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Host-Pathogen Interactions

Edited by
Ivan M. Dubovskiy

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Insects vs. Fungi

Editor

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About the Editor

Ivan M. Dubovskiy specializes in insect immune, detoxification and antioxidant systems, coevolution of host–parasite systems—especially microevolution of insect defense reactions— and in developing new approaches for increasing bio insecticides' effectivity against insect pests.

He is a member of the Russian Entomology Society, the Society for Invertebrate Pathology, and the Russian Parasitology Society.

Journal of *Fungi*

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Editorial

Host–Pathogen Interactions: Insects vs. Fungi

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Although many insects successfully live in dangerous environments exposed to diverse communities of microbes, they are often exploited and killed by specialist pathogens. In the process of co-evolution of insects and entomopathogenic microorganisms, they develop various adaptive systems that determine the sustainable existence of dynamic host–parasite interactions at both the organismic and population levels. Many different species of fungi are associated with insects. It should be noted that the diversity of fungi largely depends on the specific insect–fungus system. Thus, in the population of *Chilo suppressalis*, a serious pest of rice, in northern Iran *Beauveria bassiana*, *Akanthomyces lecanii*, *Akanthomyces muscarious*, *Metarhizium anisopliae*, *Hirsutella subulata*, and *Trichoderma* sp. persisted [1]. Lepidopteran forest-pest species *Ematurga atomaria*, *Cabera pusaria*, *Hypomecis punctinalis*, and *Orthosia gothica* were associated with members of Cordycipitaceae (*Akanthomyces muscarius* and *Cordyceps farinosa*) and fungi from families *Aspergillaceae*, *Nectriaceae*, *Mortierellaceae*, *Hypocreaceae*, etc. [2]. The host defences are designed to exclude the pathogen or mitigate the damage inflicted, while the pathogen counters with immune evasion and utilization of host resources. Transcriptome (RNAseq) analysis of immune response uncovers new abilities to study host–parasite systems. Study of cricket *Gryllus bimaculatus* transcriptome demonstrated high tissue-specific variety in inducing antifungal immune factors [3]. Entomopathogenic fungi (EPF) neutralize their immediate surroundings on the insect integument and benefit from the physiochemical properties of the cuticle and its compounds that exclude competing microbes. Interestingly, in some cases EPF have low virulence because plant phytochemicals can demonstrate antimicrobial activity on insects cuticle [4]. EPF interplay host defence with factors which regulate adhesion to the cuticle, cuticle degradation, stress management and toxins [5]. Thus *B. bassiana* express bassianolide and beauvericin toxins during infection of the bug *Triatoma infestans* [6] and proteases, chitinases and lipase in the presence of *C. suppressalis* cuticle probably to pass the insects defence faster [1]. It was found that EPF peroxisome-type and hexagonal crystal-like organelles (Woronin bodies) are required for appressorium differentiation and the topical infection of insect hosts [7]. Insects' immune, detoxification, and antioxidant systems work synergistically to combat infections and mitigate stress. Some proteins demonstrate multifunctional properties, participating in metabolism, homeostasis, and pathogen recognition [8]. Besides, insect hormones such as juvenile hormone [9] and dopamine [10] have been suggested to be a potential mediator in the insects' immunity against fungi.

The application of EPF in the field needs high-quality scientific support to establish the mechanisms of action and ways to improve fungal biological preparations [11,12]. There are some cases in which an insect's microbiota [13] and nematodes [14] may influence the development of fungal infections. These facts could open new abilities for the development of a complex approach to plant biological protection.

I would like to thank all contributors to this Special Issue on "Host–Pathogen Interactions: Insects vs. Fungi" for their significant contributions to this Special Issue and for making it a highly successful and timely collection of studies. I am extremely happy that we received eleven reviews/original papers for publication.

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Article

Fungi Recorded on Folivorous Lepidoptera: High Diversity Despite Moderate Prevalence

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Abstract: The knowledge about the diversity and ecological role of entomopathogenic fungi is primarily based on agroecosystems whereas information derived from natural insect populations is much more limited. To contribute to filling this gap, we recorded the prevalence of fungal infections in laboratory rearing experiments with five species of Lepidoptera, and in a field rearing experiment including one of these moths. The diversity of detected fungi was found to be high; we isolated 25 species of fungi from insects that had died in the course of these experiments. Six species belonged to the family Cordycipitaceae known to include unambiguous insect pathogens. The trophic niche of the representatives of other taxa is less clear and requires further studies. Regarding the most abundant species, *Cordyceps farinosa*, in which this question could be addressed, there was no indication of specialization on particular insect hosts, whereas several of the less common species may have been recorded from lepidopteran hosts for the first time. Across the subsets of the data, the prevalence of fungal infections generally remained below 5%. Our results are thus consistent with the idea that entomopathogenic fungi are always present in insect populations but rarely reach epizootic levels. The detected species richness shows that much is to be gained from mapping the diversity of fungal species associated with folivorous insects in natural populations.

Keywords: Geometridae; Hypocreales; mortality; moth; larva; pupa; entomopathogen

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1. Introduction

Focusing on the interactions of insects with plants and microbes has been considered the key to better understand various mechanisms behind ecosystem functioning [1]. The significance of insects is modulated by their abundance and the diversity of biotic interactions, most prominently of those with various natural enemies. As insects cause great losses in forestry and agriculture, the understanding of the biology of their natural enemies is of high importance also in the applied context [2–4]. Thus far, parasitoids and vertebrates have received most research effort in the context of ecological studies on insects [5,6]. Naturally, pathogens have also not escaped attention as antagonists of insects [7–9]; among those, viruses have had a central role in ecological studies [10]. Moreover, from the late 1990s, there has been an exponential growth in studies focusing on the use of fungi as biocontrol agents [2,11]. However, the knowledge about the role of entomopathogenic fungi in natural settings is still scarce, despite the almost two centuries long awareness about these organisms in the scientific community [12].

To contribute to filling this gap, we recorded the diversity and prevalence of lethal fungal pathogens in laboratory rearing of lepidopteran larvae that represented offspring of field-collected females, fed with field-collected host plants. The assemblage of fungal pathogens in the laboratory was compared to that recorded in a field experiment in which

moth larvae were reared in a seminatural setting. The focus was set on plant- and airborne fungal infections relevant for folivorous larvae, with the interactions of soil-dwelling pupae [2,3] remaining beyond the scope of the present paper. We discuss the patterns of diversity and abundance of insect-associated fungi on lepidopteran hosts, and report taxa not recorded on this group of insects previously.

2. Materials and Methods

2.1. Laboratory Rearing

Data on fungal pathogens present in laboratory rearing of Lepidoptera were obtained as a by-product of ecological experiments performed at the University of Tartu, Estonia, in 2014-2017. Four lepidopteran species—*Ematurga atomaria* L., *Cabera pusaria* L., *Hypomecis punctinalis* Scopoli (Geometridae), and *Orthosia gothica* L. (Noctuidae)—were subjected to identical experimental design ([13–15], and unpublished). Specifically, in order to record growth rates, developmental periods, and final weights, we reared the larvae from eggs to pupae singly in 50 mL plastic vials at different temperatures and weighed them periodically. Within each combination of year and species, the insects were reared simultaneously under common garden design, while the timing of experiments with different species did not coincide due to natural phenological differences of the insects. The data on the fifth species, *Chiasmia clathrata* L. (Geometridae), were obtained from a technically similar study in which growth parameters of selection lines were compared (Välimäki et al., unpublished). The larvae were fed with leaves of host plants collected in the field in Tartu, or surroundings of the town. The larvae of *E. atomaria* were fed on *Trifolium repens* L. (Fabaceae), *Vaccinium myrtillus* L. (Ericaceae) and *Salix alba* L. (Salicaceae); *C. pusaria* on *Alnus glutinosa* (L.) Gaertn. (Betulaceae); *H. punctinalis* on *Betula pendula* Roth. (Betulaceae), *Tilia cordata* Mill. (Tiliaceae), and *Quercus robur* L. (Fagaceae); *O. gothica* on *B. pendula*; and *C. clathrata* on *Lathyrus pratensis* L. (Fabaceae).

