effects of tick infestations on cattle is to reduce free-living populations. *M. anisopliae* conidia can be applied to livestock farm pastures (as in crops) to control free-living stages. In Mexico, the aerial dispersal of *M. anisopliae* conidia in pastures reduced the number of *R. microplus* larvae from 40 to 100% (Ángel-Sahagún et al., 2010; Ojeda-Chi et al., 2010). However, it is necessary to investigate the acaricidal effect using another genus of EPF (e.g., *B. bassiana, I. fumosorosea* or *P. lilacinum*). Recently, our research group evaluated the acaricidal activity of about 60 EPF strains isolated from paddocks against *R. microplus* in the

Mexican tropics (Fernández-Salas et al., 2017, 2018). EPF adapted both to the regional environmental conditions in which they were used, as well to the ticks that served as substrate for fungi development. Therefore, these EPF strains could also be useful in the implementation of biological control programs against ticks. In addition, several isolates showed thermotolerance and resistance to UV-R, which makes them possible candidates for field evaluation. Likewise, it is necessary to evaluate the fungal strains with high efficiency against other genera of ticks (*A. mixtum* and *R. annulatus*) present in pastures of cattle farms in Mexico.

Most *in vivo* Studies Have Demonstrated the Efficacy of *M. anisopliae* s.l. Applied to Naturally Infested Cattle

Most of the field studies carried out in Mexico showed that when EPF conidia were sprayed on cattle, high control percentages against parasitic stages of *R. microplus* were observed (**Table 7**). However, it is known that EPF could decrease its acaricidal efficacy against ticks under field conditions due to biotic and abiotic factors that affect its virulence and pathogenicity. Fungal growth, conidia production, survival, germination, pathogenicity, virulence and the production of bioactive compounds can be strongly influenced by exposure to ultraviolet solar radiation (Wong et al., 2019).

Therefore, it is recommended that when using EPF for tick control in the field, strategies to counteract the negative effects of these factors need to be considered in order to improve the tickcide effect. Among the main strategies are the use of protectors and dispersers of conidia from highly virulent and pathogenic isolates, the selection of isolates adapted to the climatic conditions where they will be used (i.e., native isolates with a greater natural tolerance to UV-R) and proper application of EPF in order to avoid high temperatures and UV-R.

No Adverse Effects Were Reported in Animals and/or Operators

An important point that should be taken into account when using EPF is the safety in their use. None of the field studies conducted has reported adverse reactions in cattle or operators. This is consistent with Zimmermann (2007), who mentioned that EPF do not pose risks to animals, humans or the environment, concluding that this control method is a safe and sustainable alternative. On the contrary, the use of chemical acaricides can be highly harmful to beneficial species or non-target organisms, humans, animals and the environment (Fernández-Salas et al., 2012a). Therefore, having an alternative control will help reduce the use of these chemicals and keep these situations at a low risk of presentation, based on the premise that EPF with affinity for a target organism are less capable of causing harm to non-target organisms (Goettel and Johnson, 1992). However, in Mexico, it is recommended to complement field studies with evaluations of the beneficial or negative impact of EPF on the ecosystems of livestock farms.

Integrated Pest Management of Livestock Ticks

Through many years of experience in treating ticks, studies have concluded that applying a single treatment will not maintain efficient and sustainable control in the long term. Invariably, the product used will exhibit inefficiencies in killing ticks due to their ability to become resistant. In Mexico, it has been mentioned that none of the previous strategies (chemical and non-chemical) by themselves have been sufficient to sustainably control ticks, such as *R. microplus* (Romo-Martínez et al., 2013; Fernández-Salas et al., 2019). Therefore, it is necessary to integrate two or more methodologies in order to be able to attack ticks on several fronts and take care, among themselves, of the effectiveness of the products or techniques used. Integrated pest management is defined as the systematic application of two or more technologies that are compatible with each other, with the environment and that are profitable to control populations of arthropod pests that negatively affect livestock (Bram, 1994). EPF are compatible with various tick control products, including chemical acaricides, without losing their acaricidal capacity (Sousa et al., 2011; Kiss et al., 2012; Romo-Martínez et al., 2013). Therefore, the integration of EPF in a tick control scheme is totally feasible.

Proposals for Integrated Tick Control in Mexico

Research evaluating integrated pest management schemes, including the EPF for tick control in Mexico, is scarce. There is a very important gap that requires more research, since it has been mentioned that integrated tick control is the best way to establish sustainable and successful livestock in order to increase the productive capacity of the animals (Rodríguez-Vivas et al., 2014b).

Proposals for the use of EPF within an integrated tick pest management should be designed according to several factors, listed below.

The Climatological Characteristics of the Region Where the Control Will Be Implemented

The population dynamics of ticks depends mainly on the conditions of temperature, relative humidity and rainfall. Therefore, the distribution of ticks throughout the year can be predicted since climatic factors are responsible for this characteristic.

The Ticks Present in the Control Area

Different tick genera may show differences in the biological cycles, so their presence in the bovine body and in the pastures is distinct throughout the year (e.g., *R. microplus* and *A. mixtum*, which coexist in the Mexican tropics).

The Toxicological Response/Behavior of Ticks

It is necessary to know the susceptibility status of the ticks to be treated, since, as mentioned above, chemical acaricides are and will be the basis of tick control programs, including those of integral management and the success of the establishment of these protocols will depend on their proper use.

The Availability and Compatibility of Various Methods for Tick Control

It is important to consider all available and proven alternatives for tick treatment and use them in combination with each other and with chemical acaricides.

One of the main advantages that is present in Mexico for the design of tick control protocols through integrated management is that climatic characteristics of the country have well-defined patterns (Estrada-Peña et al., 2006). However, there are few studies where the population dynamics of ticks of livestock importance have been determined in Mexico through the seasons of the year and in different states (Estrada-Peña et al., 2006; Alonso-Díaz et al., 2007; González-Cerón et al., 2009; Almazán et al., 2016). For this reason, adequate proposal designs for integrated tick management for all ecological regions of the country are limited.

CONCLUSIONS

EPF have been shown to have good acaricidal effectiveness against ticks of livestock importance and their different biological stages, both in the laboratory and in the field. However, the vast majority of studies have focused on the *R. microplus* tick. In accordance with the economic and sanitary importance of other ticks such as *A. mixtum* and *R. annulatus* in Mexico, it is also recommended to test the efficacy of these fungi against these ticks. Furthermore, the EPF used have been shown to be biologically safe when applied to animals and pastures, including the safety operators. The lack of information on the mechanisms (molecular, genetic, immunological and physiological interactions) involved in the virulence of EPF in ticks was also identified. Most of the information has been obtained on insects, which are taxonomically different from ticks, so these mechanisms may be different. According to the results of the acaricidal efficacy shown by EPF against ticks, they could be considered within an integrated management of these pests. However, it is highly recommended that more studies be carried out on the population dynamics of ticks in the different agroecological regions of the country, more evaluations of tick susceptibility to all available chemical acaricides, and the probable synergy or antagonism of EPF with other alternative control methods, since a paucity of information on these characteristics of ticks and EPF has also been identified.

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MAA-D: investigation, methodology, writing original draft, supervision, and conceptualization. AF-S: investigation, methodology, writing original draft, supervision, and conceptualization. All authors took part in reviewing and editing of the final manuscript.

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REFERENCES

Aboelhadid, S. M., Arafa, W. M., Mahrous, L. N., Fahmy, M. M., and Kamel, A. A. (2018). Molecular detection of *Rhipicephalus* (*Boophilus*) *annulatus* resistance against deltamethrin in middle Egypt. *Vet. Parasitol. Reg. Stud. Rep*. 13, 198–204. doi: 10.1016/j.vprsr.2018.06.008

Adames, M., Fernández-Ruvalcaba, M., Peña-Chora, G., and Hernández-Velázquez, V. M. (2011). Effects of passages through a suitable host of the fungus, *Metarhizium anisopliae*, on the virulence of acaricide-susceptible and resistant strains of the tick, *Rhipicephalus microplus*. *J. Insect. Sci*. 11:21. doi: 10.1673/031.011.0121

Aguilar-Domínguez, M., Sánchez-Montes, S., Esteve-Gassent, M. D., Barrientos-Salcedo, C., de León, A. P., and Romero-Salas, D. (2019). Genetic structure analysis of *Amblyomma mixtum* populations in Veracruz State, Mexico. *Ticks Tick Borne Dis*. 10, 86–92. doi: 10.1016/j.ttbdis.2018.09.004

Aguilar-Tipacamú, G., Nazar, P. M., Sesma, B. R., Llaven, M. Á. O., Martínez, C. E. I., and Trujillo, G. U. B. (2009). Resistencia al amitraz de *Rhipicephalus* (*Boophilus*) *microplus* en unidades de producción del estado de Chiapas. *Quehacer Científico en Chiapas* 1, 16–22. Availbale online at: https://dgip.unach.mx/images/pdf-REVISTA-QUEHACERCIENTIFICO/QUEHACER-CIENTIFICO-2009-ener-jun/resistencia-al-amitraz_de_rhipicephalus.pdf (accesed November 15, 2020).

Alcalá-Gómez, J., Cruz-Vázquez, C., Fernández-Ruvalcaba, M., Ángel-Sahagún, C., Vitela-Mendoza, I., and Ramos-Parra, M. (2017). Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* isolates and the effects of fungal infection on the reproduction potential of *Rhiphicephalus microplus* engorged females. *Biocontrol. Sci. Techn*. 27, 931–939. doi: 10.1080/09583157.2017.1366422

Alegría-López, M. A., Rodríguez-Vivas, R. I., Torres-Acosta, J. F. J., Ojeda-Chi, M. M., and Rosado-Aguilar, J. A. (2015). Use of ivermectin as endoparasiticide in tropical cattle herds generates resistance in gastrointestinal nematodes and the tick *Rhipicephalus microplus* (Acari: Ixodidae). *J. Med. Entomol*. 52, 214–221. doi: 10.1093/jme/tju025

Almazán, C., Torres-Torres, A., Torres-Rodríguez, L., Soberanes-Céspedes, N., and Ortiz-Estrada, M. (2016). Aspectos biológicos de *Amblyomma mixtum* (Koch, 1844) en el noreste de México. *Quehacer Científico en Chiapas* 11, 10–19. Available online at: https://www.dgip.unach.mx/images/pdf-REVISTA-QUEHACERCIENTIFICO/2016-jul-dic/Aspectos_biologicos_de_Amblyomma_mixtum_.pdf (accessed December 1, 2020).

Alonso-Díaz, M. A., Fernández-Salas, A., and Basurto, C. H. (2013b). *Manual Técnico: La Garrapata Rhipicephalus (Boophilus) Microplus: su Comportamiento, Control y Resistencia a los Acaricidas en el Trópico Mexicano: 21° Día del Ganadero*. Martínez de la Torre: COFUPRO - FUNPROVER - UNAM. pp. 19–30.

Alonso-Díaz, M. A., Fernández-Salas, A., Martínez-Ibáñez, F., and Osorio-Miranda, J. (2013a). *Amblyomma cajennense* (Acari: Ixodidae) tick populations susceptible or resistant to acaricides in the Mexican Tropics. *Vet. Parasitol*. 197, 326–331. doi: 10.1016/j.vetpar.2013.06.004

Alonso-Díaz, M. Á., Silva, B. J. L., de Magalhães Labarthe, A. C. L., and Rodríguez-Vivas, R. I. (2007). Infestación natural de hembras de *Boophilus microplus* Canestrini, 1887 (Acari: Ixodidae) en dos genotipos de bovinos en el trópico húmedo de Veracruz, México. *Vet. Méx*. 38, 503–509. Available online at: http://www.redalyc.org/articulo.oa?id=42338410

Alonso-Díaz, M. A., Torres-Acosta, J. F. J., Sandoval-Castro, C. A., and Bruce-Campbell, W. (2014). "Controlling the introduction and augmentation of parasites in and on domesticated livestock," in *Sustainable Food Production Includes Human and Environmental Health*, eds W. B. Campbell, and S. López-Ortíz (Netherlands: Springer), 191–228. doi: 10.1007/978-94-007-7454-4_5

Ángel-Sahagún, C. A., Lezama-Gutiérrez, R., Molina-Ochoa, J., Pescador-Rubio, A., Skoda, S. R., Cruz-Vázquez, C., et al. (2010). Virulence of Mexican isolates of entomopathogenic fungi (Hypocreales: Clavicipitaceae) upon *Rhipicephalus*= *Boophilus microplus* (Acari: Ixodidae) larvae and the efficacy of conidia formulations to reduce larval tick density under field conditions. *Vet. Parasitol.* 170, 278–286. doi: 10.1016/j.vetpar.2010.02.037

Araújo, J. P., and Hughes, D. P. (2016). Diversity of entomopathogenic fungi: which groups conquered the insect body? *Adv. Genet.* 94, 1–39. doi: 10.1016/bs.adgen.2016.01.001

Araya-Anchetta, A., Busch, J. D., Scoles, G. A., and Wagner, D. M. (2015). Thirty years of tick population genetics: a comprehensive review. *Infect. Genet. Evol.* 29, 164–179. doi: 10.1016/j.meegid.2014.11.008

Armendáriz-González, I. (2003). Informe de un caso de resistencia múltiple a ixodicidas en *Boophilus microplus* Canestrini (Acari: Ixodidae) en Tamaulipas, México. *Vet. Méx.* 34, 397–401. Available online at: http://www.redalyc.org/articulo.oa?id=42334408

Augustyniuk-Kram, A., and Kram, K. J. (2012). "Entomopathogenic fungi as an important natural regulator of insect outbreaks in forests," in Forest ecossistems-more than just trees, ed J. A. Blanco, and L. Yueh-Hsin (Rijeka, Croatia: In Tech Press), 265–294. doi: 10.5772/30596

Barré, N., and Uilenberg, G. (2010). Spread of parasites transported with their hosts: case study of two species of cattle tick. *Rev. sci. tech. Off. int. Epiz.* 29, 149–160. doi: 10.20506/rst.29.1.1969

Bautista-Gálvez, A. B., Segura, R. P., and Gómez-Vázquez, A. (2017). Biological Control of *Rhicephalus* (*Boophilus*) *microplus* with Entomopathogenic Fungi. *CIBA Rev. Iberoam. Cienc. Biol. Agropecuarias* 6, 33–62. doi: 10.23913/ciba.v6i12.68

Beys da Silva, W. O., Santi, L., Corrêa, A. P. F., Silva, L. A. D., Bresciani, F. R., and, Schrank, A., et al. (2010a). The entomopathogen *Metarhizium anisopliae* can modulate the secretion of lipolytic enzymes in response to different substrates including components of arthropod cuticle. *Fungal Biol.* 114, 911–916. doi: 10.1016/j.funbio.2010.08.007

Beys da Silva, W. O., Santi, L., Schrank, A., and Vainstein, M. H. (2010b). *Metarhizium anisopliae* lipolytic activity plays a pivotal role in *Rhipicephalus* (*Boophilus*) *microplus* infection. *Fungal Biol.* 114, 10–15. doi: 10.1016/j.mycres.2009.08.003

Beys-da-Silva, W. O., Rosa, R. L., Berger, M., Coutinho-Rodrigues, C. J., Vainstein, M. H., Schrank, A., et al. (2020). Updating the application of *Metarhizium anisopliae* to control cattle tick *Rhipicephalus microplus* (Acari: Ixodidae). *Exp. Parasitol.* 208:107812. doi: 10.1016/j.exppara.2019.107812

Bischoff, J. F., Rehner, S. A., and Humber, R. A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101, 512–530. doi: 10.3852/07-202

Bram, R. A. (1994). Integrated control of ectoparasites. *Rev. Off. Int. Epizoot.* 13, 1357–1365. doi: 10.20506/rst.13.4.822

Brunner-Mendoza, C., Reyes-Montes, M. D. R., Moonjely, S., Bidochka, M. J., and 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. Techn.* 29, 83–102. doi: 10.1080/09583157.2018.1531111

Cabrera-Jimenez, D., Rodríguez-Vivas, R. I., and Rosado-Aguilar, J. A. (2008). Evaluation of cypermethrin resistance in *Boophilus microplus* strains from cattle farms in the State of Yucatán, Mexico. *Téc. Pecu. Méx.* 46, 439–447. Available online at: https://cienciaspecuarias.inifap.gob.mx/index.php/Pecuarias/article/view/1793 (accessed November 24, 2020).

Campos, R. A., Arruda, W., Boldo, J. T., Silva, M. V., Barros, N. M., Azevedo, J. L., et al. (2005). *Boophilus microplus* infection by *Beauveria amorpha* and *Beauveria bassiana*: SEM analysis and regulation of subtilisin-like proteases and chitinases. *Curr. Microbiol.* 50, 257–261. doi: 10.1007/s00284-004-4460-y

Cárdenas-Canales, E. M., Ortega-Santos, J. A., Campbell, T. A., García-Vázquez, Z., Cantú-Covarrubias, A., Figueroa-Millán, J. V., et al. (2011). Nilgai antelope in northern Mexico as a possible carrier for cattle fever ticks and *Babesia bovis* and *Babesia bigemina*. *J. Wildl. Dis.* 47, 777–779. doi: 10.7589/0090-3558-47.3.777

Castillo-Gallegos, E., de la Mora, B. V., Mannetje, L. T., and Schunemann, A. A. (2005). Efecto de introducir *Arachis pintoi* sobre variables del suelo de pasturas de grama nativa del trópico húmedo mexicano. *Téc. Pec. Méx.* 43, 287–295. Available online at: http://www.redalyc.org/articulo.oa?id=61343214

CFSPH (The Center For Food Security and Public Health) (2007). *Rhipicephalus (Boophilus) microplus*. Iowa State University. P. 3. Available online at: http://www.cfsph.iastate.edu/Factsheets/es/boophilus_microplus-es.pdf (accessed October 24, 2020).

Chen, A., Wang, Y., Shao, Y., Zhou, Q., Chen, S., Wu, Y., et al. (2018). Genes involved in *Beauveria bassiana* infection to *Galleria mellonella*. *Arch. Microbiol.* 200, 541–552. doi: 10.1007/s00203-017-1456-0

Cruz-Avalos, A., Cruz-Vázquez, C., Lezama-Gutiérrez, R., Vitela-Mendoza, I., and Angel-Sahagún, C. (2015). Selección de aislados de hongos entomopatógenos para el control de *Rhipicephalus microplus* (Acari: Ixodidae). *Trop. Subtrop. Agroecosyst.* 18, 175–180.

Cumming, G. S., and Van Vuuren, D. P. (2006). Will climate change affect ectoparasite species ranges? *Glob. Ecol. Biogeogr.* 15, 486–497. doi: 10.1111/j.1466-822X.2006.00241.x

De Alva, R. (1995). "Creating new products for animal health," in *Tercer Seminario Internacional de Parasitologia Animal*, ed S. Rodríguez-Camarillo, and H. Fragoso-Sánchez (Acapulco), 86–87.

De Castro, J. J. (1997). Sustainable tick and tickborne disease control in livestock improvement in developing countries. *Vet. Parasitol.* 71, 77–97. doi: 10.1016/S0304-4017(97)00033-2

Díaz, V. M., Izaguirre, F. F., Martínez, T. J. J., Aguirre, M. J. F., Posada, C. S., García, C. C. G., et al. (2014). Efecto de tres cepas de *Metarhizium anisopliae* (Metch) Sor sobre la mortalidad de *Rhipicephalus (Boophilus) microplus* Canestrini en condiciones de laboratorio. *Livestock Res. Rural. Dev.* 26:2014. Available online at: http://www.lrrd.org/lrrd26/9/diaz26163.html (accessed November 24, 2020).

Domínguez-García, D. D., Agatón, F. T., and Rosario-Cruz, R. (2016). Evaluación económica del control de garrapatas *Rhipicephalus microplus* en México/Economic evaluation of tick (*Rhipicephalus microplus*) control in Mexico. *CIBA Rev. Iberoam. Cienc. Biol. Agropecuarias* 5, 43–52. doi: 10.23913/ciba.v5i9.49

Esteve-Gasent, M. D., Rodríguez-Vivas, R. I., Medina, R. F., Ellis, D., Schwartz, A., Cortés Garcia, B., et al. (2020). Research on integrated management for cattle fever ticks and bovine babesiosis in the United States and Mexico: current status and opportunities for binational coordination. *Pathogens* 9:871. doi: 10.3390/pathogens9110871

Esteve-Gassent, M. D., Pérez de León, A. A., Romero-Salas, D., Feria-Arroyo, T. P., Patino, R., Castro-Arellano, I., et al. (2014). Pathogenic landscape of transboundary zoonotic diseases in the Mexico–US border along the Rio Grande. *Front. Public. Health*. 2:177. doi: 10.3389/fpubh.2014.00177

Estrada-Peña, A. (2008). Tick-borne pathogens, transmission rates and climate change. *Front. Biosci.* 14, 2674–2687. Available online at: https://pdfs.semanticscholar.org/9990/3f0755d290a56049042279ed183025d999a9.pdf (accessed December 4, 2020).

Estrada-Peña, A., García, Z., and Sánchez, H. F. (2006). The distribution and ecological preferences of *Boophilus microplus* (Acari: Ixodidae) in Mexico. *Exp. Appl. Acarol.* 38, 307–316. doi: 10.1007/s10493-006-7251-2

Estrada-Peña, A., Guglielmone, A. A., and Mangold, A. J. (2004). The distribution and ecological 'preferences' of the tick *Amblyomma cajennense* (Acari: Ixodidae), an ectoparasite of humans and other mammals in the Americas. *Ann. Trop. Med. Parasitol.* 98, 283–292. doi: 10.1179/000349804225003316

Estrada-Peña, A., and Venzal, J. M. (2006). High-resolution predictive mapping for *Boophilus annulatus* and *B. microplus* (Acari: Ixodidae) in Mexico and Southern Texas. *Vet. Parasitol.* 14, 350–358. doi: 10.1016/j.vetpar.2006.07.003

Falvey, J. L. (2015). Food security: the contribution of livestock. *Chiang Mai Univ. J. Nat. Sci.* 14, 103–117. doi: 10.12982/CMUJNS.2015.0074

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., et al. (2005). Cloning of *Beauveria bassiana* chitinase gene *Bbchit1* and its application to improve fungal strain virulence. *Appl. Environ. Microbiol.* 71, 363–370. doi: 10.1128/AEM.71.1.363-370.2005

Fernandes, É. K., Bittencourt, V. R., and Roberts, D. W. (2012). Perspectives on the potential of entomopathogenic fungi in biological control of ticks. *Exp. Parasitol.* 130, 300–305. doi: 10.1016/j.exppara.2011.11.004

Fernandes, É. K. K., and Bittencourt, V. R. E. P. (2008). "Entomopathogenic fungi against South American tick species," in *Diseases of Mites and Ticks*, eds J. Bruin, and L. P. S. van der Geest (Dordrecht: Springer), 71–93. doi: 10.1007/978-1-4020-9695-2_8

Fernández-Ruvalcaba, M., Zhioua, E., and García-Vázquez, Z. (2005). Infectividad de *Metarhizium anisopliae* en contra de cepas de garrapata *Boophilus microplus* sensible y resistente a los organofosforados. *Téc. Pecu. Méx*. 43, 433–440. Available online at: http://www.redalyc.org/articulo.oa?id=61343313

Fernández-Salas, A., Alonso-Díaz, M. A., and Alonso-Morales, R. A. (2019). Effect of entomopathogenic native fungi from paddock soils against *Rhipicephalus microplus* larvae with different toxicological behaviors to acaricides. *Exp. Parasitol*. 204:107729. doi: 10.1016/j.exppara.2019.107729

Fernández-Salas, A., Alonso-Díaz, M. A., Alonso-Morales, R. A., Lezama-Gutiérrez, R., Rodríguez-Rodríguez, J. C., and Cervantes-Chávez, J. A. (2017). Acaricidal activity of *Metarhizium anisopliae* isolated from paddocks in the Mexican tropics against two populations of the cattle tick *Rhipicephalus microplus*. *Med. Vet. Entomol*. 31, 36–43. doi: 10.1111/mve.12203

Fernández-Salas, A., Alonso-Díaz, M. Á., Morales, R. A. A., Lezama-Gutiérrez, R., and Cervantes-Chávez, J. A. (2018). Phylogenetic relationships and acaricidal effects of *Beauveria bassiana* obtained from cattle farm soils against *Rhipicephalus microplus*. *J. Parasitol*. 104, 275–282. doi: 10.1645/17-162

Fernandez-Salas, A., Alonso-Morales, R. A., and Alonso-Díaz, M. Á. (2020). Distribution of entomopathogenic fungi in soils of cattle farms and factors associated with their presence in the Mexican tropics. *Trop. Subtrop. Agroecosyst*. 23, 1–12. Available online at: http://www.revista.ccba.uady.mx/urn:ISSN:1870-0462-tsaes.v23i3.3293

Fernández-Salas, A., Rodríguez-Vivas, R. I., and Alonso-Díaz, M. Á. (2012a). Resistance of *Rhipicephalus microplus* to amitraz and cypermethrin in tropical cattle farms in Veracruz, Mexico. *J. Parasitol*. 98, 1010–1014. doi: 10.1645/GE-3074.1

Fernández-Salas, A., Rodríguez-Vivas, R. I., and Alonso-Díaz, M. A. (2012c). First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics. *Vet. Parasitol*. 183, 338–342. doi: 10.1016/j.vetpar.2011.07.028

Fernández-Salas, A., Rodríguez-Vivas, R. I., Alonso-Díaz, M. A., and Basurto-Camberos, H. (2012b). Ivermectin resistance status and factors associated in *Rhipicephalus microplus* (Acari: Ixodidae) populations from Veracruz, Mexico. *Vet. Parasitol*. 190, 210–215. doi: 10.1016/j.vetpar.2012.06.003

Freimoser, F. M., Hu, G., and St Leger, R. J. (2005). Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. *Microbiology* 151, 361–371. doi: 10.1099/mic.0.27560-0

Gao, B. J., Mou, Y. N., Tong, S. M., Ying, S. H., and Feng, M. G. (2020). Subtilisin-like Pr1 proteases marking the evolution of pathogenicity in a wide-spectrum insect-pathogenic fungus. *Virulence* 11, 365–380. doi: 10.1080/21505594.2020.1749487

Giles, J. R., Peterson, A. T., Busch, J. D., Olafson, P. U., Scoles, G. A., Davey, R. B., et al. (2014). Invasive potential of cattle fever ticks in the southern United States. *Parasites Vectors* 7:189. doi: 10.1186/1756-3305-7-189

Goettel, M. S., and Johnson, D. L. (1992). *Environmental Impact and Safety of Fungal Biocontrol Agents*. Available online at: https://agris.fao.org/agris-search/search.do?recordID=GB9124449 (accessed January 7, 2021).

Golo, P. S., Angelo, I. C., Camargo, M. G., Perinotto, W. M. S., and Bittencourt, V. R. E. P. (2011). Effects of destruxin A on *Rhipicephalus (Boophilus) microplus* ticks (Acari: Ixodidae). *Rev. Bras. Parasitol. Vet*. 20, 338–341. doi: 10.1590/S1984-29612011000400015

Golo, P. S., Santos, H. A., Perinotto, W. M., Quinelato, S., Angelo, I. C., Camargo, M. G., et al. (2015). The influence of conidial Pr1 protease on pathogenicity potential of *Metarhizium anisopliae* senso latu to ticks. *Parasitol. Res*. 114, 2309–2315. doi: 10.1007/s00436-015-4426-y

González-Cerón, F., Becerril-Pérez, C. M., Torres-Hernández, G., Díaz-Rivera, P., Santellano-Estrada, E., and Rosendo-Ponce, A. (2009). Infestación natural por *Amblyomma cajennense* y *Boophilus microplus* en bovinos criollo lechero tropical durante la época de lluvias. *Agrociencia* 43, 577–584. Available online at: http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S1405-31952009000600003 (accessed April 17, 2021).

González-Padilla, E., Lassala, A., Pedernera, M., and Gutierrez, C. G. (2019). Cow-calf management practices in Mexico: farm organization and infrastructure. *Vet. Méx*. 6, 1–17. doi: 10.22201/fmvz.24486760e.2019.3.677

Grisi, L., Leite, R. C., Martins, J. R. D. S., Barros, A. T. M. D., Andreotti, R., Cançado, P. H. D., et al. (2014). Reassessment of the potential economic impact of cattle parasites in Brazil. *Rev. Bras. Parasitol. Vet*. 23, 150–156. doi: 10.1590/S1984-29612014042

Higa, L. D. O. S., Piña, F. T. B., da Silva Rodrigues, V., Garcia, M. V., Salas, D. R., Miller, R. J., et al. (2020). Evidence of acaricide resistance in different life stages of *Amblyomma mixtum* and *Rhipicephalus microplus* (Acari: Ixodidae) collected from the same farm in the state of Veracruz, Mexico. *Prev. Vet. Med*. 174:104837. doi: 10.1016/j.prevetmed.2019.104837

Humber, R. A. (2008). Evolution of entomopathogenicity in fungi. *J. Invert. Pathol*. 98, 262–266. doi: 10.1016/j.jip.2008.02.017

Jaber, L. R., and Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control*. 116, 36–45. doi: 10.1016/j.biocontrol.2017.01.018

Jiménez-Ruíz, M. (2015). *Efecto acaricida in vitro de hongos entomopatógenos nativos de Veracruz, México, contra larvas de garrapata Amblyomma cajennense (Acari: Ixodidae)* (Dissertation/Bachelor's thesis). Oaxaca: Universidad Autónoma Benito Juarez de Oaxaca.

Kirkland, B. H., Cho, E. M., and Keyhani, O. N. (2004). Differential susceptibility of *Amblyomma maculatum* and *Amblyomma americanum* (Acari: Ixodidea) to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Biol. Control*. 31, 414–421. doi: 10.1016/j.biocontrol.2004.07.007

Kiss, T., Cadar, D., and Spînu, M. (2012). Tick prevention at a crossroad: new and renewed solutions. *Vet. Parasitol*. 187, 357–366. doi: 10.1016/j.vetpar.2012.02.010

Klafke, G. M., Moreno, H. C., Tidwell, J. P., Miller, R. J., Thomas, D. B., Feria-Arroyo, T. P., et al. (2020). Partial characterization of the voltage-gated sodium channel gene and molecular detection of permethrin resistance in *Rhipicephalus annulatus* (Say, 1821). *Ticks Tick Borne Dis*. 11:101368. doi: 10.1016/j.ttbdis.2019.101368

Kutz, S., Jenkins, E., Veitch, A., Ducrocq, J., Polley, L., and Elkin, B. (2009). The Arctic as a model for anticipating, preventing, and mitigating climate change impacts on host–parasite interactions. *Vet. Parasitol*. 163, 217–228. doi: 10.1016/j.vetpar.2009.06.008

Li, A. Y., Davey, R. B., Miller, R. J., and George, J. E. (2003). Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. *J. Med. Entomol*. 40, 482–490. doi: 10.1603/0022-2585-40.4.482

Li, A. Y., Davey, R. B., Miller, R. J., and George, J. E. (2004). Detection and characterization of amitraz resistance in the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *J. Med. Entomol*. 41, 193–200. doi: 10.1603/0022-2585-41.2.193

Lohmeyer, K. H., Pound, J. M., May, M. A., Kammlah, D. M., and Davey, R. B. (2011). Distribution of *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) annulatus* (Acari: Ixodidae) infestations detected in the United States along the Texas/Mexico border. *J. Med. Entomol*. 48, 770–774. doi: 10.1603/ME10209

Maina, U. M., Galadima, I. B., Gambo, F. M., and Zakaria, D. (2018). A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *J. Entomol. Zool. Stud*. 6, 27–32. Available online at: https://www.entomoljournal.com/archives/2018/vol6issue1/PartA/5-5-367-216.pdf

Mantzoukas, S., and Eliopoulos, P. A. (2020). Endophytic entomopathogenic fungi: a valuable biological control tool against plant pests. *Appl. Sci*. 10:360. doi: 10.3390/app10010360

Martínez, I. F., De Labra, V. G., and Osorio, M. J. (2019). "Toxicological response of different genera and species of ixodide ticks collected in Mexico," in *Memorias del XI Congreso Nacional de Parasitología Veterinaria* (Monterrey), 139–145.

Merino, O., De la Cruz, N. I., Martinez, J., de León, A. P., Romero-Salas, D., Esteve-Gassent, M. D., et al. (2020). Molecular detection of Rickettsia species in ticks collected in the Mexico-USA transboundary region. *Exp. Appl. Acarol*. 80, 559–567. doi: 10.1007/s10493-020-00483-5

Miller, R. J., Almazán, C., Ortíz-Estrada, M., Davey, R. B., George, J. E., and De León, A. P. (2013). First report of fipronil resistance in *Rhipicephalus* (*Boophilus*) *microplus* of Mexico. *Vet. Parasitol*. 191, 97–101. doi: 10.1016/j.vetpar.2012.08.011

Miller, R. J., Li, A. Y., Tijerina, M., Davey, R. B., and George, J. E. (2008). Differential response to diazinon and coumaphos in a strain of *Boophilus microplus* (Acari: Ixodidae) collected in Mexico. *J. Med. Entomol*. 45, 905–911. doi: 10.1603/0022-2585(2008)45[905:DRTDAC]2.0.CO;2

Miller, R. J., Rentaria, J. A. E., Martinez, H. Q., and George, J. E. (2007). Characterization of permethrin-resistant *Boophilus microplus* (Acari: Ixodidae) collected from the state of Coahuila, Mexico. *J. Med. Entomol*. 44, 895–897. doi: 10.1603/0022-2585(2007)44[895:COPBMA]2.0.CO;2

Montero, E., González, L. M., Chaparro, A., Benzal, J., Bertellotti, M., Masero, J. A., et al. (2016). First record of *Babesia* sp. in Antarctic penguins. *Ticks. Tick. Borne. Dis.* 7, 498–501. doi: 10.1016/j.ttbdis.2016.02.006

Mora, M. A. E., Castilho, A. M. C., and Fraga, M. E. (2017). Classification and infection mechanism of entomopathogenic fungi. *Arq. Inst. Biol.* 84:e0552015. doi: 10.1590/1808-1657000552015

Morais-Urano, R. P., Chagas, A. C., Berlinck, R. G. (2012). Acaricidal action of destruxins produced by a marine-derived Beauveria felina on the bovine tick Rhipicephalus (Boophilus) microplus. *Exp. Parasitol.* 132, 362–366. doi: 10.1016/j.exppara.2012.08.011

Morillón, D., Silva Casarín, R., and Valdés, H. P. (2018). *Atlas del Impacto del Océano en el Clima en México*. Campeche: Cemie-Océano, Universidad Autónoma de Campeche. p. 128.

NASA-GCC (2019). *Overview: Weather, Global Warming and Climate Change*. Global Warming vs Climate Change. Resource 32. Available online at: https://climate.nasa.gov/resources/global-warming-vs-climate-change/ (accessed December 14, 2020).

Needham, G. R., and Teel, P. D. (1991). Off-host physiological ecology of ixodid ticks. *Ann. Rev. Entomol.* 36, 659–681. doi: 10.1146/annurev.en.36.010191.003303

Ojeda-Chi, M. M., Rodríguez-Vivas, R. I., Galindo-Velasco, E., Lezama-Gutiérrez, R., and Cruz-Vázquez, C. (2011). Control de *Rhipicephalus microplus* (Acari: Ixodidae) mediante el uso del hongo entomopatógeno *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae): Revisión. *Rev. Mex. Cienc. Pec.* 2, 177–192. Available online at: http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S2007-11242011000200005

Ojeda-Chi, M. M., Rodriguez-Vivas, R. I., Galindo-Velasco, E., and Lezama-Gutiérrrez, 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. doi: 10.1016/j.vetpar.2010.02.022

Omkar, K. K. (2016). *Ecofriendly Pest Management for Food Security*. Cambridge, MA: Elsevier.

Ortiz, E. M., Santamaría, V. M., Ortiz, N. A., Soberanes, C. N., Osorio, M. J., Franco, B. R., et al. (1995). "Caracterización de la resistencia de *Boophilus microplus* a ixodicidas en México," in *Memorias de IV Seminario Internacional de Parasitología Animal, Resistencia y Control en Garrapatas y Moscas de Importancia Veterinaria* (Acapulco), 58–66

Ortiz-Urquiza, A., and Keyhani, N. O. (2013). Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects* 4, 357–374. doi: 10.3390/insects4030357

Pérez de León, A. A., Mitchell, R. D., and Watson, D. W. (2020). Ectoparasites of cattle. *Vet. Clin. North Am. Food Anim. Pract.* 36, 173–185. doi: 10.1016/j.cvfa.2019.12.004

Pérez de León, A. A., Teel, P. D., Auclair, A. N., Messenger, M. T., Guerrero, F. D., Schuster, G., et al. (2012). Integrated strategy for sustainable cattle fever tick eradication in USA is required to mitigate the impact of global change. *Front. Physiol.* 3:195. doi: 10.3389/fphys.2012.00195

Perez-Cogollo, L. C., Rodriguez-Vivas, R. I., Ramirez-Cruz, G. T., and Miller, R. J. (2010a). First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Vet. Parasitol.* 168, 165–169. doi: 10.1016/j.vetpar.2009.10.021

Perez-Cogollo, L. C., Rodriguez-Vivas, R. I., Ramirez-Cruz, G. T., and Rosado-Aguilar, J. A. (2010b). Survey of *Rhipicephalus microplus* resistance to ivermectin at cattle farms with history of macrocyclic lactones use in Yucatan, Mexico. *Vet. Parasitol.* 172, 109–113. doi: 10.1016/j.vetpar.2010.04.030

Perinotto, W. M. S., Angelo, I. C., Golo, P. S., Quinelato, S., Camargo, M. G., Sá, F. A., et al. (2012). Susceptibility of different populations of ticks to entomopathogenic fungi. *Exp. Parasitol.* 130, 257–260. doi: 10.1016/j.exppara.2011.12.003

Polar, P., Kairo, M. T., Peterkin, D., Moore, D., Pegram, R., and John, S. A. (2005). Assessment of fungal isolates for development of a myco-acaricide for cattle tick control. *Vector Borne Zoonotic Dis.* 5, 276–284. doi: 10.1089/vbz.2005.5.276

Polley, L., and Thompson, A. (2009). Parasite zoonoses and climate change: molecular tools for tracking shifting boundaries. *Trends Parasitol.* 25, 285–291. doi: 10.1016/j.pt.2009.03.007

Porfirio, N. I., and Schwentesius, R. R. (2016). *Control Biológico de Garrapata* (*Boophilus microplus*), *con Microorganismos*. Available online at: https://www.researchgate.net/profile/Rita_Rindermann/publication/289768055_Control_biologico_de_Garrapata_con_Microorganismos/links/569282aa08aed0aed8167e2b.pdf (accessed April 17, 2021).

Pound, J. M., George, J. E., Kammlah, D. M., Lohmeyer, K. H., and Davey, R. B. (2010). Evidence for role of white-tailed deer (Artiodactyla: Cervidae) in epizootiology of cattle ticks and southern cattle ticks (Acari: Ixodidae) in reinfestations along the Texas/Mexico border in south Texas: a review and update. *J. Econ. Entomol.* 103, 211–218. doi: 10.1603/EC09359

Prieto, G. (2005). *El Clima de México a Través de los Mapas*. Geografía infinita. Available online at: https://www.geografiainfinita.com/2015/07/el-clima-de-mexico-a-traves-de-los-mapas/ (accessed March 3, 2021).

Racelis, A. E., Davey, R. B., Goolsby, J. A., De León, A. P., Varner, K., and Duhaime, R. (2012). Facilitative ecological interactions between invasive species: arundo donax stands as favorable habitat for cattle ticks (Acari: Ixodidae) along the US–Mexico border. *J. Med. Entomol.* 49, 410–417. doi: 10.1603/ME11104

Rajula, J., Rahman, A., and Krutmuang, P. (2020). Entomopathogenic fungi in Southeast Asia and Africa and their possible adoption in biological control. *Biol. Control.* 151:104399. doi: 10.1016/j.biocontrol.2020.104399

Rehner, S. A., Minnis, A. M., Sung, G. H., Luangsa-ard, J. J., Devotto, L., and Humber, R. A. (2011). Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycol.* 103, 1055–1073. doi: 10.3852/10-302

Rivera-Cervantes, M., Vargas-Sandoval, M., Ramos-Lima, M., de Jesús, J., Ayala-Ortega, M., Lara-Chávez, B. N., et al. (2017). Acción de *Metarhizuim anisopliae* (Metschnikoff) en *Rhipicephalus* (*B.*) *microplus* (Canestrini) (Acari: Ixodidae) sobre ganado bovino en Coalcomán, Michoacán. *Entomol. Mexicana* 4, 213–219. Available online at: http://entomologia.socmexent.org/revista/2017/CB/EM2792017_213-219.pdf (accessed January 2, 2021).