The larvae were allowed to pupate in moist *Sphagnum* moss, known for its antiseptic properties. This should have minimized the insects' risk of being infected during the pupal period, allowing us to focus on infections acquired during the larval stage. The pupae were kept overwinter in thermoregulated chambers at about 0 °C. In spring, adult moths were allowed to eclose at room temperature. The pupae that failed to eclose and eventually died were inspected for visual signs of fungal infection.

2.2. Field Experiment

To record the community of insect-associated fungi in near natural conditions, we reared larvae of *C. pusaria* on living wild host plants in mixed forest fragments in the surroundings of Tartu (between 58°26′ N, 26°30′ E and 58°24′ N, 26°39′ E), Estonia, in the course of 2 years. Newly hatched larvae were enclosed in 50 × 30 cm^2 polyester bags (N = 81), in which they remained until pupation. Ten larvae per bag were placed on 3 different host plants of the moth—grey alder (*Alnus incana* (L.) Moench), silver birch (*Betula pendula* Roth.), and downy birch (*B. pubescens* Ehrh.)—in the first half of July 2016 and 2017 and were checked weekly for pupation. After pupation, the insects were placed individually into Empera 124 N polystyrene vials with sterilized peat moss (*Sphagnum* sp.). The moss was sterilized by keeping it at 100 °C for 4 h. Vials were sterilized with 10% NaOCl. Pupae overwintered in thermoregulated chambers at 2 °C for 3 months. In January, adult moths were allowed to eclose at 24 °C and 12:12 h of light/darkness cycle. Insects that died during the pupal period were inspected for visual signs of fungal infection.

2.3. Identification of Fungi and Their Host Ranges

To identify the fungi and preserve these as pure living cultures, we inoculated visible fungal material (only anamorphs were encountered) to Petri dishes with 2% malt extract agar (Oxoid, Cambridge, UK) supplemented with antibiotics (1% of streptomycin and tetracycline). After a few weeks of growth in culture, the fungi were identified on the basis of morphological traits using keys provided by Domsch et al. [16], Samson et al. [12], and

Seifert et al. [17]. A culture isolate representing each morphotype was subjected to DNA extraction to confirm the identification.

The procedures of growing the mycelium, extracting DNA, conducting PCR, and sequencing followed the protocols described by Põldmaa et al. [18]. Ribosomal DNA full ITS and partial LSU sequences were obtained from 91 fungal isolates. The sequences along with their metadata were uploaded in PlutoF, a data management and publishing platform [19], and made available via UNITE database [20]. The ITS rDNA sequences were incorporated in the UNITE species hypotheses (SH), which served as the basis for species identification, by choosing an appropriate distance threshold value in each case (Table 1). The Basic Local Alignment Search Tool (blastn) at National Centre for Biotechnology Information [21] was used to check for similar sequences not yet incorporated in the UNITE database.

Table 1. Species of fungi isolated from lepidopteran hosts (the field experiment data in brackets). UNITE species hypothesis (SH) codes are presented to facilitate communication on detected fungi [22].

Order/Family	Species	SH DOI *	Hosts **
Hypocreales/Cordycipitaceae	*Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha	SH1886969.08FU	EA 8 CP 4(+25) HP 1
	Cordyceps bifusispora O.E. Erikss.	SH1887323.08FU	CP (2) EA 1
	Cordyceps farinosa (Holmsk.) Kepler, B. Shrestha & Spatafora	SH1524463.08FU	EA 29 CP 6(+10) HP 7 UP 1
	Lecanicillium praecognitum Gorczak & Kisło	SH1524455.08FU	CC 1 EA 4
	Simplicillium aogashimaense Nonaka, Kaifuch i & Masuma	SH1988378.08FU	EA 1
	Simplicillium lanosoniveum (J.F.H Beyma) Zare & W. Gams	SH1988383.08FU	OG 1 HP 1
Clavicipitaceae	*Metapochonia bulbillosa* (W.Gams & Malla) Kepler, S.A.Rehner & Humber	SH1930500.08FU	CP 1(+2) EA 1
Hypocreaceae	*Trichoderma* cf. *aethiopicum* Mullaw, C.P. Kubicek & Samuels	SH1568015.08FU	CP (7)
	Trichoderma koningii Oudem.	SH2303517.08FU	CP 1 EA 1
	Trichoderma trixiae/virilente/viridescens	SH2303501.08FU	CP 1
	Trichoderma viride Pers.	SH2303512.08FU	EA 1
Nectriaceae	*Mariannaea camptospora* Samson	SH1506679.08FU	EA 1
	Fusarium cf. *sporotrichioides* Sherb.	SH2456045.08FU	EA 1 OG 1
	Fusarium solani species complex	SH2228332.08FU	HP 1
	Fusarium tricinctum (Corda) Sacc.	SH1919083.08FU	EA 2 HP 1
Tilachlidiaceae	*Tilachlidium brachiatum* (Batsch) Petch	SH1513367.08FU	CP (5) CC 1 EA 2
Eurotiales/Aspergillaceae	*Penicillium thomii* Maire	SH2189918.08FU	EA 1 CP (12)
	Penicillium sp.	SH2283940.08FU	CP 1 EA 4 UP 1
	Penicillium paczoskii K.W. Zaleski	SH2189912.08FU	CP 1
Dothideales/Saccotheciaceae	*Aureobasidium pullulans* (de Bary) G. Arnaud	SH1872652.08FU	EA 2
Capnodiales/Cladosporiaceae	*Cladosporium delicatulum* Link	SH2320203.08FU	HP 1
Mucorales/Mucoraceae	*Mucor hiemalis* Wehmer	SH1989679.08FU	CP (17)
	Mucor plumbeus Bonord.	-	CP (25)
Mortierellales/Mortierellaceae	*Mortierella humilis* Linnem. ex W. Gams	SH2444324.08FU	EA 4 CP (1)
Umbelopsidales/Umbelopsidaceae	*Umbelopsis* sp.	-	EA 1

* DOI = digital object identifier, SH DOI is displayed here as a short code; ** insect hosts abbreviated as EA—*Ematurga atomaria*, CP—*Cabera pusaria*, HP—*Hypomecis punctinalis*, CC—*Chiasmia clathrata*, OP—*Orthosia gothica*, UP—unidentified pupa.

All pupae infected with a fungus were deposited at the TU fungarium (accession numbers TU133001-133196) and representative isolates at the TFC culture collection (TFC202234-202344) in the Natural History Museum and Botanical Garden, University of Tartu.