Rodríguez-Alcocer, U. J., Rodríguez-Vivas, R. I., Ojeda-Chi, M. M., Galindo-Velasco, E., and Lezama-Gutiérrez, R. (2014). Eficacia de la mezcla de dos cepas de *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) para el control de *Rhipicephalus microplus* en infestaciones naturales en bovinos. *Trop. Subtrop. Agroecosyst.* 17, 223–229. Available online at: http://www.redalyc.org/articulo.oa?id=93931761008

Rodriguez-Vivas, R. I., Alonso-Díaz, M. A., Rodríguez-Arevalo, F., Fragoso-Sanchez, H., Santamaria, V. M., and Rosario-Cruz, R. (2006a). Prevalence and potential risk factors for organophosphate and pyrethroid resistance in *Boophilus microplus* ticks on cattle ranches from the state of Yucatan, Mexico. *Vet. Parasitol.* 136, 335–342. doi: 10.1016/j.vetpar.2005.05.069

Rodríguez-Vivas, R. I., Grisi, L., de León, A. A. P., Villela, H. S., de Jesús Torres-Acosta, J. F., Sánchez, H. F., et al. (2017). Potential economic impact assessment for cattle parasites in Mexico. *Review. Rev. Mex. Cienc. Pecuarias.* 8, 61–74. doi: 10.22319/rmcp.v8i1.4305

Rodriguez-Vivas, R. I., Hodgkinson, J. E., and Rosado-Aguilar, J. A., Villegas-Perez, S. L., Trees, A. J. (2012). The prevalence of pyrethroid resistance phenotype and genotype in *Rhipicephalus* (*Boophilus*) *microplus* in Yucatan, Mexico. *Vet. Parasitol.* 184,221–229. doi: 10.1016/j.vetpar.2011.09.017

Rodriguez-Vivas, R. I., Jonsson, N. N., and Bhushan, C. (2018). Strategies for the control of *Rhipicephalus microplus* ticks in a world of conventional acaricide and macrocyclic lactone resistance. *Parasitol. Res.* 117, 3–29. doi: 10.1007/s00436-017-5677-6

Rodríguez-Vivas, R. I., Li, A. Y., Ojeda-Chi, M. M., Trinidad-Martinez, I., Rosado-Aguilar, J. A., Miller, R. J., et al. (2013b). *In vitro* and *in vivo* evaluation of cypermethrin, amitraz, and piperonyl butoxide mixtures for the control of resistant *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in the Mexican tropics. *Vet. Parasitol.* 197, 288–296. doi: 10.1016/j.vetpar.2013.07.018

Rodríguez-Vivas, R. I., Ojeda-Chi, M. M., Rosado-Aguilar, J. A., Trinidad-Martínez, I. C., Torres-Acosta, J. F. J., Ticante-Perez, V., et al. (2013a). Red deer (*Cervus elaphus*) as a host for the cattle tick *Rhipicephalus microplus* (Acari: Ixodidae) in Yucatan, Mexico. *Exp. Appl. Acarol.* 60, 543–552. doi: 10.1007/s10493-013-9672-z

Rodríguez-Vivas, R. I., Pérez-Cogollo, L. C., Rosado-Aguilar, J. A., Ojeda-Chi, M. M., Trinidad-Martinez, I., Miller, R. J., et al. (2014a). Rhipicephalus (Boophilus) microplus resistant to acaricides and ivermectin in cattle farms of Mexico. *Braz. J. Vet. Parasitol.* 23, 113–122. doi: 10.1590/S1984-29612014044

Rodríguez-Vivas, R. I., Quiñones, A. F., and Fragoso-Sánchez, H. (2005). "epidemiolgía y control de la garrapata *Boophilus* en México," in *Enfermedades*

de Importancia Económica en Producción Animal, ed R. I. Rodríguez-Vivas (México, DF: McGraw-Hill-UADY), 571–592.

Rodriguez-Vivas, R. I., Rivas, A. L., Chowell, G., Fragoso, S. H., Rosario, C. R., Garcia, Z., et al. (2007). Spatial distribution of acaricide profiles (*Boophilus microplus* strains susceptible or resistant to acaricides) in southeastern Mexico. *Vet. Parasitol*. 146, 158–169. doi: 10.1016/j.vetpar.2007.01.016

Rodriguez-Vivas, R. I., Rodriguez-Arevalo, F., Alonso-Díaz, M. A., Fragoso-Sanchez, H., Santamaria, V. M., and Rosario-Cruz, R. (2006b). Prevalence and potential risk factors for amitraz resistance in *Boophilus microplus* ticks in cattle farms in the State of Yucatan, Mexico. *Prev. Vet. Med*. 75, 280–286. doi: 10.1016/j.prevetmed.2006.04.001

Rodríguez-Vivas, R. I., Rosado-Aguilar, J. A., Ojeda-Chi, M. M., Pérez-Cogollo, L. C., Trinidad-Martínez, I., and Bolio-González, M. E. (2014b). Control integrado de garrapatas en la ganadería bovina. *Ecosistemas Recur. Agropecuarios* 1, 295–308.

Romero-Pérez, J. B. (2020). *Aislamiento e identificación de hongos entomopatógenos y su evaluación garrapaticida in vitro mediante manipulación de su desarrollo en diferentes sustratos* (Dissertation/Bachelor's thesis). Xicotepetl: Universidad de Xicotepetl.

Romo-Martínez, A., Fernández-Ruvalcaba, M., Hernández-Velázquez, V. M., Peña-Chora, G., Lina-García, L. P., and Osorio-Miranda, J. (2013). Evaluation of natural origin products for the control of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) on cattle artificially infested. *Basic. Res. J. Agric. Sci. Rev*. 2, 64–79. Available online at: https://www.researchgate.net/publication/280309624_Evaluation_of_natural_origin_products_for_the_control_of_Rhipicephalus_Boophilus_microplus_Acari_Ixodidae_on_cattle_artificially_infested

Rosado-Aguilar, J. A., Rodriguez-Vivas, R. I., Garcia-Vazquez, Z., Fragoso-Sanchez, H., Ortiz-Najera, A., and Rosario-Cruz, R. (2008). Development of amitraz resistance in field populations of *Boophilus microplus* (Acari: Ixodidae) undergoing typical amitraz exposure in the Mexican tropics. *Vet. Parasitol.* 152:349–353. doi: 10.1016/j.vetpar.2007.12.026

Rosario-Cruz, R., Guerrero, F. D., Miller, R. J., Rodriguez-Vivas, R. I., Tijerina, M., Dominguez-Garcia, D. I., et al. (2009). Molecular survey of pyrethroid resistance mechanisms in Mexican field populations of *Rhipicephalus (Boophilus) microplus*. *Parasitol. Res*. 105, 1145–1153. doi: 10.1007/s00436-009-1539-1

Samish, M., Ginsberg, H., and Glazer, I. (2004). Biological control of ticks. *Parasitology* 129:S389. doi: 10.1017/S0031182004005219

Sánchez-Montes, D., Ríos-Muñoz, C., Espinoza-Martínez, D., Guzmán-Cornejo, C., Berzunza-Cruz, M., and Becker, I. (2016). First Report of "*Candidatus Rickettsia amblyommii*" in West Coast of México. *Ticks Tick. Borne. Dis*. 7, 1139–1145. doi: 10.1016/j.ttbdis.2016.08.007

Schrank, A., and Vainstein, M. H. (2010). *Metarhizium anisopliae* enzymes and toxins. *Toxicon* 56, 1267–1274. doi: 10.1016/j.toxicon.2010.03.008

SEMARNAT (Secretaría de Medio Ambiente y Recursos Naturales). (2003). *Atlas Digital Geográfico*. Climas de México. Available online at: http://gisviewer.semarnat.gob.mx/aplicaciones/Atlas2015/atm_climas.html (accessed March 10, 2021).

Semenova, T. A., Dunaevsky, Y. E., Beljakova, G. A., and Belozersky, M. A. (2020). Extracellular peptidases of insect-associated fungi and their possible use in biological control programs and as pathogenicity markers. *Fungal Biol*. 124, 65–72. doi: 10.1016/j.funbio.2019.11.005

SENASICA (2013). *Distribución y Diversidad de la Garrapata Boophilus spp. SAGARPA-Boletín Técnico*. Available online at: https://www.aphis.usda.gov/import_export/downloads/presentations/Boophilus-tick.pdf (accessed January, 5, 2021).

SENASICA-SAGARPA (2015). *Distribución y Diversidad de la Garrapatas Boophilus spp*. Available online at: https://www.aphis.usda.gov/import_export/downloads/presentations/Boophilus-tick.pdf (accessed March 1, 2021).

SIAP (Servicio de Información Agroalimentaria y Pesquera) (2018). *La ganadería: Símbolo de Fortaleza del Campo Mexicano*. Día Nacional de la Ganadería. Gobierno de México. Available online at: https://www.gob.mx/siap/articulos/la-ganaderia-simbolo-de-fortaleza-del-campo-mexicano (accessed December 4, 2020).

SIAP (Servicio de Información Agroalimentaria y Pesquera) (2020). *La Ganadería: Sustento de la Economía y Vida del País*. Día de la ganadería en México. SADER-CDMX. Available online at: https://www.gob.mx/agricultura/cdmx/articulos/dia-de-la-ganaderia-en-mexico?idiom=es (accessed December 11, 2020).

Skinner, M., Parker, B. L., and Kim, J. S. (2014). Role of entomopathogenic fungi in integrated pest management. *J. Integ. Pest. Manag.* 10, 169–191. doi: 10.1016/B978-0-12-398529-3.00011-7

Snelson, J. T. (1975). Animal ectoparasites and disease vector causing major reduction in world food supplies. *FAO Plant Prot. Bull.* 13, 103–114.

Soberanes, N. C., Vargas, M. S., Sánchez, H. F., and Vázquez, Z. G. (2002). Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. *Téc. Pec. Méx*. 40, 81–92. Available online at: http://www.redalyc.org/articulo.oa?id=61340106

Sousa, L. A. D., Pires Júnior, H. B., Soares, S. F., Ferri, P. H., Ribas, P., Lima, E. M., et al. (2011). Potential synergistic effect of *Melia azedarach* fruit extract and *Beauveria bassiana* in the control of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in cattle infestations. *Vet. Parasitol*. 175, 320–324. doi: 10.1016/j.vetpar.2010.10.012

Srinivasan, R., Sevgan, S., Ekesi, S., and Tamó, M. (2019). Biopesticide based sustainable pest management for safer production of vegetable legumes and brassicas in Asia and Africa. *Pest. Manag. Sci*. 75, 2446–2454. doi: 10.1002/ps.5480

St. Leger, R. J., Staples, R. C., and Roberts, D. W. (1992). Cloning and regulatory analysis of starvation-stress gene, ssgA, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* 120, 119–124. doi: 10.1016/0378-1119(92)90019-L

Valero-Jiménez, C. A., Wiegers, H., Zwaan, B. J., Koenraadt, C. J., and van Kan, J. A. (2016). Genes involved in virulence of the entomopathogenic fungus *Beauveria bassiana*. *J. Invertebr. Pathol*. 133, 41–49. doi: 10.1016/j.jip.2015.11.011

Wang, C., and St Leger, R. J. (2007). The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. *Eukaryotic Cell*. 6, 808–816. doi: 10.1128/EC.00409-06

Webb, S. L., Demarais, S., Zaiglin, R. E., Pollock, M. T., and Whittaker, D. G. (2010). Size and fidelity of home ranges of male white-tailed deer (Odocoileus virginianus) in southern Texas. *Southw. Naturalist*. 55, 269–273. doi: 10.1894/TAL-10.1

Webster, A., Pradel, E., Souza, U. A., Martins, J. R., Reck, J., Schrank, A., et al. (2017). Does the effect of a *Metarhizium anisopliae* isolate on *Rhipicephalus microplus* depend on the tick population evaluated? *Ticks. Tick. Borne. Dis*. 8, 270–274. doi: 10.1016/j.ttbdis.2016.11.012

Webster, A., Reck, J., Santi, L., Souza, U.A., Dall'Agnol, B., Klafke, G.M., et al. (2105). Integrated control of an acaricide-resistant strain of the cattle tick *Rhipicephalus microplus* by applying *Metarhizium anisopliae* associated with cypermethrin and chlorpyriphos under field conditions. *Vet. Parasitol*. 207, 302–308. doi: 10.1016/j.vetpar.2014.11.021

Wong, H. J., Mohamad-Fauzi, N., Rizman-Idid, M., Convey, P., and Alias, S. A. (2019). Protective mechanisms and responses of micro-fungi towards ultraviolet- induced cellular damage. *Polar Sci*. 20, 19–34. doi: 10.1016/j.polar.2018.10.001

Ziapour, S. P., Kheiri, S., Fazeli-Dinan, M., Sahraei-Rostami, F., Mohammadpour, R. A., Aarabi, M., et al. (2017). Pyrethroid resistance in Iranian field populations of *Rhipicephalus* (*Boophilus*) *annulatus*. *Pestic. Biochem. Physiol*. 136, 70–79. doi: 10.1016/j.pestbp.2016.08.001

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol. Sci. Tech*. 17, 553–596. doi: 10.1080/09583150701309006

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Tolerance to Abiotic Factors of Microsclerotia and Mycelial Pellets From *Metarhizium robertsii*, and Molecular and Ultrastructural Changes During Microsclerotial Differentiation

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Metarhizium species fungi are able to produce resistant structures termed microsclerotia, formed by compact and melanized threads of hyphae. These propagules are tolerant to desiccation and produce infective conidia; thus, they are promising candidates to use in biological control programs. In this study, we investigated the tolerance to both ultraviolet B (UV-B) radiation and heat of microsclerotia of *Metarhizium robertsii* strain ARSEF 2575. We also adapted the liquid medium and culture conditions to obtain mycelial pellets from the same isolate in order to compare these characteristics between both types of propagules. We followed the peroxisome biogenesis and studied the oxidative stress during differentiation from conidia to microsclerotia by transmission electron microscopy after staining with a peroxidase activity marker and by the expression pattern of genes potentially involved in these processes. We found that despite their twice smaller size, microsclerotia exhibited higher dry biomass, yield, and conidial productivity than mycelial pellets, both with and without UV-B and heat stresses. From the 16 genes measured, we found an induction after 96-h differentiation in the oxidative stress marker genes *MrcatA*, *MrcatP*, and *Mrgpx*; the peroxisome biogenesis factors *Mrpex5* and *Mrpex14/17*; and the photoprotection genes *Mrlac1* and *Mrlac2*; and *Mrlac3*. We concluded that an oxidative stress scenario is induced during microsclerotia differentiation in *M. robertsii* and confirmed that because of its tolerance to desiccation, heat, and UV-B, this fungal structure could be an excellent candidate for use in biological control of pests under tropical and subtropical climates where heat and UV radiation are detrimental to entomopathogenic fungi survival and persistence.

Keywords: entomopathogenic fungi, UV-B radiation, thermotolerance, oxidative stress, gene expression

INTRODUCTION

Fungal microsclerotia (from hereafter referred to as MS) are hardened masses of pigmented hyphal aggregates (50–600 μm), serving as survival structures and first described in the entomopathogenic fungi *Metarhizium* species when grown dimorphically in submerged liquid cultures. This dimorphism in *Metarhizium* lies in the transition from conidia to myceliogenic growth followed by the development of dense and compact hyphal threads forming MS. This type of overwintering fungal structure is remarkably tolerant to desiccation and capable of producing infective conidia without exogenous carbon source due to their own endogenous reserves; thus, they stand out high potential to be used as mycoinsecticide in biological control programs (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Behle et al., 2013; Mascarin et al., 2014; Goble et al., 2016; Song et al., 2017). However, filamentous fungi can also grow as pellets by mycelial formation in submerged cultures (from hereafter referred to as P) (Nair et al., 2016; Zhang and Zhang, 2016; Veiter et al., 2018), which can be slightly distinct in morphology and probably less tolerant to environmental stresses in relation to MS.

Abiotic factors such as ultraviolet (UV) radiation and heat stress determine fungal propagule survival and persistence, and both are more pronounced in tropical and subtropical regions. These environmental factors can alter molecular structures and trigger the production of reactive oxygen species (ROS) that induce damage, reduce fungal viability, and even provoke cell death (Braga et al., 2001; Nascimento et al., 2010; Zhang et al., 2017). The UV spectrum is divided into three wavelength intervals: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (100–280 nm). The effects of UV-B tolerance are commonly reported to propagules as conidia, mycelium, or blastospores of entomopathogenic fungi (Fernandes et al., 2007, 2015; Braga et al., 2015; Rangel et al., 2015; Brancini et al., 2018; Zhao et al., 2019; Bernardo et al., 2020; Corval et al., 2021). Temperature can be also a limiting factor during morphogenesis, germination, and fungal metabolic processes (Abrashev et al., 2008; Zhang et al., 2017). In this sense, heat stress can delay and reduce the effectiveness of conidia germination, sporulation, and growth of thermosensitive entomopathogenic fungi (Fernandes et al., 2008, 2010; Paixão et al., 2019).

Cell development in microbial eukaryotes shows a correlation between ROS generation and the upregulation of specific antioxidant enzymes, such as superoxide dismutases (SODs), catalases (CATs), CAT–peroxidases, glutathione peroxidases (GPxs), and peroxiredoxins (Aguirre et al., 2005, 2006). An imbalance between ROS and antioxidant enzymes response can cause detrimental effects in cell morphology, growth rate, metabolism, and protein secretion, among others (Aguirre et al., 2006). On the other hand, ROS generation and oxidative stress are associated with sclerotia maturation in the filamentous fungi *Sclerotium rolfsii* (Georgiou et al., 2006) and *Rhizoctonia solani* (Liu et al., 2018) and with MS and MS-like propagules development in the entomopathogenic fungi *Metarhizium* species (Song et al., 2013, 2018; Liu et al., 2014) and *Beauveria bassiana* (Huarte-Bonnet et al., 2019), respectively. Peroxisome biogenesis has also been observed in *B. bassiana* during mycelial pellet formation when cultivated in liquid medium supplemented with hydrocarbons (Huarte-Bonnet et al., 2018) and during MS-like development (Huarte-Bonnet et al., 2019), accompanied by induction of oxidative stress marker genes and *pex* genes encoding for peroxins, proteins involved in the transport of matrix proteins from the cytosol into peroxisome lumen.

In this study, we grew the entomopathogenic fungus *Metarhizium robertsii* in two different culture media to obtain two fungal propagules (MS and P) with similar morphology but encompassing differences in biomass production and propagule size. As scarce information is available regarding microsclerotial tolerance to abiotic factors (Corval et al., 2021), the aims of this study were to compare the effect of UV-B radiation and heat on both MS and P tolerance and to characterize for the first time the expression pattern of genes potentially involved in oxidative stress, pigmentation, and peroxisome biogenesis during *M. robertsii* microsclerotial differentiation.

MATERIALS AND METHODS

Production and Characterization of Microsclerotia and Mycelial Pellets From *M. robertsii*

The entomopathogenic fungus *M. robertsii* strain ARSEF 2575 was used in this study. It is deposited at the US Department of Agriculture (USDA)–ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY. Conidia were obtained from fungal cultures grown within 10 days onto potato dextrose agar medium (Merck, Darmstadt, Germany) supplemented with 1 g L^{-1} yeast extract (Oxoid, Hampshire, England) (PDAY) in the dark at 27 ± 1°C. Ten milliliters of conidial suspensions were prepared and adjusted to 5 × 10^7 conidia mL^{-1} with 0.05% Tween 80 [polyoxyethylene sorbitan monooleate] (Sigma–Aldrich, USA) solution, vortexed, and inoculated into Erlenmeyer flasks (250 mL), containing 90 mL of different basal media (**Table 1**) to produce either MS (Mascarin et al., 2014) or P (Mapari et al., 2008). Cultures were set at 27 ± 1°C in a rotary shaker incubator at 250 rpm (Certomat BS-1, Sartorius, Germany). Aliquots (1 mL) were obtained from each culture medium at different time intervals. At the last time point (96 h), the pH of the cultures was measured. In order to evaluate the accumulation of biomass, aliquots were dewatered on filter paper discs (80 g m^{-2}) previously weighed. Samples were dried at 32°C for 2 days to obtain the dry biomass (Jackson and Jaronski, 2009). For evaluation of propagule concentration, 9 mL of 0.05% Tween 80 was added to 1 mL of liquid culture, and 100 μL of this suspension was placed between slides (76.2 × 25.4 mm) and coverslips (24 × 24 mm). The suspension was quantified by optical microscope at 40× magnification (Jackson and Jaronski, 2009), and propagule diameter was measured with a Leica ICC50 HD camera and Leica 201 LAZ EZ software version 3.0.0. Three tests were conducted in different days.

TABLE 1 | Components of basal media used to obtain microsclerotia and pellets.

Component	Molecular formula	Concentration (g/L)	
		Microsclerotia	Pellets
Anhydrous dextrose	$C_6H_{12}O_6$	200	20
Malt extract	—	—	20
Yeast extract	—	15	—
Casein (acid hydrolyzate)	—	—	1
Monobasic potassium phosphate	KH_2PO_4	4	—
Calcium chloride	$CaCl_2{\cdot}2H_2O$	0.8	—
Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	0.6	—
Ferrous sulfate	$FeSO_4{\cdot}7H_2O$	0.1	—
Manganese sulfate	$MnSO_4{\cdot}H_2O$	0.016	—
Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	0.014	—

Microscopy Images

Optical Microscopy of Microsclerotia and Mycelial Pellets

For optical microscopy, samples of either MS or P propagules were taken at 24, 48, 72, and 96 h post-inoculation of liquid media, centrifuged to recover propagules, washed twice with sterile water, and observed with a Nikon eclipse e200 optical microscope (Nikon, Japan) at 100× magnification.

Transmission Electron Microscopy of Microsclerotia

Two-day-old MS cultures were used for transmission electron microscopy (TEM) images following the protocol described by Huarte-Bonnet et al. (2018). Briefly, MS were washed, fixed in glutaraldehyde 2% for 2 h with soft vacuum, and washed three times with phosphate buffer. Then, MS were stained overnight with 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, USA) and washed again with the same buffer. DAB is a chemical used for determining peroxidase activity, usually employed as a peroxisome marker in microscopy images (Fahimi, 2017). Post-fixation was performed with 1% osmium tetroxide at 4°C for 1 h, followed by dehydration with a series of alcohols in a vacuum chamber. Samples were finally infiltrated with epoxy resin, and thin sections of ∼70 nm were cut. Samples were observed using TEM JEM 1200 EX II (JEOL, Japan) and photographed; images were captured with an ES1000W Erlangshen CCD Camera (Gatan, USA).

Tolerance to UV-B Radiation of Microsclerotia and Mycelial Pellets

Four-day-old propagules from cultures of both MS and P were washed and suspended in sterile water. Aliquots from each suspension containing a total of 100 propagules were inoculated onto water agar 2% (wt/vol) medium in Petri dish (80 × 10 mm) and exposed to UV-B radiation as described by Fernandes et al. (2007) and Pereira-Junior et al. (2018). The plates were irradiated at 1,283.38 mW m^{-2} of Quaite-weighted irradiance (Quaite et al., 1992) in a chamber containing 4 UV lamps (UVB-313 EL/40W; Q-Lab Corporation, Westlake, USA) for 0.5, 1, 2, 3, 4, and 5 h, which corresponded to the doses of 2.31, 4.62, 9.24, 13.86, 18.48, and 23.10 kJ m^{-2}, respectively. All plates previously open were covered with a 0.13-mm-thick cellulose diacetate film (JCS Industries, La Mirada, USA). Cellulose diacetate blocks UV-C radiation (<280 nm) and short-wavelength UV-B (280–290 nm) but allows the passage of UV-B radiation (290–320 nm) and minimal UV-A (320–400 nm) emitted by the lamps. Spectral irradiance was measured with a USB 2000+ Rad spectroradiometer (Ocean Optics, Dunedin, USA). Control plates were covered with aluminum foil to block all UV radiation (Pereira-Junior et al., 2018). After UV-B exposure, the plates were incubated for 10 days at 27 ± 1°C in the dark to produce conidia from either MS or P; the conidial production and viability were then determined as described below. Three tests (two repetitions each) were conducted in different days.

Tolerance to Heat of Microsclerotia and Mycelial Pellets

One-milliliter aliquots containing 100 propagules of either MS and P were prepared as described above into glass test tube with rubber stoppers (16 × 100 mm) and exposed to 45°C in a thermostatic bath for 0.5, 1, 2, 3, 4, and 5 h. Control tubes remained at 27°C. After each time exposure, the samples were centrifuged, and then 500 μL of supernatant was removed. The remaining volume was inoculated onto water agar 2% (wt/vol) medium in Petri dish (80 × 10 mm). After inoculation, the plates were incubated for 10 days at 27 ± 1°C in the dark to produce conidia from either MS or P propagules; the conidial production and viability were then determined as described below. Three tests (two repetitions each) were conducted in different days.

Evaluation of Conidial Production and Viability After Exposure of Microsclerotia and Mycelial Pellets to UV-B Radiation and Heat

Conidial production and viability were assayed in MS and P propagules exposed to either UV-B radiation or heat. Conidia produced 10 days post-inoculation on water agar plates were harvested using 0.05% Tween 80 solution. By serially diluting conidial suspension, the production of conidia was quantified in each sample using a hemocytometer under optical microscope at 400× magnification. Conidial viability was assayed by inoculation of 20 μL of each propagule suspension in the center of a Petri dish (35 × 10 mm) containing 8 mL PDAY plus 0.002% (vol/wt) benomyl (50% active ingredient; Benlate®, DuPont, São Paulo, Brazil) (Braga et al., 2001) and 0.05% (vol/wt) chloramphenicol (INLab Confiança, Diadema, SP, Brazil). Plates were incubated for 48 h at 27 ± 1°C in the dark. Two drops of cotton blue were applied with a Pasteur pipette over the inoculum in each plate, and germination was immediately assessed at 400× magnification. A minimum of 300 conidia were evaluated per plate as germinated or non-germinated, and the relative percent viability of conidia was calculated according to Braga et al. (2001).

TABLE 2 | Oligonucleotides used in this study.

Gene (acronym used)	Forward (5′-3′)	Reverse (5′-3′)
Peroxin 5 (*Mrpex5*)	TTTGTCCGGGCTCGCTACAATC	ATTTCGTGCGCCTTGCTTCG
Peroxin 7 (*Mrpex7*)	CCTGGCTTGGTCGGAAATCAAC	TGTTTCGCGCTTGTGTTCGTG
Peroxin 14/17 (*Mrpex14/17*)	AGGTCCAAAGGCATCAGCGAAG	TGAGCGTTGCCGAGTTGTGC
Peroxin 19 (*Mrpex19*)	ATGCCGCTCCCAAGGAATCC	TCAAACTGCTGCTGCATTTCCG
Glutathione peroxidase (*Mrgpx*)	GGGCAAAGTCGTCCTCATCGTC	TGGCCGCCAAACTGGTTACAG
Hydrophobin *(MrssgA*)	GTGTATTGCTGCAACAAAG	AGACCATTTTGCTGGACATTG
Superoxide dismutase 1 (*Mrsod1*)	CCAATGGCTGCACTTCTGCTGG	TGTGAGGGCCGATGAGCTTGAC
Superoxide dismutase 2 (*Mrsod2*)	CCAGCATCTCGGCGCAAATC	CCAGCATCTCGGCGCAAATC
Catalase A (*MrcatA*)	GTCGGCGCACAACAACTTCTG	CCAGTCGAACTTGACGACGTGC
Catalase B (*MrcatB*)	ACAGGATCAGCCACGACATCGC	TCCTTGAGAGCGTTCGCCTGAG
Catalase P (*MrcatP*)	TGCCCAATGGAGCCACAACTTC	GCAAAGGCATCGGCGAACTG
Polyketide synthase 1 (*Mrpks1*)	CATTCCGCCTCTCTCATTGCC	TGTGCGGCGCATGATATGG
Polyketide synthase 2 (*Mrpks2*)	CATCAGCGCCATCGGTTTAGAC	CGGGATAGGGATTGGTTTGTGG
Laccase 1 (*Mrlac1*)	AGGGAGACCGCACAGGATTGTG	ACTGGCTCCAATCCGACACGAC
Laccase 2 (*Mrlac2*)	TCCCTGGGTCAACGAAAGCC	CGCCGCGATAAAGTTCATGC
Laccase 3 (*Mrlac3*)	TCGGCTCAAGTGTCGTGTCCAC	CCGATCCTGTTGCCCAAACG
Glyceraldehyde-3-phosphate dehydrogenase *(Mrgpd)**	GACTGCCCGCATTGAGAAG	AGATGGAGGAGTTGGTGTTG

**Housekeeping gene.*

Gene Expression Analysis

MS cultures were sampled at 24, 48, 72, and 96 h for RNA extraction and two-step real-time polymerase chain reaction (RT-PCR) analysis. Each aliquot (10 mL) was collected into screwed 15-mL centrifuge plastic tube and centrifuged for 3 min at 7,500 rpm. The supernatant was discarded, and the centrifugation pellet was washed with sterile water. The supernatant was again discarded, and the process was repeated three times. Then, MS propagules were harvested with a microbiological loop, and exposed to liquid nitrogen. Samples were ground with mortar and pestle, and 100 mg of each sample immediately transferred to 2 mL microcentrifuge tubes containing 1 mL Trizol (Invitrogen, USA). Total RNA extraction was performed according to manufacturer instructions. Total RNA samples were treated with DNase by using the Turbo DNA-free Kit (Ambion, USA). RT-PCR was carried out with iScript cDNA Synthesis Kit and iQSYBR Green Supermix (Bio-Rad, USA). Amplification was performed on a StepOne Plus equipment (Applied Biosystems, USA). In order to confirm that only single products were amplified, a temperature-melting step was then performed. The primer sequences used are listed in **Table 2**. Glyceraldehyde-3-phosphate dehydrogenase gene (*Mrgpd*) was used as housekeeping gene. Relative expression ratio of each target gene was calculated with the $\Delta\Delta$Ct approach, using MS harvested after 24-h growth as control. Three independent biological replicates were tested, with technical duplicates for each sample.

Statistical Analyses

Statistical software R v.3.6.1 (R Core Team, 2018) was used in all analyses performed. Log2-fold data on gene expression level were fitted to a linear mixed model with normal distribution and fixed effects attributed to gene class, evaluation time, and their interaction term, and random effect was attributed to replicate to account for repeated measures over time. As interaction was significant, then means of gene expression levels were compared for each time interval and also among time intervals for each gene class. Count data on propagule yields (MS and P) were fitted to generalized linear model (GLM) with negative binomial distribution with log link function, whereas the biomass production data were fitted to a linear model (LM) with normal distribution, both models including a fixed effect for "propagule type" in the linear predictor. Similarly, LM with normal distribution was fitted to propagule size.

Regarding the UV-B tolerance assay, $\log_{10}$-transformed conidial production data were fitted to a LM with normal distribution. For heat tolerance, conidial production was fitted to a GLM with quasi-Poisson distribution and log link function. Fixed effects in both models were attributed to "propagule type," "time of exposure," and their interaction term in the linear predictor. Furthermore, analysis of variance (type II tests) and analysis of deviance (type II tests) were performed to assess for significance of fixed factors in these models, respectively. Pairwise mean comparisons of two samples (MS vs. P) were conducted via contrast estimates with *t* ratio test, whereas multiple pairwise comparisons of means were carried out with Tukey honestly significant difference (HSD) test, all at significance of 5%. Packages "emmeans" (Lenth, 2020), "mass" (Venables and Ripley, 2002), and "ggplot2" (Wickham, 2016) were employed in these analyses.

RESULTS

Characterization of Microsclerotia and Mycelial Pellets

M. robertsii strain ARSEF 2575 produced either MS or P propagules in liquid cultures (**Figure 1**). The initial pH value of both culture media used was 5.0 and varied between 4.0 and

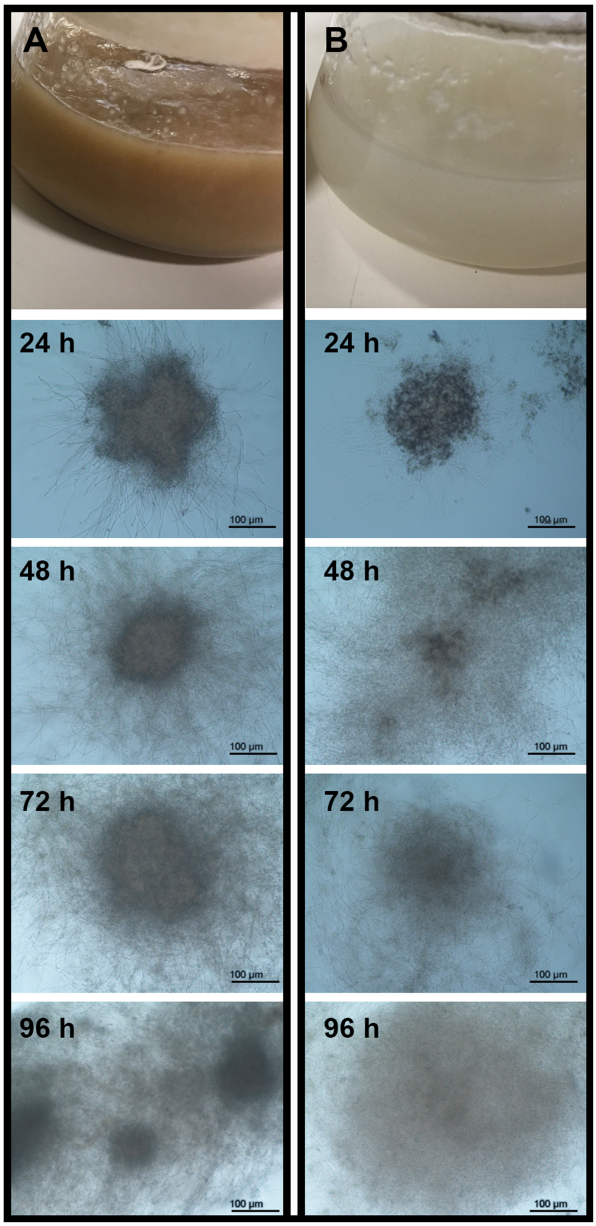

FIGURE 1 | Microsclerotia **(A)** and mycelial pellets **(B)** of *Metarhizium robertsii* ARSEF 2575 produced in submerged liquid medium. From top to bottom is shown: liquid cultures in Erlenmeyer flasks at the end of fermentation (96 h), and the propagule formation registered at 100× magnification with a light microscope at 24, 48, 72, and 96 h.

4.5 during fermentation. After 96-h cultivation, both propagules differed significantly on yield (MS = 700 propagules mL^{-1}, P = 90 propagules mL^{-1}) [$\chi^2 = 83.64$; degrees of freedom (df) = 1, 14; $p < 0.05$] and biomass production (MS = 0.053 g mL^{-1}, P = 0.015 g mL^{-1}) ($F = 362.2$; df = 1, 14; $p < 0.05$) (**Figure 2**). MS, but not P, showed a more compact hyphal aggregation with formation of a central medulla of thin-walled hyphae (**Figure 1**). Germinated conidia differentiated into MS between 6 and 18 h after inoculation, forming visible hyphal aggregates at 24 h, and compact and mature MS at 96 h with typical darker pigmentation than mycelial pellets. Optical images after 96-h growth exhibited P propagules with larger size (263–485 µm) than MS (174–226 µm); however, the latter showed higher density with more distinct curve of distribution size ($F = 164.9$; df = 1, 118; $p < 0.0001$, **Figure 2**).

Tolerance to UV-B Radiation and Heat of Microsclerotia and Mycelial Pellets

Fungi exposed to UV-B radiation (1,283.38 mW m^{-2}) had their conidial production decreased in a time-dependent manner; however, both MS and P resulted to be tolerant after exposure to heat (45°C). Comparing both types of propagules, conidial productivity of MS was higher than those of P even without treatment by UV-B or heat at all exposition times assayed, except after 5-h exposure ($F = 12.38$; df = 6, 70; $p < 0.0001$; and $F = 3.24$; df = 6, 70; $p = 0.007$, respectively) (**Figure 3**). The effect of UV-B radiation and heat on MS sporogenesis was evident resulting in significant reduction on conidial production after 2- and 4-h exposures, respectively, in relation to its initial production from unexposed MS (**Figure 3**). Overall, the susceptibility of P was considerably higher than MS after exposure to UV-B ($F = 52.78$; df = 1, 70; $p < 0.0001$) and heat stress ($F = 74.74$; df = 1, 70; $p < 0.0001$). The mean viability of conidia produced from UV-B- or heat-stressed MS or P was higher than 97% in each test, regardless of the exposure time.

Ultrastructural and Gene Expression Analyses During Microsclerotial Differentiation

TEM images revealed differences in cell wall configuration between conidia (rodlet layer is observed) and MS (single-layered cell wall is observed) (**Figure 4**). The organelles observed in MS comprised several mitochondria, lipid droplets, and peroxisomes located next to hyphal septa, suggesting Woronin bodies (WBs), a peroxisome-derived, dense core microbody with a unit membrane found near the septae that divide hyphal compartments in filamentous Ascomycota. Also, high peroxidase activity was detected in MS but not in conidia. Peroxidase activity was demonstrated as small black dots due to DAB reaction with H_2O_2 inside the cells and also in hyphal apex (**Figure 4**).

The expression pattern of 16 genes putatively involved in MS formation was analyzed (**Figure 5**). Based on previous studies (Huarte-Bonnet et al., 2018, 2019, 2020; Pereira-Junior et al., 2018), the selected genes were those associated with oxidative stress (*MrcatA*, *MrcatB*, *MrcatP*, *Mrsod1*, *Mrsod2*, *Mrgpx*), peroxisome biogenesis (*Mrpex5*, *Mrpex7*, *Mrpex14/17*, *Mrpex19*), pigmentation (*Mrpks1*, *Mrpks2*, *Mrlac1*, *Mrlac2*, *Mrlac3*), and hydrophobin rodlet layer (*MrssgA*). For each gene, the expression was measured at 48, 72, and 96 h after start of fermentation and was normalized with values measured at 24 h (control). For CAT family, *MrcatA* and *MrcatP* expression at 96 h post-inoculation was significantly higher (2.2- and 3.9-fold increase, respectively) than those found at both 48 and 72 h. The most expressed gene at

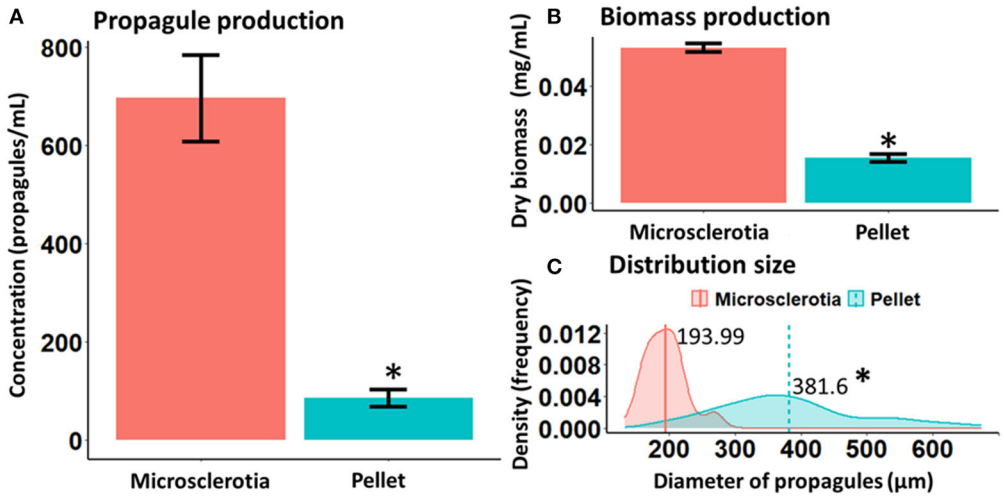

FIGURE 2 | Yield **(A)**, biomass **(B)**, and distribution size **(C)** of microsclerotia and mycelial pellets obtained from 96-h-old liquid cultures of *Metarhizium robertsii* ARSEF 2575. *$p < 0.05$.

96 h was *Mrgpx* (5.3-fold induction), significantly higher ($p < 0.01$) than the previous point at 72 h (2.6-fold induction) (**Figure 5**). No induction over time was observed for SOD family. Within the peroxin family, *Mrpex14/17* (1.5-fold induction) and *Mrpex5* (2.6-fold induction) were observed at 96 h, the same as for the pigmentation-associated genes *Mrlac1* (2.4-fold induction), *Mrlac2* (2.6-fold induction), and *Mrlac3* (2.4-fold induction) ($p < 0.001$).

DISCUSSION

Nutritional conditions in filamentous fungal fermentation strongly influence growth and morphology of the resulting propagules (Cox and Thomas, 1992), as well as pH and aeration rate (Nair et al., 2016; Veiter et al., 2018). Fermentation conditions used in this study were favorable to produce either MS or mycelial pellets (P) from *M. robertsii* ARSEF 2575. MS development resumed with hyphal aggregates at 18 h post-inoculation, with compact structures appearing at 24 h, and mature pigmented MS propagules at 96 h. This process bears out with MS definition in entomopathogenic fungi, which consists in the formation of dark pigmented and compact hyphal threads with or without a distinct core (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Behle et al., 2013; Mascarin et al., 2014; Goble et al., 2016; Song et al., 2016a,b; Xin et al., 2020). Pellet (P) formation was obtained in a liquid culture medium considered poor by its composition, consisting in a basal medium devoid of salts but constituted of three elements that provided the source of carbon and nitrogen required for propagule development. Some parameters, such as pH, oxygen level, temperature, and agitation speed, are very important for the growth and maturation of these propagules (Zhang and Zhang, 2016). The pH of the culture medium used during MS production in *Metarhizium* species was shown to be initially acid (pH 5.5) (Jackson and Jaronski, 2012; Behle et al., 2013; Song et al., 2015, 2017). In concordance with reports from *Metarhizium* (formerly *Nomuraea*) *rileyi* (Song et al., 2015), we found that pH dropped to 4.0 during MS development, likewise during P formation. The pH is the driving factor for electrostatic and hydrophobic interactions on specific aggregation and pellet morphology (Zhang and Zhang, 2016; Veiter et al., 2018). The medium composition was determinant for P formation with bigger diameter than MS. However, even at the same agitation speed and temperature conditions, we found higher biomass accumulation and propagules concentration in MS than in P. This result corroborates with studies in *Metarhizium anisopliae*, which previously demonstrated that liquid cultures with carbon-rich media accumulated more biomass and yielded more MS numbers (Jackson and Jaronski, 2009), while fermentation media with higher nitrogen concentrations also resulted in greater biomass accumulation and MS yields than those grown in nitrogen-deficient medium (Behle and Jackson, 2014). Nutritional and environmental growth conditions play an essential role in fungal development by providing energy source and cofactors for biochemical reactions, and these factors also exert a remarkable impact on the formation and quality attributes of a variety of propagules employed for different purposes in pest control (Jaronski and Mascarin, 2016). For *Metarhizium* strains, reduced culture viscosity during the MS formation in a nutrient-poor medium was observed, because of rapid exhaustion of nutrients (Mascarin et al., 2014). Accordingly, we found that the culture medium for MS production appeared more liquid and less thick than the culture medium for P production. Lower viscosity is also associated with higher oxygen supply in fungal submerged liquid cultures, which can enhance fungal growth and specific propagule yields, as reported before (Mascarin et al., 2015).

In previous studies using other fungal propagules such as blastospores or aerial conidia, the effects of abiotic factors were addressed to assess tolerance and survival of some fungi

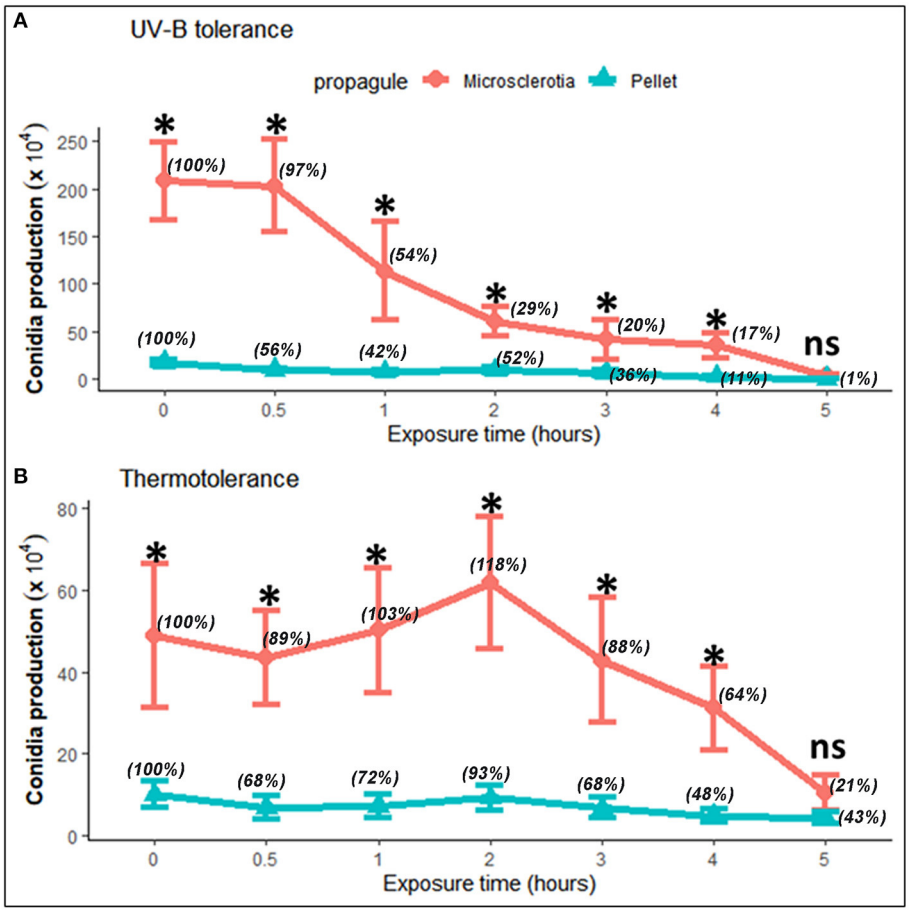

FIGURE 3 | Tolerance of microsclerotia and mycelial pellets of *Metarhizium robertsii* ARSEF 2575 to artificial UV-B radiation [1,283.38 mW m^{-2} of Quaite-weighted irradiance (Quaite et al., 1992) for 0 (control), 0.5, 1, 2, 3, 4, and 5 h, which corresponded to the doses of 0 (control), 2.31, 4.62, 9.24, 13.86, 18.48, and 23.10 kJ m^{-2}, respectively] **(A)**, and heat (45°C) **(B)**. Values indicate mean and standard error. Relative percentage values for each type of propagule are shown in parentheses. At each exposure time, the significant differences between propagules are shown with an asterisk ($p < 0.05$). ns = not significant.

commonly used in biological control (Braga et al., 2001, 2015; Paixão et al., 2017; Pereira-Junior et al., 2018; Bernardo et al., 2020). Conidia from *Metarhizium* species exposed to UV-B radiation (irradiances between 920 and 1,200 mW m^{-2}) had both germination and survival affected in a dose- and time-dependent manner (Braga et al., 2001). Conidia from the same strain used in this study (ARSEF 2575) exposed to 866.7 mW m^{-2} Quaite-weighted irradiance showed a certain degree of UV-B tolerance in relation to the dose used (3.9–6.2 kJ m^{-2}) (Pereira-Junior et al., 2018). Conidia and blastospores from *M. robertsii* IP 146 were equally tolerant to UV-B radiation (743.7 mW m^{-2} irradiance); however, the relative viability was lower than in aerial conidia when exposed to 45°C (Bernardo et al., 2020). Recently, the viability of MS exposed to UV-B radiation (4.0 kJ m^{-2}) varied greatly among *Metarhizium* spp. isolates, from very susceptible to quite tolerant ones (Corval et al., 2021). In the present study, we demonstrated for the first time the comparative tolerance of MS and P from *M. robertsii* ARSEF 2575 to UV-B radiation (1,283.38 mW m^{-2}). Although the effects of either UV-B or heat exposure did not inhibit conidial production (sporogenesis) by MS or P post-inoculation on water agar medium, we found that MS possess the ability to withstand better both stressful conditions as shown by their higher conidial production than P. Furthermore, we can infer that based on the number of conidia produced, the heat stress scenario (45°C) has a stronger detrimental effect on both MS and P propagules than UV-B radiation. Conidial production was evaluated after 10 days of incubation, and such period of time was necessary to promote complete sporulation in MS and P propagules under a highly humid microenvironment generated by the water agar medium used as an artificial substrate.

TEM microscopy revealed differences between conidia and MS in some intracellular structures such as cell wall, peroxisomes, and WBs, as well as peroxidase activity. WBs are organelles found exclusively in mycelium of filamentous fungi, and their function is sealing the septal pore in response to injury, allowing the rest of the mycelium to continue growth and to confer stress resistance, among other proposed functions (Liu et al.,

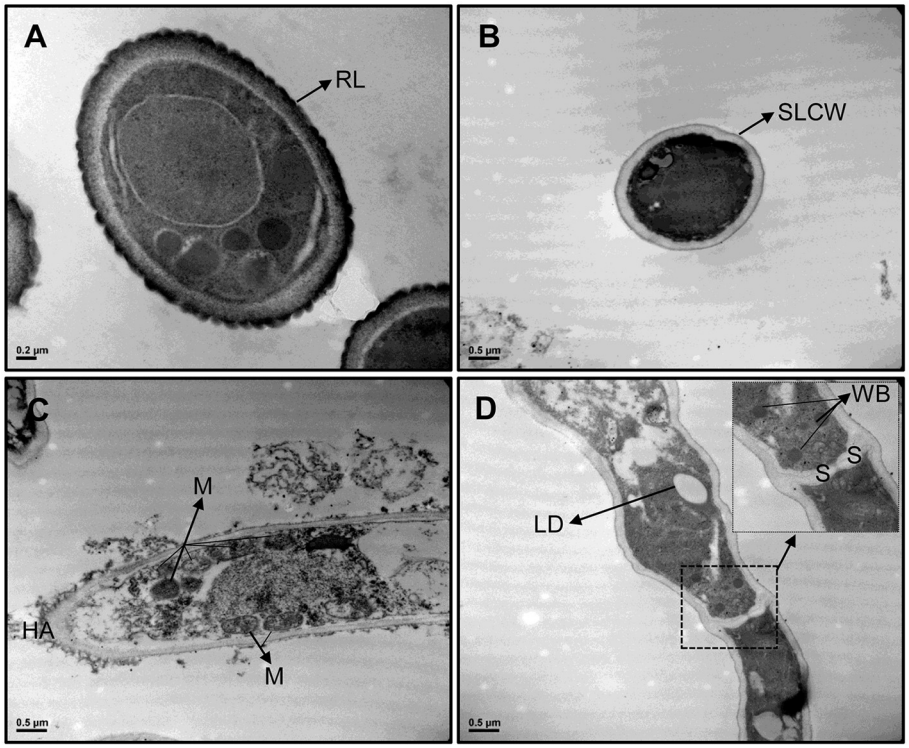

FIGURE 4 | TEM images from *Metarhizium robertsii* ARSEF 2575 conidia **(A)** and microsclerotial propagules **(B–D)** at 30,000× magnification stained with DAB. Cross section of both propagules showed differences in cell wall and ultrastructure morphology. Because of DAB reaction, microsclerotia exhibited black dots and black areas inside the cells, in hyphal apex, and in cell interconnections. Several mitochondria, peroxisomes, and Woronin bodies are visible and strongly stained in microsclerotia, but not in conidia. RL, rodlet layer; SLCW, single layered cell wall; HA, hyphal apex; M, mitochondrium; LD, lipid droplet; WB, Woronin body; S, septum.

2008). This organelle is, in fact, a special type of peroxisome found at the cell periphery or in association with the septum (Jedd and Chua, 2000; Liu et al., 2011). Thus, two types of peroxisomes can be found in filamentous fungi; one type is immobile sealing pores between hyphal cells, and the other is mobile and actively inserted into growing hyphae (Knoblach and Rachubinski, 2016). Both types are associated with anabolic and catabolic pathways, peroxide metabolism, oxidation of fatty acids, and the biosynthesis of phospholipids (Jedd and Chua, 2000). Recently, the protein forming hexagonal crystals inside WBs (HEX1) was functionally characterized in the mycelium of *M. robertsii*; MrHex1 was responsible for WB formation and involved in sealing septal pores, but unexpectedly, it does not seem to have any function regarding stress tolerance and virulence (Tang et al., 2020). For the first time, we noted the presence of peroxisomes in *M. robertsii* MS by TEM after DAB fixation, indicating high peroxidase activity inside cells and in-cell interconnections during MS formation. Most peroxisomes were found near the septa of hyphae, suggesting that they might be, in fact, WB. Peroxisomes and peroxidase activity were studied on mycelial pellets and MS-like pellets of *B. bassiana* (Huarte-Bonnet et al., 2018, 2019). Both studies, in accordance with this study on *M. robertsii*, also reported induction of *pex* genes encoding proteins named peroxins (PEXs). In general, PEXs are associated with peroxisomes biogenesis, and most of them are involved in the transport of matrix proteins from the cytosol into the peroxisome lumen (Kiel et al., 2006; Opaliński et al., 2010; Pieuchot and Jedd, 2012). The characterization of *pex* genes was done in other filamentous fungi such as *Magnaporthe oryzae* (*MoPex7,* expressed during short-chain fatty acid metabolism and pathogenesis) (Goh et al., 2011) and *Fusarium graminearum* (*FgPex4*, involved in regulation of hyphal growth, sexual and asexual reproduction, virulence, cell wall integrity, and elimination of ROS) (Zhang et al., 2019). The gene family *Pex14/17* (also known as *Pex33*) has been identified as fungal-specific gene encoding peroxin associated with conidiospore formation and peroxisome biogenesis and encoding a protein located in peroxisomal membrane (Managadze et al., 2010; Opaliński et al., 2010). Some orthologs of *Pex14/17* have been also characterized in *M. oryzae*, associated with conidial germination, germ tube elongation, and initial emergence of appressoria and as docking receptor peroxisomal membrane protein (Li et al., 2017). In *B. bassiana*, *Bbpex14/17* but also *Bbpex5*, *Bbpex7*, and *Bbpex19* have been shown to be induced during formation of MS-like pellets (Huarte-Bonnet et al., 2019). In this study, the genes *Mrpex5* and *Mrpex14/17* were the only upregulated at 96 h post-inoculation in liquid medium. Although in eukaryotic cells both *Pex5* and *Pex7* have been reported as cycling cytosolic receptors recognizing the peroxisomal targeting signals PTS1 and PTS2, respectively (Pieuchot and Jedd, 2012); in *Neurospora crassa*,

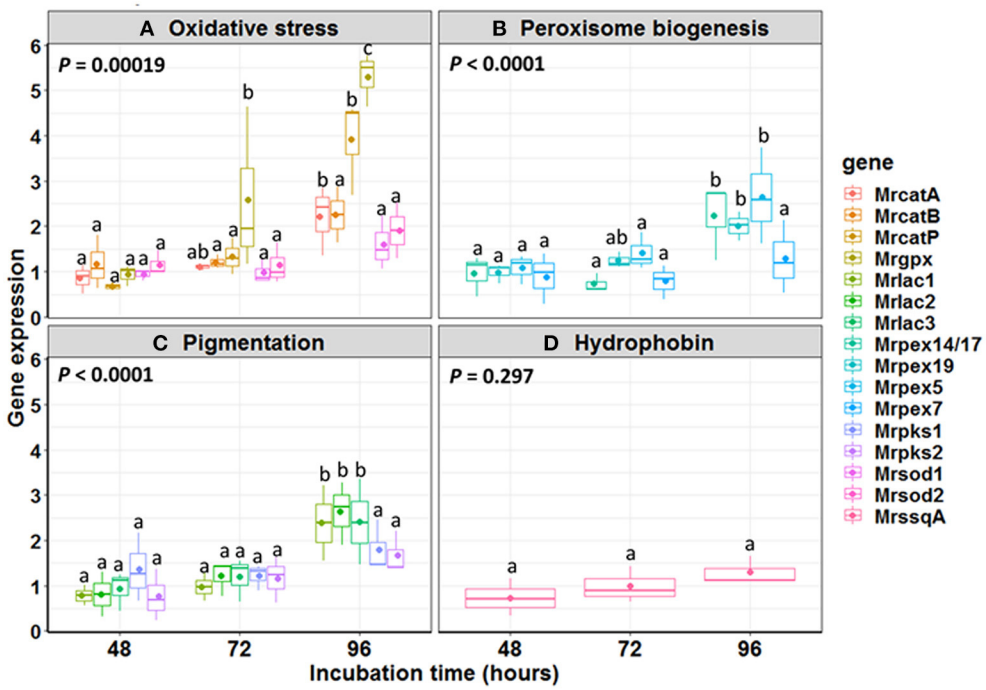

FIGURE 5 | Relative expression ratio of genes involved in **(A)** oxidative stress, **(B)** peroxisome biogenesis, **(C)** pigmentation, and **(D)** hydrophobin during *Metarhizium robertsii* microsclerotial differentiation. The boxes include 25–75% of the values; the line inside the boxes is the median (50%) of the distribution of all values, and the vertical bars represent the non-outlier range. Mean values are indicated by dots inside each boxplot. Mean comparisons were made between time intervals within each gene class, and different letters highlight significant differences between the gene expression. Significance level used: $\alpha = 0.05$ according to Tukey HSD test.

the docking complex of peroxisomal matrix protein import is composed of PEX14/17 (PEX33) interacting with itself and with the PTS1-receptor PEX5 (Managadze et al., 2010). This result suggests that both genes might be expressing and acting together in this function during the development and aging of *M. robertsii* MS.

We also found an oxidative stress scenario during MS differentiation. ROS production has been reported during cell differentiation in fungi and specifically for sclerotia differentiation (Georgiou et al., 2006). Studies on the hyphal aggregation processes indicate they are mediated by reduced oxygen entrance inside the cell due to a decreased surface-volume ratio and thus might be a mechanism of fungal mycelial adaptation to an increment of ROS in the microenvironment (Gessler et al., 2007). Antioxidant enzymes such as SODs, CATs, and GPxs act as the first line of cell defense against ROS (Aguirre et al., 2005; Song et al., 2013; Liu et al., 2018; Huarte-Bonnet et al., 2019). SODs decompose superoxide anion and singlet oxygen into oxygen and H_2O_2, and depending on the cofactors used, this family comprises three types of isoforms, i.e., Cu^{2+}/Zu^{2+}, Mn^{2+}, and Fe^{2+} (Culotta et al., 2006). In structure and phylogeny, SODs from *M. robertsii* are homologous to those from *B. bassiana* and other filamentous fungi, but some of them are functionally distinct (Zhu et al., 2018). SOD gene expression was reported during differentiation of microesclerotia-like pellets in *B. bassiana* (Huarte-Bonnet et al., 2019) and MS development in *M. rileyi* (Song et al., 2013). Nevertheless, it was found that both *Mrsod1* (Cu^{2+}/Zu^{2+} SOD) and *Mrsod2* (Mn^{2+}SOD) were not induced during MS formation. Fungal CATs act during germination and growth in response to oxidative stress (Michán et al., 2002; Pedrini et al., 2006; Wang et al., 2013; Song et al., 2018; Huarte-Bonnet et al., 2019). In this study, we measured the expression pattern of two cytosolic CATs (*cat*A and *cat*B) and one peroxisomal CAT (*cat*P) (Wang et al., 2013). Only *MrcatP* was induced in mature MS, in agreement with the previous result reported on mycelial pellets and MS-like pellets of *B. bassiana* (Huarte-Bonnet et al., 2018, 2019). A peroxisomal CAT gene was also expressed during vegetative growth and sclerotial developmental stages of *Sclerotinia sclerotiorum* (Yarden et al., 2014). Interestingly, the most expressed gene from oxidative stress response was *Mrgpx*, which was induced in MS at 72 and 96 h. GPxs are known to reduce either H_2O_2 or organic hydroperoxides to water or their corresponding alcohols using reduced glutathione and glutathione disulfide (Margis et al., 2008; Huarte-Bonnet et al., 2015). As GPxs and CAT used both H_2O_2 as substrate, it might occur that H_2O_2 can be reduced by induction of one of the two detoxification systems. In *B. bassiana* mycelial and MS-like pellets, CATs (*BbcatA-C* and *BbcatP*) were strongly induced, but *Bbgpx* was low or not induced (Huarte-Bonnet et al., 2018, 2019), just as the opposite we found in this study for *M. robertsii* MS. Taken together, our values of *Mrgpx*, *MrcatP*, and *Mrsod1* expression measured at 96 h showed similarities with the study by Michiels et al. (1994), reporting that Gpx has a

high protective behavior, CAT has an intermediate behavior, and Cu/Zn-SOD has a very small protective effect to protect cells against free radicals.

Hydrophobins are surface active proteins produced by filamentous fungi. They are proteins involved in the growth and morphogenetic processes (Wösten, 2001), and in *M. anisopliae* s.l., the hydrophobin-encoding gene *ssgA* was linked with appressorium development (St. Leger et al., 1992). Although earlier studies reported hydrophobin genes playing important roles in MS development of *Verticillium dahliae* (Klimes and Dobinson, 2006) and pellet formation via hydrophobic interactions in filamentous fungi (Zhang and Zhang, 2016), we have not found induction for *MrssgA* in any of the fermentation timepoints during MS development. Regarding the identification of potential pigmentation mechanisms, laccases (LACs) and polyketide synthases (PKSs) have been linked with fungal pigmentation. LACs are multicopper oxidases that catalyze the transformation of aromatic and non-aromatic compounds with the reduction of molecular oxygen to water. There are various isoforms due to the diverse physiological functions during the fungal life cycle (Rivera-Hoyos et al., 2013). In *Metarhizium* spp., *M. anisopliae* LAC (*Mlac1*) is expressed during isotropic growth and has been proposed to provide tolerance to abiotic stress during conidial pigmentation (Fang et al., 2010). *M. robertsii* and *M. acridum* LACs (*lac1*, *lac2*, and *lac3*) were upregulated when exposed to UV-B irradiation (Pereira-Junior et al., 2018). In this study, all three *Mrlac1*, *Mrlac2*, and *Mrlac3* were also induced in mature MS. On the other hand, PKS is an enzyme family implicated in the biosynthesis of polyketides with several biological activities, including pigment and biosynthesis of mycotoxins. *Pks1* and *Pks2* have been reported to be expressed in *Metarhizium* spp. *Pks1* is involved in conidial pigmentation and tolerance to environmental stresses, and *Pks2* is related to pathogenicity (Pereira-Junior et al., 2018; Zeng et al., 2018). In *M. rileyi*, the expression pattern of *pks* has been increased between initial stage and mature MS (Song et al., 2013); however, in this study, neither *Mrpks1* nor *Mrpks2* genes were induced during MS formation. As hyphal aggregation process is accompanied by the biosynthesis of pigment molecules in the mycelium (Gessler et al., 2007), and all LACs but not *pks* genes were induced at 96 h; thus, we hypothesize that the LAC pathway might be responsible for MS pigmentation. However, additional functional studies are needed to confirm this hypothesis. As a conclusion, looking at the expression pattern of genes involved in oxidative stress response, peroxisome biogenesis, and pigmentation, the upregulation of all of them was documented only at 96 h, that is, in mature MS.

In summary, *M. robertsii* is able to produce different propagules under different fermentation conditions, i.e., favoring the development of either MS or P. Despite their size twice smaller, MS exhibited higher dry biomass and concentration than P. The microsclerotial differentiation process includes at least a mechanism triggering oxidative stress, high peroxidase activity, and active peroxisome biogenesis. We propose that because of its tolerance to desiccation, heat, and UV-B, MS of this isolate could be an excellent candidate to be used in biological control of pests under environmental tropical and subtropical conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ÉF and NP conceived the project. FP, ÉF, CH-B, GM, and NP designed the experiments. FP, CR-S, and CH-B performed the experiments. FP, GM, and NP analyzed the data. FP and NP wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Abrashev, R. I., Pashova, S. B., Stefanova, L. N., Vassilev, S. V., Dolashka-Angelova, P. A., and Angelova, M. B. (2008). Heat-shock-induced oxidative stress and antioxidant response in *Aspergillus niger* 26. *Can. J. Microbiol.* 54, 977–983. doi: 10.1139/W08-091

Aguirre, J., Hansberg, W., and Navarro, R. (2006). Fungal responses to reactive oxygen species. *Med. Mycol.* 44, 101–107. doi: 10.1080/13693780600900080

Aguirre, J., Ríos-Momberg, M., Hewitt, D., and Hansberg, W. (2005). Reactive oxygen species and development in microbial eukaryotes. *Trends Microbiol.* 13, 111–118. doi: 10.1016/j.tim.2005.01.007

Behle, R. W., and Jackson, M. A. (2014). Effect of fermentation media on the production, efficacy, and storage stability of *Metarhizium brunneum* microsclerotia formulated as a prototype granule. *J. Econ. Entomol.* 107, 582–590. doi: 10.1603/EC13426

Behle, R. W., Jackson, M. A., and Flor–Weiler, L. B. (2013). Efficacy of a granular formulation containing *Metarhizium brunneum* F52 (Hypocreales: Clavicipitaceae) microsclerotia against nymphs of *Ixodes scapularis* (Acari: Ixoididae). *J. Econ. Entomol.* 106, 57–63. doi: 10.1603/EC12226

Bernardo, C. C., Pereira-Junior, R. A., Luz, C., Mascarin, G. M., and Fernandes, É. K. K. (2020). Differential susceptibility of blastospores and aerial conidia of entomopathogenic fungi to heat and UV-B stresses. *Fungal Biol.* 124, 714–722. doi: 10.1016/j.funbio.2020.04.003

Braga, G. U., Flint, S. D., Miller, C. D., Anderson, A. J., and Roberts, D. W. (2001). Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitudes from 61 N to 54 S. *J. Invertebr. Pathol.* 78, 98–108. doi: 10.1006/jipa.2001.5048

Braga, G. U., Rangel, D. E., Fernandes, É. K., Flint, S. D., and Roberts, D. W. (2015). Molecular and physiological effects of environmental UV radiation on fungal conidia. *Curr. Genet.* 61, 405–425. doi: 10.1007/s00294-015-0483-0

Brancini, G. T., Bachmann, L., Ferreira, M. E. D. S., Rangel, D. E., and Braga, G. Ú. (2018). Exposing *Metarhizium acridum* mycelium to visible light up-regulates a photolyase gene and increases photoreactivating ability. *J. Invertebr. Pathol.* 152, 35–37. doi: 10.1016/j.jip.2018.01.007

Corval, A. R. C., Mesquita, E., Corrêa, T. A., Silva, C. S. R., Bitencourt, R. O. B., Fernandes, É. K. K., et al. (2021). UV-B tolerances of conidia, blastospores, and microsclerotia of *Metarhizium* spp. entomopathogenic fungi. *J. Basic Microbiol.* 61, 15–26. doi: 10.1002/jobm.202000515

Cox, P. W., and Thomas, C. R. (1992). Classification and measurement of fungal pellets by automated image analysis. *Biotechnol. Bioeng.* 39, 945–952. doi: 10.1002/bit.260390909

Culotta, V. C., Yang, M., and O'Halloran, T. V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. *Biochim. Biophys. Acta Mol. Cell. Res.* 1763, 747–758. doi: 10.1016/j.bbamcr.2006.05.003

Fahimi, H. D. (2017). Cytochemical detection of peroxisomes in light and electron microscopy with 3,3′-diaminobenzidine. *Methods Mol. Biol.* 1595, 93–100. doi: 10.1007/978-1-4939-6937-1_10

Fang, W., Fernandes, É. K., Roberts, D. W., Bidochka, M. J., and Leger, R. J. S. (2010). A laccase exclusively expressed by *Metarhizium anisopliae* during isotropic growth is involved in pigmentation, tolerance to abiotic stresses and virulence. *Fungal Genet. Biol.* 47, 602–607. doi: 10.1016/j.fgb.2010.03.011

Fernandes, É. K., Keyser, C. A., Chong, J. P., Rangel, D. E., Miller, M. P., and Roberts, D. W. (2010). Characterization of *Metarhizium* species and varieties based on molecular analysis, heat tolerance and cold activity. *J. Appl. Microbiol.* 108, 115–128. doi: 10.1111/j.1365-2672.2009.04422.x

Fernandes, É. K., Rangel, D. E., Braga, G. U., and Roberts, D. W. (2015). Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation. *Curr. Genet.* 61, 427–440. doi: 10.1007/s00294-015-0492-z

Fernandes, E. K., Rangel, D. E., Moraes, Á. M., Bittencourt, V. R., and Roberts, D. W. (2007). Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. *J. Invertebr. Pathol.* 96, 237–243. doi: 10.1016/j.jip.2007.05.007

Fernandes, É. K., Rangel, D. E., Moraes, Á. M., Bittencourt, V. R., and Roberts, D. W. (2008). Cold activity of *Beauveria* and *Metarhizium*, and thermotolerance of *Beauveria*. *J. Invertebr. Pathol.* 98, 69–78. doi: 10.1016/j.jip.2007.10.011

Georgiou, C. D., Patsoukis, N., Papapostolou, I., and Zervoudakis, G. (2006). Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. *Integr. Comp. Biol.* 46, 691–712. doi: 10.1093/icb/icj034

Gessler, N. N., Aver'yanov, A. A., and Belozerskaya, T. A. (2007). Reactive oxygen species in regulation of fungal development. *Biokhimiya* 72, 1091–1109. doi: 10.1134/S0006297907100070

Goble, T. A., Gardescu, S., Fisher, J. J., Jackson, M. A., and Hajek, A. E. (2016). Conidial production, persistence and pathogenicity of hydromulch formulations of *Metarhizium brunneum* F52 microsclerotia under forest conditions. *Biol. Control.* 95, 83–93. doi: 10.1016/j.biocontrol.2016.01.003

Goh, J., Jeon, J., Kim, K. S., Park, J., Park, S. Y., and Lee, Y. H. (2011). The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in *Magnaporthe oryzae*. *PLoS ONE* 6:e28220. doi: 10.1371/journal.pone.0028220

Huarte-Bonnet, C., Juárez, M. P., and Pedrini, N. (2015). Oxidative stress in entomopathogenic fungi grown on insect-like hydrocarbons. *Curr. Genet.* 61, 289–297. doi: 10.1007/s00294-014-0452-z

Huarte-Bonnet, C., Paixão, F. R., Mascarin, G. M., Santana, M., Fernandes, É. K. K., and Pedrini, N. (2019). The entomopathogenic fungus *Beauveria bassiana* produces microsclerotial-like pellets mediated by oxidative stress and peroxisome biogenesis. *Environ. Microbiol. Rep.* 11, 518–524. doi: 10.1111/1758-2229.12742

Huarte-Bonnet, C., Paixão, F. R., Ponce, J. C., Santana, M., Prieto, E. D., and Pedrini, N. (2018). Alkane-grown *Beauveria bassiana* produce mycelial pellets displaying peroxisome proliferation, oxidative stress, and cell surface alterations. *Fungal Biol.* 122, 457–464. doi: 10.1016/j.funbio.2017.09.003

Huarte-Bonnet, C., Pereira-Junior, R. A., Paixão, F. R., Braga, G. U. L., Roberts, D. W., Luz, C., et al. (2020). *Metarhizium robertsii* and *M. acridum* conidia produced on riboflavin-supplemented medium have increased UV-A tolerance and upregulated photoprotection and photoreactivation genes. *BioControl* 65, 211–222. doi: 10.1007/s10526-019-09990-w

Jackson, M. A., and 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. doi: 10.1016/j.mycres.2009.03.004

Jackson, M. A., and Jaronski, S. T. (2012). Development of pilot-scale fermentation and stabilisation processes for the production of microsclerotia of the entomopathogenic fungus *Metarhizium brunneum* strain F52. *Biocontrol Sci. Technol.* 22, 915–930. doi: 10.1080/09583157.2012.696578

Jaronski, S. T., and Jackson, M. A. (2008). Efficacy of *Metarhizium anisopliae* microsclerotial granules. *Biocontrol Sci. Technol.* 18, 849–863. doi: 10.1080/09583150802381144

Jaronski, S. T., and Mascarin, G. M. (2016). "Mass production of fungal entomopathogens," in *Microbial Agents for Control of Insect Pests: From Theory to Practice, 1st Edn*, ed L. A. Lacey (Elsevier; Academic Press) Link: http://store.elsevier.com/product.jsp?locale=en_US&isbn=9780128035276

Jedd, G., and Chua, N. H. (2000). A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. *Nat. Cell Biol.* 2, 226–231. doi: 10.1038/35008652

Kiel, J. A., Veenhuis, M., and van der Klei, I. J. (2006). *PEX* genes in fungal genomes: common, rare or redundant. *Traffic* 7, 1291–1303. doi: 10.1111/j.1600-0854.2006.00479.x

Klimes, A., and Dobinson, K. F. (2006). A hydrophobin gene, *VDH1*, is involved in microsclerotial development and spore viability in the plant pathogen *Verticillium dahliae*. *Fungal Genet. Biol.* 4, 283–294. doi: 10.1016/j.fgb.2005.12.006

Knoblach, B., and Rachubinski, R. A. (2016). How peroxisomes partition between cells. A story of yeast, mammals and filamentous fungi. *Curr. Opin. Cell Biol.* 41, 73–80. doi: 10.1016/j.ceb.2016.04.004

Lenth, R. (2020). *Emmeans: Estimated Marginal Means, aka Least-Squares Means*. R package. Version 1.4.8.

Li, L., Wang, J., Chen, H., Chai, R., Zhang, Z., Mao, X., et al. (2017). Pex14/17, a filamentous fungus-specific peroxin, is required for the import of peroxisomal matrix proteins and full virulence of *Magnaporthe oryzae*. *Mol. Plant Pathol.* 18, 1238–1252. doi: 10.1111/mpp.12487

Liu, B., Wang, H., Ma, Z., Gai, X., Sun, Y., He, S., et al. (2018). Transcriptomic evidence for involvement of reactive oxygen species in *Rhizoctonia solani* AG1 IA sclerotia maturation. *PeerJ* 6:e5103. doi: 10.7717/peerj.5103

Liu, F., Lu, Y., Pieuchot, L., Dhavale, T., and Jedd, G. (2011). Import oligomers induce positive feedback to promote peroxisome differentiation and control organelle abundance. *Dev. Cell.* 21, 457–468. doi: 10.1016/j.devcel.2011.08.004

Liu, F., Ng, S. K., Lu, Y., Low, W., Lai, J., and Jedd, G. (2008). Making two organelles from one: Woronin body biogenesis by peroxisomal protein sorting. *J. Cell Biol.* 180, 325–339. doi: 10.1083/jcb.200705049

Liu, J., Yin, Y., Song, Z., Li, Y., Jiang, S., Shao, C., et al. (2014). NADH: flavin oxidoreductase/NADH oxidase and ROS regulate microsclerotium development in *Nomuraea rileyi*. *World J. Microbiol. Biotechnol.* 30, 1927–1935. doi: 10.1007/s11274-014-1610-7

Managadze, D., Würtz, C., Wiese, S., Schneider, M., Girzalsky, W., Meyer, H. E., et al. (2010). Identification of PEX33, a novel component of the peroxisomal docking complex in the filamentous fungus *Neurospora crassa*. *Eur. J. Cell Biol.* 89, 955–964. doi: 10.1016/j.ejcb.2010.07.003

Mapari, S. A., Meyer, A. S., and Thrane, U. (2008). Evaluation of *Epicoccum nigrum* for growth, morphology and production of natural colorants in liquid media and on a solid rice medium. *Biotechnol. Lett.* 30, 2183–2190. doi: 10.1007/s10529-008-9798-y

Margis, R., Dunand, C., Teixeira, F. K., and Margis-Pinheiro, M. (2008). Glutathione peroxidase family–an evolutionary overview. *FEBS J.* 275, 3959–3970. doi: 10.1111/j.1742-4658.2008.06542.x

Mascarin, G. M., Jackson, M. A., Kobori, N. N., Behle, R. W., Dunlap, C. A., and Delalibera, I. (2015). 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. doi: 10.1007/s00253-015-6620-3

Mascarin, G. M., Karobi, N. N., Vital, R. C. J., Jackson, M. A., and Quintela, E. D. (2014). Production of microsclerotia by Brazilian strains of *Metarhizium* spp. using submerged liquid culture fermentation. *World J. Microbiol. Biotechnol.* 30, 1583–1590. doi: 10.1007/s11274-013-1581-0

Michán, S., Lledias, F., Baldwin, J. D., Natvig, D. O., and Hansberg, W. (2002). Regulation and oxidation of two large monofunctional catalases. *Free Radic. Biol. Med.* 33, 521–532. doi: 10.1016/S0891-5849(02)00909-7

Michiels, C., Raes, V., Toussaint, O., and Remacle, J. (1994). Importance of Cu/Zn-SOD for Se-glutathione cell survival peroxidase, catalase, and against oxidative stress. *Free Radic. Biol. Med.* 17, 235–248. doi: 10.1016/0891-5849(94)90079-5

Nair, R. B., Lennartsson, P. R., and Taherzadeh, M. J. (2016). Mycelial pellet formation by edible ascomycete filamentous fungi, *Neurospora intermedia*. *AMB Exp*. 6, 1–10. doi: 10.1186/s13568-016-0203-2

Nascimento, É., Da Silva, S. H., dos Reis Marques, E., Roberts, D. W., and Braga, G. U. (2010). Quantification of cyclobutane pyrimidine dimers induced by UVB radiation in conidia of the fungi *Aspergillus fumigatus*, *Aspergillus nidulans*, *Metarhizium acridum* and *Metarhizium robertsii*. *Photochem. Photobiol.* 86, 1259–1266. doi: 10.1111/j.1751-1097.2010.00793.x

Opaliński, Ł., Kiel, J. A., Homan, T. G., Veenhuis, M., and van der Klei, I. J. (2010). *Penicillium chrysogenum* Pex14/17p–a novel component of the peroxisomal membrane that is important for penicillin production. *FEBS J.* 277, 3203–3218. doi: 10.1111/j.1742-4658.2010.07726.x

Paixão, F. R., Fernandes, É. K. K., and Pedrini, N. (2019). "Thermotolerance of Fungal Conidia" in *Microbes for Sustainable Insect Pest Management,* eds M. Khan, and W. Ahmad (Switzerland: Springer), 185–196. doi: 10.1007/978-3-030-23045-6_6

Paixão, F. R. S., Muniz, E. R., Barreto, L. P., Bernardo, C. C., Mascarin, G. M., Luz, C., et al. (2017). Increased heat tolerance afforded by oil-based conidial formulations of *Metarhizium anisopliae* and *Metarhizium robertsii*. *Biocontrol Sci. Technol.* 27, 324–337. doi: 10.1080/09583157.2017.1281380

Pedrini, N., Juárez, M. P., Crespo, R., and de Alaniz, M. J. (2006). Clues on the role of *Beauveria bassiana* catalases in alkane degradation events. *Mycologia* 98, 528–534. doi: 10.3852/mycologia.98.4.528

Pereira-Junior, R. A., Huarte-Bonnet, C., Paixão, F. R., Roberts, D. W., Luz, C., Pedrini, N., et al. (2018). Riboflavin induces *Metarhizium* spp. to produce conidia with elevated tolerance to UV-B, and upregulates photolyases, laccases and polyketide synthases genes. *J. Appl. Microbiol.* 125, 159–171. doi: 10.1111/jam.13743

Pieuchot, L., and Jedd, G. (2012). Peroxisome assembly and functional diversity in eukaryotic microorganisms. *Annu. Rev. Microbiol.* 66, 237–263. doi: 10.1146/annurev-micro-092611-150126

Quaite, F. E., Sutherland, B. M. and Sutherland, J. C. (1992). Action spectrum for DNA damage in alfalfa lowers predicted impact of ozone depletion. *Nature* 358, 576–578. doi: 10.1038/358576a0

R Core Team (2018). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Vienna. Available online at: https://www.r-project.org (accessed April 16, 2021).

Rangel, D. E., Braga, G. U., Fernandes, É. K. K., Keyser, C. A., Hallsworth, J. E., and Roberts, D. W. (2015). Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. *Curr. Genet.* 61, 383–404. doi: 10.1007/s00294-015-0477-y

Rivera-Hoyos, C. M., Morales-Álvarez, E. D., Poutou-Piñales, R. A., Pedroza-Rodríguez, A. M., RodrÍguez-Vázquez, R., and Delgado-Boada, J. M. (2013). Fungal laccases. *Fungal Biol. Rev.* 27, 67–82. doi: 10.1016/j.fbr.2013.07.001

Song, Z., Lin, Y., Du, F., Yin, Y., and Wang, Z. (2017). Statistical optimisation of process variables and large-scale production of *Metarhizium rileyi* (Ascomycetes: Hypocreales) microsclerotia in submerged fermentation. *Mycology* 8, 39–47. doi: 10.1080/21501203.2017.1279688

Song, Z., Shen, L., Yin, Y., Tan, W., Shao, C., Xu, J., et al. (2015). Role of two *Nomuraea rileyi* transmembrane sensors *Sho1p* and *Sln1p* in adaptation to stress due to changing culture conditions during microsclerotia development. *World J. Microbiol. Biotechnol.* 31, 477–485. doi: 10.1007/s11274-015-1801-x

Song, Z., Shen, L., Zhong, Q., Yin, Y., and Wang, Z. (2016a). Liquid culture production of microsclerotia of *Purpureocillium lilacinum* for use as bionematicide. *Nematology* 18, 719–726. doi: 10.1163/15685411-00002987

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

Song, Z., Yin, Y., Lin, Y., Du, F., Ren, G., and Wang, Z. (2018). The *bZip* transcriptional factor activator protein-1 regulates *Metarhizium rileyi* morphology and mediates microsclerotia formation. *Appl. Microbiol. Biotechnol.* 102, 4577–4588. doi: 10.1007/s00253-018-8941-5

Song, Z., Zhong, Q., Yin, Y., Shen, L., Li, Y., and Wang, Z. (2016b). The high osmotic response and cell wall integrity pathways cooperate to regulate morphology, microsclerotia development, and virulence in *Metarhizium rileyi*. *Sci. Rep.* 6:38765. doi: 10.1038/srep38765

St. Leger, R. J., Staples, R. C., and Roberts, D. W. (1992). Cloning and regulatory analysis of starvation-stress gene, *ssgA*, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* 120, 119–124. doi: 10.1016/0378-1119(92)90019-L

Tang, G., Shang, Y., Li, S., and Wang, C. (2020). MrHex1 is required for Woronin body formation, fungal development and virulence in *Metarhizium robertsii*. *J. Fungi*. 6:172. doi: 10.3390/jof6030172

Veiter, L., Rajamanickam, V., and Herwig, C. (2018). The filamentous fungal pellet-relationship between morphology and productivity. *Appl. Microbiol. Biotechnol.* 102, 2997–3006. doi: 10.1007/s00253-018-8818-7

Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics With S. 4th Edn*. New York, NY: Springer. doi: 10.1007/978-0-387-21706-2

Wang, Z. L., Zhang, L. B., Ying, S. H., and Feng, M. G. (2013). Catalases play differentiated roles in the adaptation of a fungal entomopathogen to environmental stresses. *Environ. Microbiol.* 15, 409–418. doi: 10.1111/j.1462-2920.2012.02848.x

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer. doi: 10.1007/978-3-319-24277-4

Wösten, H. A. (2001). Hydrophobins: multipurpose proteins. *Annu. Rev. Microbiol.* 55, 625–646. doi: 10.1146/annurev.micro.55.1.625

Xin, C., Xing, X., Wang, F., Liu, J., Ran, Z., Chen, W., et al. (2020). MrMid2, encoding a cell wall stress sensor protein, is required for conidium production, stress tolerance, microsclerotium formation and virulence in the entomopathogenic fungus *Metarhizium rileyi*. *Fungal Genet. Biol.* 134, 1–9. doi: 10.1016/j.fgb.2019.103278

Yarden, O., Veluchamy, S., Dickman, M. B., and Kabbage, M. (2014). *Sclerotinia sclerotiorum* catalase SCAT1 affects oxidative stress tolerance, regulates ergosterol levels and controls pathogenic development. *Physiol. Mol. Plant Pathol.* 85, 34–41. doi: 10.1016/j.pmpp.2013.12.001

Zeng, G., Zhang, P., Zhang, Q., Zhao, H., Li, Z., Zhang, X. Wang, C., et al. (2018). Duplication of a *Pks* gene cluster and subsequent functional diversification facilitate environmental adaptation in *Metarhizium* species. *PLoS Genet.* 14:e1007472. doi: 10.1371/journal.pgen.1007472

Zhang, J., and Zhang, J. (2016). The filamentous fungal pellet and forces driving its formation. *Crit. Rev. Biotechnol.* 36, 1066–1077. doi: 10.3109/07388551.2015.1084262

Zhang, L., Wang, L., Liang, Y., and Yu, J. (2019). FgPEX4 is involved in development, pathogenicity, and cell wall integrity in *Fusarium graminearum*. *Curr. Genet.* 65, 747–758. doi: 10.1007/s00294-018-0925-6

Zhang, X., Leger, R. J. S., and Fang, W. (2017). Pyruvate accumulation is the first line of cell defense against heat stress in a fungus. *MBio* 8, 1–12. doi: 10.1128/mBio.01284-17

Zhao, T., Tian, H., Xia, Y., and Jin, K. (2019). MaPmt4, a protein O-mannosyltransferase, contributes to cell wall integrity, stress tolerance and virulence in *Metarhizium acridum*. *Curr. Genet.* 65, 1025–1040. doi: 10.1007/s00294-019-00957-z

Zhu, X. G., Tong, S. M., Ying, S. H., and Feng, M. G. (2018). Antioxidant activities of four superoxide dismutases in *Metarhizium robertsii* and their contributions to pest control potential. *Appl. Microbiol. Biotechnol.* 102, 9221–9230. doi: 10.1007/s00253-018-9302-0

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Interactive Gene Expression Between *Metarhizium anisopliae* JEF-290 and Longhorned Tick *Haemaphysalis longicornis* at Early Stage of Infection

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The longhorned tick, *Haemaphysalis longicornis* (Acari: Ixodidae), is a hard tick and a vector for severe fever with thrombocytopenia syndrome (SFTS) virus. The number of patients infected with SFTS is rapidly increasing. Recently, the invertebrate pathogen *Metarhizium anisopliae* JEF-290 was reported to be useful to control the tick as an alternative to chemical acaricides, which are not easily applicable in human living areas where the tick is widely spread. In this study, we analyzed how the tick and the fungal pathogen interact at the transcriptional level. Field-collected tick nymphs were treated with JEF-290 conidia at 1×10^8 conidia/ml. In the early stage of infection with 2.5% mortality, the infected ticks were subjected to RNA sequencing, and non-infected ticks and fungal masses served as controls. Fungus and tick genes were mostly up-regulated at the early stage of infection. In the gene set enrichment analysis of the infecting fungus, catabolic processes that included lipids, phospholipids, and detoxification processes, the response to oxidative stress, and toxic substances were significantly up-regulated. In this fungal up-regulation, various lipase, antioxidant enzyme, and hydrolase genes were highly transcribed. The gene set enrichment analysis of the infected tick showed that many peptide synthesis processes including translation, peptide metabolism, ribonucleotide metabolism, and energy production processes that included ATP generation and ADP metabolism were significantly up-regulated. Structurally, mitochondria and ribosome subunit genes in ticks were highly transcribed to upregulate these processes. Together these results indicate that JEF-290 initiates process that infects the tick while the tick actively defends against the fungal attack. This work provides background to improve our understanding of the early stage of fungal infection in longhorned tick.