3. Results

3.1. Fungi from the Laboratory Experiments and Their Prevalence

Data on 2978 lepidopteran pupae were obtained from the laboratory rearing experiments (Table 2). Fungi were detected from 82 pupae (2.8%) and identified as representing 23 species from 10 families and 6 orders (Table 1). The prevalence of fungal infections differed among the 3 years but remained below 10% (Table 2, note the small sample sizes of *C. pusaria* and *H. punctinalis* in 2016, associated with atypically high prevalence). The majority of the fungi belonged to Cordycipitaceae (59 pupae infected), followed by Aspergillaceae (8), Nectriaceae (7), Mortierellaceae (4), Hypocreaceae (4), Tilachlidiaceae (3), Clavicipitaceae (2), Saccotechiaceae (2), Umbelopsidaceae (1), and Cladosporiaceae (1).

Table 2. Incidence of fungal infection in lab reared moths in different years.

Host Species	Year	Pupae	Fungal Prevalence %
Ematurga atomaria	2014	383	2.3
	2015	327	5.2
	2016	554	6
Cabera pusaria	2014	179	0
	2015	286	3.1
	2016	13	53.8
Chiasmia clathrata	2014	409	0.5
Orthosia gothica	2014	462	0.4
Hypomecis punctinalis	2015	360	1.7
	2016	5	60

On the basis of previous knowledge [12], all of the Cordycipitaceae (six species, overall prevalence 2.2%) were considered to unambiguously represent entomopathogens infecting living hosts. The most abundant of such species, *Cordyceps farinosa*, was found in total on 43 pupae of 3 moth species out of 5. While present each year, it was the most prevalent entomopathogen in 2015 and 2016. Species from other families should be considered potential (opportunistic) pathogens of Lepidoptera as most of them belong to large genera including saprotrophs and pathogens of various hosts, with some representatives occasionally found also on insects [23–25]. There are exceptions from this general scheme, however. In particular, while several species of Clavicipitaceae are known as entomopathogens, the genus *Metapochonia* has been mainly found on nematodes. The genus is represented here by the nematode and rotifer pathogen *Metapochonia bulbillosa* [26], whereas the respective UNITE SH (Table 1) also includes a few sequences obtained from Coleoptera. Moreover, *Tilachlidium brachiatum* has been known to grow only on decaying fungi (K. Põldmaa, personal observation). In addition, our data may include the first records on Lepidoptera/insects for some species from the genera *Mariannaea*, *Mortierella*, *Simplicillium*, *Trichoderma*, and *Umbelopsis*. However, the respective host associations need further investigation as species concepts in these groups are changing as a consequence of advances in molecular systematics.

Lepidopteran species differed in the prevalence of infection—*O. gothica* had the lowest rate (0.4%), while *E. atomaria* and *C. pusaria* had the highest (5% and 3.3%, respectively). There was also a difference between the 3 years of study, showing a trend of increase in the fungal prevalence (Table 2). However, given the somewhat non-systematic character of the data (rearing experiments were not designed to study the prevalence of infections), we refrain here from presenting formal statistical analyses.

3.2. Field Experiment

Of the 868 first instar *C. pusaria* caterpillars released for the experiment, 296 (34.1%, Table 3) insects reached pupation. Fungi were detected on 87 out of the 191 dead pupae (45.5%). However, if we consider only Cordycipitaceae (3 species on 37 pupae), the average prevalence of fungal infections drops to 17.3% (2.2% in 2016 and 24% in 2017). The prevalence of fungi was thus considerably higher in the field compared to the lab rearings. The detected taxa (10 species, Table 1) overlapped with those that were identified in the laboratory rearing, except for two species of Mucorales, known as ubiquitous saprotrophs (but see also [24]). The 19 pupae, infected by a member of Cordycipitaceae plus another fungus, suggest that the latter may represent saprotrophs exploiting the already dead moth tissue.

Table 3. Demographic parameters of *C. pusaria* in the field experiment. Total sample size is shown in brackets.

Year	Survived until Pupation	Pupal Mortality	
		Fungi	Cause Unknown
2016	55.7% (140)	13.3% (45)	86.7% (45)
2017	29.9% (728)	55.5% (146)	44.5% (146)

4. Discussion

Our results suggest that potentially entomopathogenic fungi are invariably present in insect populations. Fungal infections were recorded in all subsets of our laboratory rearing data (host species * year), with just one exception (*C. pusaria* in 2014, but note the low samples size, Table 2). The detected diversity of fungi was notably high. Altogether, 25 species of fungi from 7 orders were isolated from the laboratory and the field experiments, with two-thirds (16 species) belonging to the Hypocreales (Table 1). Six of the collected fungi could not be unambiguously affiliated to a described species on the basis of their ITS DNA sequences (Table 1), and UNITE species hypotheses with respective DOI codes [22] are used for communicating on these. This might have been due to the possibility that cryptic undescribed species were involved, or that voucher specimens of known species have not yet been sequenced, or the inapplicability of the ITS region for discriminating sibling species. Greatest fungal diversity was found on *Ematurga atomaria* (17 spp.), followed by *Cabera pusaria* (11), *Hypomecis punctinalis* (6), *Chiasmia clathrata* (2), and *Orthosia gothica* (2). However, the numbers of species recorded for each host are well consistent with species-specific sample sizes (Table 2) so that these figures should not be interpreted as an indication of differences in the community of fungi associated with different moth species.

Among the unambiguously entomopathogenic fungi, here defined as members of Cordycipitaceae, we identified two abundant (*Akanthomyces muscarius* and *Cordyceps farinosa*) and five scarce species (Table 1). In addition, members of the families Hypocreaceae, Nectriaceae, Aspergillaceae, and Mucoraceae were frequently found growing on dead insect pupae. These fungi were especially common in the field experiment, often accompanying a species of the Cordycipitaceae. Therefore, we may consider such fungi to primarily take advantage of pupae killed by other pathogens, but it cannot by any means be excluded that some of these may still possess thus far unrecognized opportunistic abilities to cause the death of the insects (see [24]). Further studies are needed to establish the nutritional strategies of fungi that are repeatedly found on dead insects.

Our study focused on fungal infections of folivorous larvae, which can only be brought about by plant- and air-borne propagules. In particular, the hosts were not in contact with soil and leaf litter, which are the environments considered to constitute reservoirs for entomopathogenic fungi [2,4]. This may explain why our samples did not include some well-known and common entomopathogens, such as *Beauveria* and *Metarhizium* spp., and suggests that the full spectrum of the fungi associated with natural populations of the

studied insects may be considerably broader than recorded in the present study. Several of the presumable saprotrophs/potential entomopathogens, for which we have identified no or just very few previous records on insects, have been reported from plants, soil, or also from air and water. The fact that such fungi were more common on pupae from the field than from the lab experiment suggests that prolonged exposure to the natural environment favors the deposition of different fungi on insects and/or their host plants (but see also [2]).

Our laboratory-derived data do not suggest any strong specialization of the fungi to particular host species. However, the just moderate amount of data available did not allow us to perform any formal analyses of specialization patterns. Indeed, 9 of the 13 fungal species that were found only on one host were represented by just a single observation. Nevertheless, it should be noted that the most abundant species—*Cordyceps farinosa* and *Akanthomyces muscarius*—were both found as readily infecting all the three most numerous hosts (*C. pusaria*, *E. atomaria*, and *H. punctinalis*), with no evidence of preferring one species over the other.

The overall prevalence of fungal infections in our laboratory rearings varied among different subsets of the data from 0 to 6% (excluding subsamples with less than 100 pupae). This value was 13.3 and 55.5% for the 2 years of the field experiment (0 to 6% vs. 2.2 and 24%, if to consider Cordycipitaceae only), indicating 10 times higher incidence of fungal infections than in the laboratory rearings. These values of prevalence can alone be interpreted as evidence of a non-negligible role of the insect–fungus interactions in the ecology of studied moths.