Keywords: ***Metarhizium anisopliae***, ***Haemaphysalis longicornis***, **transcription, catabolic process, peptides synthesis**

INTRODUCTION

Haemaphysalis longicornis (Acari: Ixodidae), the longhorned tick is an important vector for human disease, and known to transmit severe fever with thrombocytopenia syndrome virus (SFTSV), *Rickettsia japonica*, and *Coxiella burnetii* (Mahara, 1997). SFTSV, which belongs to the Phlebovirus genus in the family Bunyaviridae, was first reported in China in 2009, and then spread throughout Asia with reports in North America also (Denic et al., 2011; McMullan et al., 2012). SFTSV causes vomiting, diarrhea, high fever, and thrombocytopenia, and the mortality ranges from 2% to as high as 30% (Gai et al., 2012). Previous studies confirmed that infection occurs person to person through blood contact or bodily secretions from infected patients (Liu et al., 2012). The longhorned tick is also an important pest for livestock such as deer, sheep, and cattle. In New Zealand and Australia, the tick can reduce cattle production by 25% (Heath, 2016). This tick also mediates cattle diseases from Theileria species, which are threats to the livestock industry (Hammer et al., 2015).

The longhorned tick is a three-host ixodid tick, which takes blood meals on three different hosts at each developmental stage until engorged. The engorged adult females lay more than 2,000 eggs in the late spring and early summer. The eggs hatch into six-legged larvae in late summer and early autumn. The ticks overwinter at the nymphal stage, and become active in the following spring. The ticks suck blood for 5–7 days, and they can survive for many month without blood meal at all stages (Heath, 2016). The longhorned tick consists of a diploid and triploid population which are bisexual and obligatory parthenogenetic, respectively, and an aneuploidy population is capable of both bisexual and parthenogenetic reproduction which make longhorned tick a very interesting case of tick cytogenetics and reproduction (Oliver, 1977).

Several management tools are available to control tick (Lee et al., 2015), but most of them are not environmentally sound or effective. Most of the commonly used pesticides against ticks include pyrethroid and carbamate. Pyrethroid insecticides have high insecticidal activity but have a negative impact on the environment. In addition, mite resistance to pyrethroid-based acaricides has been recently reported (Hernandez et al., 2018). Carbamate insecticides are also highly toxic to beneficial insects, such as bees, so their use is limited. ticks inhabit parks, mountains, and lakesides, and it is difficult to spray acaricides near areas of human habitation. For this reason, various methods using biological control agents have recently been studied. Cymbopogon citratus essential oil has a relatively high mortality rate in longhorned ticks (Agwunobi et al., 2020). Another report showed that the antlion Euroleon coreaus could become a new predator for the ticks (Nwanade et al., 2020). However, control methods using predators and plant extracts have disadvantages as they are difficult to apply in large areas and rarely show high control activity (George et al., 2004; Quadros et al., 2020).

Entomopathogenic fungi may be an alternative strategy to control ticks. This fungal group is a facultative pathogen to arthropods, causes pathogenicity specifically to insects, mites and ticks, and has been used to control various pests (Charnley and Collins, 2007; Fernandes et al., 2012). Although studies on entomopathogenic fungi–mediated control of tick are still lacking, the possible effects of entomopathogenic fungi on other ticks have been shown. *Dermacentor variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* showed relatively high susceptibility when Beauveria bassiana or *Metarhizium anisopliae* was applied to target ticks (Kirkland et al., 2004). In addition, entomopathogenic fungi have high virulence against engorged females and tick eggs (Perinotto et al., 2012). Recently, studies have shown that selected B. bassiana strains show high virulence against longhorned tick, and confirmed the characteristics of the strains (Zhendong et al., 2019). However, the biological control of longhorned tick using entomopathogenic fungi is unknown.

A previous study of transcriptome analysis of small planthopper, *Laodephax striatellus* has provided the information about changes of gene expression pattern upon chemical pesticide-treatment, and demonstrated that suppression of the upregulated genes synergistically improved the insecticidal effect of the pesticide (Fang et al., 2020). In considering the use of microbials on targeting pests, it is also important to understand how the two organisms respond to each other at transcriptional level because it will provide information of pathogenicity and immunity related genes which might help study of putative virulence-modulating genes. Many studies have used transcriptome sequencing of ticks to determine their defense mechanisms when parasitize their hosts (Charrier et al., 2018; Araujo et al., 2019). In addition, tick-borne diseases have been studied at the transcriptional level. In a study in Australia, RNA sequencing was conducted to study the diseases caused by various ticks (Harvey et al., 2019). In Europe, various studies have been conducted to identify diseases that ticks can carry (Vayssier-Taussat et al., 2013; Pettersson et al., 2017). However, little research has been done on the defense mechanisms in ticks during fungal infection and the fungal mechanisms during tick infection.

In this study, the entire genome of JEF-290 was sequenced by PacBio sequencing technology, application of *M. anisopliae* JEF-290 for the control of longhorned tick was evaluated, and the interaction between tick and fungal pathogen at the early stage of infection was investigated by using transcriptome *analyses*. To compare the gene expression level of JEF-290 and longhorned tick under infection, differently expressed genes (DEGs), gene ontology (GO), and gene set enrichment *analyses* were conducted, and the results were analyzed to identify potentially important tick genes for defense and fungal genes for infection. This study will help clarify and modulate how the longhorned tick and JEF-290 respond to each other at the onset of fungal infection.

MATERIALS AND METHODS

Fungal Isolate

M. anisopliae JEF-290 was obtained from the Insect Microbiology and Biotechnology Laboratory (IMBL), Jeonbuk National University, South Korea. The fungal isolate was grown on quarter strength Sabouraud dextrose agar (1/4SDA; Difco,

United States) in the dark at 25 ± 1°C and stored in 20% (v/v) glycerol at −80°C. This isolate has high acaricidal activity against the longhorned tick (Lee et al., 2019).

Fungal Whole Genome Sequencing

For whole genome sequencing of *M. anisopliae* JEF-290 at Macrogen[1] (Macrogen Inc., Seoul, South Korea), genomic DNA and RNA were extracted from 7-day-old fungal mycelia. DNA quantity was assessed by using Victor 3 fluorometry (Perkin-Elmer) with Pico-green® fluorescent nucleic acid stain (Thermo Fisher Scientific). To assess DNA quality, gel electrophoresis was performed. The concentration of genomic DNA was measured using a Nano Drop spectrophotometer (Thermo Fisher Scientific) and a Qubit fluorometer (Life Technology). For PacBio RS sequencing, 8 μg of input genomic DNA was used for 20 kb library preparation. For gDNA with a size range less than 17 Kb, the Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, Cambridge, United States) was used to determine the actual size distribution. Genomic DNA was sheared with g-TUBE (Covaris Inc., Woburn, MA, United States) and purified using AMPure PB magnetic beads (Beckman Coulter Inc., Brea, CA, United States). The gDNA concentration was measured using both a Nano Drop spectrophotometer and a Qubit fluorometer, and approximately 200 ng μl^{-1} of gDNA was run on a field-inversion gel. A total of 10 μl of library was prepared using the PacBio DNA Template Prep Kit 1.0 (for 3∼10 Kb). SMRT bell templates were annealed using the PacBio DNA/Polymerase Binding Kit P6. The PacBio DNA Sequencing Kit 4.0 and eight SMRT cells were used for sequencing. Subsequent steps were based on the PacBio Sample Net-Shared Protocol[2]. The genome sequences of entomopathogenic isolates such as *M. anisopliae, M. rileyi*, *M. acridum*, and *M. robertsii* were subjected to orthologous analysis. Orthologous and paralogous gene clustering analysis was performed using OrthoMCL (v.2.0.3). Data were analyzed in blastp (v2.2.25 +) (E-value 1e-5), and sequences with less than 10 amino acids or stop codon ratios exceeding 20% were excluded.

Longhorned Tick

Wild populations of tick nymphs were collected several times from a grassland field near a rural community in Seongnam City, Korea using a carbon dioxide trapping method (Miles, 1968) from April until July in 2017 and 2018. Dry ice (2.5 kg) was placed into traps (36 × 40 cm) and traps were left in the grass field for 7 days. The collected ticks were identified based on a tick handling manual and training handbook (Yamaguti et al., 1971). Because the collected ticks consisted of approximately 95 percent of longhorned tick and less than five percent of *H. flava*, the larvae which are difficult to identify morphologically were removed from the collected colony, and only the nymphs were subjected to identification to collect ticks.

RNA Extraction

Seventy microliter of conidial suspension of *M. anisopliae* JEF-290 (1×10^8 conidia/ml) was spread on a 1/4SDA plate, and cultured at 27°C for 14 days to harvest conidia by vortexing from a sporulated agar disc (5 mm diameter) in 1.0 ml of 0.03% siloxane solution for 60 sec. The concentration of newly harvested conidia was adjusted to 1×10^8 conidia/ml. Approximately 180 nymphs were dipped into the conidial suspension for approximately 30 sec, placed on a filter paper-layered petri dish, and incubated at 27°C. The ticks were collected when the tick mortality rate reached 3.0%.

Longhorned tick RNA was extracted from the infected 180 nymphs, and same number of non-infected nymphs. On the nitrocellulose membrane liner covering 1/4SDA culture petri-dishes, JEF-290 was inoculated as described above. Five days after inoculation, actively proliferating mycelia and conidia were harvested for RNA extraction as controls. Total RNAs of these samples were extracted with TRIzol reagent (Invitrogen Life Technologies, CA, United States) following the manufacturer's instructions. RNA purity and integrity were quantified by an ASP-2680 spectrophotometer (ACTGene, Piscataway, NJ, United States) and an Agilent 2100 Bionalyzer system.

RNA Sequencing and *de novo* Assembly

Libraries of infected tick, non-infected tick and non-infecting fungus were made using the Truseq RNA kit (Illumina, San Diego, CA, United States) following the manufacturer's protocol at Macrogen.

The first step was to purify mRNA containing poly-A using poly-T oligo-attached magnetic beads and fragment the samples into small pieces at elevated temperature with divalent cations. The cleaved RNA fragments were reverse-transcribed into first strand cDNA with random primers.

Ampure XP beads were used in the second strand reaction mix to generate double-stranded cDNA by removing the RNA template and synthesizing the replacement strand. The 3′ overhang was removed, and polymerase was used to fill the 5′ overhang using an End Repair (ERP) mix. The adapter was then ligated to the fragment by attaching the "A" nucleotide to the 3′ end of the blunt fragment and giving a core reaction single "T" nucleotide at the 3′ end of the adapter. Multiple indexing adapters were ligated to the ends of the double-stranded cDNA and then enriched by PCR to create DNA library templates. In each isolate, infecting and non-infecting samples were sequenced in parallel using an Illumina HiSeq 2000 sequencer with a read length of 101 bp. The read quality was verified by fastQC v.0.11.8 (Andrews, 2010) and the quality was filtered to remove low quality sequences with a Phred score of 30 or less using NGS QC Toolkit v.2.3.3 (Patel and Jain, 2012). For efficient and robust *de novo* reconstruction of transcriptomes, Trinity (ver 2.8.3) was used[3]. TransDecoder (version 5.5.0) was used to identify candidate coding regions within the transcription sequence. Contigs with more than 90% of sequence identities were clustered using cd-hit-est v.4.8.1 (Fu et al., 2012) to remove isoforms, and the *in silico* cDNA library was constructed.

[1] www.macrogen.com

[2] http://pacificbiosciences.com/

[3] https://github.com/trinityrnaseq/

Differentially Expressed Gene and Gene Ontology Analyses

To quantify transcript abundances, Kallisto (ver 0.45.0) was used to build an index form from the fasta form of target sequences and non-infecting and infecting libraries were compared. Transcripts per million (TPM) of non-infecting and infecting samples was calculated. Raw signals were normalized using a $\log_2$-based transformation. Fold-change statistical tests were performed and $\log_2|FC| \geq 2$ was defined as statistically significant differential expression. Contigs were blasted using the Blast2Go program with a local blast. The statistical significance threshold was 1.0E-10 and the number of blast hits was set to one. GO analysis of up- and down-regulated contigs was performed using InterPro (online) in the Blast2Go program. The public EMBL-EBI database was used to scan sequences against InterPro's signatures. Up- and down-regulated contigs were annotated at GO level 2. In addition, using the Blast2Go program, the tick contigs were mapped to the local immune database that based on ImmunoDB[4] was prepared by downloading immune genes of *Drosophila melanogaster* from NCBI to analyze the up-regulation of immune-related contigs in the infected tick.

Validation of RNA-Sequencing

Six and five randomly selected genes of tick and *M. anisopliae* JEF-290 were validated using qRT-PCR, respectively. The RNA samples from infected tick and non-infected tick, and non-infecting JEF-290 were subjected to reverse transcription (RT) using an AccuPower® RT PreMix (Bioneer, Daejeon, South Korea) with oligo (dT) 15 primer (Promega, MI, United States). The qRT-PCR primers were designed at SnapDragon[5]. qRT-PCR was performed using Thunderbird® SYBR® qPCR mix (QPS-201, TOYOBO, Japan) in a 96-well Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, United States). PCR conditions were as follows: denaturation for 1 min at 95°C, and then 40 cycles of 15 s at 95°C, 1 min at 60°C followed by melting, increased 0.5°C per 5 s started from 65 to 95°C. The total RNA without reverse transcription was used as a negative control, and different *actin* genes from tick and JEF-290 were independently used as an internal control to normalize relative expression level. In this validation, other housekeeping genes were tried but they worked well, so *actin* genes were finally used. All experiments were performed in triplicate. $\triangle$Ct (threshold cycle) was calculated as (Ct value of up-regulated genes)–(Ct value of *actin*) and subjected to the calculation of fold change value ($2^{-\Delta\Delta Ct}$).

Gene Set Enrichment Analysis

DEG contigs of infecting *M. anisopliae* JEF-290 and infected longhorned tick with more than twofold change difference were subjected to GO enrichment analysis to identify the significantly involved functional gene groups in infection and defense. Blastn was performed with an *E*-value of 1.0E-10 and non-blasted and non-GO ID contigs were removed. As a reference, *Ixodes scapularis* and *Beauveria bassiana* were used. Functional genes set enrichment was performed using the g:Profiler web server[6]. The related *p*-value was corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) procedure with a threshold of 0.05.

RESULTS

Whole Genome of *M. anisopliae* JEF-290

As a result of whole genome sequencing of *M. anisopliae* JEF-290, a total of 11,868,389,082 bases were identified with N50 of 26,851 bp (**Supplementary Table 1**). The number of reads was 746,811 and the mean subread length was 15.9 Kb. A total of 18 contigs were assembled and the whole genome size was analyzed to be 42.85 Mb with 13,634 coding genes, which was larger than other *Metarhizium* species (**Table 1**). Comparison of the orthologs of the six different genus of entomopathogenic fungi revealed that all strains shared 5,320 genes, and JEF-290, M. robertstii, M. riley, and *M. acridum* shared 698 genes (**Supplementary Figure 1**).

Infection of *M. anisopliae* JEF-290 to Longhorned Tick

Under laboratory conditions, application of *M. anisopliae* JEF-290 to nymphs resulted in high control efficacy and mycosis (**Figure 1**). The survival rate of nymphs in the non-treated control was 90.0% at 15 days post-application, but JEF-290 treatment resulted in approximately 66.7% mortality at 15 days and >90% of mortality at 30 days [$F_{(2,\ 143)} = 241.9$, $p < 0.001$]. Fungal mycosis was observed on the cadavers of infected nymphs.

Comparative Differentially Expressed Genes of *M. anisopliae* JEF-290 and Longhorned Tick

From analyzing the RNA sequences, 8.767 Gb (longhorned tick), 8.549 Gb (JEF-290) and 11.224 Gb (infected tick) of raw sequences were identified (**Supplementary Table 2**). Filtering and *de novo* assembly were performed based on the raw data above to obtain a sequence of 27.2, 16.5, and 28.9 Mb, respectively. The assembled contigs of longhorned tick, JEF-290 and infected tick were clustered with more than 90% identity using the CD-hit-est program, and the numbers of clustered contigs were 33,099 (longhorned tick), 15,044 (JEF-290), and 36,292 (infected tick), respectively. The N50 values of the samples were 1101 b (longhorned tick), 1554 b (JEF-290), and 1056 b (infected tick). In the DEG analysis, relatively large numbers of tick and fungal genes were up-regulated when the tick was infected by the fungus (**Figure 2**). Among the tick genes, 1216 genes were up-regulated and 165 genes were down-regulated ($|FC| > 2$). Among the fungal genes, 137 genes were up-regulated and 11 genes were down-regulated ($|FC| > 2$) (**Supplementary Table 3**). qRT-PCR was performed on the randomly selected genes (**Supplementary Table 4**), and the results confirmed that gene expression levels determined by qRT-PCR and RNA-seq analysis results were

[4]http://cegg.unige.ch/Insecta/immunodb

[5]http:/www.flyrnai.org/snapdragon

[6]http://bitt.cs.ut.ee/gprofiler/

TABLE 1 | Genome features of *M. anisopliae* JEF-290 and other entomopathogenic fungi.

Features	*Ma* JEF-290	*M. anisopliae*	*M. robertsii*	*M. acridum*	*M. rileyi*	*Bb* JEF-007	*C. militaris*
Size (Mb)	42.8	38.5	39	38.1	32.0	36.5	32.2
Coverage (fold)	237X	98.3X	100X	107X	107.3X	105.1X	147X
Scaffold No. (>1 kb)	18	74	176	241	389	39	13
Scaffold N50 (Mb)	6.25	2.04	1.96	0.33	0.89	3.12	4.55
% G + C content	50.9	50.7	51.5	50	49.3	48	51.4
% G + C in coding gene	54.3	54.5	54.4	54.1	54.3	57.1	58.6
% Repeat rate	1.32	0.89	0.98	1.52	1.51	1.71	3.04
Protein-coding genes	13654	10891	10,582	9,849	8764	10,857	9,684
Protein families (protein no.)	4848 (8310)	6401 (8554)	2,797[b] (7,556)	2,746[b] (6,948)	6261 (6852)	1,284[a] (4,282)	2,736[b] (6,725)
Gene density (gene per Mb)	319	283	271	259	274	297	301
Exons per gene	2.2	2.6	2.8	2.7	2.8	2.3	3
% Secreted proteins	16.2	19.5	17.6	15.1	19.5	18.9	16.2
tRNA	274	152	141	122	113	140	136
NCBI Accession No.	PRJNA530366	AZNF00000000.1	ADNJ00000000	ADNI00000000	AZHC00000000.1	PRJNA352877	AEVU00000000

[a]*MAKER analysis.* [b]*InterProScan analysis.*

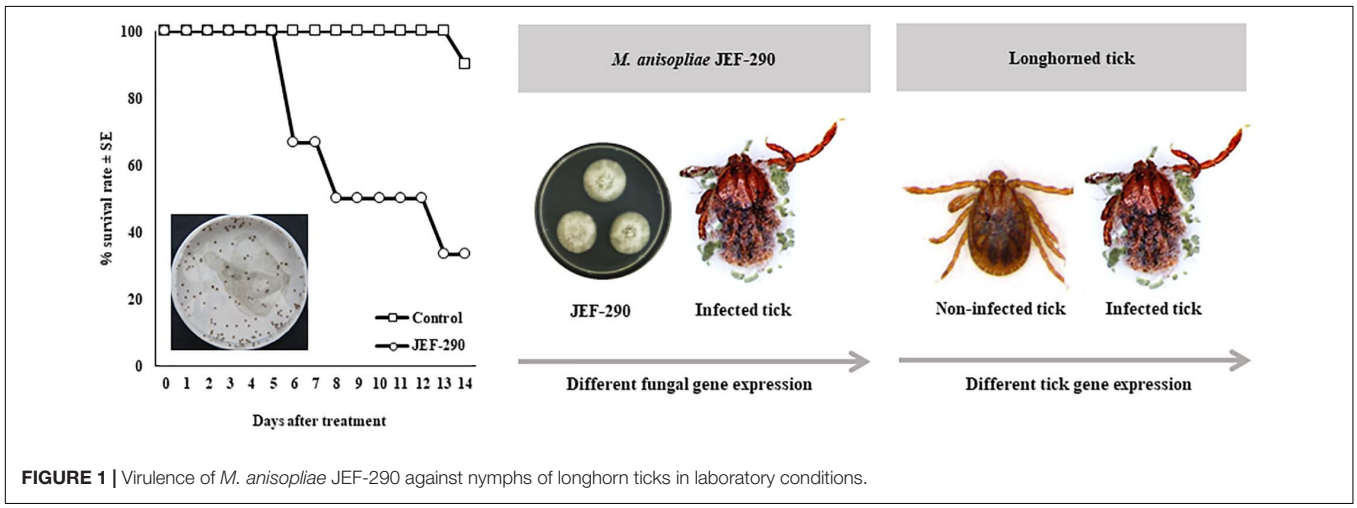

FIGURE 1 | Virulence of *M. anisopliae* JEF-290 against nymphs of longhorn ticks in laboratory conditions.

similar to each other although limited numbers of internal control genes were used (**Supplementary Figure 2**).

Gene Ontology of Differentially Expressed Genes

In the GO analysis of the infected tick, fungal GO terms were almost up-regulated, but GO terms of longhorned tick were mostly up-regulated with partial down-regulation (**Figure 2**). In the GO of longhorned tick genes, 1,381 DEGs were classified in the following three GO terms: biological process, cellular components, and molecular function (**Figure 2A**). Many longhorned tick contigs belonging to biological process and molecular function showed a trend that more of these contigs were up-regulated while the contigs belonging cellular component showed relatively less changes of gene expression.

The up-regulated longhorned tick GO terms (FC > 2) included 1,216 genes that included biological process (54%), molecular function (36%), and cellular component (11%), and these GO terms were mainly metabolism, catalytic activity, and bindings. The contigs annotated as being involved in metabolic process (GO:0008152), cellular process (GO:0009987), and organic substance metabolic process (GO:0071704), primary metabolic process (GO:0044238), catalytic activity (GO:0003824), hydrolase activity (GO:0016787), small molecule binding (GO:0036094), anion binding (GO:0043168), and nucleotide binding (GO:0000166) were upregulated noteworthily. The down-regulated longhorned tick GO terms (FC < 2) included 165 genes that included biological process (59%), molecular function (29%), and cellular component (11%). The contigs annotated as being involved in organic cyclic compound binding (GO:0097159), heteroclyclic compound binding (GO:1901363), nucleic acid binding (GO:0003676), and protein binding (GO:0005515) were down-regulated noteworthily.

In the GO of *M. anisopliae* JEF-290, 295 DEGs were categorized as three GO types: biological process, cellular component, and molecular function (**Figure 2B**). The up-regulated fungal GO terms (FC > 2) included 137 genes that included biological process (53%), molecular function

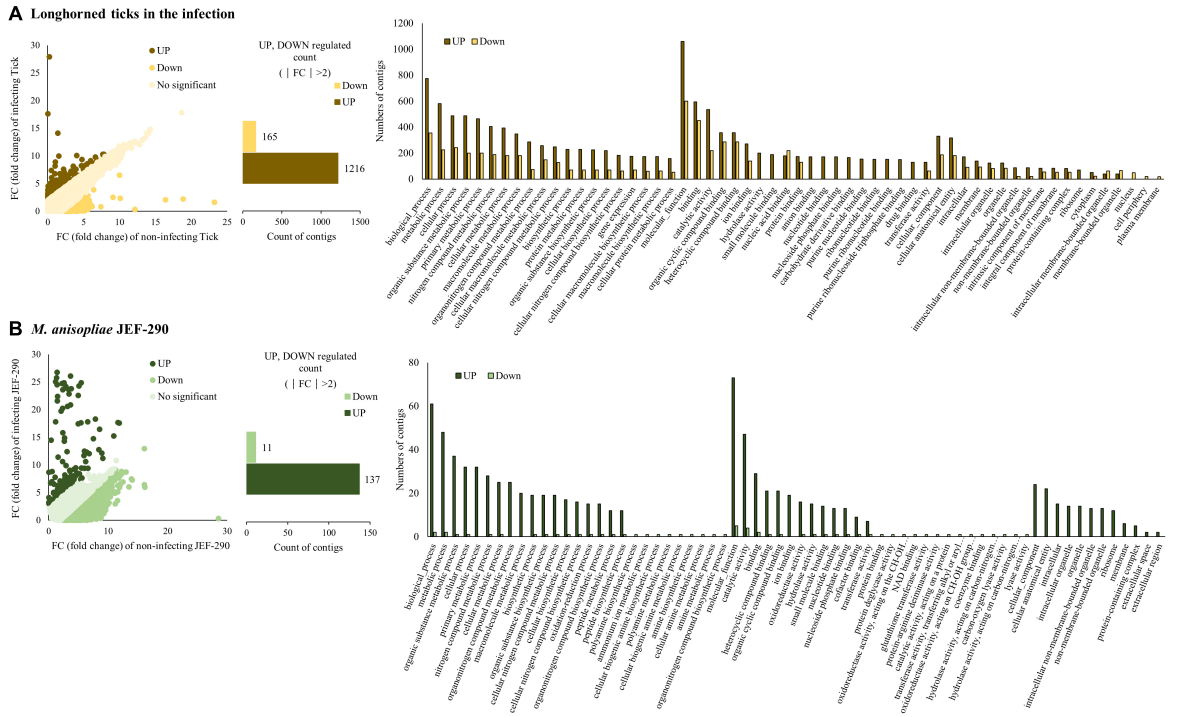

FIGURE 2 | Differentially expressed genes (DEG) of **(A)** longhorned tick and **(B)** *M. anisopliae* JEF-290 at early stage of infection. Longhorned ticks were exposed to 14 days old fungus cultured plates for 14 days. As a non-infecting control, 5 days cultured fungus was used for RNA extraction. In each isolate, the numbers of up- and down-regulated contigs (|fold change| > 2) were analyzed and GO *analyses* of DEGs were conducted using Blast2Go program.

(36%) and cellular component (12%). The up-regulated GO terms were mainly metabolic process and binding. The contigs annotated as being involved in organic substance metabolic process (GO:0071704), nitrogen compound metabolic process (GO:0006807), macromolecule metabolic process (GO:0043170), cellular metabolic process (GO:0044237), biosynthetic process (GO:0009058), oxidoreductase activity (GO:0016491), hydrolase activity (GO:0016787), transferase activity (GO:0016740), cellular anatomical entity (GO:0110165), and intracellular organelle (GO:0043229) were up-regulated noteworthily. The down-regulated fungal GO terms (FC < 2) included 11 genes that included biological process (43%) and molecular function (57%).

Immune-Related Upregulated Longhorned Tick Genes

Mapping of the longhorned tick contigs to a local immune database from ImmunoDB revealed that three serpin genes were up-regulated at the early stage of fungal infection (**Table 2**). Other immune-related genes did not show any significant up- and down-regulations. From the mapping, two uncharacterized genes (Infect_tick_13855 and Infect_tick_25741) and three serpin genes (Infect_tick_1165, Infect_tick_1329 and Infect_tick_13414) were upregulated when the tick was infected by *M. anisopliae* JEF-290. Other immune-related genes were mapped to the database, but most of the genes were not strongly affected by the fungal infection (|FC| < 1): *D. melanogaster cactus*, *pelle* and *caspar* genes, and *peroxidasin*, *thioredoxin peroxidase*, and other serpin genes. *Serpin 28DC, 55B,* and *43Ab* genes were upregulated, but the other mapped serpin genes (*serpin 27A, 28F, 28B, 42Dc, 42De, 43Aa, 43Ad, 77Ba,* and *100A*) were not significantly changed.

Gene Ontology Enrichment Analysis

Gene set enrichment analysis of the DEGs showed that the GO terms related to peptide synthesis processes and cellular energy production of longhorned tick were over-represented while those of catabolic processes were over-represented in *M. anisopliae* JEF-290 was working on catabolic processes to degrade metabolites (**Figure 3**). In the infected tick, seven tick pathways and 30 tick GO terms were significantly involved in the fungal infection (**Figure 3A**). The major up-regulated GO terms were peptide biosynthetic process (GO:0043043), translation (GO:0006412), amide biosynthetic processes (GO:0043604), and peptide metabolic processes (GO:0006518). Up-regulated longhorned tick genes were related to the following pathways: 18 genes were related to carbon metabolism (KEGG:01200), nine genes to glycolysis/gluconeogenesis (KEGG:00010), 14 genes to ribosomes (KEGG:03010), nine genes to biosynthesis of amino acids (KEGG:01230), seven genes to pyruvate metabolism (KEGG:00620), six genes to the citrate cycle (KEGG:00020), and senven genes to glyoxylate/dicarboxylate metabolism (KEGG:00630). In the enriched pathways of infected tick, the RNA-binding translation regulator IRP (FC = 6.0) and enolase (FC = 5.) were highly up-regulated in the TCA

TABLE 2 | Immune-related genes of infected longhorned tick at early stage of infection.

Contig No.	length	Immune group	Description	*e*-value	sim mean (%)	log_2FC value
Infect_tick_20112	2557	IMDPATH	Drosophila melanogaster caspar (casp), transcript variant A, mRNA	3.44E-78	68.42	0.370982
Infect_tick_11706	2488	IMDPATH	Drosophila melanogaster I-kappaB kinase beta (IKKbeta), mRNA	5.91E-36	63.51	0.451297
Infect_tick_11636	3249	IMDPATH	Drosophila melanogaster TAK1-associated binding protein 2 (Tab2), transcript variant A, mRNA	6.65E-12	56.76	−0.12631
Infect_tick_17827	3391	IMDPATH	Drosophila melanogaster TGF-beta activated kinase 1 (Tak1), transcript variant A, mRNA	3.75E-89	81.25	−0.43068
Infect_tick_14945	5055	JAKSTATs	Drosophila melanogaster hopscotch (hop), mRNA	9.36E-43	60.36	−0.59627
Infect_tick_4516	3595	JAKSTATs	Signal-transducer and activator of transcription protein at 92E (Stat92E), transcript variant B, mRNA	4.09E-89	76.25	−0.31486
Infect_tick_11603	4388	LYSs	Uncharacterized protein (CG8492 LYS-long), mRNA	4.22E-38	79.71	0.626852
Infect_tick_13855	615	LYSs	Uncharacterized protein (CG16756), mRNA	6.33E-26	74.36	1.656939
Infect_tick_25189	641	PRDXs	Uncharacterized protein (CG15116), mRNA PRDX9	4.84E-20	62.75	0.40434
Infect_tick_25741	5000	PRDXs	Uncharacterized protein, transcript variant A (CG10211)	6.5E-113	68.94	1.184511
Infect_tick_28125	2086	PRDXs	Transcript variant B (CG4009), mRNA PRDX13	5.64E-47	62.26	0.625927
Infect_tick_10092	5336	PRDXs	Dual oxidase, transcript variant B (Duox), mRNA PRDX20	0	88.49	−0.15924
Infect_tick_19211	5126	PRDXs	Peroxidasin, transcript variant A (Pxn), mRNA PRDX15	0	75.29	0.824367
Infect_tick_34328	1010	PRDXs	Thioredoxin peroxidase 1 (Jafrac1), transcript variant A, mRNA PRDX8	1.4E-105	88.42	0.33423
Infect_tick_18570	1047	PRDXs	Thioredoxin peroxidase 2, transcript variant A (Jafrac2), mRNA PRDX7	1.3E-104	83.84	0.51408
Infect_tick_2240	848	PRDXs	Uncharacterized protein (CG12896), mRNA PRDX5	1.66E-70	68.24	0.699156
Infect_tick_9192	1017	PRDXs	Peroxiredoxin 3 (Prx3), mRNA PRDX6	3.1E-85	78.49	0.423467
Infect_tick_35967	1633	SRPNs	Necrotic (nec), mRNA	2.76E-47	53.99	0.268997
Infect_tick_18887	1195	SRPNs	Serpin 27A, transcript variant A (Spn27A), mRNA	2.68E-46	55.26	0.108819
Infect_tick_22860	1213	SRPNs	Serpin 28F (Spn28F), mRNA	1.03E-46	61.46	0.540716
Infect_tick_28060	1248	SRPNs	Serpin 28B, transcript variant A (Spn28B), mRNA	8.31E-39	52.46	0.080996
Infect_tick_13414	1792	SRPNs	Serpin 28Dc (Spn28Dc), mRNA	5.75E-20	49.18	2.346453
Infect_tick_18886	1621	SRPNs	Serpin 42Dc, transcript variant A (Spn42Dc), mRNA	2.87E-45	66.27	0.233116
Infect_tick_28061	1311	SRPNs	Serpin 42De, transcript variant A (Spn42De), mRNA	3.21E-48	55.38	0.481642
Infect_tick_12140	1294	SRPNs	Serpin 43Aa (Spn43Aa), mRNA	1.49E-43	52.7	0.455747
Infect_tick_1165	1297	SRPNs	Serpin 55B (Spn55B), mRNA	4.82E-38	55.68	1.233754
Infect_tick_1166	1992	SRPNs	Serpin 77Ba, transcript variant A (Spn77Ba), mRNA	1.56E-18	46.32	0.795475
Infect_tick_2329	2038	SRPNs	Serpin 100A (Spn100A), mRNA	1.66E-23	50	0.536968
Infect_tick_13636	1329	SRPNs	Serpin 43Ab (Spn43Ab), mRNA	8.86E-29	52.26	1.765842
Infect_tick_12140	1432	SRPNs	Serpin 43Ad (Spn43Ad), mRNA	4.96E-19	49.34	0.455747
Infect_tick_16761	1893	TOLLPATH	Drosophila melanogaster cactus (cact), transcript variant A, mRNA	2.36E-29	57.02	−0.05294
Infect_tick_33902	1931	TOLLPATH	Drosophila melanogaster pelle (pll), transcript variant A, mRNA	4.42E-62	74.47	−0.30926
Infect_tick_30771	1608	AMP	Drosomycin-like1	5E-31	100	1.56264

cycle and glycolysis pathways, respectively (**Figure 4**). The infected tick also significantly upregulated peptide production and energy production pathways. The 26 GO terms in JEF-290 were significantly involved in the fungal infection (**Figure 3B**). The major up-regulated GO terms were lipid catabolic processes, phospholipid catabolic processes, cellular oxidant detoxification, cellular detoxification, and response to oxidative stress. JEF-290 significantly up-regulated lipid degradation and detoxification against the host defense (**Figure 3B**).

DISCUSSION

Representative Features of *M. anisopliae* JEF-290 Genome

In this RNA-sequencing study, the whole genome of *M. anisopliae* was very useful for fungal gene characterization. The genome size of *M. anisopliae* JEF-290 is 42.8 Mb, which is larger than those of other *Metarhizium* species (32∼39 Mb). JEF-290 was found to contain 99 specific genes those were not found in the

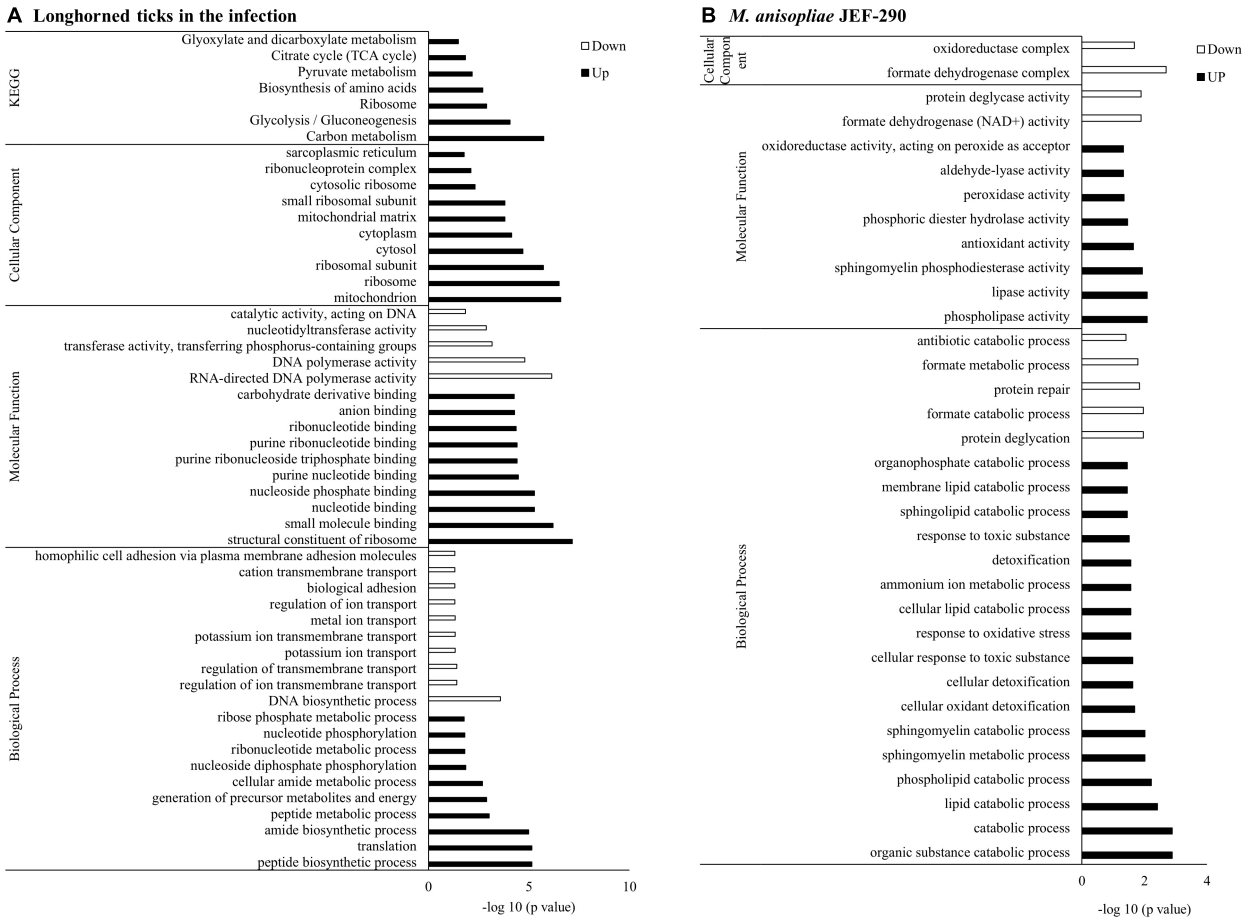

FIGURE 3 | GO enrichment of longhorned tick **(A)** and *M. anisopliae* JEF-290 **(B)** DEGs at early stage of infection. Longhorned tick in the infection and *M. anisopliae* JEF-290 that showed more than ± 2 fold change difference were subjected to GO enrichment analysis using g:Profiler to investigate significant functional differences when infecting ticks and *M. anisopliae* JEF-290. The *M. anisopliae* does not provide enough gene IDs for enrichment analysis, so alternatively Beauveria bassiana with much larger gene IDs was used as a reference and the *p*-value was corrected for multiple testing using the FDR procedure with a threshold of 0.05.

other analyzed species. Many JEF-290 genes were shared with other *Metarhizium* species and other genus of entomopathogenic fungi, however, this isolate has some unique genes which were found to be involved in a variety of fungal life activities. Alpha-beta hydrolase is a structure-related enzyme with various catalytic functions (Holmquist, 2000). In general, the alpha-beta hydrolase enzyme is involved in the breakdown of cellular metabolites (Hotelier et al., 2004). The unique ankyrin repeat protein is involved in protein-protein interactions. It exists in most organisms and is involved in cell cycle control, transcriptional regulation, cell signaling, development, differentiation, apoptosis, cellular scaffolding, and plant defense (Li et al., 2006). The ubiquitin-protein ligase in JEF-290 is actively involved in the protein conversion mechanisms that play an important role in the regulation of various cellular functions. Ubiquitin-protein ligase is also involved in activating toll-like receptors (Chuang and Ulevitch, 2004). The ribonuclease H-like (RNHL) superfamily group is involved in nucleic acid-related biological processes including replication, homologous recombination, DNA repair, transposition, and RNA interference (Majorek et al., 2014). Transposase-like proteins are generally known as mobile genetic units and have the potential to be mobilized by stress.