The lab-based estimates can underestimate the prevalence of entomopathogens in nature as the insects in the laboratory culture should be less exposed to various potential sources of infection than in the field, e.g., soil or infected insects [23,27]. Alternatively, laboratory mass rearings of insects might be prone to disease outbreaks, leading to higher prevalence values in the lab compared to the field. This appears not to have been the case, as epizootic levels were not reached in any of the subsets of our data. Additionally, the diversity of fungal pathogens recorded in the lab, as well as their similarity with field collections, provides evidence against outbreak of a particular fungus in our experimental facilities. The observation that fungal pathogens are always present at low frequencies is well consistent with the decades-long experience of insect rearing by some of the authors (but see [28]). Such a pattern might indicate that the presence of fungal conidia is not a limiting determinant of the prevalence of fungal diseases (see also [29]) but instead, the condition of the host may be decisive—only the weakest individuals are unable to resist the infection [4,30].

Currently, ecological knowledge about entomopathogenic fungi is primarily based on studying a few well-known species of fungi and isolating entomopathogens mainly from soil rather than describing complete fungal communities of particular insect species [3]. This has produced a skew in our knowledge, with the conclusions mainly based on a few members of the Hypocreales such as *Beauveria* and *Metarhizium* species [8]. The present study is one of the first that has aimed to document a full set of fungi isolated from several lepidopteran species (see also [24,25]). The detected diversity should inspire further studies—in addition to considerable bionomic data to be gained, the deeper knowledge would allow us to address the thus far little understood ecological role of pathogenic fungi in natural insect populations.

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Screening and Virulence of the Entomopathogenic Fungi Associated with *Chilo suppressalis* Walker

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Abstract: The current study aimed to explore the entomopathogenic fungi associated with the larvae of *Chilo suppressalis* Walker, a serious pest of rice, in northern Iran. The collected specimens were cultured and identified through morphological and molecular methods. The 38 specimens were identified by microscopic examination and genetic sequencing of the ITS region as follows: twenty-one isolates of *Beauveria bassiana*, five isolates of *Akanthomyces lecanii,* four isolates of *Akanthomyces muscarious*, three isolates of *Metarhizium anisopliae*, two isolates *of Hirsutella subulata*, two isolates of *Trichoderma* sp. and one isolate of *Aspergillus* sp. All the identified isolates were treated on the larvae through bioassay, evaluating the amount of hydrophobin and the activities of proteases, chitinases and lipase to find their virulence. Moreover, the percentage of thermotolerant and cold activity of the isolates were tested to determine their environmental persistence. The overall results revealed the isolates of *B. bassiana,* including BBRR1, BBAL1 and BBLN1 as the most virulent and environmental adaptive isolates among the fungi associated with *C. suppressalis*.

Keywords: entomopathogenic fungi; *Chilo suppressalis*; isolation; identification; pathogenicity

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1. Introduction

The rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Crambidae), is an economic pest of rice, annually causing significant losses in Asia, southern America and northern Africa [1]. The larvae feed intensively on rice stems and cause "whitehead" and "dead-heart" of the seedlings, which directly decrease the overall yield of rice [2]. The main control measure to suppress the *C. suppressalis* population is the wide-spraying of synthetic insecticides, including diazinon, Padan® and Reagent®. Nevertheless, *C. suppressalis* has developed resistance to these insecticides on one hand and resulted in environmental pollution, food residuals and toxicity on non-target organisms on the other hand [3,4]. These concerns should change the management strategies of chemical insecticides toward biocontrol agents like entomopathogens. Among the entomopathogens used to manage the population of insect pests, entomopathogenic fungi cause epizootics among insect pests and appear as the prevalent natural pathogens to regulate population fluctuations of pests and subsequent losses [5]. Their presence in almost all terrestrial and aquatic ecosystems, as well as way of infection by producing different extracellular enzymes and by releasing toxic secondary metabolites, has led to the success of entomopathogenic fungi to affect noxious arthropods in agriculture, forestry and livestock [5,6].

There are many reports on the efficacy of different entomopathogenic fungi, including *Akanthomyces lecanii*, *Akanthomyces muscarious*, *Aspergillus* spp., *Beauveria bassiana*, *Isaria fumosorosea*, *Isaria sinclairii*, *Metarhizium anisopliae*, *Metarhizium rileyi*, *Nomuraea rileyi*, *Pecilomyces lilacinus* and *Purpureocillium lilacinum* against lepidopteran pests such as *Chilo suppressalis*, *Spodoptera litura*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Ostrinia nubilalis*,

Helicoverpa armigera, *Helicoverpa zea*, *Plutella xylostella*, *Duponchelia fovealis*, *Agrotis ipsilon*, *Pieris rapae*, *Trichoplusia ni*, *Ocinara varians*, *Galleria mellonella*, *Plodia interpunctella*, *Ephestia kuehniella* [7–19]. These agents have generally shown to be safe for humans with the least effects on non-targets while they are relatively sensitive to environmental conditions, mainly heat, cold and UV radiation, so it is imperative to find isolates adaptable to these constraints for formulation and field application [20–23].

Exogenous isolates of the entomopathogenic fungi that were commercialized as pest biocontrol agents in different countries may be ineffective on some pests due to environmental suitability and strain differences related to the host [24]. Therefore, the application of local isolates may be a promising choice mainly in case of ecological suitability with pest species and lower hazards on non-target organisms compared to exotic strains [22,25–27]. These points were verified by several studies that demonstrate the virulence of isolates belonging to the same fungal species could be different because of genetic variations occurring in a specific geographical distribution [28–30]. The provinces of Guilan and Mazandaran are located in the north of Iran with high humidity, moderate annual temperatures and heavy rainfall, in which these conditions are appropriate for entomopathogenic fungi [5]. The rice fields of northern Iran, known as a reservoir of *C. suppressalis* [31], can represent ideal sites to study the existence of entomopathogenic fungi with natural enzootics to *C. suppressalis*, so the aims of our study were to; (a) isolate and identify different entomopathogenic fungi from fungus-infected *C. suppressalis* larvae, (b) evaluate the virulence of these fungi against the larvae of *C. suppressalis*, (c) examine the infection process of these isolates by the production of extracellular secretions and (d) compare the conidial germination of the fungal isolates after exposure to heat and cold.

2. Materials and Methods

2.1. Collection and Morphological Identification

The collection sites were all the municipal regions of Guilan and Mazandaran provinces in the north of Iran (Mazandaran and Guilan, Iran) with the highly cultivated area of rice. In each site, the remained stems of rice within the paddy fields were opened, and the infected larvae of *C. suppressalis* were collected and kept in sterile centrifuge tubes. The infected larvae were recognized according to the mycelial growth outside the larval body. Once the samples were transferred to the laboratory, the larvae were surface disinfected with sodium hypochlorite (2%) for 3 min and rinsed three times in sterile distilled water [27]. The larvae were then transferred on potato dextrose agar (PDA, Merck) plates and incubated at 25 °C for 2–4 days for fungal development. Afterward, the fungal mycelia were picked up and transferred to fresh PDA plates for purification. Finally, single-spore cultures were gathered according to the method described by Fang [32] and cultured on PDA slants. All collected specimens were inoculated on PDA plates and incubated at 25 °C in the dark for 14 days. For microscopic examination, mycelia and conidia from fungal specimens were mounted on a sliding glass and observed at 100× magnification on a phase-contrast microscope (Canon INC DS126311, Taiwan). Morphological identification of the specimens was made based on conidial morphology, shape, color and size based on the following literature: *Akanthomyces* spp. isolates [28,33,34], *Beauveria* spp. Isolates [28,34,35], *Hirsutella* spp. isolates [34,36–38] and *Metarhizium* spp. isolates [34,39].

2.2. Genomic DNA Extraction and PCR

DNA extraction was done using the protocol of Montero-Pau et al. [40]. Briefly, the mass mycelia of the specimen grown in PDA media were transferred to the 1.5 mL tubes containing 100 µL of alkaline lysis buffer (0.2 mM disodium ethylene diamide tetraacetic acid, 25 mM NaOH, pH 8.0, Merck) and centrifuged for 30 min at 2000× *g*. Then, the tubes were incubated at 95 °C for 30 min and cooled on ice for five min. Finally, 100 µL of Tris-HCl solution (Sigma-Aldrich, Vienna, Austria; 40 mM, pH 5.0) was added to the tubes, vortexed and maintained at −20 °C. The extracted solution was used as a template for PCR.

To amplify the internal transcribed spacers (ITS5-5.8S-ITS4), ITS5 (5′GGAAGTAAAAG TCGTAACAAGG3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers were synthesized as previously described [41]. The PCR reaction mixture consisted of 12.5 µL of master mix (Including 10× PCR buffer. MgCl2, dNTPS TaqPolymerase, CinnaClone, Tehran, Iran), 7.5 µL of double-distilled H_2O, 1 µL of each primer and 3 µL of DNA solution. PCR was carried out using a thermal cycler (Eppendorf Personal, Darmstadt, Germany) with the following reaction parameters: an initial denaturation for 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min and a final extension for 5 min at 72 °C. Amplified PCR products were visualized by electrophoresis on 1% agarose gels. The PCR products were sent to a sequencing service company (Royan Zistagene Co., Tehran, Iran) for purification and sequencing. Finally, sequences were compared with other fungi using the BLAST search tool in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Insect Rearing

The stock population of *C. suppressalis* was established by collecting the egg patches from rice fields of Amol, northern Iran. The eggs were kept in a growth chamber at 25 ± 2 °C, 85 ± 5% R.H. and 16:8 (L:D) h of photoperiod. The newly hatched larvae were transferred to the tubes (20 × 15 cm) supplied by rice seedlings of Hashemi variety. Quality of food was monitored every day, and the old cutting stems were replaced by the fresh ones [42]. Rearing was continued for three generations at the same controlled conditions.

2.4. Bioassay

Conidia of the two-week-old PDA cultures of the identified isolates were removed by a scalpel then suspended in sterile distilled water containing Tween-80 (0.02%). The concentrations of 10^2 to 10^8 conidia/mL from each isolate were separately prepared based on the preliminary tests. Early fourth instar larvae of *C. suppressalis* were randomly selected and separately dipped in the serial concentrations of each isolate while the control larvae were dipped in an aqueous solution of 0.02% Tween-80 alone. The bioassays were done in three replicates with ten larvae per replication, and the larvae were maintained at the rearing condition for the whole bioassay period. Mortality was recorded within 7 days, and LC_{50} values were determined using POLO-Plus software. For calculation of LT_{50}, mortality was recorded until the death of all larvae at 10^8 conidia/mL concentration.

2.5. Hydrophobin Protein Extraction and Estimation

Hydrophobin content was determined according to the method described by Ying and Feng [43]. Conidia from the two-week-old cultures were added to 1 mL of 2% SDS aqueous solution containing β-mercaptoethanol (5%, Merck) and incubated in a boiling water bath for 10 min before being centrifuged at 22,000× *g* and 4 °C for 10 min. The supernatant was removed, and conidia were rinsed twice in distilled water to eliminate SDS (Merck) soluble proteins. Samples were incubated in 1 mL formic acid at zero temperature for 2 h before being centrifuged at 22,000× *g* and 4 °C for 10 min. The supernatant was transferred into fresh tubes, and 0.5 mL of distilled water was added to the samples. Afterward, 0.75 mL of 45% NaOH solution added to the mixture and maintained at 4 °C overnight. The proteins were separated from the supernatant by centrifugation at 22,000× *g* and 4 °C for 10 min. The extractable proteins of formic acid were rinsed twice with ethanol solution (3:1, *v*/*v*) and then dissolved in 2% SDS to quantitatively determine the amount of protein as mg/mL of conidia using the procedure of Lowry et al. [44].

2.6. Liquid Culture for Enzyme Production of the Isolates

The liquid media used for biochemical production of the extracellular enzymes contained; 0.02% of KH_2PO_4; 0.01% of $CaCl_2$; 0.01% of $MgSO_4$; 0.02% of Na_2HPO_4; 0.01% of $ZnCl_2$ and 0.01% of yeast extract (Merck). The media were inoculated with 1 mL of 10^8 conidia/mL concentration of each isolate separately and 5% (weight) of larval cuticle was

added to each flask containing liquid media. Then the flasks were kept on a rotatory shaker (70 rev/min) for 8 days at 25 ± 1 °C [3].

2.7. Sample Preparations for Enzymatic Assays

After 8 days, the culture flasks were harvested by centrifugation at 10,000× *g* for 30 min and washed in ice-cold Tris-HCl (25 mM, pH 8). Weighed mycelia were ground to a fine powder, suspended in DW, homogenized and centrifuged at 22,000× *g* and 4 °C for 30 min to obtain the supernatant of enzyme assay [12].

2.7.1. Assay of Proteases

Activities of subtilisin-like (Pr1) and trypsin-like (Pr2) as the two key fungal proteases were determined by the specific substrates of succinyl-(alanine) 2-prolinephenylalanine-*p*-nitroanilide and benzoylphenylalanine-valine-arginine-*p*-nitroanilide (Sigma-Aldrich, Co., Vienna, Austria), respectively. The reaction mixture contained 100 µL of Tris-HCl buffer (20 mM, pH 8), 30 µL of each substrate separately and 20 µL of enzyme solution. The mixture was incubated at 25 °C for 10 min, then 100 µL of trichloroacetic acid (TCA, 30%) was added, and the absorbance was recorded at 405 nm [3].

2.7.2. Lipase Assay

Lipase assay was done using the method of Tsujita et al. [45]. Fifty microliters of *p*-nitrophenyl butyrate (27 mM, Sigma-Aldrich, Co., Vienna, Austria) as substrate, 20 µL enzyme solution and 100 µL of Tris-HCl buffer (20 mM, pH 7) were incorporated and incubated at 37 °C for 5 min. Then, 100 µL of NaOH (1 N) was added to each tube, and the absorbance was recorded at 405 nm.

2.7.3. Endochitinase Assay

Twenty microliters of the enzyme solution were added to 50 µL of 0.5% colloidal chitin (Sigma-Aldrich, Co., Vienna, Austria) as substrate and 100 µL of Tris-HCl buffer (20 mM, pH 7). Then, the samples were incubated in a water bath of 30 °C for 60 min. Then, 100 µL of dinitrosalisylic acid (DNS, Sigma-Aldrich, Vienna, Austria) was added, the incubation was prolonged for 10 min at boiling water, and the absorbance was recorded at 545 nm [46].