Host and Fungal Gene Expression at the Onset of Infection

In the early infection stage, JEF-290 possibly initiates infection steps while the longhorned tick recognizes the fungal infection, and is attempting to defend against infection. However, once the infected hosts died, the fungus becomes saprophytic. Comparative transcriptome analysis of the early stage of fungal infection may provide more informative interactions between the tick and entomopathogenic fungus, including fungal initiation of infection and host response to the fungal infection. On the other hand, it would not be possible to observe much of an interactive response of ticks against fungal infection in the late infection stage showing more than 90% of host mortality, therefore RNA-sequencing would not provide meaningful information of the interaction.

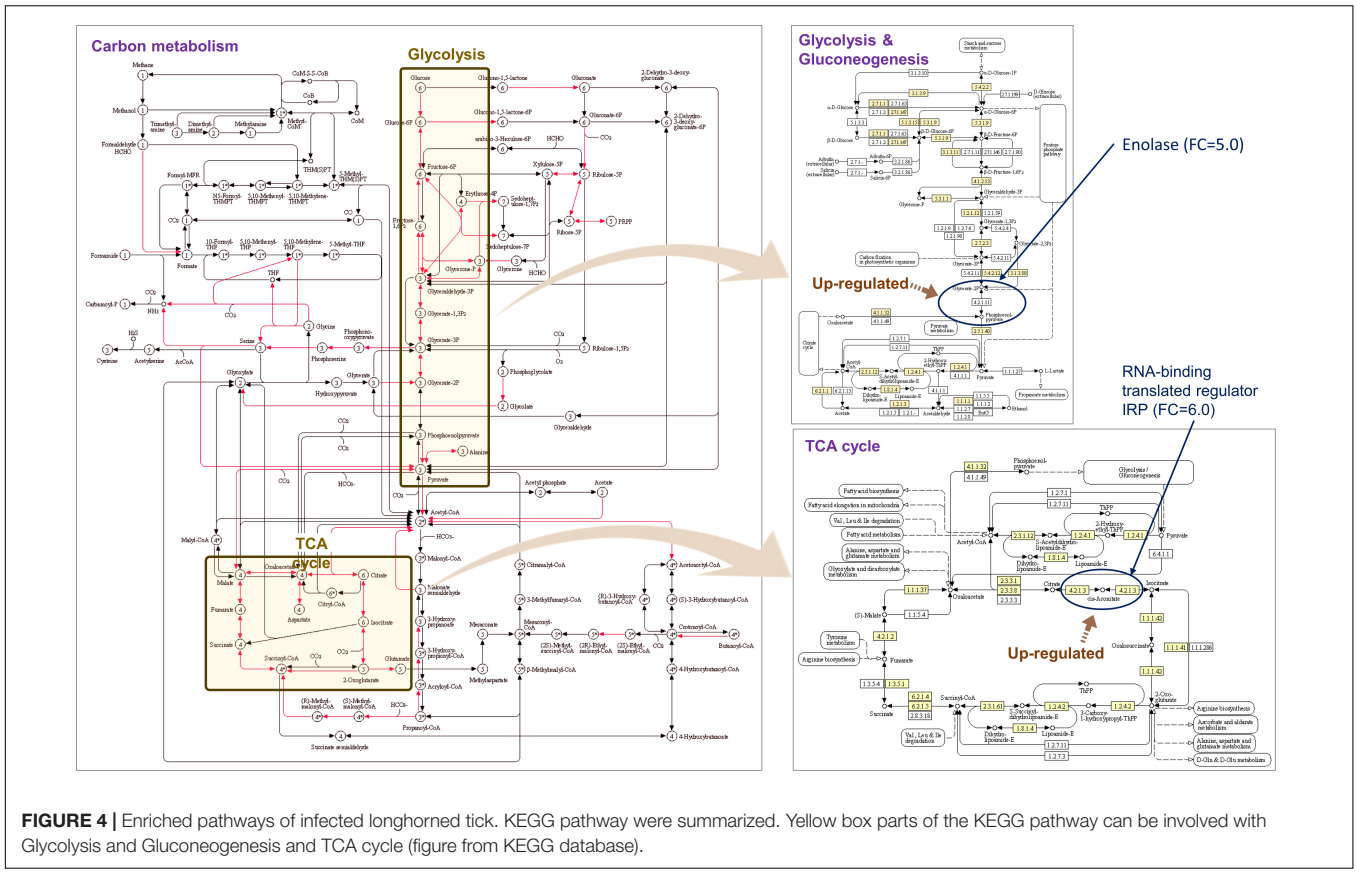

FIGURE 4 | Enriched pathways of infected longhorned tick. KEGG pathway were summarized. Yellow box parts of the KEGG pathway can be involved with Glycolysis and Gluconeogenesis and TCA cycle (figure from KEGG database).

In the analysis of longhorned tick and JEF-290 transcripts, many up-regulated contigs were found in both pathogen and host at the early infection stage. Our results showed that the longhorned tick recognizes JEF-290 infection, and increases the expression of defense and immune related contigs. However, the contigs which are supposed to transcribe very short mRNAs, such as antimicrobial peptide genes were not be able to be analyzed properly because the standard sequencing library preparation protocol does not target such short mRNAs. Therefore the library preparation protocol need to be modified for further study of low molecules. Additionally, cloning and sequencing of the transcripts is indispensable for reliable validation of Illumina sequencing results and further functional study using qPCR.

From the mapping of longhorned tick contigs to the immunoDB, three serpin genes were identified as up-regulated. Serpin is a well-known family of serine protease inhibitors that are typically around 45 kDa. Serpin regulates the arthropod immune reaction through the inhibition of serine protease reactions that initiate the melanization cascade and antimicrobial peptide production (Reichhart et al., 2011; Meekins et al., 2016), therefore the insects, ticks, and mites have serpin genes in their genomes (Mulenga et al., 2009; Rodriguez-Valle et al., 2015; Rider et al., 2015). Forty five serpin genes were found in the blacklegged tick *Ixodes scapularis*, 22 in the cattle tick Rhipicephalus microplus, and only 10 in the scabies mite Sarcoptes scabiei. Some immune-responsive serpins function at the early stage of microbial infection, and are highly up-regulated in the response to microbial infection or physical injury (Meekins et al., 2016). Several studies on arthropod serpins have been reported, but still an interactive response between arthropods and entomopathogenic fungi did not fully explain the up- and down-regulation of serpin gene-expression.

Infection-Specific Gene Expressions of the Fungus and Tick

In the gene set enrichment analysis of longhorned tick, pathways related to protein synthesis and energy production such as translation, peptide metabolism, ribonucleotide metabolism, energy production process including ATP generation, and ADP metabolism were up-regulated. These results suggest that the longhorned tick actively secreted protective substances against the invasion of JEF-290. Of the several pathways obtained from the enrichment analysis of longhorned tick, the RNA-binding translational regulator IRP gene was significantly involved in the management of fungal challenge in most of the significant pathways. When the longhorned tick was infected by the fungus, this gene was highly up-regulated (FC = 6.0). RNA binding proteins (RBP) regulate the translation of mRNA by interacting with 5' and 3' untranslated regions (UTR) of mRNA (Harvey et al., 2018). RBP is involved in the assembly of mRNA to the ribosomes and regulates protein synthesis or alternatively suppresses translation (Moore and Lindern, 2018). Among the

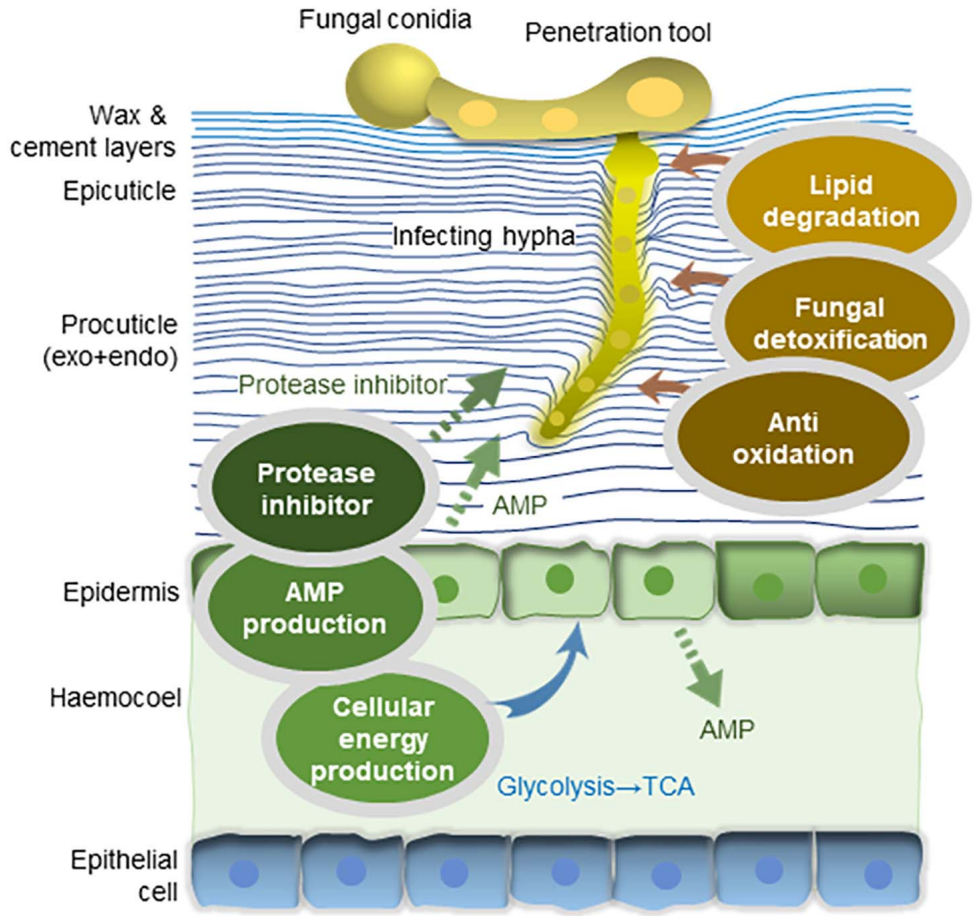

FIGURE 5 | Interaction of *M. anisopliae* JEF-290 and longhorned tick at early stage of fungal infection. Genes from the longhorned tick and JEF-290 fungus were mostly up-regulated at the early stage of fungal infection. Our results indicate that JEF-290 initiates infection of the longhorned tick by degrading host cuticles with a detoxification tool, while the longhorned tick was actively defending against the fungal attack by producing a large amount of energy by inducing catabolism processes (original illustration).

RBPs, iron regulatory protein (IRP) binds to RNA stem-loops of UTR to manipulate mRNA translation (Zhang et al., 2002) and maintains homeostasis of iron concentrations in mammalians including in response to blood-sucking ticks (Anderson et al., 2012). IRP regulates not only iron homeostasis-related mRNAs, but also other mRNAs such as mitochondrial acotinase (ACO2) mRNA, which has an iron responsive element (IRE) and encodes a protein involved in the tricarboxylic acid cycle (TCA) catalyzing citrate to isocitrate (Wang and Pantopoulos, 2011). Acotinase has a 4Fe-4S iron-sulfur cluster as an IRE that directly interacts with the IRP. We speculate that when longhorned tick is infected by JEF-290, IRP possibly binds to the IRE of acotinase mRNA of tick for gene regulation, however, clear evidence remains to be demonstrated.

In the gene set enrichment analysis of JEF-290, fungal pathways such as catabolic processes including lipid, phospholipid, sphingomycelin, and sphingolipid pathways, and detoxification processes including response to oxidative stress and toxic substances were to be up-regulated. We speculate that JEF-290 easily degrades insect cuticles during penetration of the host insect body. In addition, degrading host defense substances possibly makes the fungal invasion much easier. In entomopathogenic fungi, the cytochrome P450 (CYP450) gene encodes a protein that is involved in the degradation of lipid and wax layers of arthropods (Shin et al., 2020). In RNA-sequencing of western flower thrips infected with B. bassiana ERL836 or JEF-007, two different CYP genes (CYP539B1 and CYP655C1) were identified in the infecting fungal isolates (Kim et al., 2020). CYP52 and CYP53 degrade insect lipid layers and assimilate cuticular hydrocarbons (Huarte-Bonnet et al., 2018). In fungal detoxification, most substrate-degrading fungi contain a detoxification system, named the xenomic network, which includes the cytochrome P450 (CYP450) family (Phase I) and glutathione transferase (GST) family (Phase II) (Schrand et al., 2010; Meux et al., 2011). These proteins are involved in the modification of exogenous compounds through oxidative reaction and conjugation, respectively. As discussed above, entomopathogenic fungi produce various CYPs, and they are involved in both fatty acid degradation and fungal detoxification against the arthropod immune response perhaps to work as a

cuticle penetrator and defender against arthropods. More studies need to be conducted to explore this potential mechanism.

In summary, genes from the longhorned tick and JEF-290 fungus were mostly up-regulated at the early stage of fungal infection. Our results indicated that JEF-290 initiates infection of the longhorned tick by degrading host cuticles with a detoxification tool while the longhorned tick was actively defending against the fungal attack by producing a large amount of energy by inducing catabolism processes (**Figure 5**). These findings may provide a strong background to understand the early stage of fungal infection of the longhorned tick. Comparative transcriptome analysis needs to be combined with genetic variation study for better understanding of the tick and fungus interaction, and searching for virulence-related marker genes of both pathogen and host.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ML designed this work and analyzed RNA-sequencing raw data. JK and WK analyzed the RNA-sequencing data. ML and SP extracted DNA and RNA from the samples. D-HL collected longhorned tick from fields and identified. JK designed the whole experiments and wrote the manuscript. All authors approved the submission of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.643389/full#supplementary-material

Supplementary Figure 1 | Reciprocal analysis of *M. anisopliae* JEF-290 and other entomopathogenic fungi.

Supplementary Figure 2 | Validation of RNA-sequencing data using qRT-PCR.

Supplementary Table 1 | Raw data and assembly of *M. anisopliae* JEF-290 contigs for whole genome prediction. The sequencing of whole genomes using Pac Bio RSII technology with error correction.

Supplementary Table 2 | Summary of the longhorned tick, *M. anisopliae* JEF-290 and infected longhorned tick *in silico* cDNA library.

Supplementary Table 3 | TPM values and fold changes of JEF-290 and longhorned tick contigs.

Supplementary Table 4 | Primers used in qRT-PCR for validation of RNA-sequencing.

REFERENCES

Agwunobi, D. O., Pei, T., Yang, J., Wang, X., Lv, L., Shen, R., et al. (2020). Expression profiles of glutathione S-transferases genes in semi-engorged Haemaphysalis longicornis (Acari: Ixodidae) exposed to Cymbopogon citratus essential oil. *Syst. Appl. Acarol.* 25, 918–930. doi: 10.11158/saa.25.5.12

Anderson, C. P., Shen, M., Eisenstein, R. S., and Leibold, E. A. (2012). Mammalian iron metabolism and its control by iron regulatory proteins. *Biochim. Biophys. Acta* 1823, 1468–1483. doi: 10.1016/j.bbamcr.2012.05.010

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]*. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Araujo, R. N., Silva, N. C., Mendes-Sousa, A., Paim, R., Costa, G. C., Dias, L. R., et al. (2019). RNA-seq analysis of the salivary glands and midgut of the Argasid tick Ornithodoros rostratus. *Sci. Rep.* 9, 1–13. doi: 10.1038/s41598-019-42899-z

Charnley, A. K., and Collins, S. A. (2007). 10 Entomopathogenic Fungi and Their Role in Pest Control. *Environ. Microb. Relationsh.* 4:159. doi: 10.1007/978-3-540-71840-6_10

Charrier, N. P., Couton, M., Voordouw, M. J., Rais, O., Durand-Hermouet, A., Hervet, C., et al. (2018). Whole body transcriptomes and new insights into the biology of the tick Ixodes ricinus. *Parasit. Vectors* 11:364. doi: 10.1186/s13071-018-2932-3

Chuang, T. H., and Ulevitch, R. J. (2004). Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat. Immunol.* 5, 495–502. doi: 10.1038/ni1066

Denic, S., Janbeih, J., Nair, S., Conca, W., Tariq, W. U., and Al-Salam, S. (2011). Acute thrombocytopenia, leucopenia, and multiorgan dysfunction: the first case of SFTS Bunyavirus outside China? *Case Rep. Infect. Dis.* 2011:204056. doi: 10.1155/2011/204056

Fang, Y., Park, M. G., Choi, J. Y., Park, D. H., Wang, M., Kim, H. J., et al. (2020). Insecticidal and synergistic activity of dsRNAs targeting buprofezin−specific genes against the small brown planthopper, Laodelphax striatellus. *Arch. Insect Biochem. Physiol.* 105:e21739. doi: 10.1002/arch.21739

Fernandes, ÉK., Bittencourt, V. R., and Roberts, D. W. (2012). Perspectives on the potential of entomopathogenic fungi in biological control of ticks. *Exp. Parasitol.* 130, 300–305. doi: 10.1016/j.exppara.2011.11.004

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next generation sequencing data. *Bioinformatics* 28:3150. doi: 10.1093/bioinformatics/bts565

Gai, Z. T., Zhang, Y., Liang, M. F., Jin, C., Zhang, S., Zhu, C. B., et al. (2012). Clinical progress and risk factors for death in severe fever with thrombocytopenia syndrome patients. *J. Infect. Dis.* 206, 1095–1102. doi: 10.1093/infdis/jis472

George, J. E., Pound, J. M., and Davey, R. B. (2004). Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology* 129, S353–S366. doi: 10.1017/S0031182003004682

Hammer, J. F., Emery, D., Bogema, D. R., and Jenkins, C. (2015). Detection of *Theileria orientalis* genotypes in *Haemaphysalis longicornis* ticks from southern Australia. *Parasit. Vectors* 8:229. doi: 10.1186/s13071-015-0839-9

Harvey, E., Rose, K., Eden, J. S., Lo, N., Abeyasuriya, T., Shi, M., et al. (2019). Extensive diversity of RNA viruses in Australian ticks. *J. Virol.* 93:18. doi: 10.1128/JVI.01358-18

Harvey, R. F., Smith, T. S., Mulroney, T., Queiroz, R. M. L., Pizzinga, M., Dezi, V., et al. (2018). Trans-acting translational regulatory RNA binding proteins. *Wiley Interdiscip. Rev. RNA* 9:e1465. doi: 10.1002/wrna.1465

Heath, A. (2016). Biology, ecology and distribution of the tick, *Haemaphysalis longicornis* Neumann (Acari: Ixodidae) in New Zealand. *N Z. Vet. J.* 64, 10–20. doi: 10.1080/00480169.2015.1035769

Hernandez, E. P., Kusakisako, K., Talactac, M. R., Galay, R. L., Hatta, T., Fujisaki, K., et al. (2018). Glutathione S-transferases play a role in the detoxification of flumethrin and chlorpyrifos in Haemaphysalis longicornis. *Parasit. Vectors* 11, 1–14. doi: 10.1186/s13071-018-3044-9

Holmquist, M. (2000). Alpha/beta-hydrolase fold enzymes structures, functions and mechanisms. *Curr. Protein Pept. Sci.* 1, 209–235. doi: 10.2174/1389203003381405

Hotelier, T., Renault, L., Cousin, X., Negre, V., Marchot, P., and Chatonnet, A. (2004). ESTHER, the database of the α/β−hydrolase fold superfamily of proteins. *Nucleic Acids Res.* 32, 145–147. doi: 10.1093/nar/gkh141

Huarte-Bonnet, C., Kumar, S., Saparrat, M. C., Girotti, J. R., Santana, M., Hallsworth, J. E., et al. (2018). Insights into hydrocarbon assimilation by Eurotialean and Hypocrealean Fungi: roles for CYP52 and CYP53 clans of cytochrome P450 genes. *Appl. Biochem. Biotechnol.* 184, 1047–1060. doi: 10.1007/s12010-017-2608-z

Kim, S., Kim, J. C., Lee, S. J., Lee, M. R., Park, S. E., Li, D., et al. (2020). *Beauveria bassiana* ERL836 and JEF-007 with similar virulence show different gene expression when interacting with cuticles of western flower thrips, Frankniella occidentalis. *BMC Genomics* 21:1–12. doi: 10.1186/s12864-020-07253-y

Kirkland, B. H., Westwood, G. S., and Keyhani, N. O. (2004). Pathogenicity of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to Ixodidae tick species *Dermacentor variabilis, Rhipicephalus sanguineus*, and *Ixodes scapularis*. *J. Med. Entomol.* 41, 705–711. doi: 10.1603/0022-2585-41.4.705

Lee, D. W., Chang, K. S., Kim, M. J., Ahn, Y. J., Jo, H. C., and Kim, S. I. (2015). Acaricidal activity of commercialized insecticides against Haemaphysalis longicornis (Acari: Ixodidae) nymphs. *J. Asia Pacific Entomol.* 18, 715–718. doi: 10.1016/j.aspen.2015.09.004

Lee, M. R., Li, D., Lee, S. J., Kim, J. C., Kim, S., Park, S. E., et al. (2019). Use of Metarhizum aniopliae s.l. to control soil-dwelling longhorned tick, Haemaphysalis longicornis. *J. Invertebr. Pathol.* 166:107230. doi: 10.1016/j.jip.2019.107230

Li, J., Mahajan, A., and Tsai, M. D. (2006). Ankyrin repeat: a unique motif mediating protein- protein interactions. *Biochemistry* 45, 15168–15178. doi: 10.1021/bi062188q

Liu, Y., Li, Q., Hu, W., Wu, J., Wang, Y., Mei, L., et al. (2012). Person-to-person transmission of severe fever with thrombocytopenia syndrome virus. *Vector Borne Zoonotic Dis.* 12, 156–160. doi: 10.1089/vbz.2011.0758

Mahara, F. (1997). Japanese spotted fever: report of 31 cases and review of the literature. *Emerg. Infect. Dis.* 3, 105–111. doi: 10.3201/eid0302.970203

Majorek, K. A., Dunin-Horkawicz, S., Steczkiewicz, K., Muszewska, A., Nowotny, M., Ginalski, K., et al. (2014). The RNase H-like superfamily: new members, comparative structural analysis and evolutionary classification. *Nucleic Acids Res.* 42, 4160–4179. doi: 10.1093/nar/gkt1414

McMullan, L. K., Folk, S. M., Kelly, A. J., MacNeil, A., Goldsmith, C. S., Metcalfe, M. G., et al. (2012). A new phlebovirus associated with severe febrile illness in Missouri. *N. Engl. J. Med.* 367, 834–841. doi: 10.1056/NEJMoa1203378

Meekins, D. A., Kanost, M. R., and Michel, K. (2016). Serpins in arthropod biology. *Semin. Cell Dev. Biol.* 62, 105–119. doi: 10.1016/j.semcdb.2016.09.001

Meux, E., Prosper, P., Ngadin, A., Didierjean, C., Morel, M., Dumarçay, S., et al. (2011). Glutathione Transferases of Phanerochaete chrysosporium S-glutathionyl-p-hydroquinone reductase belongs to a new structural class. *J. Biol. Chem.* 286, 9162–9173. doi: 10.1074/jbc.M110.194548

Miles, V. I. (1968). A carbon dioxide bait trap for collecting ticks and fleas from animal burrows. *J. Med. Entomol.* 5, 491–495. doi: 10.1093/jmedent/5.4.491

Moore, K., and Lindern, M. V. (2018). RNA Binding Proteins and Regulation of mRNA Translation in Erythropoiesis. *Front. Physiol.* 9:910. doi: 10.3389/fphys.2018.00910

Mulenga, A., Khumthong, R., and Chalaire, K. C. (2009). *Ixodes scapularis* tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analysis. *BMC Genomics* 10:217. doi: 10.1186/1471-2164-10-217

Nwanade, C. F., Yang, X., Bao, R., Yu, Z., and Liu, J. (2020). A case of rapid predation of the tick Haemaphysalis longicornis (Acari: Ixodidae) by antlion Euroleon coreaus. *Int. J. Acarol.* 46, 1–2. doi: 10.1080/01647954.2020.1765868

Oliver, J. H. Jr. (1977). Cytogenetics of mites and ticks. *Annu. Rev. Entomol.* 22, 407–429. doi: 10.1146/annurev.en.22.010177.002203

Patel, R. K., and Jain, M. (2012). NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PLoS One* 7:e30619. doi: 10.1371/journal.pone.0030619

Perinotto, W. M. S., Angelo, I. C., Golo, P. S., Quinelato, S., Camargo, M. G., Sá, F. A., et al. (2012). Susceptibility of different populations of ticks to entomopathogenic fungi. *Exp. Parasitol.* 130, 257–260. doi: 10.1016/j.exppara.2011.12.003

Pettersson, J. H. O., Shi, M., Bohlin, J., Eldholm, V., Brynildsrud, O. B., Paulsen, K. M., et al. (2017). Characterizing the virome of Ixodes ricinus ticks from northern Europe. *Sci. Rep.* 7, 1–7. doi: 10.1038/s41598-017-11439-y

Quadros, D. G., Johnson, T. L., Whitney, T. R., Oliver, J. D., and Oliva Chávez, A. S. (2020). Plant-Derived Natural Compounds for Tick Pest Control in Livestock and Wildlife: Pragmatism or Utopia? *Insects* 11:490. doi: 10.3390/insects11080490

Reichhart, J. M., Gubb, D., and Leclerc, V. (2011). The Drosophila serpins: multiple functions in immunity and morphogenesis. *Methods Enzymol.* 499, 205–225. doi: 10.1016/B978-0-12-386471-0.00011-0

Rider, S. D. Jr., Morgan, M. S., and Arlian, L. G. (2015). Draft genome of the scabies mite. *Parasit. Vectors* 8, 1–14. doi: 10.1186/s13071-015-1198-2

Rodriguez-Valle, M., Xu, T., Kurscheid, S., and Lew-Tabor, A. E. (2015). Rhipicephalus microplus serine protease inhibitor family: annotation, expression and functional characterisation assessment. *Parasit. Vectors* 8, 1–9. doi: 10.1186/s13071-014-0605-4

Schrand, A. M., Rahman, M. F., Hussain, S. M., Schlager, J. J., Smith, D. A., and Syed, A. F. (2010). Metal−based nanoparticles and their toxicity assessment. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2, 544–568. doi: 10.1002/wnan.103

Shin, T. Y., Lee, M. R., Park, S. E., Lee, S. J., Kim, W. J., and Kim, J. S. (2020). Pathogenesis−related genes of entomopathogenic fungi. *Arch. Insect Biochem. Physiol.* 105:e21747. doi: 10.1002/arch.21747

Vayssier-Taussat, M., Moutailler, S., Michelet, L., Devillers, E., Bonnet, S., Cheval, J., et al. (2013). Next generation sequencing uncovers unexpected bacterial pathogens in ticks in western Europe. *PLoS One* 8:e81439. doi: 10.1371/journal.pone.0081439

Wang, J., and Pantopoulos, K. (2011). Regulation of cellular iron metabolism. *Biochem. J.* 434, 365–381. doi: 10.1042/BJ20101825

Yamaguti, N., Tipton, V. J., Keegan, H. L., and Toshioka, S. (1971). Ticks of Japan, Korea, and the Ryukyu islands. *Biol. Ser.* 15, 8–1971.

Zhang, D., Dimopoulos, G., Wolf, A., Minana, B., Kafatos, F. C., and Winzerling, J. J. (2002). Cloning and molecular characterization of two mosquito iron regulatory proteins. *Insect. Biochem. Mol. Biol.* 32, 579–589. doi: 10.1016/s0965-1748(01)00138-2

Zhendong, H., Guangfu, Y., Zhong, Z., and Ruiling, Z. (2019). Phylogenetic relationships and effectiveness of four *Beauveria bassiana* sensu lato strains for control of Haemaphysalis longicornis (Acari: Ixodidae). *Exp. Appl. Acarol.* 77, 83–92. doi: 10.1007/s10493-018-0329-9

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ORIGINAL RESEARCH
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The Msn2 Transcription Factor Regulates Acaricidal Virulence in the Fungal Pathogen *Beauveria bassiana*

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Beauveria bassiana holds promise as a feasible biological control agent for tick control. The *B. bassiana* stress–response transcription factor Msn2 is known to contribute to fungal growth, conidiogenesis, stress–response and virulence towards insects; however, little is known concerning whether Msn2 is involved in infection across Arthropoda classes. We evaluated the effects of Msn2 on *B. bassiana* virulence against *Rhipicephalus microplus* (Acari, Ixodidae) using wild-type, targeted gene knockout (*ΔBbmsn2*) and complemented mutant (*ΔBbmsn2/Bbmsn2*) strains. Reproductive parameters of *R. microplus* engorged females treated topically or by an intra-hemocoel injection of conidial suspensions were assessed. Treated cuticles of engorged females were analyzed by microscopy, and proteolytic activity of *B. bassiana* on cuticles was assessed. Topically treated engorged females showed high mean larval hatching (>84%) in control and *ΔBbmsn2* treatments, whereas treatment with the wild-type or *ΔBbmsn2/Bbmsn2* strains resulted in significantly decreased (lowered egg viability) larval hatching. Percent control of *R. microplus* topically treated with *ΔBbmsn2* was lower than in the groups treated with wild-type (56.1%) or *ΔBbmsn2/Bbmsn2* strains. However, no differences on reproductive parameters were detected when *R. microplus* were treated by intra-hemocoel injection using low (800 conidia/tick) doses for all strains tested; *R. microplus* injected with high doses of wild-type or mutant strains (10^6 conidia/tick) died before laying eggs (~48 h after treatment). SEM analyses of *B. bassiana* infection showed similar conidial germination and formation of pseudo-appressoria on tick cuticle. Histological sections of ticks treated with the wild-type or *ΔBbmsn2/Bbmsn2* strains showed fungal penetration through the cuticle, and into the tick interior. Hyphae of *ΔBbmsn2*, however, did not appear to penetrate or breach the tick exocuticle 120 h after treatment. Protease activity was lower on tick cuticles treated with *ΔBbmsn2* than those treated with the wild-type or *ΔBbmsn2/Bbmsn2* strains. These data show that loss of the Msn2 transcription factor reduced *B. bassiana* virulence against *R. microplus*, but did not interfere with conidial germination, appressoria formation or sporulation on tick cadavers, and plays only a minimal role once the cuticle is breached. Our results indicate that the BbMsn2 transcription factor acts mainly during the fungal penetration process

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and that decreased protease production may be one mechanism that contributes to the inability of the mutant strain to breach the tick cuticle.

Keywords: tick, biological control, entomopathogenic fungi, cuticle, virulence, *Beauveria bassiana*, *Rhipicephalus microplus*, Msn2

INTRODUCTION

Beauveria bassiana (Hypocreales: Cordycipitaceae) is one of the most widely studied entomopathogenic fungi for applied tick control (Kirkland et al., 2004a; Kirkland et al., 2004b; Fernandes et al., 2012). The potential of this fungus to control *Rhipicephalus microplus* (Acari: Ixodidae) has been shown in laboratory assays, with variable virulence among *B. bassiana* isolates (Posadas and Lecuona, 2009; Campos et al., 2010; Fernandes et al., 2011; Sun et al., 2013). According to Fernandes et al. (2011), the mean lethal concentration of *B. bassiana* to kill 50% (LC_{50}) of *R. microplus* engorged female can vary from 10^7 to 10^9 conidia ml^{-1}.

B. bassiana conidia infect ticks through their cuticle or natural openings (Bernardo et al., 2018). The process of fungal infection on ticks is thought to be similar to that known for insects (Arruda et al., 2005), and includes conidial adhesion on the host cuticle, production of germ tube, differentiation into appressorium (something not seen for all *B. bassiana* isolates), penetration through the host cuticle by enzymatic action (e.g., lipases, proteases and chitinases) and mechanical pressure, and growth within the host integument and hemocoel (colonization stage) (Ortiz-Urquiza and Keyhani, 2016). The host dies by tissue destruction and by action of toxins from fungi (Schrank and Vainstein, 2010). However, ticks are known to potentially display significantly higher natural resistances to insect pathogenic fungi, and acaricidal specific factors may be produced by these fungi (Kirkland et al., 2004a, Kirkland et al., 2004b, Kirkland et al., 2005).

As mentioned, the virulence of *B. bassiana* is influenced by the production of proteases, particularly the subtilisin-like protease called Pr1 (Joshi et al., 1995), chitinases and lipases (Fang et al., 2005) and, specifically against ticks, the metabolite oxalic acid has been shown to be important (Kirkland et al., 2005). In the last fifteen years, many studies have contributed to understanding the network of genes which are related to the virulence of *B. bassiana* (Jin et al., 2010; Ortiz-Urquiza and Keyhani, 2013; Wang et al., 2013; Valero-Jiménez et al., 2016). Fang et al. (2005) demonstrated that overproduction of the *Bbchit1* chitinase enhanced the virulence of *B. bassiana* against aphids, as indicated by the significantly lower LC_{50} and mean lethal time to kill 50% (LT_{50}) of target insects of the mutant strain compared to the wild-type strain. Another study using hybrid chitinase gene (Bb*chit1*–Bm*ChBD*) showed a 23% reduction of the LT_{50} to kill aphids (*Myzus persicae*) when they were treated with the mutant strain of *B. bassiana* in comparison to its wild-type (Fan et al., 2007).

Transcriptional regulation of effector genes in eukaryotic cells is one of the fundamental mechanisms involved in cellular responses to stress and/or virulence signaling pathways (Ortiz-Urquiza and Keyhani, 2015). In entomopathogenic fungi, the transcription factor Msn2 regulates the conidiogenesis of *B. bassiana* and *Metarhizium robertsii*; a knockout strain of each species (*ΔBbmsn2* and *ΔMrmsn2*, respectively) reduced 43 and 39% the conidial production in comparison to their respective wild-type strains (Liu et al., 2013). Also, *ΔBbmsn2* and *ΔMrmsn2* strains had a reduced cell tolerance to chemical and environmental stresses. Decreased virulence of *ΔBbmsn2* and *ΔMrmsn2* against *Spodoptera litura* (Lepidoptera: Noctuidae) second-instar larvae and *Tenebrio molitor* (Coleoptera: Tenebrionidae) third-instar larvae was recorded; the LT_{50} values were 28 and 25% longer than the control strains of *B. bassiana* (5.5 days) and *M. robertsii* (4.8 days), respectively. In *Galleria mellonella* (Lepidoptera: Pyralidae) larvae, the LT_{50} significantly increased when treated with the knockout strain (LT_{50} = 3.4 ± 0.08 days) in comparison to the wild-type strain (2.71 ± 0.03 days) (Luo et al., 2015). These data confirmed that Msn2 is critical for entomopathogenic fungal infection of insects; however, any similar role towards Acari has not been investigated. Furthermore, to date, no histological investigations related to *Msn2* on infection or effects on reproduction have been performed on insects or ticks. Overall, little is known about the molecular basis of virulence in *B. bassiana* towards ticks. Here, we examined the consequences of loss of *Msn2* on *B. bassiana* infection towards ticks combining genetic characterization with enzymatic and histological approaches. Our data indicate *BbMsn2* is critical for penetration but not subsequent growth and proliferation once the tick cuticle has been breached. Furthermore, important effects were seen with respect to lowered female fertility, indicating potential added benefits in the application of *B. bassiana* for tick control.

MATERIAL AND METHODS

Beauveria bassiana Strains, Conidial Suspensions, and Viability

The strains of *B. bassiana* knockout *ΔBbmsn2* and complemented (*ΔBbmsn2*/*Bbmsn2*) were constructed and initially characterized by Luo et al. (2015). *ΔBbmsn2* and the complemented strain were obtained from *B. bassiana* Bb0062. *Beauveria bassiana* strains were cultivated on potato dextrose agar (PDA, Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L^{-1} yeast extract (Bacto™ Yeast Extract, Sparks, MD, USA) (PDAY) in Petri plates (90 × 15 mm) and incubated in the dark for 15 days at 26 ± 1°C and relative humidity (RH) ≥90%. Temperature and relative humidity in the incubator were monitored with a data logger HOBO H8® (Onset Computer Corporation, Bourne, MA, USA). Fresh conidia from each strain were harvested using a spatula, suspended in 0.01% (v/v) Tween 80® (Labsynth Prod.

Lab. Ltda, Diadema, SP, Brazil) and filtered through cheesecloth to remove mycelia. Conidial suspensions were quantified in hemocytometer at 400× magnification in a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany), and the concentration was adjusted to 2.0×10^8 conidia ml^{-1} or as indicated. To assess conidial viability, 20 µl of each conidial suspension were inoculated onto 8 ml of PDAY medium supplemented with 0.002% (w/v) Benomyl (50% active ingredient; Benlate®, DuPont, São Paulo, SP, Brazil) (Braga et al., 2001) and 0.05% (w/v) chloramphenicol (Sigma-Aldrich, Steinheim, Germany) in Petri plates (35 × 9 ×10 mm). The plates were incubated at 26 ± 1°C for 48 h. After incubation, a drop of lactophenol and cotton blue solution and coverslip were applied over the inoculum; a minimum of 300 conidia per plate was evaluated, and the percent relative germination was determined (Braga et al., 2001). Conidia were considered germinated when the germ tube was longer than the maximum conidial diameter. Conidia were used only if viability was assessed to be >98% in all experiments.

Fungal Bioassays Using *Rhipicephalus microplus* and Measurement of Tick Reproductive Parameters

Rhipicephalus microplus engorged females were collected from artificially infested cattle at Universidade Federal de Goiás (UFG, Goiânia, Brazil). In the laboratory, ticks were washed in tap water, immersed in 1% (v/v) hypochlorite for 1 min, rinsed in sterile distilled water for 1 min and dried with sterile paper towels. The females were homogeneously distributed by weight (160-315 mg) into four treatment groups: control, *BbWT*, *ΔBbmsn2/Bbmsn2* and *ΔBbmsn2*; each group had 10 individuals.

Two infection protocols were evaluated: (i) topical and (ii) intra-hemocoel injection. For topical assays, ticks were individually immersed in 1 ml of Tween 80® 0.01% (control) or in the conidial suspensions at 2.0×10^8 conidia ml^{-1} for 3 min. In addition, ticks were individually inoculated by intra-hemocoel injection with 5 µl of Tween 80® 0.01% (control), or 5 µl of conidial suspension at 1.6×10^5 conidia ml^{-1} (800 conidia/tick), 1.6×10^6 conidia ml^{-1} (8,000 conidia/tick), 1.6×10^7 conidia ml^{-1} (80,000 conidia/tick) or 2.0×10^8 conidia ml^{-1} (10^6 conidia/tick); injections were performed in the foramen located between the capitulum and the dorsal scutum of engorged females using a stereomicroscope and a 0.3 mm insulin syringe (Angelo et al., 2010). After treatment, ticks were individually placed in each well of 24-well cell culture plates (Corning Brasil Indústria e Comércio Ltda., Suzano, SP, Brazil), incubated at 26 ± 1°C, RH ≥90% and 12 h photophase. The egg mass from each female was collected at the end of oviposition, weighed, transferred to an individual glass tube (16 × 125 mm), closed with a cotton plug, and incubated at 26 ± 1°C and RH ≥90% for assessment of larval hatching. The eggs were observed daily and the larval hatching for each tube was visually estimated through microscopic examination, and values were assigned in percentages ranging from 0 to 100% by intervals of 5% in relation to the total mass of eggs (Drummond et al., 1971; Barreto et al., 2016; Bernardo et al., 2018).

The following reproductive parameters were investigated: estimated reproduction (ER) and percent control (PC) (Drummond et al., 1971). The effectiveness of treatment was measured by the effect on the ER of the engorged females; in this equation, 20,000 is the estimate of the number of larvae that normally hatch from 1 g of eggs of *R. microplus*; therefore, the percent control of ER estimates the treatment efficacy to decrease the tick population in an infested environment. Bioassays were repeated three times on different days, and with new batches of conidia. The ER and PC were calculated by the Equations (1) and (2), respectively.