2.7.4. Exochitinase Assay

The activity of exochitinase was assayed by 200 µL of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (pNPg; Sigma-Aldrich, Co., Vienna, Austria) solution (1 mg pNPg per mL of distilled water) as substrate, 25 µL of enzyme solution and 500 µL of Tris-HCl (25 mM, pH 7) which was incubated at 40 °C for 20 h. Then, the mixture was centrifuged at 13,000 rpm at 4 °C, and the supernatant was added to 200 µL of sodium tetraborate-NaOH buffer (125 mM, pH 10) before to read the absorbance at 400 nm. The extinction coefficient of 18.5 Mm^{-1} $-cm^{-1}$ was considered for activity calculation based on the following formula:

Volume activity (U/mL) = [ΔOD (OD test–OD blank) × V_t × df]/(18.5 × t × 1.0 × Vs)

where, V_t = total volume; Vs = sample volume; 18.5 = millimolar extinction coefficient of *p*-nitrophenol under the assay condition; 1.0 = lightpath length (cm); t = reaction time; and df = dilution factor [46].

2.8. Protein Assay

The method of Lowry et al. [44] was used to determine the content of protein in the provided samples. Twenty microliters of the enzyme solution were added into 100 µL of reagent (Ziest Chem. Co., Tehran, Iran) and incubated for 30 min before reading the absorbance at 545 nm.

2.9. Effects of Thermotolerance and Cold Activity on Conidial Germination

To measure thermotolerance for conidial germination, 100 µL of conidial suspensions (5×10^6 conidia/mL) from each isolate was transferred to 1.5 mL tubes and kept in a

thermal cycler adjusted to 45 °C. After 1 h and 2 h, 20 µL of conidial suspensions were removed and plated (without spreading) on PDA. Finally, plates were maintained at 25 °C, and conidial germination was counted after 24 h by microscopic observation. Moreover, 20 µL of a conidial suspension (5 × 106 conidia/mL) was plated (without spreading) on PDA and kept at 5 °C to determine the germination after 7 and 14 days in cold condition. In both experiments, the conidial control suspensions were inoculated on PDA at 25 °C. The relative percent germination was estimated by comparing conidial germination to untreated isolates, and at least 100 conidia were counted for each treatment in every test [27].

2.10. Statistical Analysis

Probit analysis was done to determine LC_{50} and LT_{50} values at the corresponding 95% confidence interval (CI) values by using POLO-Plus software. Biochemical data and germination of conidia were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences among control and treatments were statistically considered at a probability of less than 5% and marked by different letters.

3. Results and Discussion

3.1. Screening and Identification of Fungi

A total of 38 fungal specimens were collected from *C. suppressalis* larvae, which were naturally infected by fungi in the rice fields of northern Iran. The specimens were morphologically identified as *Akanthomyces lecanii* (×5 isolates), *Akanthomyces muscarius* (×4 isolates), *Aspergillus* sp. (×1 isolate), *Beauveria bassiana* (×21 isolates), *Hirutella subulata* (×2 isolates), *Metarhizium anisopliae* complex (×3 isolates) and *Trichoderma* sp. (×2 isolates) (Table 1, Figure 1). Among all specimens, it was the first report of the natural occurrence of *H. subulata* in Iran. In *A. lecanii*, conidiogenous cells were phialidic, phialides approximately small, length size of 11–16 µm and width size of 1.4–2 µm, aculeate and strongly tapering, solitary or in whorls 3–6, conidial shape ellipsoidal-cylindrical, length size 4.1–5.2 µm and width size of 1.3–2.1 µm (Table 1, Figure 1a). In *A. muscarious*, conidiogenous cells were phialidic; phialides burned straight on prostrate hyphae or on secondary branches, phialides generally tall and slender and longer than *A. lecanii*, length size of 28–35 µm and width size of 1.6–2 µm, conidial shape cylindrical and longer than *A. lecanii*, length size 6.5–9.5 µm and width size of 1.5–1.9 µm (Table 1, Figure 1b). The major difference between the species of *Akanthomyces* is the shape and size of phialides that our specimens matched accurately match the description given by Zare and Gams [33]. In *Aspergillus* sp. conidial shape was globose, length size 1.8–2.3 µm and width size of 1.8–2.3 µm (Table 1, Figure 1c). In addition, the Conidial shape of *B. bassiana* isolates was globose with a length size of 2.1–3.2 µm and width size of 2–3 µm; conidiogenous cells were phialidic; the phialides were flask-shaped, swollen at the base or near the base and tapering at the apex. In addition, the conidiogenous cells were usually solitary or in a cluster of up to five (Table 1, Figure 1d), which appeared typical of those described by other researchers [28,34,35]. The major difference between the species of *Beauveria* is the shape and size of conidia [28]. In *H. subulata*, conidiogenous cells were phialidic, phialides scattered, and the lower phialides were narrow ellipsoid; the conidial shape was ovoid and in a chain, length size 5.5–6.9 µm and width size of 3.9–5.1 µm (Table 1, Figure 1e). Our isolates were compared with Yoon et al. [37], and significant differences were not observed among them. The conidial shape of *M. anisopliae* isolates was oblong oval with a length size of 6.8–7.8 µm and a width size of 2.6–3.7 µm (Table 1, Figure 1f). Conidia were the only morphological particular that reliably distinguishes several *Metarhizium* species [39]. In *Trichoderma* sp. conidial shape was globose, length size 2.2–3.1 µm and width size of 2.1–2.9 µm (Table 1, Figure 1g).

Table 1. Morphological characteristics and GenBank accession number of the collected fungi from the larvae of *Chilo suppressalis*.

Identification	Isolates	Conidia Size (um)	Shape of Conidia	Color of Conidia or Colony	Gene Bank Accession No.	Ident (%)
Akanthomyces lecanii	ALRR	4.3 ± 0.07 × 1.9 ± 0.04	Ellipsoidal-Cylindrical	White	MW143527	99.44
	ALFN	4.9 ± 0.1 × 1.7 ± 0.04	Ellipsoidal-Cylindrical	White	MW143528	99.08
	ALAL	4.5 ± 0.06 × 1.8 ± 0.04	Ellipsoidal-Cylindrical	White	MW143529	99.08
	ALRT	4.8 ± 0.06 × 1.6 ± 0.05	Ellipsoidal-Cylindrical	White	MW143531	99.44
	ALLN	4.5 ± 0.07 × 1.5 ± 0.03	Ellipsoidal-Cylindrical	White	MW143530	99.44
Akanthomyces muscarius	AMRT	8 ± 0.09 × 1.7 ± 0.04	Cylindrical	White	MW143523	99.82
	AMAI	7.8 ± 0.08 × 1.8 ± 0.03	Cylindrical	White	MW143524	98.40
	AMAL	8.2 ± 0.04 × 1.8 ± 0.05	Cylindrical	White	MW143525	99.80
	AMBL	7.5 ± 0.02 × 1.9 ± 0.06	Cylindrical	White	MW143526	99.61
Aspergillus sp.	ASAI	2 ± 0.05 × 2 ± 0.04	Globose	Dark green	MW143532	99.82
Beauveria bassiana	BBAL1	2.7 ± 0.07 × 2.5 ± 0.07	Globose	White	MW143537	99.81
	BBAL2	2.8 ± 0.07 × 2.5 ± 0.05	Globose	White	MW143538	100
	BBAL3	3 ± 0.05 × 2.8 ± 0.09	Globose	White	MW143539	99.81
	BBAL4	2.9 ± 0.04 × 2.8 ± 0.08	Globose	White	MW143540	100
	BBBL1	2.9 ± 0.06 × 2.6 ± 0.06	Globose	White	MW143541	99.61
	BBBL2	3.3 ± 0.05 × 2.9 ± 0.09	Globose	White	MW143542	99.61
	BBLN1	2.8 ± 0.08 × 2.6 ± 0.08	Globose	White	MW143546	99.63
	BBLN2	2.9 ± 0.05 × 2.8 ± 0.07	Globose	White	MW143547	99.81
	BBLN3	2.5 ± 0.06 × 2.4 ± 0.04	Globose	White	MW143548	99.63
	BBLD1	2.8 ± 0.05 × 2.6 ± 0.05	Globose	White	MW143549	99.43
	BBLD2	2.9 ± 0.04 × 2.7 ± 0.07	Globose	White	MW143550	99.44
	BBLD3	2.6 ± 0.08 × 2.4 ± 0.05	Globose	White	MW143551	99.26
	BBLD4	2.9 ± 0.04 × 2.5 ± 0.06	Globose	White	MW143552	99.26
	BBLD5	2.8 ± 0.05 × 2.6 ± 0.09	Globose	White	MW143553	99.81
	BBRT1	2.9 ± 0.05 × 2.7 ± 0.09	Globose	White	MW143533	100
	BBRT2	2.8 ± 0.04 × 2.7 ± 0.04	Globose	White	MW143534	100
	BBRR1	2.7 ± 0.08 × 2.4 ± 0.06	Globose	White	MW143535	100
	BBRR2	2.9 ± 0.08 × 2.7 ± 0.07	Globose	White	MW143536	100
	BBSI	2.9 ± 0.06 × 2.7 ± 0.07	Globose	White	MW143544	98.90
	BBFN	2.9 ± 0.04 × 2.7 ± 0.08	Globose	White	MW143543	100
	BBAI	3 ± 0.06 × 2.9 ± 0.08	Globose	White	MW143545	100