Equation (1):

$$ER = \frac{weight\ of\ egg\ mass\ (g)}{initial\ weight\ of\ engorged\ female\ (g)} \times percentage\ of\ larval\ hatch \times 20,000$$

Equation (2):

$$PC = \frac{mean\ ER\ of\ the\ control\ group - mean\ ER\ of\ the\ treated\ group}{mean\ ER\ of\ control\ group} \times 100$$

Scanning Electron and Light Microscopy of Tick's Cuticle

Initial steps of the *B. bassiana* infection process were examined by scanning electron microscopy (SEM) and light microscopy. Six *R. microplus* engorged females were individually treated with *BbWT*, *ΔBbmsn2/Bbmsn2* or *ΔBbmsn2* by topical application of 2.5 µl conidial suspension at 2.0×10^8 conidia ml^{-1}. The treated females were incubated at 26 ± 1°C, with RH ≥90% and 12 h photophase, for 48 or 120 h. Then, approximately 50 µl of fixative [2% (v/v) glutaraldehyde (Impex, Labimpex Ind. Com. de Prods. Lab. Ltda., Diadema, SP, Brazil), 2% (v/v) paraformaldehyde (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil), 3% (w/v) sucrose (Sigma-Aldrich, Steinheim, Germany) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, Steinheim, Germany), pH 7.2] was injected into each female using an insulin syringe according to Barreto et al. (2016). Each female was placed in a 15-ml centrifuge tube containing 2 ml of fixative and maintained for 10 days at 4°C in a refrigerator.

Ticks incubated for 48 h were examined by SEM (n = 3). The dorsal cuticle of females was dissected and removed, and then washed three times (15 min each time) in sodium cacodylate buffer (0.1 M, pH 7.2). Cuticles were dehydrated in a graded series of ethanol solutions (30, 50, 70, 80 and 90%), held for 15 min in each solution and passed twice in 100% ethanol for 15 min. Subsequently, the cuticles were individually placed in micro-centrifuge tubes containing 300 µl of hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA, USA) and maintained immersed for 5 min (Barreto et al., 2016). After drying, the samples proceeded to metallization. Accordingly, the samples were placed on a stub and coated with gold in a sputter-applicator (Denton Vacuum Desk V). The cuticles were analyzed and electro micrographs were obtained with a scanning electron microscope (Jeol JSM 6610) at an accelerating voltage of 20 kV. The images were analyzed qualitatively (conidial adhesion, size of

germinative tubes and presence of appressoria) and quantitatively (number of germinated conidia).

Ticks incubated for 120 h were prepared for histological analyses of their cuticle. After 10 d in fixative, treated ticks (n = 3) were longitudinally cut, dehydrated as described with SEM samples and embedded in resin (Historesin®, Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions. Sections of 4 μm were made in Microtome (Leica Biosystems, Wetzlar, Germany), stained with Periodic Acid-Schiff (PAS) and Green light (Arruda et al., 2005) and assessed by using a light microscope (Nikon E200). Images (n = 5) were captured with a high-definition microscope camera Leica ICC50 HD, with resolution of 1,280 × 720 p (HD ready). The experiments were conducted three times on different days with three replicates in each treatment group.

Protease Assay

Cuticles of *R. microplus* engorged females were dissected (n = 10), washed in distilled water and immersed in 10 ml conidial suspension of the strain *BbWT*, *ΔBbmsn2/Bbmsn2* or *ΔBbmsn2*, at 2.0×10^8 conidia ml^{-1} for 3 min. Cuticles were incubated at 26°C and RH ≥90% for 120 h. After incubation, the pool of cuticles was macerated in 1 ml distilled water and centrifuged at 25,000 RCF for 5 min at 4°C. Protease activity was measured by azocasein hydrolysis as described by Segers et al. (1994) and Phillips et al. (1984) with the following modifications: commercial azocasein (Sigma Chemical Co., St. Louis, MO, USA) was dissolved at 1% (w/v) in 0.1 M Tris–HCl buffer, pH 8.5. Briefly, 1,000 μl of each supernatant sample were incubated with 500 μl de azocasein 1% at 28°C for 60 min. This reaction was stopped by adding 1 ml of 10% TCA and maintaining it for 15 min at 4°C, and followed by centrifugation at 25,000 RCF for 10 min to remove the precipitated protein. Six hundred milliliters of the supernatant were neutralized by adding 700 μl of 1M NaOH, and absorbance at 450 nm was recorded with Enzyme-Linked Immunosorbent Assay (ELISA). One unit of enzyme activity (U) was calculated by the Equation (3):

Equation (3):

$$UA = \frac{\text{control absorbance} - \text{sample absorbance}}{0.001} \times \frac{1}{60}$$

Statistical Analyses

All data sets were previously checked for normality and homoscedasticity with Shapiro–Wilk and Bartlett tests, respectively. Normally distributed data (engorged female initial weight, egg mass weight, and conidial germination) were fitted to a parametric model and then were submitted to ANOVA followed by an SNK test for multiple comparisons. Non-normally distributed data (percentage of larval hatch) were fitted to a non-parametric model and then analyzed by a Kruskal–Wallis test, followed by an FDR test. Protease activity of mutant strains was compared with the *BbWT* strain by applying the Dunnett's test. Analyses were performed in the statistical environment R (R Team C, 2018). *P*-values less than 0.05 were considered as significant.

RESULTS

Measurement of Reproductive Parameters and Virulence Assays

Reproductive parameters of *R. microplus* engorged females treated topically with different strains (*BbWT*, *ΔBbmsn2*, and *ΔBbmsn2/Bbmsn2*) of *B. bassiana* were examined 15 d post-infection after immersion in 2.0×10^8 conidia ml^{-1} as detailed in the *Material and Methods* section (**Table 1**). Because of the difficulty in determining the exact timing of tick death, lethal mortality times could not be accurately determined; however, reproductive parameters that would critically inform on successful biological control efforts were measured. The weight of egg mass from females treated with any of the *B. bassiana* conidial suspensions tested (i.e., either wild-type, mutant, or complemented mutant) was similar and lower (~50%) than that of control untreated ticks ($F_{3,118} = 18.33$; $P < 0.001$). High mean larval hatching (~90%) was observed in the control group, which was slightly reduced after treatment with the *ΔBbmsn2* strain (down to ~84%, $P = 0.006$). However, engorged females treated with *BbWT* or *ΔBbmsn2/Bbmsn2* had significantly decreased larval hatching (10–20%, $\chi^2 = 17.38$; $df = 3$; $P = 0.006$), indicating lower egg viability using the wild-type and complemented *B. bassiana* strains. Overall, the percent control of ticks was higher in the groups treated with *BbWT* (56.1%) or *ΔBbmsn2/Bbmsn2* (58.7%) as compared to untreated engorged females or ticks treated with the *ΔBbmsn2* mutant strain (39.7%).

TABLE 1 | Biological parameters of *Rhipicephalus microplus* engorged females treated by immersion in Tween 80® 0.01% (control) or in conidial suspension (2.0×10^8 conidia ml^{-1}) of *Beauveria bassiana* strains (*BbWT*, *ΔBbmsn2/Bbmsn2* or *ΔBbmsn2*) incubated at 26 ± 1°C and RH ≥ 90%.

Treatment by immersion	Engorged female weight (mg)	Egg mass weight (mg)	Larval hatch (%)	ER†	Percent control (%)‡ (n = 30)
Control	232.1 ± 16[a]	126.3 ± 15[a]	89.2 ± 3.2[a]	968,730.1	–
BbWT	231.2 ± 16[a]	66.4 ± 13[b]	78.5 ± 3.0[bc]	447,094.8	56.1
ΔBbmsn2/Bbmsn2	233.4 ± 14[a]	68.2 ± 15[b]	70.9 ± 4.9[c]	406,020.6	58.7
ΔBbmsn2	232.5 ± 16[a]	77.6 ± 16[b]	84.0 ± 2.2[ab]	554,807.5	39.7

(†) ER: estimated reproduction [ER = weight of egg mass (g)/initial weight of engorged female (g) × percentage of larval hatch × 20,000] (Drummond et al., 1971).

(‡) Percent control [ER of the control group – mean ER of the treated group/mean ER of the control group × 100] (Drummond et al., 1971).

Means are followed by standard errors of 10 replicates per bioassay, in three independent trials. Means followed by the same letter in the same column did not differ significantly (P > 0.05) according to the Student–Newman–Keuls (engorged female weight and egg mass weight) or Kruskal–Wallis test (larval hatch).

Despite being unable to determine exact time of death, all ticks showed eventual mycosis and sporulation of the fungus on the tick cadavers.

For intra-hemocoel injection assays (that would bypass the need to cuticle penetrations), a concentration range of fungal conidia (800, 80,000, 80,000, and 10^6 conidia/tick) was tested (**Table 2**). Engorged females of *R. microplus* treated by intra-hemocoel injection of 800 conidia/tick showed decreased (50–60%) mean egg mass weights for all strains tested ($F_{3,55} = 24.05$; $P < 0.001$) as compared to control uninfected ticks, with only slight differences seen between the wild-type, *ΔBbmsn2*, and *ΔBbmsn2/Bbmsn2* strains (**Table 2**). In addition, a decreased mean oviposition period from 7 to 2 d was seen after injection of 800 conidia/tick of any of the *B. bassiana* strains tested ($F_{3,55} = 27.66$; $P < 0.001$) in comparison to control untreated ticks. Ticks treated by intra-hemocoel injection at concentrations ≥8,000 conidia/tick died before laying their eggs (i.e., within 48 h after treatment), while in control group the oviposition period was 7.9 ± 1 d (**Table 2**), and thus had essentially no oviposition period. No differences in sporulation were observed on the cadaver of ticks between the wild-type, *ΔBbmsn2*, and *ΔBbmsn2/Bbmsn2* strains at 5 d after infection (**Figure 1**).

Infection of *B. bassiana* Strains on Tick Cuticle

The absence of the Msn2 transcription factor did not interfere with conidial germination or adhesion to the tick cuticle. Electron micrographs showed similar ($F_{2,6} = 0.0638$; $P = 0.9388$) conidial germination between the *B. bassiana* strains on the tick cuticle at 48 h incubation: *BbWT* = 84.33 ± 13.2%, *ΔBbmsn2/Bbmsn2* = 82.33 ± 11.20% and *ΔBbmsn2* = 76.33 ± 22.67% (**Figure 2**). Most conidia from *BbWT* (**Figure 3A**), *ΔBbmsn2/Bbmsn2* (**Figures 3B, C**) and *ΔBbmsn2* (**Figure 3D**) appeared to have germinated, and appressoria-like tip structures could be seen.

Conidia from *BbWT* and *ΔBbmsn2/Bbmsn2* were capable of germinating and penetrating through the cuticle (**Figures 4A, C**, respectively). Hyphae of *BbWT* (**Figures 4A, B**) and *ΔBbmsn2/Bbmsn2* were also observed inside the tick body, with fungal development in adjacent tissues (**Figures 4C, D**). However, the hyphae of the *ΔBbmsn2* strain appeared to continue to grow on the surface/exocuticle of the tick with fewer instances of penetrative hypha seen at 120 h incubation as compared to the wild-type and complemented strains (**Figure 4E**).

Protease Activity

The proteolytic activity of the *B. bassiana* strains on *R. microplus* cuticle was measured using azoalbumin as detailed in the *Material and Methods* section. Protease activity was significantly lower in the *ΔBbmsn2* strain (1.91 ± 0.29 U ml^{-1}) than in the *BbWT* (9.88 ± 4.08 U ml^{-1}; $P = 0.0113$) and *ΔBbmsn2/Bbmsn2* strains (8.16 ± 1.59 U ml^{-1}; $P = 0.0150$) (**Figure 5**).

DISCUSSION

Our data indicate that the Msn2 transcription factor significantly contributes to the ability of *B. bassiana* to infect *R. microplus via* the "natural" cuticle-penetration requiring route. Engorged females treated topically with the *ΔBbmsn2* strain showed a significantly decreased percent control of ticks in comparison to the wild-type and complemented strains. However, the mortality of ticks reached 100% when engorged females were injected, thus by-passing the requirement for cuticle penetration, with *ΔBbmsn2* (≥8,000 conidia/tick) conidial suspension by intra-hemocoel injection. Even at the lowest dose injected (800 conidia/tick), reduced reproductive

TABLE 2 | Biological parameters of *Rhipicephalus microplus* engorged females treated by an intra-hemocoel injection with 5 µl of Tween 80® 0.01% (control), or 5 µl of conidial suspension of *Beauveria bassiana* strains (*BbWT*, *ΔBbmsn2/Bbmsn2* and *ΔBbmsn2*) at 1.6 × 10^5 conidia ml^{-1} (800 conidia/tick), 1.6 × 10^6 conidia ml^{-1} (8,000 conidia/tick), 1.6 × 10^7 conidia ml^{-1} (80,000 conidia/tick) or 2.0 × 10^8 conidia ml^{-1} (10^6 conidia/tick), and incubated at 26 ± 1°C and RH ≥ 90%.

Treatment by inoculation	Dosage (conidia/tick)	Engorged female weight (mg)	Egg mass weight (mg)	Oviposition period (days)*
Control	0	249.1 ± 0.6[a]	78.1 ± 19 (n = 30)	7.9 ± 1
BbWT	10^6	249.0 ± 1.1[a]	0 (n = 0)	–
ΔBbmsn2/Bbmsn2	10^6	250.4 ± 1.6[a]	0 (n = 0)	–
ΔBbmsn2	10^6	251.6 ± 0.9[a]	0 (n = 0)	–
Control	0	236.1 ± 6.0[a]	76.4 ± 3.9 (n = 30)	7.9 ± 0.4
BbWT	8 × 10^4	239.3 ± 6.6[a]	0 (n = 0)	–
ΔBbmsn2/Bbmsn2	8 × 10^4	237.5 ± 5.7[a]	0 (n = 0)	–
ΔBbmsn2	8 × 10^4	232.3 ± 6.4[a]	0 (n = 0)	–
Control	0	235.6 ± 6.8[a]	77.8 ± 5 (n = 30)	6.7 ± 0.1
BbWT	8 × 10^3	239.2 ± 7.0[a]	0 (n = 0)	–
ΔBbmsn2/Bbmsn2	8 × 10^3	236.4 ± 7.8[a]	0 (n = 0)	–
ΔBbmsn2	8 × 10^3	237.1 ± 6.6[a]	0 (n = 0)	–
Control	0	237.9 ± 8.2[a]	76.8 ± 5a (n = 30)	7.2 ± 0.5[a]
BbWT	800	237.6 ± 9.1[a]	32.7 ± 6b (n = 7)	2.3 ± 0.1[b]
ΔBbmsn2/Bbmsn2	800	239.6 ± 9.0[a]	11.0 ± 1b (n = 11)	2.0 ± 0.0[b]
ΔBbmsn2	800	238.9 ± 9.0[a]	14.6 ± 5b (n = 12)	2.1 ± 0.05[b]

(*) Mean number of days engorged females laid their eggs.

Means are followed by standard errors of 30 replicates per bioassay. Means followed by the same letter in the same column, and in the same dosage tested, did not differ significantly (P > 0.05) according to the Student–Newman–Keuls test.

FIGURE 1 | Fungal sporulation on *Rhipicephalus microplus* engorged females' cadavers at day 5 post treatment by an intra-hemocoel injection with 5-μl conidial suspension at 2.0×10^8 conidia ml^{-1}.

parameters of engorged females was seen for all of the *B. bassiana* strains tested. In a previous study, *S. litura* and *T. molitor* larvae treated with *ΔBbmsn2* conidia had, respectively, LT_{50} values 28 and 25% higher (decreased virulence) than the complemented strain (Liu et al., 2013). In addition, *G. mellonella* larvae treated topically with *ΔBbmsn2* conidia also had an increased LT_{50} detected; however, no difference in LT_{50} values among the mutant and wild-type strains was observed when *G. mellonella* larvae were treated by injecting conidial suspension into the hemocoel (Luo et al., 2015). These results and our data lead us to conclude that the transcription factor Msn2 acts predominantly during the penetration of *B. bassiana* through the tick cuticle, with retention of the ability of the fungus to evade immune systems once inside the host.

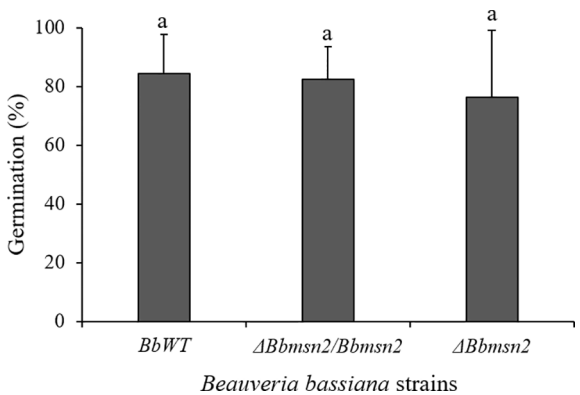

FIGURE 2 | Germination (%) of conidia of *Beauveria bassiana* strains: *BbWT*, *ΔBbmsn2/Bbmsn2* and *ΔBbmsn2* on the cuticle of *Rhipicephalus microplus* engorged females incubated at 26 ± 1°C and RH ≥90% for 48 h. Bars with the same letter do not differ significantly ($P \geq 0.05$) among treatments. Standard errors are based on three independent trials.

The first step to successful infection of entomopathogenic fungi is the adhesion of fungal propagules (conidia or blastospores) on the host cuticle. Accordingly, high fungal virulence is directly related to increased adhesion to the host cuticle (Butt and Goettel, 2000). Adhesion is considered to follow a two-step process involving initial attachment followed by consolidation of adhesion, influenced by the surface characteristics of the fungal cells and the target substrata (Holder et al., 2007). The *B. bassiana* conidial surface is composed of a layer of hydrophobic rod-like proteins (hydrophobins, *hyd1* and *hyd2*), which mediate, in part, attachment to the host cuticle that is also hydrophobic (Holder and Keyhani, 2005; Ortiz-Urquiza and Keyhani, 2016). The deletion of *hyd1* gene in *B. bassiana* decreased the conidia hydrophobicity and virulence, although it did not interfere in its adhesion capacity. The deletion of the *hyd2* gene caused changes in the structure of the conidia cell wall, and consequently diminished its adhesion capacity and hydrophobicity but without altering its virulence (Zhang et al., 2011). Liu et al. (2013) have shown that the hydrophobin genes (*hyd1* and *hyd2*) were repressed by 66–68% in a *ΔBbmsn2* mutant strain, but it did not affect the *mad1* and *mad2* expressions, additional factors important for facilitating conidial adhesion to arthropods and plants, respectively (Broetto et al., 2010). Microscopic analyses were used in order to probe effects of BbMsn2 on adhesion to tick surfaces, and these data showed that conidia of *ΔBbmsn2* were equivalent to the wild-type (*BbWT*) and complemented strains (*ΔBbmsn2/Bbmsn2*) in size and ability to adhere to the tick cuticle. These data indicate that the impaired virulence seen in topical assays is not likely due to impaired adhesion to the host surface.

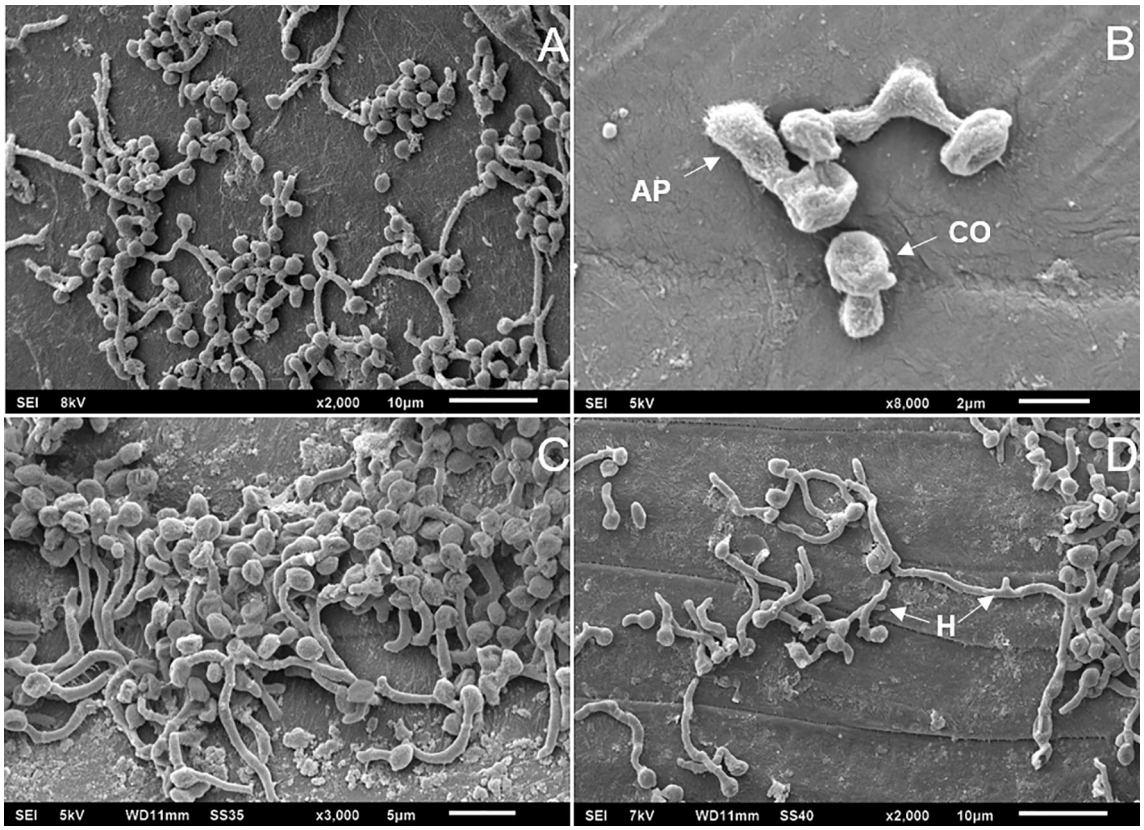

FIGURE 3 | Electron micrographs of *Rhipicephalus microplus* engorged females treated topically with conidia of *Beauveria bassiana* strains: *BbWT*, *ΔBbmsn2/Bbmsn2* or *ΔBbmsn2*, and incubated for 48 h at 26 ± 1°C and RH ≥ 90%. Germinating conidia of *BbWT* on the tick cuticle **(A)** Germinating conidia of *ΔBbmsn2/Bbmsn2* with developed appressorium on the tick cuticle **(B)** Germinating conidia of *ΔBbmsn2/Bbmsn2* on the tick cuticle **(C)** Germinated conidia of *ΔBbmsn2* [some with long germ tubes (hyphae)] on the tick cuticle **(D)**. CO, conidia; AP, appressorium; H, hyphae.

The tick cuticle is divided in several layers; from the outside to inside: epicuticle, exocuticle, endocuticle and epidermis (Hackman, 1982). The epicuticle layer is composed of lipids, long chain alkenes, esters and fatty acids (Ali et al., 2009). In particular, *R. microplus* females, during the engorgement process, have altered epicuticle composition that includes an almost 20% increase in surface lipids; with the most commonly found including alcohol and fatty acids combined by esterification (Hackman, 1982). To penetrate the host cuticle, entomopathogenic fungi use mechanical pressure and secrete proteases, chitinases, and lipases, which degrade their main constituents (proteins, chitin, and lipids) to allow the hyphae to penetrate through the exoskeleton. *B. bassiana* secretes lipases which potentiate the degradation of the arthropod wax layer (Sánchez-Pérez et al., 2014). However, Feng (1998) indicated that lipase activity of several *B. bassiana* s.l. isolates had little correlation with their virulence. Luo et al. (2015) demonstrated that proteinase and lipase activities were similar in *ΔBbmsn2* colonies grown on skim milk agar plates and in the wild-type. However, in the present study, our data show that the protease activity of *B. bassiana ΔBbmsn2* on *R. microplus* cuticle decreased in the Msn2 mutant. It is known that the production of (total) protease activity can vary significantly according to the composition of host cuticle (Cito et al., 2016), which may induce or inhibit fungal development (Ment et al., 2012). Additionally, Santi et al. (2019) identified 50 proteins involved in the infection process, which were produced by *B. bassiana* cultured in media supplemented with cuticles derived from *R. microplus*, but not produced in media supplemented with glucose only. Our results showed a decreased penetration of *ΔBbmsn2* hyphae through the cuticle in comparison to the wild-type and complemented strains at 120 h post-infection. This inability to trespass the tick cuticle may be due to the reduced capacity to degrade chitin, proteins, and/or lipids that constitute the cuticle composition of *R. microplus* (Hackman, 1982). In addition, impairments related to the fungal response to the osmotic, oxidative and/or nutrient stress that occurs on the cuticle, and are mediated by *Msn2*, may also contribute to the decreased virulence (Luo et al., 2015).

The production of oxalic acid is also an important virulence factor in *B. bassiana* against ticks. Direct treatment of ticks with oxalate at pH 4.0 resulted in almost 80% mortality in adults of the tick *Amblyomma americanum* within 14 days after treatment (Kirkland et al., 2005). Oxalate production reduces extracellular

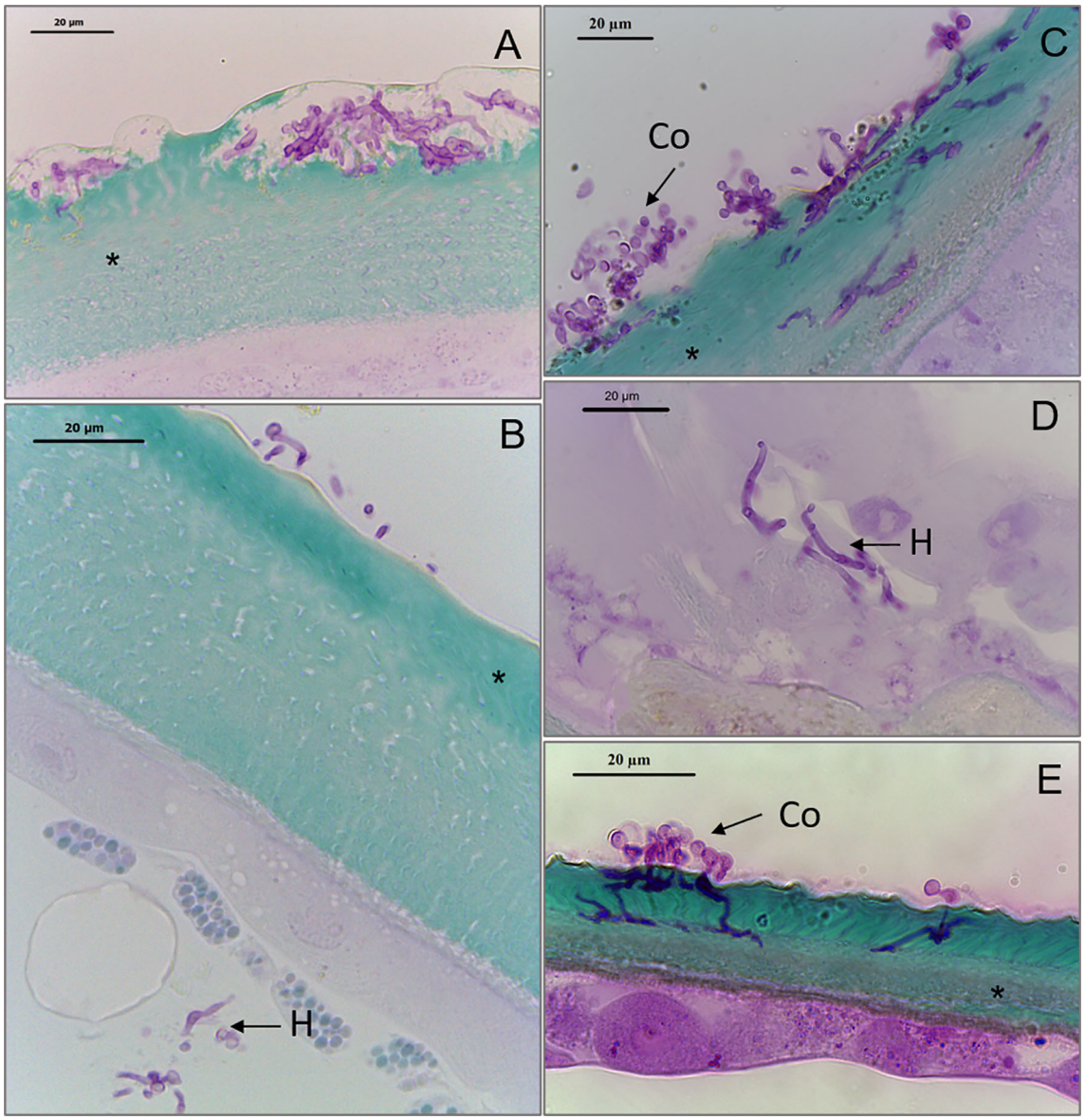

FIGURE 4 | Sagittal sections of *Rhipicephalus microplus* engorged females treated with conidia of *Beauveria bassiana* strains: *BbWT*, *ΔBbmsn2/Bbmsn2* or *ΔBbmsn2*, and incubated for 120 h at 26 ± 1°C and RH ≥90%. Germinating conidia of *BbWT* penetrates through the tick cuticle **(A)** Germinating conidia of *BbWT* attaches to the tick cuticle and fungal hyphae infecting the tick interior **(B)** Germinating conidia of *ΔBbmsn2/Bbmsn2* penetrates through all the layers of the tick cuticle **(C)** Hyphae of *ΔBbmsn2/Bbmsn2* infects the interior tick tissues **(D)** Incomplete penetration of *ΔBbmsn2* hyphae through the tick cuticle **(E)**. Co, germinated conidia; H, hyphae inside tick tissue; asterisk (*) cuticle.

pH and, consequently, acts to facilitate degradation of components of the host cuticle, e.g., chitin, elastin and collagen (Bidochka and Khachatourians, 1991). *M. anisopliae* mutants unable to acidify the culture medium (i.e., that produced less metabolic acids) also show a decreased protease activity (St Leger et al., 1999). In *B. bassiana*, Luo et al. (2015) demonstrated that the *ΔBbmsn2* strain had little to no radial growth at pH 4.1 or 4.7 (although conidiation still occurred), and its growth was reduced in comparison to the wild-type strain. In addition, oosporein production is impaired in the Msn2 mutant (Luo et al., 2015), however its contribution to infection may be limited (Fan et al., 2017). A critical difference with respect to Msn2 function between insects and ticks may be that in insects Msn2 appears to contribute to both topical and intra-hemocoel infection (Luo et al., 2015), whereas our data indicate that for ticks, Msn2 is essentially only required for full virulence *via* the topical route of infection. While topical infection is the "natural" route of infection, these data imply important downstream immune system differences in dealing with invading (fungi) microbes between ticks and insects.

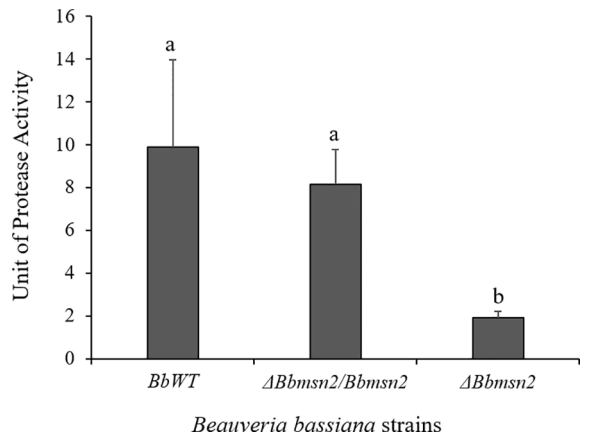

FIGURE 5 | Protease activity on the cuticle of *Rhipicephalus microplus* treated by an immersion in a conidial suspension of *Beauveria bassiana* strains: *BbWT, ΔBbmsn2/Bbmsn2* or *ΔBbmsn2*, and incubated for 120 h at 26 ± 1°C and RH ≥90%. Bars with different letters, i.e. "a" and "b", indicate significant difference ($P < 0.05$). Standard errors are based on three independent trials.

Effects on reproduction can be critical to the biological control potential of insect pathogenic fungi. Reduced fecundity and lack of resistance development have been reported in using *Cordyceps fumosorosea* (formerly, *Isaria fumosorosea*) against *Bemisia tabaci* (whitefly) (Gao et al., 2017), as well as in *B. bassiana* (Bernardo et al., 2018) or *M. anisopliae* (Bittencourt et al., 1994; Muniz et al., 2020) infecting *R. microplus* engorged females. After penetrating the cuticle of *R. microplus*, *M. anisopliae* reaches the hemocoel and can be found colonizing the hemolymph (Bittencourt et al., 1995) and internal organs, including the ovary tissues (Paulo et al., 2018) thus contributing to the reduced fecundity of engorged females seen after infection. *B. bassiana* infection towards *Argas persicus* (Acari: Argasidae) also directly affects the female reproductive system and causes damages to the ovaries, inhibiting vitellogenesis (Marzouk et al., 2020). Our data show that *B. bassiana* infection reduced fecundity of *R. microplus* engorged females by reducing ovipositing and larval hatching, with loss of Msn2 impairing these effects. For successful control, even if entomopathogenic fungi may not (quickly) kill engorged females, any reduction in fecundity can be crucial for controlling *R. microplus* infestations, because fully engorged females naturally drop off the host and lay thousands of eggs on the ground before dying and completing their life cycle.

In recent years, studies on the identification of genes and their functions in *B. bassiana* have revealed a wide range of mechanisms involved in the infection process (Butt et al., 2016); however, most of these studies have focused on insect larvae that are naturally more susceptible to infection by these fungi (Ortiz-Urquiza and Keyhani, 2016), and the extent to which these results can be extrapolated to other targets within Arthropoda remains to be determined. Our data indicate some shared contributions as well as potential differences. In addition, our results indicate that conidial germination, appressorial differentiation, and even fungal colonization on the host surface may be poor indicators of successful tick control (mortality), which requires host penetration (Ment et al., 2012).

CONCLUSIONS

Our results indicate that the absence of Msn2 transcription factor reduced the virulence of *B. bassiana* s.l. against *R. microplus* demonstrated by the delayed fungal penetration and decreased protease production on the tick cuticle. Results on tick reproduction revealed potential effects beyond direct virulence that can impact biological control efforts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Commission on Animal Use of Universidade Federal de Goiás (CEUA, protocol #057/16). The access to Brazilian genetic heritage was approved by the Genetic Heritage Management Council (CGen) of Brazil (protocol #A420934).

AUTHOR CONTRIBUTIONS

EM, NK and ÉF designed the experiments and wrote the manuscript. EM and CR-S performed the bioassays, histology of ticks and protease activity assays. EM and WA performed scanning electron microscopy and histology of ticks. NK inspired co-authors to investigate this subject and produced the mutant strains of *B. bassiana*. All authors contributed to the article and approved the submitted version.

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REFERENCES

Ali, S., Huang, Z., and Ren, S. (2009). Production and Extraction of Extracellular Lipase From the Entomopathogenic Fungus *Isaria Fumosoroseus* (Cordycipitaceae; Hypocreales). *Biocontrol Sci. Technol.* 19 (1), 81–89. doi: 10.1080/09583150802588524

Angelo, I. C., Gôlo, P. S., Camargo, M. G., Kluck, G. E. G., Folly, E., and Bittencourt, V. R. E. P. (2010). Haemolymph Protein and Lipid Profile of *Rhipicephalus (Boophilus) microplus* Infected by Fungi. *Transbound Emerg. Dis.* 57 (1-2), 79–83. doi: 10.1111/j.1865-1682.2010.01119.x

Arruda, W., Lubeck, I., Schrank, A., and Vainstein, M. H. (2005). Morphological Alterations of *Metarhizium anisopliae* During Penetration of *Boophilus microplus* Ticks. *Exp. Appl. Acarol.* 37 (3-4), 231–244. doi: 10.1007/s10493-005-3818-6

Barreto, L. P., Luz, C., Mascarin, G. M., Roberts, D. W., Arruda, W., and Fernandes, É. K. K. (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. doi: 10.1016/j.jip.2016.06.007

Bernardo, C. C., Barreto, L. P., Ribeiro-Silva, C. S., Luz, C., Arruda, W., and Fernandes, É. K. K. (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*. *Ticks Tick-Borne Dis.* 9 (5), 1334–1342. doi: 10.1016/j.ttbdis.2018.06.001

Bidochka, M. J., and Khachatourians, G. G. (1991). The Implication of Metabolic Acids Produced by *Beauveria bassiana* in Pathogenesis of the Migratory Grasshopper, *Melanoplus sanguinipes*. *J. Invertebr. Pathol.* 58, 106–117. doi: 10.1016/0022-2011(91)90168-P

Bittencourt, V. R. E. P., Massard, C. L., and Lima, A. F. (1994). Ação do Fungo *Metarhizium anisopliae* Sobre a Fase Não Parasitária do Ciclo Biológico de *Boophilus microplus*. *Rev. Univ. Rur. Sér. Ciênc. Vida* 16:1-2, 49–55.

Bittencourt, V. R. E. P., Massard, C. L., and Lima, A. F. (1995). Dinâmica Da Infecção do Fungo *Metarhizium anisopliae* (Metschnikof) Sorokin Sobre O Carrapato *Boophilus microplus* (Canestrini). *Rev. Univer. Rur. Sér. Ciênc. Vida.* 17, 83–88.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J., and Roberts, D. W. (2001). Variability in Response to UV-B Among Species and Strains of *Metarhizium* Isolated From Sites at Latitudes From 61°N to 54°S. *J. Invertebr. Pathol.* 78 (2), 98–108. doi: 10.1006/jipa.2001.5048

Broetto, L., Da Silva, W. O. B., Bailão, A. M., Soares, C. A., Vainstein, M. V., and Schrank, A. (2010). Glyceraldehyde-3-Phosphate Dehydrogenase of the Entomopathogenic Fungus Cell-Surface Localization and Role in Host Adhesion. *FEMS Microbiol. Let.* 312 (2), 101–109. doi: 10.1111/j.1574-6968.2010.02103.x

Butt, T. M., Coates, C. J., Dubovskiy, I. M., and Ratcliffe, N. A. (2016). Entomopathogenic Fungi: New Insights Into Host–Pathogen Interaction. *Adv. Genet.* 94, 307–364. doi: 10.1016/bs.adgen.2016.01.006

Butt, T. M., and Goettel, M. S. (2000). "Bioassays of Entomogenous Fungi," in *Bioassays of Entomopathogenic Microbes and Nematodes*. Eds. A. Navon and K. R. S. Ascher (Wallingford, UK: CAB International), 141–195.

Campos, R. A., Boldo, J. T., Pimentel, I. C., Dalfovo, V., Araújo, W. L., Azevedo, J. L., et al. (2010). Endophytic and Entomopathogenic Strains of *Beauveria* sp. to Control the Bovine Tick *Rhipicephalus (Boophilus) microplus*. *Genet. Mol. Res.* 9 (3), 1421–1430 doi: 10.4238/vol9-3gmr884

Cito, A., Barzanti, G. P., Strangi, A., Francardi, V., Zanfini, A., and Dreassi, E. (2016). Cuticle-Degrading Proteases and Toxins as Virulence Markers of *Beauveria bassiana* (Balsamo) Vuillemin. *J. Basic Microbiol.* 56 (9), 941–948. doi: 10.1002/jobm.201600022

Drummond, R. O., Gladney, W. J., Whetstone, T. M., and Ernst, S. E. (1971). Laboratory Testing of Insecticides for Control of the Winter Tick. *J. Econ. Entomol.* 64 (3), 686–688. doi: 10.1093/jee/64.3.686

Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., et al. (2007). Increased Insect Virulence in *Beauveria bassiana* Strains Overexpressing an Engineered Chitinase. *Appl. Environ. Microbiol.* 73 (1), 295–302. doi: 10.1128/AEM.01974-06

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., et al. (2005). Cloning of *Beauveria bassiana* Chitinase Gene Bbchit1 and Its Application to Improve Fungal Strain Virulence. *Appl. Environ. Microbiol.* 71 (1), 363–370. doi: 10.1128/AEM.71.1.363-370.2005

Fan, Y., Liu, X., Keyhani, N. O., Tang, G., Pei, Y., Zhang, W., et al. (2017). Regulatory Cascade and Biological Activity of *Beauveria bassiana* Oosporein That Limits Bacterial Growth After Host Death. *Proc. Natl. Acad. Sci.* 114, E1578–E1586. doi: 10.1073/pnas.1616543114

Feng, M. G. (1998). Reliability of Extracellular Protease and Lipase Activities of *Beauveria bassiana* Isolates Used as Their Virulence Indices. *Acta Microbiol. Sin.* 38 (6), 461–467.

Fernandes, É. K. K., Angelo, I. C., Rangel, D. E. N., Bahiense, T. C., Moraes, A. M. L., Roberts, D. W., et al. (2011). An Intensive Search for Promising Fungal Biological Control Agents of Ticks, Particularly *Rhipicephalus microplus*. *Vet. Parasitol.* 182 (4), 307–318. doi: 10.1016/j.vetpar.2011.05.046

Fernandes, É. K. K., Bittencourt, V. R. E. P., and Roberts, D. W. (2012). Perspectives on the Potential of Entomopathogenic Fungi in Biological Control of Ticks. *Exp. Parasitol.* 130 (3), 300–305. doi: 10.1016/j.exppara.2011.11.004

Gao, T., Wang, Z., Huang, Y., Keyhani, N. O., and Huang, Z. (2017). Lack of Resistance Development in *Bemisia tabaci* to *Isaria fumosorosea* After Multiple Generations of Selection. *Sci. Rep.* 7, 42727. doi: 10.1038/srep42727

Hackman, R. H. (1982). Structure and Function in Tick Cuticle. *Ann. Rev. Entomol.* 27, 75–95. doi: 10.1146/annurev.en.27.010182.000451

Holder, D. J., and Keyhani, N. O. (2005). Adhesion of the Entomopathogenic Fungus *Beauveria* (*Cordyceps*) *bassiana* to Substrata. *Appl. Environ. Microbiol.* 71 (9), 5260–5266. doi: 10.1128/aem.71.9.5260-5266.2005

Holder, D. J., Kirkland, B. H., Lewis, M. W., and Keyhani, N. O. (2007). Surface Characteristics of the Entomopathogenic Fungus *Beauveria* (Cordyceps) *bassiana*. *Microbiology* 153, 3448–3457. doi: 10.1099/mic.0.2007/008524-0

Jin, K., Zhang, Y., Fang, W., Luo, Z., Zhou, Y., and Pei, Y. (2010). Carboxylate Transporter Gene JEN1 From the Entomopathogenic Fungus *Beauveria bassiana* Is Involved in Conidiation and Virulence. *Appl. Environ. Microbiol.* 76, 254–263. doi: 10.1128/AEM.00882-09

Joshi, L., St leger, R. J., and Bidochka, M. J. (1995). Cloning of a Cuticle-Degrading Protease From the Entomopathogenic Fungus, *Beauveria bassiana*. *FEMS Microbiol. Lett.* 125 (2-3), 211–218. doi: 10.1111/j.1574-6968.1995.tb07360.x

Kirkland, B. H., Cho, E., and Keyhani, N. O. (2004a). Differential Susceptibility of *Amblyomma maculatum* and *Amblyomma americanum* (Acari: Ixodidae) to the Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Biol. Control* 31 (3), 414–421. doi: 10.1016/j.biocontrol.2004.07.007

Kirkland, B. H., Eisa, A., and Keyhani, N. O. (2005). Oxalic Acid as a Fungal Acaracidal Virulence Factor. *J. Med. Entomol.* 42 (3), 346–351. doi: 10.1093/jmedent/42.3.346

Kirkland, B. H., Westwood, G. S., and Keyhani, N. O. (2004b). Pathogenicity of Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae* to Ixodidae Tick Species *Dermacentor variabilis*, *Rhipicephalus sanguineus*, and *Ixodes scapularis*. *J. Med. Entomol.* 41 (4), 705–711. doi: 10.1603/0022-2585-41.4.705

Liu, Q., Ying, S. H., Li, J. G., Tian, C. G., and Feng, M. G. (2013). Insight Into the Transcriptional Regulation of Msn2 Required for Conidiation, Multi-Stress Responses and Virulence of Two Entomopathogenic Fungi. *Fungal Genet. Biol.* 54, 42–51. doi: 10.1016/j.fgb.2013.02.008

Luo, Z., Li, Y., Mousa, J., Bruner, S., Zhang, Y., Pei, Y., et al. (2015). Bbmsn2 Acts as a pH-Dependent Negative Regulator of Secondary Metabolite Production in the Entomopathogenic Fungus *Beauveria bassiana*. *Environ. Microbiol.* 17 (4), 1189–1202. doi: 10.1111/1462-2920.12542

Marzouk, A. S., Swelim, H. H., and Ali, A. A. B. (2020). Ultrastructural Changes Induced by the Entomopathogenic Fungus *Beauveria bassiana* in the Ovary of the Tick *Argas* (Persicargas) *persicus* (Oken). *Ticks Tick-Borne Dis.* 11 (6), 101507. doi: 10.1016/j.ttbdis.2020.101507

Ment, D., Churchill, A. C. L., Gindin, G., Belausov, E., Glazer, I., Rehner, S. A., et al. (2012). Resistant Ticks Inhibit *Metarhizium* Infection Prior to Haemocoel Invasion by Reducing Fungal Viability on the Cuticle Surface. *Environ. Microbiol.* 14, 1570–1583. doi: 10.1111/j.1462-2920.2012.02747.x

Muniz, E. R., Paixão, F. R. S., Barreto, L. P., Luz, C., Arruda, W., Angelo, I. C., et al. (2020). Efficacy of *Metarhizium anisopliae* Conidia in Oil-In-Water Emulsion Against the Tick *Rhipicephalus microplus* Under Heat and Dry Conditions. *BioControl* 65, 339–351. doi: 10.1007/s10526-020-10002-5

Ortiz-Urquiza, A., and Keyhani, N. O. (2013). Action on the Surface: Entomopathogenic Fungi Versus the Insect Cuticle. *Insects* 4 (3), 357–374. doi: 10.3390/insects4030357

Ortiz-Urquiza, A., and Keyhani, N. O. (2015). Stress Response Signaling and Virulence: Insights From Entomopathogenic Fungi. *Curr. Genet.* 61 (3), 239–249. doi: 10.1007/s00294-014-0439-9

Ortiz-Urquiza, A., and Keyhani, N. O. (2016). Molecular Genetics of *Beauveria bassiana* Infection of Insects. *Adv. Genet.* 94, 165–249. doi: 10.1016/bs.adgen.2015.11.003

Paulo, J. F., Camargo, M. G., Coutinho-Rodrigues, C. J. B., Marciano, A. F., Freitas, M. C., da Silva, E. M., et al. (2018). *Rhipicephalus microplus* Infected by *Metarhizium*: Unveiling Hemocyte Quantification, GFP-Fungi Virulence, and Ovary Infection. *Parasitol. Res.* 117 (6), 1847–1856. doi: 10.1007/s00436-018-5874-y

Phillips, P. K., Prior, D., and Dawes, B. (1984). A Modified Azoalbumin Technique for the Assay of Proteolytic Enzymes for Use in Blood Group Serology. *J. Clin. Pathol.* 37 (3), 329–331. doi: 10.1136/jcp.37.3.329

Posadas, J. B., and Lecuona, R. E. (2009). Selection of Native Isolates of *Beauveria bassiana* (Ascomycetes: Clavicipitaceae) for the Microbial Control of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *J. Med. Entomol.* 46 (2), 284–291. doi: 10.1603/033.046.0213

R Team C (2018). *R: A Language and Environment for Statistical Computing* (Vienna: R Foundation for Statistical Computing).

Sánchez-Pérez, L. D. C., Barranco-Florido, J. B., Rodriguez-Navarro, S., Cervantes-Mayagoitia, J. F., and Ramos-Lopez, M. A. (2014). Enzymes of Entomopathogenic Fungi, Advances and Insights. *Adv. Enzyme Res.* 2, 65–76. doi: 10.4236/aer.2014.22007

Santi, L., Coutinho-Rodrigues, C. J. B., Berger, M., Klein, L. A. S., De Souza, E., Rosa, R. L., et al. (2019). Secretomic Analysis of *Beauveria bassiana* Related to Cattle Tick, *Rhipicephalus microplus*, Infection. *Folia Microbiol.* 64, 361–372. doi: 10.1007/s12223-018-0659-3

Schrank, A., and Vainstein, M. H. (2010). *Metarhizium anisopliae* Enzymes and Toxins. *Toxicon.* 56 (7), 1267–1274. doi: 10.1016/j.toxicon.2010.03.008

Segers, R., Butt, T. M., Kerry, B. R., and Peberdy, J. F. (1994). The Nematophagous Fungus *Verticillium chlamydosporium* Produces a Chymoelastase-Like Protease Which Hydrolyses Host Nematode Proteins In Situ. *Microbiology* 140 (Pt 10), 2715–2723. doi: 10.1099/00221287-140-10-2715

St Leger, R. J., Nelson, J. O., and Screen, S. E. (1999). The Entomopathogenic Fungus *Metarhizium anisopliae* Alters Ambient pH, Allowing Extracellular Protease Production and Activity. *Microbiology* 145, 2691–2699. doi: 10.1099/00221287-145-10-2691

Sun, M., Ren, Q., Guan, G., Li, Y., Han, X., Ma, C., et al. (2013). Effectiveness of *Beauveria bassiana* sensu lato Strains for Biological Control Against *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in China. *Parasitol. Int.* 62 (5), 412–415. doi: 10.1016/j.parint.2013.04.008

Valero-Jiménez, C. A., Wiegers, H., Zwaan, B. J., Koenrradt, C. J. M., and Kan, J. A. (2016). Genes Involved in Virulence of the Entomopathogenic Fungus *Beauveria bassiana*. *J. Invertebr. Pathol.* 133, 41–49. doi: 10.1016/j.jip.2015.11.011

Wang, X. X., Ji, X. P., Li, J. X., Keyhani, N., Feng, M. G., and Ying, S. H. (2013). A Putative Alpha-Glucoside Transporter Gene BbAGT1 Contributes to Carbohydrate Utilization, Growth, Conidiation and Virulence of Filamentous Entomopathogenic Fungus *Beauveria bassiana*. *Res. Microbiol.* 164 (5), 480–489. doi: 10.1016/j.resmic.2013.02.008

Zhang, S., Xia, Y. X., Kim, B., and Keyhani, N. O. (2011). Two Hydrophobins are Involved in Fungal Spore Coat Rodlet Layer Assembly and Each Play Distinct Roles in Surface Interactions, Development and Pathogenesis in the Entomopathogenic Fungus, *Beauveria bassiana*. *Mol. Microbiol.* 80 (3), 811–826. doi: 10.1111/j.1365-2958.2011.07613.x

Conflict of Interest: ÉF is Associate Editor for the section Fungi-Animal Interactions in Frontiers in Fungal Biology, and Guest Associate Editor for the section Invertebrate Physiology, in the research topic: Entomopathogenic Fungi for the Control of Arthropods - Frontiers in Physiology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Discovery of Novel Entomopathogenic Fungi for Mosquito-Borne Disease Control

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The increased application of chemical control programs has led to the emergence and spread of insecticide resistance in mosquitoes. Novel environmentally safe control strategies are currently needed for the control of disease vectors. The use of entomopathogenic fungi could be a suitable alternative to chemical insecticides. Currently, *Beauveria* spp. and *Metarhizium* spp. are the most widely used entomopathogenic fungi for mosquito control, but increasing the arsenal with additional fungi is necessary to mitigate the emergence of resistance. Entomopathogenic fungi are distributed in a wide range of habitats. We have performed a comprehensive screen for candidate mosquitocidal fungi from diverse outdoor environments in Maryland and Puerto Rico. An initial screening of 22 fungi involving exposure of adult *Anopheles gambiae* to 2-weeks-old fungal cultures identified five potent pathogenic fungi, one of which is unidentified and the remaining four belonging to the three genera *Galactomyces* sp., *Isaria* sp. and *Mucor* sp. These fungi were then screened against *Aedes aegypti,* revealing *Isaria* sp. as a potent mosquito killer. The entomopathogenic effects were confirmed through spore-dipping assays. We also probed further into the killing mechanisms of these fungi and investigated whether the mosquitocidal activities were the result of potential toxic fungus-produced metabolites. Preliminary assays involving the exposure of mosquitoes to sterile filtered fungal liquid cultures showed that *Galactomyces* sp., *Isaria* sp. and the unidentified isolate 1 were the strongest producers of factors showing lethality against *An. gambiae*. We have identified five fungi that was pathogenic for *An. gambiae* and one for *Ae. aegypti*, among these fungi, four of them (two strains of *Galactomyces* sp., *Mucor sp.,* and the unidentified isolate 1) have never previously been described as lethal to insects. Further characterization of these entomopathogenic fungi and their metabolites needs to be done to confirm their potential use in biologic control against mosquitoes.

Keywords: *Anopheles gambiae*, *Aedes aegypti*, entomopathogenic fungi, biopesticides, mosquito control, vector-borne diseases

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INTRODUCTION

Vector-borne disease accounts for more than 17% of all infectious disease, causing more than 700,000 deaths annually. *Anopheles gambiae* is the most important malaria vector, and *Aedes aegypti* is responsible for the transmission of dengue fever, Zika virus disease, chikungunya, Rift Valley fever, and yellow fever, among others [World Health Organization (WHO), 2020]. Current

mosquito vector control strategies are primarily based on synthetic insecticides. However, the increased implementation of chemical control programs has led to the emergence and spread of insecticide resistance in *Anopheles* sp. *(An.)* and *Aedes* sp. *(Ae.)* populations and is also affecting non-target organisms, including humans (Moyes et al., 2017; Riveron et al., 2018).

Several studies have demonstrated the potential of using entomopathogenic fungi for controlling mosquito vectors (Charnley and Collins, 2007), as an effective and environmentally safe strategy. Since one of the mode of action of entomopathogenic fungi is mediated through surface contact with adult mosquitoes, these agents would be applicable to a variety of deployment strategies, some of which are already in use for chemical insecticides (Scholte et al., 2005; Farenhorst et al., 2011). More importantly, they are effective against mosquito strains that have developed resistance to the available chemical insecticides (Farenhorst et al., 2009, 2010; Blanford et al., 2011). Furthermore, in contrast to chemical insecticides that generally kill mosquitoes within 24 h, fungal biopesticides usually require more than a week to kill exposed mosquitoes and thereby the probability is decreased that resistance will emerge (Ffrench-Constant, 2005; Read et al., 2009).

Metarhizium spp. and *Beauveria* spp. are the best characterized and most widely used fungi in biological control programs (Scholte et al., 2005; Hancock, 2009; Knols et al., 2010; Blanford et al., 2011; Farenhorst et al., 2011). About 13 species and sub-species of both these fungi have been formulated and registered as mycoinsecticides or mycoacaricides (de Faria and Wraight, 2007). While their mode of action against adult mosquitoes is based on slow and gradual penetration and invasion through the invertebrate cuticle, the emergence of resistance is still a likely event, as has been demonstrated by Dubovskiy et al. (2013). It is therefore necessary to expand the repertoire of fungal biopesticides with additional fungi in order to mitigate the emergence of resistance; the availability of additional fungal biopesticides will potentially also enable a greater versatility of exposure routes that may be dependent of the biology of the fungus. Key stages of the infection process by entomopathogenic fungi are: (a) adhesion of infectious spores to the surface of the insect cuticle; (b) penetration of the cuticle via enzymes and mechanical force; (c) colonization of the hemocoel; and (d) emergence of the conidiophores for external sporulation on the insect cadaver (Butt et al., 2016). In addition, entomopathogenic fungi produce a variety of secondary metabolites, some of which are highly toxic to the larval, pupal, and adult mosquito stages, as well as to some pathogens such as the malaria parasite (Singh and Prakash, 2012; Niu et al., 2015; Vivekanandhan et al., 2018a, 2020a). A better understanding of the toxic metabolites produced by fungi can help unravel the relevant mosquitocidal mechanisms and enable the development of novel natural product-based mosquito control agents.

Entomopathogenic fungi are present in a wide range of habitats (Lacey and Fransen, 1996; Chandler et al., 1997; Sánchez-Peña et al., 2011). Here we aimed at discovering novel mosquitocidal fungi from diverse habitats with activity against *An. gambiae* and *Ae. aegypti* adults. We use three different ways to expose adult mosquitoes to the isolated environmental fungi, the first exposure assay was primarily used as a screening method to differentiate pathogenic from non-pathogenic environmental fungi. This method was a direct exposure of adult mosquitoes to fungal cultures on agar plates containing fungal mycelia, the growing hyphae, spores and possible toxic compounds all together. The second methodology involved the use of purified spores in solution (10^8 spores/ml) alone. The third way to expose mosquitoes, to determine whether the killing effect is mediated by toxic metabolites and/or requires actual exposure to live fungi, was sugar fed adult mosquitoes with a sugar solution containing only the fungal metabolites.

RESULTS

Fungus Sample Collection

To maximize the probability of identifying novel fungi with entomopathogenic properties, samples of fungus-containing plants, decomposing material, soil, and stagnant water were collected from outdoor mosquito habitats in Maryland (MD) and Puerto Rico (**Table 1**).

In Baltimore, MD, samples were collected from six different sources, including stagnant water from containers, a fountain, and a pond, and from soil in an outdoor pot and two tree flowers. In the rural area of Maunabo, Puerto Rico, 30 fungal samples were collected from 15 different types of leaves, five different decomposing materials, nine types of flowers, and one sample was collected from moss.

Culturing and Identification of Fungi

The 36 environmental samples were manually homogenized and plated on rich fungal solid growth medium to allow the growth of as many fungi as possible. Each morphologically distinct fungal colony was re-streaked on a new agar plate until an axenic fungal culture was attained for each of the different fungal colonies. Once these were separated and streaked on individual plates, several isolates did not grow for more than one generation and were thus excluded from the study. Possible explanations for this could be that these isolates had an obligate symbiosis relationship with microorganisms in the collected sample, or that they could not produce a sufficient number of spores necessary to propagate under laboratory conditions. The 36 collected samples generated a total of 76 fungal isolates, 22 of which could be successfully cultured under the specified laboratory conditions (**Figure 1**). DNA was extracted from these 22 fungi and used for ribosomal 18S gene amplification and sequencing for identification (**Table 1**). After the DNA extraction, ribosomal 18S gene amplification was performed using internal transcribed spacer (ITS) degenerate primers, with either of two forward primers (ITS1, ITS3) in combination with one common reverse primer (ITS4) (**Supplementary Table 1**).

The sequence identity was determined through BLAST nucleotide searches against the NCBI database (**Table 1**). The identity of the fungi was determined through analysing phylogenetic trees constructed using MEGAX software with the the Bootstrap and the Neighbor-joining statistic method (**Supplementary Files 1–3**).

TABLE 1 | Collection and identification of fungi.

Ref Nr[a]	ITS-based identification				Collection		Entomopathogenic activity	
	Fungi	Query cover (%)	Query identity (%)	Accesionnumber	Country (MD/PR)[b]	Source	Known EPF[c] (Y/N)[d]	EPF[c] against mosquitoes (Y/N)[d]
1	*Isolate* 1 sp.	99	98,9	*MT786363.1*	MD	Water pond	**N**	**N**
2	Ascomycota	95	99,8	KT315401.1	PR	Leaf	**Y**[1,2,3,4,5,6]	
3	*Aureobasidium* sp.	88	100	MK156692.1	PR	Leaf	**N**	**N**
4	*Cercospora* sp.	100	99,8	AY633838.1	PR	Leaf	**N**	**N**
5	*Cladosporium* sp.	100	99,6	MT508793.1	PR	Leaf	**Y**[7]	**N**
6	*Cladosporium* sp.	100	100	MT582794.1	MD	Container	**Y**[8]	**N**
7	*Colletotrichum* sp.	100	99,8	MN889467.1	PR	Leaf	**N**	**N**
8	*Fusarium* 1 sp.	100	99,6	MT598827.1	PR	Leaf	**Y**[9,10]	**Y**[11]
9	*Fusarium* 2 sp.	99	100	MW553789.1	PR	Moss		
10	*Fusarium* 3 sp.	100	99,6	MT603301.1	PR	Flower		
11	*Fusarium* 4 sp.	100	99,8	MW582382.1	MD	Fountain		
12	*Galactomyces* 1 sp.	99	100	MF044044.1	MD	Soil	**N**	**N**
13	*Galactomyces* 2 sp.	100	99,7	DQ907937.1	MD	Soil		
14	*Isaria* sp.	100	100	KX241857.1	MD	Container	**Y**[12,13,14,15,16]	**Y** [16,17,18,19,20,21]
15	*Mucor* 1 sp.	100	99,6	MN087659.1	PR	Dec Material[e]	**N**	**N**
16	*Mucor* 2 sp.	100	99,6	MN905930.1	MD	Container	**N**	**N**
17	*Mucor* 3 sp.	100	100	MK594384.1	MD	Fountain	**Y**[22]	**N**
18	*Penicillium* sp.	100	99,4	AF125944.1	PR	Leaf	**Y**[23]	
19	*Phomopsis* 1 sp.	100	100	MT071116.1	PR	Leaf	**Y**[24,25]	**N**
20	*Phomopsis* 2 sp.	100	99,6	MT071116.1	PR	Leaf		
21	*Pleosporales* sp.	100	100	JN851029.1	PR	Leaf	**N**	**N**
22	*Scopulariopsis* sp.	99	100	MT609891.1	PR	Dec Material[e]	**Y**[26]	**N**

[a]Ref Nr, Reference Number; [b]Locality, Samples were collected in Maryland (MD) or in Puerto Rico (PR). [c]EPF, entomopathogenic fungi; [d]Y/N, Yes or No; [e]Dec Material, Decomposing Material; [1]Farenhorst et al. (2011); [2]Hancock (2009); [3]Hancock et al. (2009); [4]Knols et al. (2010); [5]Blanford et al. (2005); [6]Scholte et al. (2005), [7]Singh et al. (2015);[8]Elbanhawy et al. (2019);[9]Chehri (2017);[10]Pelizza et al. (2011); [11]Vivekanandhan et al. (2018a); [12]United States Environmental Protection Agency (EPA). (2020); [13]European Commission (EU) (2020); [14]Ministerio de Agricultura (2020); [15]Zhang et al. (2016); [16]Xu et al. (2017); [17]Luz et al. (2007); [18]Leles et al. (2010); [19]Blanford et al. (2012); [20]Ramirez et al. (2018); [21]Banu and Balasubramanian (2014); [22]Konstantopoulou et al. (2006); [23]Maketon et al. (2014); [24]Meepagala et al. (2018); [25]Amatuzzi et al. (2018); [26]Niu et al. (2019).

As many as 20 of the 22 isolated fungi were identified down to genus level using a cut-off of 98% for query coverage and identity. One isolate (Ascomycota) was identified only at the phylum level, and the identity of another isolate was ambiguous and was therefore called fungal isolate 1. The top hit for fungal isolate 1 when BLASTed was *Aaosphaeria* sp., but as can be seen in the phylogenetic tree this result was not conclusive as several other genera appears equally identical. The 22 fungal isolates belonged to the following 12 different genera and one phylum: phylum Ascomycota, *Aureobasidium* sp., *Cercospora* sp., *Cladosporium* sp., *Colletotrichum* sp., *Fusarium* sp., *Galactomyces* sp., *Isaria* sp., *Mucor* sp., *Penicillium* sp., *Phomopsis* sp., *Pleosporales* sp. and *Scopulariopsis* sp. (ITS-based sequences are presented in **Supplementary File 1**). Some of the identified fungi have different nomenclature depending on the sexual stage; *Galactomyces*, *Isaria*, *Fusarium, Mucor* and *Phomopsis* are synonymous to *Geotricum*, *Cordyceps/Paecylomyces*, *Fusicola, Rhizomucor* and *Diaporthe,* respectively (Eliskases-Lechner et al., 2011; Gräfenhan et al., 2011; Udayanga et al., 2011; Sun et al., 2020).

The most common genus was *Fusarium* sp., being represented by four isolates that originated from both Puerto Rico and Maryland. Two fungal isolates each of *Cladosporium* sp. *Galactomyces* sp. and *Phomopsis* sp. were represented in our collection. *Cladosporium* sp. was identified in both Puerto Rico and Maryland, while *Galactomyces* sp. and *Phomopsis* sp. isolates were only identified in Maryland or Puerto Rico, respectively. Single isolates were identified for the remaining fungal genera.

Screening for Entomopathogenic Activity With a Fungus Direct-Exposure Assay Against *An. gambiae* Adults

As an initial screen to identify potential entomopathogenic isolates, our 22 isolated fungi were tested for mosquitocidal activity through a direct exposure assay against adult non-blood-fed females of the major malaria vector *An. gambiae.* This mosquito species is in general more delicate and therefore more

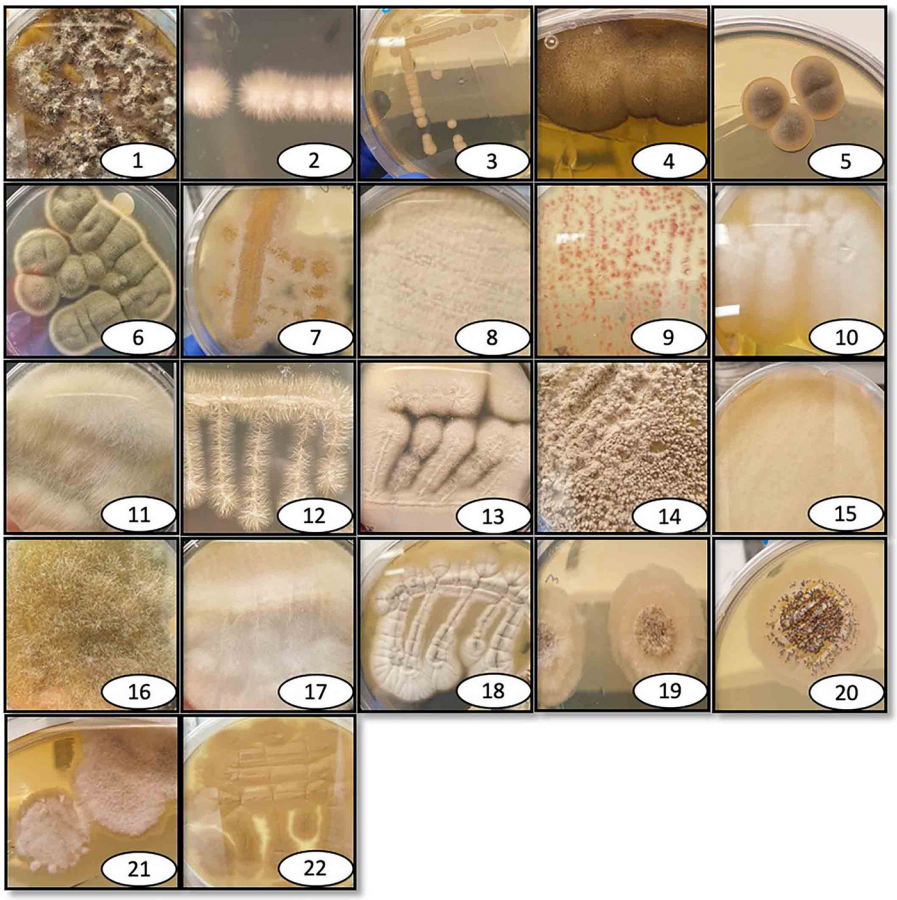

FIGURE 1 | Pictures of isolated and identified fungi. Individual photographs of the isolated fungi that are identified here, labeled with the reference number, and listed in **Table 1**. The fungi were collected at various outdoor locations and maintained in the lab on either rich growth agar medium brain heart infusion or Sabouraud dextrose, depending on the fungal species.

susceptible to pathogens than is the more robust arboviral vector *Ae. aegypti* (Beckage et al., 2004; Alkhaibari et al., 2017). In short, adult females were placed directly on the fungus culture agar plate, which was then shaken for 30 s to assure extensive mosquito surface exposure. Sterile BHI agar plates were used as a negative control. The mosquitoes were then transferred to cups, and survival was monitored for 20 days. Fungus-mediated lethality was estimated by calculating the *p*-value with a long-rank Mantel-Cox analysis, and a value of <0.0001 was considered indicative of highly potent entomopathogenic activity. From this first screening, a total of five fungal isolates (isolate 1, two *Galactomyces* sp., *Isaria* sp., *Mucor* 3 sp.) showed potent killing activity against *An. gambiae* females, with these mosquitoes having a significantly ($p < 0.0001$) shorter lifespan than did the non-fungus-exposed mosquitoes (**Table 2**, **Supplementary Figure 1**).

The median survival of the non-fungus-exposed mosquitoes was 10 days; *Mucor* 3 sp. displayed the most potent lifespan-shortening activity, resulting in a median mosquito survival period of 1 day, followed by *Isaria* sp. (3 days), *Galactomyces* 2 sp. (4 days), and fungal isolate 1 and *Galactomyces* 1 sp. (5 days). Exposure to the positive control *B. bassiana* resulted in a median mosquito survival of 2 days (**Table 2**). All non-fungus-exposed mosquitoes had died by 16 days, all isolate 1-exposed mosquitoes had died by 13 days, all mosquitoes exposed to either of the two *Galactomyces sp.* isolates had died by 9 days, those exposed to *Isaria* had died by 4 days, and the *Mucor* 3 sp.-exposed mosquitoes had died by 6 days. Mosquitoes exposed to the positive control *B. bassiana* were all dead at day 3 (**Table 2**). Three of the entomopathogenic fungi we discovered, isolate 1, *Isaria* sp., and *Mucor* 3 sp., displayed an intense fungal proliferation/growth on the dead mosquito carcasses. Mosquitoes that had succumbed after *Galactomyces* sp. exposure showed a darker coloration than that of the non-fungus-exposed mosquitoes.

The interesting findings from the screen made us re-evaluate the identification of four of the five potent fungal isolates. Based on the ITS, ribosomal small subunit (SSU) and ribosomal large subunit (LSU)-sequencing data, the phylogenetic trees (**Supplementary Files 1–3**) and spore photos of the four interesting fungi (**Supplementary Figure 2**)

TABLE 2 | Mortality of *An. gambiae* and *Ae. aegypti* mosquitoes exposed through fungal direct-exposure assay.

An. gambiae			
Fungi	***P*-value**[a]	**Mortality (days)**	
		Median[b]	**Total**[c]
Isolate 1	<0,0001****	5	13
Ascomycota	0.6	5	12
Aureobasidium sp.	0.6 NS	5	10
Cercospora sp.	0.3 NS	3	13
Cladosporium 1 sp.	0.02*	11	19
Cladosporium 2 sp.	0.0006***	4	8
Colletotrichum sp.	0.0007***	10	18
Fusarium 1 sp.	0.1 NS	3	11
Fusarium 2 sp.	0.8 NS	5	12
Fusarium 3 sp.	0.1 NS	9	15
Fusarium 4 sp.	0.0009***	7	10
Galactomyces 1 sp.	<0.0001****	5	9
Galactomyces 2 sp.	<0.0001****	4	9
Isaria sp.	<0.0001****	3	4
Mucor 1 sp.	0,1 NS	8	14
Mucor 2 sp.	0.0009***	7	12
Mucor 3 sp.	<0.0001****	1	6
Penicillium sp.	0.02*	9	17
Phomopsis 1 sp.	0.009 NS	11	19
Phomopsis 2 sp.	0.2 NS	12	19
Pleosporales sp.	0.9 NS	15	20
Scopulariopsis sp.	0.1 NS	9	14
Untreated (–ctrl)[d]	/	10	16
Beauveria bassiana (+ctrl)[e]	<0.0001****	2	3
Ae. aegypti			
Fungi	***P*-value**[a]	**Mortality**	
		Median[b]	**Total**[c]
Isolate 1	0,7 NS	14	NA
Galactomyces 1 sp.	0,6 NS	14	NA
Galactomyces 2 sp.	0,6 NS	14	NA
Isaria sp.	<0,0001****	4	6
Mucor 3 sp.	0,2 NS	17	NA
untreated (–ctrl)[d]	/	14	NA
Beauveria bassiana (+ctrl)[e]	<0,0001****	1	3

[a] *P-value calculated by comparing the negative control to each fungus, analyzed by log-rank Mantel-Cox test;* [b] *Median indicates the median number of days the mosquitoes survived after exposure;* [c] *Total indicates the day when 100% of the mosquitoes had died;* [d] *ctrl–, negative control;* [e] *ctlr+, positive control; NS, not significant; NA, not achieved, since the mosquitoes survived past day 20. The values in this table were calculated from one replicate.*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

we were able to suggest a species for four of the five fungal isolates. These are *Galactomyces candidum* 1, *Galactomyces candidum* 2, *Isaria fumosorosea,* and *Mucor hiemalis*.

Testing for Entomopathogenic Activity Against *Ae. aegypti* Adults

Next, we decided to evaluate the five fungi (isolate 1, *G. candidum* 1, *G. candidum* 2, *I. fumosorosea,* and *M. hiemalis*) that had been shown to be entomopathogenic in the *An. gambiae*-based screen against *Ae. aegypti* using the same direct exposure assay. Of these five fungi, only *I. fumosorosea* showed a highly potent entomopathogenic activity against *Ae. aegypti* that was comparable to the activity of the positive control *B. bassiana*; they both had a *p*-value of <0.0001 (**Table 2**, **Supplementary Figure 1**). While the median lifespan of the non-exposed mosquitoes was 14 days, the lifespan for those exposed to *Isaria* sp. or the positive control *B. bassiana* were 4 days and 1 day, respectively. For the *Isaria* sp.-exposed mosquitoes, 100% mortality was reached on day 6, whereas those exposed to the positive control *B. bassiana* died within a 3-day period after exposure (**Table 2**).

Determining Entomopathogenic Activity With Spore-Dipping Fungus Exposure Assays Against *An. gambiae* and *Ae. aegypti* Adults

In order to confirm the outcome of the direct-exposure mosquitocidal assays and to further explore the entomopathogenic activities of the five selected fungal isolates, We performed spore-dipping assays against *An. gambiae* and *Ae. aegypti*. This type of assay allowed us to better control the concentration of spores to which mosquitoes were exposed and thereby obtain more reproducible and comparable data. In contrast to the direct exposure assay, in this method the mosquitoes are only exposed to spores and not fungal mycelia, growing hyphae, or possible toxic compounds. We used a spore concentration corresponding to that used in previous studies (Dong et al., 2012; Vivekanandhan et al., 2020b). Adult female mosquitoes were dipped for 1 min into a spore solution, to allow the spores to come in contact with the mosquito surface. As a negative control, mosquitoes were dipped in a non-spore-containing solution. The spore-dipping experiments were performed in triplicate, and the two mosquito species were tested with the respective fungi that had shown a significant ($p < 0.0001$) killing activity in the direct-exposure assay. Five-days-old *An. gambiae* females were tested against isolate 1, *G. candidum* 1 and 2, *I. fumosorosea,* and *M. hiemalis*. All five fungi displayed a strong and significant ($p < 0.0001$) mosquitocidal activity after dipping of the mosquitoes into the spore solution (**Table 3**, **Figure 2A**). The median lifespan of the negative control cohort was 8 days, and the mosquitoes exposed to isolate 1, *G. candidum* 2, or *I. fumosorosea* displayed a 2-day median survival time. Mosquitoes exposed to spores of *G. candidum* 1 or *M. hiemalis* showed a median survival time of 4 and 3 days, respectively.

Mosquitoes exposed to the positive control *B. bassiana* had a median survival time of 3 days. The spores of these fungal isolates killed all the adults within 6–9 days, depending on the replicates, whereas the non-spore-treated mosquitoes died within 14–18 days in the three biological replicates. These findings were also confirmed with a hazard ratio analysis, which expresses the probability of the death event. For example, isolate 1-exposed mosquitoes that had all succumbed by 6 days after exposure had a hazard ratio of 4.4, indicating that death was 4.4 times more likely after exposure to this fungus than if not exposed. For *G. candidum* 1- and *G. candidum* 2-exposed mosquitoes, the hazard ratios were 3.9 and 4.5, respectively. For *I. fumosorosea*-exposed mosquitoes, which had all died by 6 days after exposure, the hazard value was 5.1, and for *M. hiemalis*-exposed mosquitoes it was 2.5. Mosquitoes exposed to the positive control *B. bassiana* showed a hazard ratio of 5.4 and 100% mortality was observed after 7 days (**Table 3**). We also screened adult female *Ae. aegypti* against *Isaria* sp. spores, and the results showed a potent reduction of the lifespan ($p < 0.0001$) (**Figure 2B**), with a median of survival time of 11 days, as compared to 16 days for the negative control and 9 days for the *B. bassiana*-exposed positive control (**Table 3**).

All *Ae. aegypti* exposed to *I. fumosorosea* had died by 16 days after exposure, yielding a hazard ratio of 2.1, whereas 57% of the non-spore-exposed control cohort had succumbed by day 20. All mosquitoes had succumbed after exposure to *B. bassiana* spores at 13 days post-treatment, yielding a hazard ratio of 4.8 (**Table 3**).

Mosquitocidal Activity of Fungus Culture Filtrates Against *An. gambiae* and *Ae. aegypti* Adults

To initiate an investigation into the nature of the entomopathogenic activity, we designed assays that would discriminate between killing mechanisms that are mediated by secreted fungus-produced metabolites and those requiring exposure to living fungi. Adult female *An. gambiae* and *Ae. aegypti* were fed on a sucrose solution containing a fungal liquid potato dextrose (PD) culture filtrate that presumably would contain the secreted factors of interest. We examined the five selected mosquitocidal fungi identified in the initial screening against adult *An. gambiae* females. As expected, both mosquito species showed an overall greater survival after ingestion of the fungal culture filtrates than after direct exposure to live fungi or spore solution. In fact, the hazard ratio of *An. gambiae* was between 0.8 and 2.4 and of *Ae. aegypti* mosquitoes <1, for the tested fungal isolates (**Table 4**, **Figure 3**).

An. gambiae mosquitoes that had ingested isolate 1 and *G. candidum* 2 culture filtrates showed a median of survival of 8 days and a significant reduction in the lifespan when compared to untreated mosquitoes ($p = 0.003$ and $p = 0.006$, respectively). *An. gambiae* mosquitoes that fed on the *I. fumosorosea* culture filtrate showed a median survival time of 6 days ($p = 0.002$), and those ingesting the *B. bassiana* culture filtrate showed a median survival of 7 days and a *p*-value of 0.002. Ingestion of *G. candidum* 1 or *M. hiemalis* culture filtrate did not have any effect on *An. gambiae* longevity when compared to the non-culture filtrate-fed control mosquitoes (**Table 4**, **Figure 3A**). All *An. gambiae* mosquitoes that were fed on isolate 1, *I. fumosorosea* or *G. candidum* 2 culture filtrates died within 14 days after ingestion, whereas those fed on *G. candidum* 1 or *M. hiemalis* culture filtrates, as well as the untreated mosquitoes, survived up to 16–18 days. The positive control mosquitoes that fed on a *B. bassiana* culture filtrate had all died at 7 days post-feeding (**Table 4**). As compared to the untreated control mosquitoes, none of the culture filtrates of the five selected fungi affected the survival of *Ae. aegypti* after ingestion, not even the *I. fumosorosea* isolate, which exerted potent killing activity against this mosquito species in the direct-exposure assay (**Table 4**, **Figure 3B**). Our results show that some of the fungi do produce and secrete factors that are toxic to the mosquitoes, but the most efficient killing requires exposure to live fungi and presumably involves an active infection process.

DISCUSSION

The failure of disease control programs resulting from insecticide resistance among mosquito populations highlights the urgent need for new tools for mosquito control, including new insecticides. In the present study, we sampled diverse habitats to find entomopathogenic fungi that could satisfy the requirements for further development into mosquitocidals for use in vector control programs. We collected a total of 36 samples from various habitats and sources in Maryland and Puerto Rico that yielded 76 fungal isolates, 22 of which were successfully cultured under laboratory conditions. We were unsuccessful to maintain all the isolated fungi in the lab for several reasons; some fungi were simply not able to survive in the lab-condition of temperature and humidity, considering that they were isolated in Puerto Rico, some of them could have been in strict symbiosis with other fungi or source like flowers or other plants, some of them were perhaps not able to produce enough spores to propagate.

Analyses of their ribosomal 18S gene, through sequences blasting and through phylogenetic trees generation, revealed that the 22 isolates belonged to 12 different genera: Ascomycota phylum, *Aureobasidium* sp., *Cercospora* sp., *Cladosporium* sp., *Colletotrichum* sp., *Fusarium* sp., *Galactomyces* sp., *Isaria* sp., *Mucor* sp., *Penicillium* sp., *Phomopsis* sp., *Pleosporales* sp. and *Scopulariopsis* sp. Members belonging to the same genus as at least eight of our isolates have previously been shown to possess entomopathogenic properties against various types of insects, including various mosquito species (Ascomycota, *Cladosporium* sp., *Diaporthe* sp., *Fusarium* sp., *Isaria* sp., *Mucor* sp., *Penicillium* sp., and *Scopulariopsis)*. These results further confirm the presence of entomopathogenic fungi in a wide variety of outdoor environments (Lacey and Fransen, 1996; Chandler et al., 1997; Sánchez-Peña et al., 2011). Within the Ascomycota phylum we find the most important entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp.) that are used a biopesticides to control mosquitoes (Blanford et al., 2005; Scholte et al., 2005; Hancock, 2009; Hancock et al., 2009; Knols et al., 2010; Farenhorst et al., 2011). Several species of *Cladosporium* sp. (also Ascomycota phylum) have been found to exert pathogenic activity against

TABLE 3 | Mortality of *An. gambiae* and *Ae. aegypti* mosquitoes exposed to spore solution in the dipping assay.