Table 1. *Cont.*

Identification	Isolates	Conidia Size (um)	Shape of Conidia	Color of Conidia or Colony	Gene Bank Accession No.	Ident (%)
Hirutella subulata	HSAL	$5.9 \pm 0.1 \times 4.5 \pm 0.06$	Ovoid	White-cream	MW143559	99.61
	HSBL	$6 \pm 0.2 \times 4.7 \pm 0.08$	Ovoid	White-cream	MW143560	99.61
Metarhizium anisopliae complex	MASA	$7.6 \pm 0.1 \times 3.2 \pm 0.07$	Oblong oval	Brown-green	MW143556	100
	MAAI	$7.4 \pm 0.8 \times 3.3 \pm 0.05$	Oblong oval	Brown-green	MW143557	99.81
	MAAL	$7.7 \pm 0.4 \times 3.6 \pm 0.05$	Oblong oval	Brown-green	MW143558	99.81
Trichoderma sp	TSRT	$2.7 \pm 0.08 \times 2.7 \pm 0.09$	Globose	Dark green	MW143555	100
	TSAH	$2.5 \pm 0.05 \times 2.3 \pm 0.06$	Globose	Dark green	MW143554	100

Note: final tested isolates were renamed after the submission of the ITS sequence to the GenBank database.

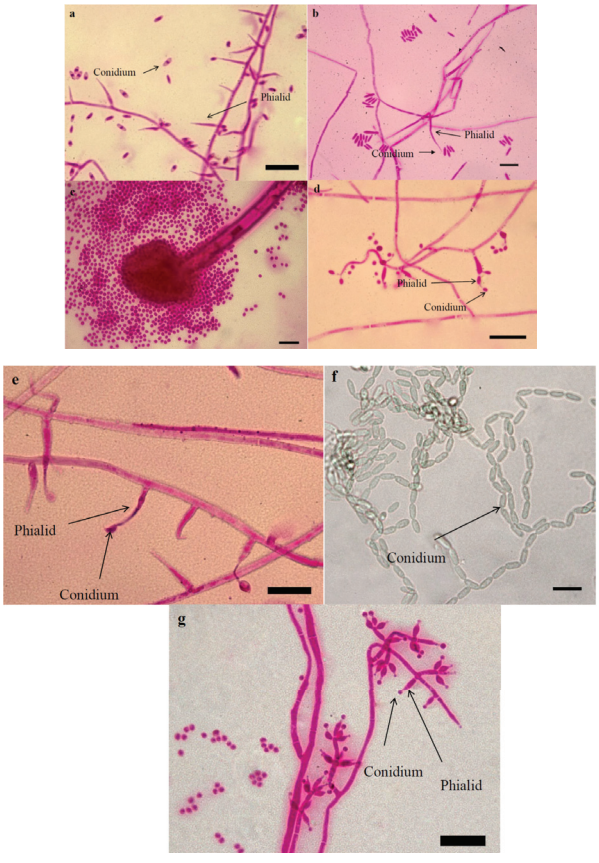

Figure 1. Morphological characteristics (conidium and phialid) of the fungus associated with the larvae of *Chilo suppressalis*. (**a**) *Akanthomyces lecanii*, (**b**) *Akanthomyces muscarius*, (**c**) *Aspergillus* sp., (**d**) *Beauveria bassiana*, (**e**) *Hirutella subulate*, (**f**) *Metarhizium anisopliae* complex, (**g**) *Trichoderma* sp. Bars are 20 micrometers.

The ITS5-5.8S-ITS4 region was used for molecular analysis, confirming identifications of the fungal isolates. The amplified ITS region from all specimens showed approximately 600 bp-sized fragments, and the samples were sequenced and compared in the GenBank database. Results of the ITS sequence data were consistent with those obtained using morphological studies. After submission to the GenBank database, the fungal isolates were renamed based on the given code (Table 1). In the current study, *B. bassiana* was the most frequent detected fungus in rice collected from fields similar to others studies that have shown *B. bassiana* as the most widespread entomopathogenic fungi in the endemic Moroccan forests of *Argania spinosa*, Switzerland, Spain, China and Southern Italy [22,47–50]. Moreover, *B. bassiana* has been reported as the major pathogen of insects in more than 200 species that have been identified from the soil and dead insects in nature [24,25,51,52].

3.2. Bioassay

Only the isolates of *B. bassiana*, *M. anisopliae and H. subulata* caused mortality against the larvae of *C. suppressalis*. A comparison of LC_{50} and LT_{50} values indicated the significant differences among the isolates. The most virulent, isolate BBLN1 (1×10^4 conidia/mL), had the least LC_{50} value, followed by BBAL1 (2.1×10^4 conidia/mL), BBRR1

(2.2×10^4 conidia/mL), BBLN2 (5.4×10^4 conidia/mL), BBAL3 (5.6×10^4 conidia/mL) and MASA (7.1×10^4 conidia/mL), while HSBL (1.6×10^6 conidia/mL), HSAL (7.9×10^5 conidia/mL) and BBLD5 (4.4×10^5 conidia/mL) showed the comparatively high LC_{50} values (Table 2). Moreover, the least LT_{50} values were obtained to be 2.71, 3.15, 3.45, 3.66 and 3.69 days for the larvae treated by BBRR1, BBLN1, BBAL1, BBAL4 and MASA, respectively (Table 3). These results revealed that BBRR1, BBLN1 and BBAL1 isolates of *B. bassiana* had higher efficacy than the other isolates on *C. suppressalis* larvae with a lesser concentration of conidia with a shorter time (days) to kill 50% of the larval population. Jandricic et al. [53] reported the higher virulence of *B. bassiana* isolates against the *Myzus persicae* Sulzer, *Aphis gossypii* Glover and *Aulacorthum solani* Kaltenbach (Hemiptera: Aphididae) compared to *M. anisopliae* isolates. Ramzi and Zibaee [12] showed that the two commercial isolates of *B. bassiana* and *B. bassiana* (BB1 and BB2) had the higher virulence against *C. suppressalis* larvae compared to *A. lecanii*, *I. fumosoroseus and M. anisopliae*. In addition, the higher virulence of the different isolates of *B. bassiana* and *M. anisopliae* has been observed on the boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) [29]. In our study, the least virulence of HSAL and HSBL as the two isolates of *H. subulata* were obtained compared to *B. bassiana* and *M. anisopliae* isolates, which may be correlated with low germination and sporulation rates in addition to the low activities of the extracellular enzymes of these isolates (see below) [54]. Finally, the isolates of *A. lecanii* and *A. muscarious* showed no mortality against *C. suppressalis* larvae. This case may be attributed to host–pathogen interaction between these isolates and the larvae of *C. suppressalis*, such as efficient attachment of conidia to the integument, negative impacts of integument composition with penetration tube of the fungi and immune responses of the larvae toward conidia. All these phenomena deserve detailed experiments to precisely elucidate the case.