An. gambiae				
Fungi	***P*-value[a]**	**Mortality (days)**		**Hazard ratio (95% CI)[d]**
		Median[b]	**Total + SD[c]**	
Isolate 1	<0.0001****	2	6 + 0.8	4.46 (3–6.6)
Galactomyces candidum 1	<0.0001****	4	8.3 + 2	3.9 (2.7–5.8)
Galactomyces candidum 2	<0.0001****	2	8.6 + 1.8	4.5 (3.1–6.67)
Isaria fumosorosae	<0.0001****	2	6.3 + 1.6	5.2 (3,4–7.9)
Mucor hiemalis	<0.0001****	3	9.6 + 3	2.5 (1.7–3.7)
Untreated (–ctrl)[e]	/	8	14.6 + 2.4	/
Beauveria bassiana (+ctrl)[f]	<0.0001****	3	7.3 + 1.2	5.4 (3.6–8,1)
Ae. aegypti				
Fungi	***P*-value[a]**	**Mortality (days)**		**Hazard ratio (95% CI)[d]**
		Median[b]	**Total + SD[c]**	
Isolate 1	ND	ND	ND	ND
Galactomyces candidum 1	ND	ND	ND	ND
Galactomyces candidum 2	ND	ND	ND	ND
Isaria fumosorosea	<0.0001****	11	16.6 + 3.9	2.1 (1.4–3.1)
Mucor hiemalis	ND	ND	ND	ND
Untreated (–ctrl)[e]	/	16	NA	/
Beauveria bassiana (+ctrl)[f]	<0.0001****	9	13.3 + 1.4	4.8 (3.3–7.1)

[a]P-value calculated by comparing the negative control to each fungus, analyzed by log-rank Mantel-Cox test; [b]Median indicates the median number of days the mosquitoes survived after exposure; [c]Total + SD indicates the day when 100% of the mosquitoes had died, with the corresponding standard error; [d]Hazard ratio (95% CI), calculated with log-rank Mantel and Haenzel analysis with the corresponding 95% interval of confidence; [e]ctrl–, negative control; [f]ctlr+, positive control; NS, not significant; NA, not achieved, since mosquitoes survived past day 20. The values in this table were calculated from three replicates.
**p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

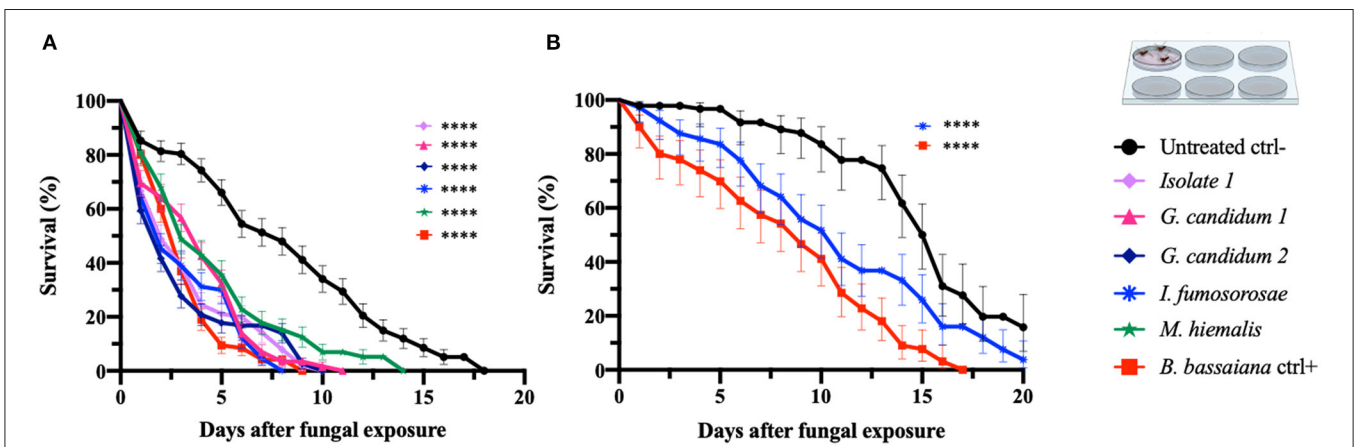

FIGURE 2 | Spore-dipping assays of *An. gambiae* and *Ae. aegypti* mosquitoes. Mosquitoes were exposed via a dipping assay to a spore solution (10^8 spores/ml) for 1 min, and the mosquito survival was monitored for 20 days. Data are here presented for *An. gambiae* **(A)** and *Ae. aegypti* **(B)**. The fungal species tested on each mosquito species were the ones identified as active in the respective direct-exposure experiment. The graphs represent the average and standard deviation of three biological replicates, $N = 30$ females per replicate. As a negative control, mosquitoes were exposed to a spore-free solution, and as a positive control they were exposed to a spore solution of *B. bassiana*. The statistical significance of the results for each fungus was compared to the negative control and analyzed by log rank-Mantel-Cox test; *p*-values are presented in **Table 3**. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

insects; for example, lab trials showed that a *C. velox* fungus is able to kill *Spodoptera litura* larvae, one of the most important insect pests of agricultural crops in tropical regions of Asia (Singh et al., 2015), and extracts of *C. cladosporioides* have been shown to kill *Aphis gossypii*, which is a major pest of the cotton industry (Elbanhawy et al., 2019). *Diaporthe* sp. filtrates have been shown

TABLE 4 | Mortality of *An. gambiae* and *Ae. aegypti* mosquitoes exposed to fungal metabolites by feeding assay.

An. gambiae				
Fungi	***P*-value[a]**	**Mortality (days)**		**Hazard ratio (95% CI)[d]**
		Median[b]	**Total + SD[c]**	
Isolate 1	0.003**	8	14.6 + 0.8	1.3 (1–1.9)
Galactomyces candidum 1	0.5 NS	9	16 + 2.8	1 (0.8–1.4)
Galactomyces candidum 2	0.006**	8	14.3 + 1.6	1.5 (1.3–2.1)
Isaria fumosorosea	0.002**	6	14.3 + 4.4	1.7 (1.3–2.4)
Mucor hiemalis	0.5 NS	9	17.3 + 1.6	1.1 (0.8–1.4)
Untreated (–ctrl)[e]	/	9	18 + 2.8	/
Beauveria bassiana (+ctrl)[f]	0.002**	7	14.6 + 1.2	1.6 (1.1–2.2)
Ae. aegypti				
Fungi	***P*-value[a]**	**Mortality (days)**		**Hazard ratio (95% CI)[d]**
		Median[b]	**Total + SD[c]**	
Isolate 1	0.6 NS	16	NA	0.8 (0.5–1.4)
Galactomyces candidum 1	0.5 NS	17	NA	0.8 (0.5–1.4)
Galactomyces candidum 2	0.1 NS	17	NA	0.8 (0.5–1.5)
Isaria fumosorosea	0.2 NS	17	NA	0.7 (0.4–1.2)
Mucor hiemalis	0.6 NS	16	NA	0.89 (0.5–1.4)
Untreated (–ctrl)[e]	/	14	NA	/
Beauveria bassiana (+ctrl)[f]	0.5 NS	16	NA	0.85 (0.5–1.4)

[a]P-value was calculated by comparing the negative control to each fungus, then analyzed by log-rank Mantel-Cox test; [b]Median indicates the median number of days the mosquitoes survived after exposure; [c]Total + SD indicates the day when 100% of the mosquitoes had died, with the corresponding standard error; [d]Hazard ratio (95% CI) is the hazard ratio with its 95% confidence interval, calculated with log-rank Mantel and Haenzel analysis; [e]ctrl–, negative control; [f]ctrl+, positive control; NS, not significant; NA, not achieved, since mosquitoes survived past day 20. The values in this table were calculated from three replicates.

**p < 0.05, **p < 0.05, ***p < 0.001, ****p < 0.0001.*

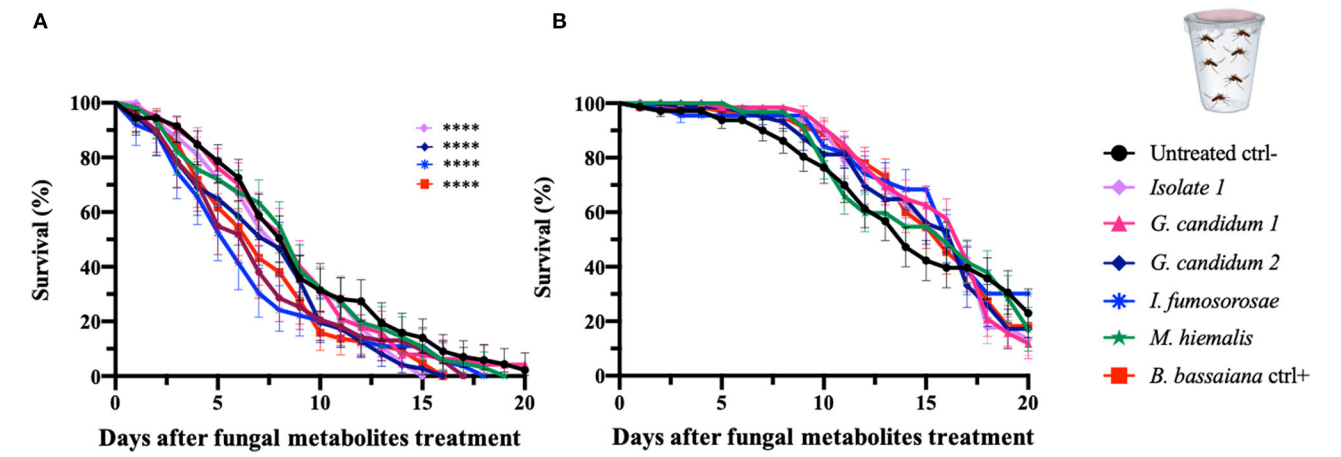

FIGURE 3 | Fungal metabolite assays of *An. gambiae* and *Ae. aegypti* mosquitoes. Mosquitoes were allowed to feed on a sucrose solution containing fungal metabolites (free from spores and mycelium) for 48 h, and the mosquito survival was monitored for 20 days. Data here are presented for *An. gambiae* **(A)** and *Ae. aegypti* **(B)**. The metabolites used in the experiments are derived from the fungi identified as active against *An. gambiae* in the initial screen (direct exposure experiment) and were collected from fungal liquid culture. The graphs represent the average and standard deviation of three biological replicates, $N = 30$ females per replicate. As a negative control, mosquitoes were fed on a metabolite-free solution, and as a positive control on metabolite solutions from *B. bassiana*. The statistical significance of the results for each fungus was compared to the negative control and analyzed by log-rank Mantel-Cox test; *p*-values are presented in **Table 4**.

to have both larvicidal and adulticidal activity against *Ae. aegypti* mosquitoes (Meepagala et al., 2018), and exposure to its conidia has been shown to have pathogenic activity against the strawberry pest *Duponchelia fovealist* (Amatuzzi et al., 2018). The *Fusarium*

sp. genus belongs to the Hypocreales order, as do *Beaveria* spp. and *Metarhizium* spp.; species within this genus have been shown to possess entomopathogenic activity against *Tribolium* sp., is an insect pest of stored grains (Chehri, 2017); a *F. verticillioides* isolate that is a grasshopper pathogen (Pelizza et al., 2011); and *F. oxysporum* extracts, which are lethal against larvae and pupae of multiple mosquito vectors such as *An. stephensi, Ae. aegypti,* and *Culex (Cx) quinquefasciatus* (Vivekanandhan et al., 2018a). *Isaria* sp. is a well-known entomopathogenic fungus, already commercialized for the control of different insect species in several countries. The only insecticidal activity of *Mucor hiemalis* has been shown to be correlated with its ability to secrete insecticidal metabolites against the adult stages of the agricultural pests *Bactrocera oleae* and *Ceratitis capitata* (Konstantopoulou et al., 2006). *Penicillium citrum* exerts lethal activity against larvae and adults of *Cx. quinquefasciatus* (Maketon et al., 2014). Finally, *Scopulariopsis* sp. has been found to be lethal to the agricultural pest *Bemisia tabaci* (Niu et al., 2019) (**Table 1**).

But among these eight already known as EPFs, we have here discovered three novel pathogenic fungi: isolate 1 that was previously unknown and un-characterized, two strains of *Galactomyces candidum* and *Mucor hiemalis*.

Our first screening for mosquitocidal activity was performed through surface exposure of female *An. gambiae* to plated 2-week-old fungal cultures that usually contained between 4×10^8 and 1×10^{10} spores/ml, depending on the fungal colony. This assay, that involves the shaking of mosquitoes on the fungal plate ensured an extensive surface exposure to not only spores, but also the fungal mycelia and hyphae and eventual toxic compounds produced by the fungi; however, the somewhat short lifespan of the control mosquitoes suggests that this procedure adversely affected *An. gambiae* viability, maybe due to the mechanical stress during the shaking on the agar plate. Nevertheless, we saw significant differences in survival between the non-exposed mosquitoes and several of the fungus-exposed cohorts, with a stringent statistical evaluation yielding a p-value of <0.0001. We identified five fungal isolates that significantly reduced the *An. gambiae* mosquitoes' lifespan and therefore were chosen for further investigation. These five were: isolate 1, *Galactomyces candidum* 1, *Galactomyces candidum* 2*, Isaria fumosorosea,* and *Mucor hiemalis. I. fumosorosea* also displayed a significant ($p < 0.0001$) killing activity against *Ae. aegypti* with the same direct exposure assay.

The isolate 1 fungus that displayed adulticidal activity against *An. gambiae* appears to be a novel mosquitocidal fungus that has not been previously investigated for pathogenic effects on insects, plants, or humans. It did display relatedness to *Aaosphaeria* sp. that has not been addressed with regards to these properties. *G. candidum* is a Saccharomycotina, a yeast-like fungus whose potential pathogenic activities have been investigated in previous studies, and recent laboratory-based experiments have shown killing activity against *Botrytis cinera,* a gray mold causing plant disease (Chen et al., 2018). Studies performed by Chen et al. have suggested that *Galactomyces sp.* is able to produce volatile compounds and chitinase enzymes that inhibit fungal growth. *Galactomyces sp.* is also used in dairy industry (Perkins et al., 2002). *I. fumosorosea* belongs to the Hypocreales order, which includes the well-studied entomopathogenic fungi *Beauveria* spp. and *Metarhizium* spp. *Isaria* sp. has been used as an environmentally friendly pest biocontrol agent in many countries, being registered as a biopesticide in the USA (United States Environmental Protection Agency (EPA)., 2020), in the European Union (European Commission (EU), 2020), and in Brazil (Ministerio de Agricultura, 2020). In China, although this fungus has not been registered as a myco-pesticide, it is widely used to control whiteflies and aphids (Zhang et al., 2016; Xu et al., 2017). Several studies have identified the genus *Isaria* sp. as a source of promising entomopathogenic candidates against adult *Ae. aegypti* and *An. stephensi* as well as *Ae. aegypti* eggs (Luz et al., 2007; Leles et al., 2010*;* Blanford et al., 2012*;* Ramirez et al., 2018). Furthermore, *Isaria* sp. mycelia extracts have been used for the development of silver nanoparticles that are able to kill *Ae. aegypti* and *Cx. quinquefasciatus* larvae (Banu and Balasubramanian, 2014). *M. hiemalis* is a saprophytic species belonging to the Zygomycetes, Mucorales. It is frequently found to infect injured insects (Heitor, 1962), but as far as we know it has not been previously studied with regard to mosquitocidal activity.

We also used a spore-based dipping assay that relied on exposure of mosquitoes to a liquid spore-containing solution to further study the fungi that had shown confirmed entomopathogenic activity in the direct-exposure assay. We reasoned that this method would enable us to expose the mosquitoes to the same concentration of spores in each replicate and that with 1 min of spore exposure we could assess the fungal infection in the mosquitoes, if the fungus was pathogenic. The actual procedure of dipping *An. gambiae* mosquitoes into the spore-free PBS solution seemed to affect mosquito viability, since the control cohort displayed a lifespan of only 14 days. A possible contributing factor to this decreased viability could have been the presence of detergent (Tween-80) that to some degree could permeabilize the external wax layer of the cuticle (Vincent, 2002). However, the generally more robust *Ae. aegypti* mosquitoes were not affected by this treatment. We found that *An. gambiae* exposed to *I. fumosorosea* as well as *M. hiemalis* and *Ae. aegypti* exposed to *I. fumosorosea* through direct exposure, when we assume a more extensive contact with spores occurred, displayed a shorter lifespan than did the mosquitoes exposed to the same fungi in the 10^8 spores/ml dipping assay. This likely spore dosage-dependent effect was also observed with *B. bassiana* fungus, for which an increased dose of spores was associated with a greater mortality in *Ae. aegypti* mosquitoes (Dong et al., 2012). These results confirm a previous study showing a dosage-dependent entomopathogenic activity of *I. fumosorosea* against *B. tabaci* nymphs (Gao et al., 2017). We also demonstrate here a spore dosage-dependence with regard to *M. hiemalis.* The killing activity caused by the fungal spores did not seem to be exposure method-dependent for all our fungal isolates. For example, exposure to isolate 1 resulted in a 100% mortality of *An. gambiae* by 6 days after dipping in the spore solution, whereas all the mosquitoes of this species treated with the direct-exposure method survived longer and died within 13 days after exposure. This observation highlights the importance of adapting the delivery, or exposure, method to the

physiological and morphological characteristics of a particular fungus. However, *An. gambiae* subjected to *G. candidum* 1 in the two different assays displayed an identical median and total mortality time-course, demonstrating a fungal killing mechanism that is not dependent on the mode of exposure. *G. candidum* 2, in contrast, showed higher mortality in the spore-dipping assay than in direct-exposure assay, and this result could potentially be explained by the presence of toxic compounds that, in the spore solution, were able to more efficiently come in contact with the mosquitoes. Mosquitoes, this result was also corroborate with the fungus-culture-filtrate experiment result. The ability of *G. candidum* to produce pathogenic compounds had already been shown in the work of Chen and co-workers, in which they demonstrated the production of volatile toxic compounds and a chitinase by *Galactomyces* that can affect the longevity of adult mosquitoes (Chen et al., 2018).

To investigate whether the mechanism of fungus-mediated killing required interaction with live fungi or was caused by a secreted fungus factor, we exposed *An. gambiae* and *Ae. aegypti* mosquitoes to a fungus-culture filtrate through a sugar meal. Our results indicated that isolate 1, *G. candidum* 2, and *I. fumosorosea* produce mosquitocidal compound(s) against *An, gambiae*. While isolate 1 has not been previously studied with regard to mosquitocidal activity, *G. candidum* and *I. fumosorosea* have previously been shown to produce entomopathogenic factors (Chen et al., 2018). Interestingly, only one of our two *Galactomyces sp.* isolates had this property, thereby demonstrating that different isolates of the same fungal species can produce different metabolites and thus display different entomopathogenic activities. *I. fumosorosea* has previously been shown to produce metabolites with antibacterial, insecticidal, antiviral, and cytotoxic activity (Weng et al., 2019). Ingestion of the fungal-culture filtrates, including that of *B. bassiana,* which is known to produce entomopathogenic metabolites (Bukhari et al., 2011; Mnyone et al., 2011; Vivekanandhan et al., 2018b), had no effect on *Ae. aegypti*. This result likely reflects the greater robustness of this mosquito species and also raises the possibility that some of the fungi-produced toxic factors were in too low concentration to exert an effect. It is also possible that a different route of exposure (i.e., surface exposure) could have yielded a different result with some of the tested fungal culture filtrates.

In summary, in this study we have isolated and characterized five potentially entomopathogenic fungi with activity against two major mosquito disease vectors. Among these fungi, four have never been tested on insect pests before: isolate 1, two strains of *Galactomyces* candidum and *Mucor hiemalis*. We further show and corroborate the particular potential of *Isaria fumosorosea* as a potential biopesticide candidate against various mosquito species.

These fungi merit further investigation as the source of novel biological agents for mosquito control.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University.

Sample Collection

Organic material with potential fungus growth was collected from different outdoor environments in Maryland and in Puerto Rico. Each sample was collected with sterile forceps, placed in a 1.5-ml tube with 200 μl of sterile 1X PBS and kept at 4°C until further use.

Culture and Isolation of Fungi

Each sample was manually homogenized with a pestle and plated on brain heart infusion (BHI) agar containing 50 μg/ml of chloramphenicol and left at room temperature until fungal growth was observed. From each plate, every different fungus that grew was single-subcultured until the fungal culture was axenic. All the fungi were maintained on BHI except for *Aaosphaeria arxii*, which was maintained on Sabouraud dextrose agar medium. *Beauveria bassiana* strain 80.2, kindly provided by Dr. Silverman, was used as a positive control and was maintained under the same conditions as the other fungi.

Identification of Isolated Fungi

DNA from 0.5 cm of fungal culture was extracted with a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The fungal biomass was homogenized for 2 min in 200 μl of lysis buffer using 0.5-mm sterile glass beads. Two pairs of degenerate fungal primers were used to amplify the ITS ribosomal 18S region: ITS1 forward and ITS3 forward (ITS1 5′-CTHGGTCATTTAGAGGAASTAA, ITS3 5′-FAHCG ATGAAGAACRYAG), and ITS4 reverse (ITS4 5′-RTCCT CCGCTTWTTGWTWTGC) (Toju et al., 2012). One primer pair was used to amplify the 18S small subunit-SSU (NS1-F 5′-GTAGTC ATATGCTTGTCTC, NS4-R 5′- CTT CCGTCAATTC CTTTAAG) and one pair for the 28S large sub unit LSU (LROR-F 5′-ACCCGCTGAACTTAAGC, LR6-R 5′-CGCCAGTTCTG CTTACC) (Raja et al., 2017).

The target amplicon was column-purified using a ZYMO kit and sent to Quintara Biosciences for Sanger sequencing, and the returned sequence was blasted in the NCBI database against fungus taxa for species identification. The ITS-18S sequences were also used to generate phylogenetic trees using the Bootstrap and Neighbor-joining statistical method with the p-distance model through the MEGAX software.

A morphological analysis of spores from a selection of fungal isolates was performed. Spores were obtained from 2 week old fungal cultures. PBS+ 0,02% tween 80 was added to the agar plate and the fungi was scraped loose, filtered through glass wool and transferred to Eppendorf tubes. Samples were centrifuged at 5,000 rpm for 5 min to remove any remaining debris. The supernatant was analyzed under bright field microscope and photos of spores were acquired at 40x magnification using a Leica DM2500 microscope and a DFC310 FX Digital Color Camera (Leica Microsystems).

Mosquito Rearing

Rockefeller strain *Ae. aegypti* and Keele strain *An. gambiae* were maintained in an insectary chamber at 27°C and 80% humidity on a 12-h light/dark cycle according to standard rearing procedures. The larvae were fed on sterile pulverized fish food, and adult mosquitoes were provided with a 10% sucrose solution. The mosquitoes for the experiments were kept in cups inside a large incubator provided with the same environmental conditions as in the insectary chamber described above.

Fungal Direct Exposure Assays

To assess the first screening experiments, 30 not blood-fed females, each 3–5 days old, were tested against fungi. Mosquitoes were cold-anesthetized, placed directly into an agar plate containing a 2-week-old fungal culture, and shaken for 30 s. Here, the mosquitoes are exposed to a complex content of fungal spores, fungal mycelia, the growing hyphae, and possible toxic compounds. Thereafter, the mosquitoes were placed in cups with 10% sucrose solution; to avoid bacterial and fungal growth, the sucrose paper was changed three times per week. A plate with *B. bassiana* was used as a positive control, and sterile BHI agar medium was used as a negative control.

All the direct exposure experiments were done once, and mosquito survival was checked daily for 20 days. For logistic reasons, the 22 fungal isolates against *An. gambiae* were tested at three different times, always including the positive and negative controls, and *p*-values were calculated against the respective negative controls.

Fungal Dipping-Based Exposure Assays

To confirm the mosquitocidal potential of the five pathogenic fungi, a fungal spore exposure assay was performed on 30 female *An. gambiae* and 30 female *Ae. aegypti* mosquitoes, only against those fungi for which a *p*-value of <0.0001 was obtained from direct exposure. The fungal spore exposure was done using purified spores and the dipping procedure (Dong et al., 2012), with the mosquitoes exposed to a solution of 10^8 spores/ml. The spores were collected from 2-week-old fungal plate cultures, counted with a Neubauer hemocytometer, and adjusted to 10^8 spores/ml. In brief, mosquitoes were placed, back first, into 2 ml of the spore solution for 1 min. Only the back of the mosquito came in contact with the spore solution. *B. bassiana* spore solution was used as a positive control, and 1X PBS with 0.02% tween-80, used to collect spores, was used as a negative control. Mosquito sugar papers were changed three times per week. The dipping experiments were done in triplicate, and mosquito survival was checked daily for 20 days.

Fungus Culture Filtrate-Feeding Assays

To discriminate the fungal killing activity as a result of fungal proliferation on mosquito bodies from the potential presence of toxic compounds, 30 females of *An. gambiae* and *Ae. aegypti* mosquitoes were exposed to fungal metabolites through a feeding assay. Fungal metabolites were collected from a 2-week-old fungal liquid culture (potato dextrose medium) (Vandermolen et al., 2013) by filtration through a 0.2-μm filter, then added to a 10% sucrose solution to give a final concentration of 5% sucrose. Metabolites produced by *B. bassiana* were used as a positive control, and filtered potato dextrose broth with sucrose was used as a negative control. Mosquitoes were fed on the metabolite/sucrose solution for 48 h, and then the solution was replaced with 10% sucrose. The sucrose papers were changed three times per week as for all other experiments. To confirm that the mosquitoes had died as a consequence of the activity of the metabolites and not because of fungal growth, dead mosquitoes were checked each week under an optical microscope to confirm the absence of fungal proliferation on the mosquito bodies. These assays were run in triplicate, and the mosquito survival rate was checked daily for 20 days.

Statistical Analysis

Mortality was expressed as a median, and the final day of mosquito survival and the hazard ratio were calculated by Manten and Haenzel analysis. The statistical significance of survival curves was set to the conventional $\alpha < 0.05$ level, calculated with a long-rank Mantel-Cox analysis and using Graphpad Prism software, version 8.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the Johns Hopkins University.

AUTHOR CONTRIBUTIONS

AA and CE performed the experiments. All authors conceived experiments, analyzed the data obtained, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2021.637234/full#supplementary-material

REFERENCES

Alkhaibari, A. M., Carolino, A. T., Bull, J. C., Samuels, R. I., and Butt, T. M. (2017). Differential pathogenicity of metarhizium blastospores and conidia against larvae of three mosquito species. *J. Med. Entomol.* 54, 696–704. doi: 10.1093/jme/tjw223

Amatuzzi, R. F., Cardoso, N., Poltronieri, A. S., Poitevin, C. G., Dalzoto, P., Zawadeneak, M. A., et al. (2018). Potential of endophytic fungi as biocontrol agents of *Duponchelia fovealis* (Zeller) (Lepidoptera:Crambidae). *Braz. J. Biol.* 78, 429–435. doi: 10.1590/1519-6984.166681

Banu, A. N., and Balasubramanian, C. (2014). Optimization and synthesis of *Isaria* delivered by silver nanoparticles resulted lethal for *Ae aegypti* and *Cx quinquefasciatus* larvae. *Parasitol. Res.* 113, 3843–3851. doi: 10.1007/s00436-014-4052-0

Beckage, N. E., Marin, K. M., Walton, W. E., Wirth, M. C., and Tan, F. F. (2004). Comparative larvicidal toxicities of three ecdysone agonists on the mosquitoes *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*. *Arch. Insect Biochem. Physiol.* 57, 111–122. doi: 10.1002/arch.20021

Blanford, S., Chan, B. H., Jenkins, N., Sim, D., Turner, R. J., Read, A. F., et al. (2005). Fungal pathogen reduces potential for malaria transmission. *Science* 308, 1638–1641. doi: 10.1126/science.1108423

Blanford, S., Jenkins, N. E., and Read, A. F. (2012). Evaluating the lethal and pre-lethal effects of a range of fungi against adult *Anopheles stephensi* mosquitoes. *Malar. J.* 11:365. doi: 10.1186/1475-2875-11-365

Blanford, S., Shi, W., Christian, R., Marden, J. H., Koekemoer, L. L., Brooke, B. D., et al. (2011). Lethal and pre-lethal effects of a fungal biopesticide contribute to substantial and rapid control of malaria vectors. *PLoS ONE* 6:e23591. doi: 10.1371/journal.pone.0023591

Bukhari, T., Takken, W., and Koenraadt, C. J. M. (2011). Development of *Metarhizium anisopliae* and *Beauveria bassiana* formulations for control of malaria mosquito larvae. *Parasit. Vectors* 4:23. doi: 10.1186/1756-3305-4-23

Butt, T. M., Coates, C. J., Dubovskiyx, I. M., and Ratcliffe, N. A. (2016). Entomopathogenic fungi: new insights into host pathogen interactions. *Adv. Genet.* 94, 307–364. doi: 10.1016/bs.adgen.2016.01.006

Chandler, D., Hay, D., and Reid, A. P. (1997). Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Appl. Soil Ecol.* 5, 133–141. doi: 10.1016/S0929-1393(96)00144-8

Charnley, A. K., and Collins, S. A. (2007). "Entomopathogenic fungi and their role in pest control," in *The Mycota IV: Environmental and Microbial Relationships, 2nd Edn*, eds C. P. Kubicek and I. S. Druzhinina (Cham: Springer), 159–187.

Chehri, K. (2017). Molecular identification of entomopathogenic *Fusarium* species associated with Tribolium species in stored grains. *J. Invertebr. Pathol.* 144, 1–6. doi: 10.1016/j.jip.2017.01.003

Chen, P. H., Chen, R. Y., and Chou, J. Y. (2018). Screening and evaluation of yeast antagonists for biological control of *Botrytis cinerea* on strawberry fruits. *Mycobiology* 46, 33–46. doi: 10.1080/12298093.2018.1454013

de Faria, M. R., and Wraight, S. P. (2007). Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biol. Control* 43, 237–256. doi: 10.1016/j.biocontrol.2007.08.001

Dong, Y., Morton, J. C., Ramirez, J. L., and Souza-Neto, J. A. (2012). The entomopathogenic fungus *Beauveria bassiana* activate toll and JAK-STAT pathway-controlled effector genes and anti-dengue activity in *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 42, 126–132. doi: 10.1016/j.ibmb.2011.11.005

Dubovskiy, M., Whitten, M. M. A., Yaroslavtseva, O. N., Greig, C., Kryukov, V. Y., Grizanova, E. V., et al. (2013). Can insects develop resistance to insect pathogenic fungi? *PLoS ONE* 8:e60248. doi: 10.1371/journal.pone.0060248

Elbanhawy, A. A., Elsherbiny, E. A., El-Mageed, A. E. A., and Abdel-Fattah, G. M. (2019). Potential of fungal metabolites as a biocontrol agent against cotton aphid, *Aphis gossypii* Glover and the possible mechanisms of action of fungal metabolites as a biocontrol agent against cotton aphid. *Pestic. Biochem. Physiol.* 159, 34–40. doi: 10.1016/j.pestbp.2019.05.013

Eliskases-Lechner, F., Guéguen, M., and Panoff, J. M. (2011). "Yeasts and molds, *Geotrichum candidum*," in *Encyclopedia of Dairy Sciences, 2nd Edn*, ed J. W. Fuquay (Amsterdam: Academic Press), 765–771.

European Commission (EU) (2020). *Pesticides*. Available online at: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/ (accessed October 13, 2020).

Farenhorst, M., Hilhorst, A., Thomas, M. B., and Knols, B. G. J. (2011). Development of fungal applications on netting substrates for malaria vector control. *J. Med. Entomol.* 48, 305–313. doi: 10.1603/ME10134

Farenhorst, M., Knols, B. G. J., Thomas, M. B., Howard, A. F. V., Takken, W., Rowland, M., et al. (2010). Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PLoS ONE* 5:e12081. doi: 10.1371/journal.pone.0012081

Farenhorst, M., Mouatcho, J. C., Kikankie, C. K., Brooke, B. D., Hunt, R. H., Thomas, M. B., et al. (2009). Fungal infection counters insecticide resistance in African malaria mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17443–17447. doi: 10.1073/pnas.0908530106

Ffrench-Constant, R. H. (2005). Something old, something transgenic, or something fungal for mosquito control? *Trends Ecol. Evol.* 20, 577–579. doi: 10.1016/j.tree.2005.08.007

Gao, T., Wang, Z., Huang, Y., Keyhani, N. O., and Huang, Z. (2017). Lack of resistance development in *Bemisia tabaci* to *Isaria fumosorosea* after multiple generations of selection. *Sci. Rep.* 7:42727. doi: 10.1038/srep42727

Gräfenhan, T., Schroers, H. J., Nirenberg, H. I., and Seifert, K. A. (2011). An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in *Cosmospora*, *Acremonium*, *Fusarium*, *Stilbella*, and *Volutella*. *Stud. Mycol.* 68, 79–113. doi: 10.3114/sim.2011.68.04

Hancock, P. A. (2009). Combining fungal biopesticides and insecticide-treated bednets to enhance malaria control. *PLoS Comput. Biol.* 5:e1000525. doi: 10.1371/journal.pcbi.1000525

Hancock, P. A., Thomas, M. B., and Godfray, H. C. J. (2009). An age-structured model to evaluate the potential of novel malaria-control interventions: a case study of fungal biopesticide sprays. *Proc. Biol. Sci.* 276, 71–80. doi: 10.1098/rspb.2008.0689

Heitor, F. (1962). Parasitisme de blessure par le champignon *Mucor hiemalis* Wehmer chez les insectes. *Ann. Epiphyt.* 13, 179–203.

Knols, B. G., Bukhari, T., and Farenhorst, M. (2010). Entomopathogenic fungi as the next-generation control agents against malaria mosquitoes. *Future Microbiol.* 5, 339–341. doi: 10.2217/fmb.10.11

Konstantopoulou, M. A., Milonas, P., and Mazomenos, B. E. (2006). Partial purification and insecticidal activity of toxic metabolites secreted by a *Mucor hiemalis* Strain (SMU-21) against adults of *Bactrocera oleae* and *Ceratitis capitata* (Diptera: Tephritidae). *J. Econ. Entomol.* 99,1657–1664. doi: 10.1093/jee/99.5.1657

Lacey, L. A., Fransen, J. J, and Carruthers, R. (1996). "Global distribution of naturally occurring fungi of Bemisia, their biologies and use as biological control agents," in *Bemisia, Taxonomy, Biology, Damage, Control and Management*, eds D. Gerling and R. T. Mayer (Andover: Intercept), 401–433.

Leles, R. N., Sousa, N. A., Rocha, L. F. N., Santos, A. H., Silva, H. H. G., and Luz, C. (2010). Pathogenicity of some hypocrealean fungi to adult *Aedes aegypti* (Diptera: Culicidae). *Parasitol. Res.* 107, 1271–1274. doi: 10.1007/s00436-010-1991-y

Luz, C., Tai, M. H. H., Santos, A. H., Rocha, L. F. N., Albernaz, D. A. S., and Silva, H. H. G. (2007). Ovicidal activity of entomopathogenic hyphomycetes on *Aedes aegypti* (Diptera: Culicidae) under laboratory conditions. *J. Med. Entomol.* 44, 799–804. doi: 10.1093/jmedent/44.5.799

Maketon, M., Amnuaykanjanasin, A., and Kaysorngup, A. (2014). A rapid knockdown effect of *Penicillium citrinum* for control of the mosquito *Culex quinquefasciatus* in Thailand. *World J. Microbiol. Biotechnol.* 30, 727–736. doi: 10.1007/s11274-013-1500-4

Meepagala, K. M., Estep, A. S., Clausen, B. M., and Becnel, J. J. (2018). Mosquitocidal activity of a naturally occurring isochroman and synthetic analogs from the plant pathogenic fungus, *Diaporthe eres* against *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* 55, 969–974. doi: 10.1093/jme/tjy016

Ministerio de Agricultura, Agrofit Consulta Aberta (2020). *Ingredientes ativos*. Available online at: http://agrofit.agricultura.gov.br/agrofit_cons/ (accessed October 13, 2020).

Mnyone, L. L., Kirby, M. J., Mpingwa, M. W., Lwetoilera, D. W., Knols, B. G. J., Takken, W., et al. (2011). Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood feeding stage. *Parasitol. Res.* 108, 317–322. doi: 10.1007/s00436-010-2064-y

Moyes, C. L., Vontas, J., Martins, A. J., Ching, N.g L., Koou, S. Y., Dusfour, I., et al. (2017). Contemporary status of insecticide resistance in the major *Aedes*

vectors of arboviruses infecting humans. *PLoS Negl. Trop. Dis*. 11:e0005625. doi: 10.1371/journal.pntd.0005625

Niu, G., Wang, B., Zhang, G., King, J. B., Cichewicz, T. R. H., and Li, J. (2015). Targeting mosquito FREP1 with a fungal metabolite blocks malaria transmission. *Sci. Rep*. 5:14694. doi: 10.1038/srep14694

Niu, X., Xie, W., Zhang, J., and Hu, Q. (2019). Biodiversity of entomopathogenic fungi in the soils of South China. *Microorganisms* 7:311. doi: 10.3390/microorganisms7090311

Pelizza, S. A., Stenglein, S. A., Cabello, M. N., Dinolfo, M. I., and Lange, C. E. (2011). First record of *Fusarium verticillioides* as an entomopathogenic fungus of grasshoppers. *J. Insect Sci.* 11:70. doi: 10.1673/031.011.7001

Perkins, V., Vignola, S., Lessard, M. H., Plante, P. L., Corbeil, J., Dugat-Bony, E., et al. (2002). Phenotypic and genetic characterization of the cheese ripening yeast *Geotrichum candidum*. *Front. Microbiol.* 11:737. doi: 10.3389/fmicb.2020.00737

Raja, H. A., Miller, A. N., Pearce, C. J., and Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *J. Nat. Prod*. 80, 756–770. doi: 10.1021/acs.jnatprod.6b01085

Ramirez, J. L., Muturi, E. J., Dunlap, C., and Rooney, A. P. (2018). Strain-specific pathogenicity and subversion of phenoloxidase activity in the mosquito *Aedes aegypti* by members of the fungal entomopathogenic genus *Isaria*. *Sci. Rep.* 8:9896. doi: 10.1038/s41598-018-28210-6

Read, A. F., Lynch, P. A., and Thomas, M. B. (2009). How to make evolution- proof insecticides for malaria malaria control. *PLoS Biol.* 7:e1000058. doi: 10.1371/journal.pbio.1000058

Riveron, J. M., Tchouaku, M., Mugenzi, L., Menze, B. D., Chiang, M. C., and Wondji, C. S. (2018). "Insecticide resistance in malaria vectors: an update at a global scale," in *Towards Malaria Elimination: A Leap Forward*, eds S. Manguin and V. Dev (London: IntechOpen Limited), 150–175. doi: 10.5772/intechopen.78375

Sánchez-Peña, S. R., Lara, J. S. J., and Medina, R. F. (2011). Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, Mexico, and their virulence towards thrips and whiteflies. *J. Insect Sci.* 11:1. doi: 10.1673/031.011.0101

Scholte, E., Ng'habi, K., Kihonda, J., Takken, W., Paaijmans, K., Abdulla, S., et al. (2005). An Entomopathogenic fungus for control of adult african malaria mosquitoes. *Science* 308, 1641–1642. doi: 10.1126/science.1108639

Singh, B., Kaur, T., Kaur, S., Manhas, R. K., and Kaur, A. (2015). Insecticidal potential of an endophytic *Cladosporium velox* against *Spodoptera litura* mediated through inhibition of alpha glycosidases. *Pestic. Biochem. Physiol.* 131, 46–52. doi: 10.1016/j.pestbp.2016.01.004

Singh, G., and Prakash, S. (2012). Lethal effects of *Aspergillus niger* against mosquitoes vector of filaria, malaria, and dengue: a liquid mycoadulticide. *Sci. World J*. 2012:603984. doi: 10.1100/2012/603984

Sun, T., Shen, Z., Shaukat, M., Du, C., and Ali, S. (2020). Endophytic isolates of *Cordyceps fumosorosea* to enhance the growth of solanum melongena and reduce the survival of whitefly (*Bemisia tabaci*). *Insects* 11:78. doi: 10.3390/insects11020078

Toju, H., Tanabe, A. S., Yamamoto, S., and Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* 7:e40863. doi: 10.1371/journal.pone.0040863

Udayanga, D., Liu, X., McKenzie, E. H. C., Chukeatirote, E., Bahkali, A. H. A., and Hyde, K. D. (2011). The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. *Fungal Divers*. 50:189. doi: 10.1007/s13225-011-0126-9

United States Environmental Protection Agency (EPA). (2020). *Eu Pesticides Database*. Available online at: https://www.epa.gov/pesticides/ (accessed October 13, 2020).

Vandermolen, K. M., Raja, H. A., El-Elimat, T., and Oberlies, N. H. (2013). Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *AMB Express*. 3:71. doi: 10.1186/2191-0855-3-71

Vincent, J. F. V. (2002). Arthropod cuticle: a natural composite shell system. *Appl. Sci. Manuf*. 33, 1311–1315. doi: 10.1016/S1359-835X(02)00167-7

Vivekanandhan, P., Bedinib, S., and Shivakumar, M. S. (2020b). Isolation and identification of entomopathogenic fungus from Eastern Ghats T of South Indian forest soil and their efficacy as biopesticide for mosquito control. *Parasitol. Int.* 76:102099. doi: 10.1016/j.parint.2020.102099

Vivekanandhan, P., Karthi, S., Shivakumar, M. S., and Benelli, G. (2018a). Synergistic effect of entomopathogenic fungus *Fusarium oxysporum* extract in combination with temephos against three major mosquito vectors. *Pathog. Glob. Health* 112, 37–46. doi: 10.1080/20477724.2018.1438228

Vivekanandhan, P., Kavitha, T., Karthi, S., Senthil-Nathan, S., and Shivakumar, M. (2018b). Toxicity of *Beauveria bassiana*-28 mycelial extracts on larvae of *Culex quinquefasciatus* mosquito (Diptera: Culicidae). *Int. J. Environ. Res. Public Health* 15:440. doi: 10.3390/ijerph15030440

Vivekanandhan, P., Swathy, K., Kalaimurugan, D., Ramachandran, M., and Yuvaraj, A. (2020a). Larvicidal toxicity of *Metarhizium anisopliae* metabolites against three mosquito species and non-targeting organisms. *PLoS ONE* 15:e0232172. doi: 10.1371/journal.pone.0232172

Weng, Q., Zhang, X., Chen, W., and Hu, Q. (2019). Secondary metabolites and the risks of *Isaria fumosorosea* and *Isaria farinose*. *Molecules* 24:664. doi: 10.3390/molecules24040664

World Health Organization (WHO). (2020). *Vector-borne diseases*. Available online at: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases (accessed October 1, 2020).

Xu, J., Xu, X., Shakeel, M., Li, S., Wang, S., Zhou, X., et al. (2017). The entomopathogenic fungi *Isaria fumosorosea* plays a vital role in suppressing the immune system of *Plutella xylostella*: Rna-seq and dge analysis of immunity-related genes. *Front. Microbiol.* 8:1421. doi: 10.3389/fmicb.2017.01421

Zhang, B., Zou, C., and Hu, Q. (2016). Effects of *Isaria fumosorosea* on TYLCV (tomato yellow leaf curl virus) accumulation and transmitting capacity of *Bemisia tabaci*. *PLoS ONE* 11:e0164356. doi: 10.1371/journal.pone.0164356

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