Table 2. LC_{50} values (conidia/mL) of the entomopathogenic fungi collected from rice fields against the fourth instar larvae of *Chilo suppressalis*.

Isolates	*N*	LC_{50} (Cl 95%) Conidia/mL	X^2 (df)	Slope ± SE
BBAL1	150	2.1×10^4 ($1.1 \times 10^3 - 1.9 \times 10^5$)	3.253 (3)	0.420 ± 0.068
BBAL2	150	2.3×10^5 ($4.7 \times 10^4 - 1.4 \times 10^6$)	0.377 (3)	0.313 ± 0.059
BBAL3	150	5.6×10^4 ($1.1 \times 10^4 - 2.4 \times 10^5$)	0.689 (3)	0.353 ± 0.062
BBAL4	150	9.6×10^4 ($1.9 \times 10^4 - 4.8 \times 10^5$)	0.820 (3)	0.331 ± 0.060
BBBL1	150	1.5×10^5 ($3.1 \times 10^4 - 7.9 \times 10^5$)	0.453 (3)	0.321 ± 0.059
BBBL2	150	3.9×10^5 ($7.2 \times 10^4 - 3.2 \times 10^6$)	0.327 (3)	0.292 ± 0.058
BBLN1	150	1×10^4 ($2.9 \times 10^3 - 9.9 \times 10^4$)	3.084 (3)	0.380 ± 0.065
BBNL2	150	5.4×10^4 ($1.1 \times 10^4 - 2.4 \times 10^5$)	2.285 (3)	0.345 ± 0.061
BBLN3	150	1.5×10^5 ($4 \times 10^4 - 6.7 \times 10^5$)	2.455 (3)	0.385 ± 0.064
BBLD1	150	1.1×10^5 ($2.3 \times 10^4 - 5.5 \times 10^5$)	1.280 (3)	0.336 ± 0.060
BBLD2	150	1×10^5 ($2.6 \times 10^4 - 4.5 \times 10^5$)	1.126 (3)	0.379 ± 0.063
BBLD3	150	9.5×10^4 ($2.3 \times 10^4 - 3.9 \times 10^5$)	1.156 (3)	0.386 ± 0.064
BBLD4	150	1.2×10^5 ($2.7 \times 10^4 - 5.8 \times 10^5$)	2.365 (3)	0.351 ± 0.061
BBLD5	150	4.4×10^5 ($9.9 \times 10^4 - 2.7 \times 10^6$)	1.126 (3)	0.336 ± 0.061
BBRT1	150	4.9×10^5 ($6.8 \times 10^4 - 6.8 \times 10^6$)	1.320 (3)	0.247 ± 0.056
BBRT2	150	3.4×10^5 ($7.8 \times 10^4 - 1.9 \times 10^6$)	0.534 (3)	0.342 ± 0.061
BBRR1	150	2.2×10^4 ($4.6 \times 10^3 - 8.8 \times 10^4$)	1.477 (3)	0.337 ± 0.064
BBRR2	150	2.4×10^5 ($6.1 \times 10^4 - 1.1 \times 10^6$)	0.776 (3)	0.375 ± 0.063
BBSI	150	1.4×10^5 ($3.2 \times 10^4 - 7.6 \times 10^5$)	0.513 (3)	0.337 ± 0.060
BBFN	150	2.3×10^5 ($4.5 \times 10^4 - 1.5 \times 10^6$)	1.470 (3)	0.307 ± 0.059
BBAI	150	1.9×10^5 (4.2×10^4–1×10^6)	0.539 (3)	0.332 ± 0.060
HSAL	150	7.9×10^5 ($1.5 \times 10^5 - 7.3 \times 10^6$)	1.985 (3)	0.309 ± 0.052
HSBL	150	1.6×10^6 ($2.4 \times 10^5 - 1.5 \times 10^7$)	0.449 (3)	0.297 ± 0.059
MASA	150	7.1×10^4 ($1.6 \times 10^4 - 2.9 \times 10^5$)	2.325 (3)	0.374 ± 0.062
MAAI	150	1.6×10^5 ($3.4 \times 10^4 - 9.4 \times 10^5$)	1.743 (3)	0.325 ± 0.060
MAAL	150	3.6×10^5 ($7.4 \times 10^4 - 2.4 \times 10^6$)	0.820 (3)	0.315 ± 0.059

Note: calculations were carried out by POLO-Plus software.

Table 3. LT_{50} values (days) of the entomopathogenic fungi collected from rice fields against the fourth instar larvae of *Chilo suppressalis*.

Isolates	LT_{50} (Cl 95%) Days	X^2 (df)	Slope ± SE
BBAL1	3.45 (2.55–4.43)	11.948 (5)	4.007 ± 0.496
BBAL2	4.16 (3.16–5.21)	20.914 (8)	3.270 ± 0.347
BBAL3	3.87 (3.28–4.45)	9.303 (7)	4.354 ± 0.465
BBAL4	4.03 (3.47–4.55)	7.305 (8)	4.12 ± 0.447
BBBL1	4.18 (3.50–4.86)	12.719 (8)	3.983 ± 0.410
BBBL2	4.63 (4.13–5.13)	0.883 (8)	3.803 ± 0.419
BBLN1	3.15 (2.36–4)	13.895 (5)	3.302 ± 0.423
BBNL2	3.70 (2.96–4.45)	12.155 (7)	3.545 ± 0.392
BBLN3	3.75 (2.96–4.54)	14.866 (7)	3.899 ± 0.416
BBLD1	4.05 (3.25–4.84)	15.98 (8)	3.654 ± 0.376
BBLD2	4.28 (3.30–5.23)	27.697 (8)	3.654 ± 0.376
BBLD3	3.81 (3.38–4.21)	6.745 (7)	4.248 ± 0.456
BBLD4	3.66 (3.24–4.05)	5.112 (8)	4.166 ± 0.420
BBLD5	4.23 (3.74–2.4.71)	3.729 (8)	3.648 ± 0.386
BBRT1	4.91 (4.03–5.78)	15.756 (8)	3.623 ± 0.396
BBRT2	4.77 (4.29–5.24)	3.858 (8)	4.141 ± 0.445
BBRR1	2.71 (2.29–3.10)	5.961 (6)	3.260 ± 0.383
BBRR2	4.16 (3.52–4.78)	12.398 (8)	4.373 ± 0.441
BBSI	4.33 (3.77–4.87)	8.335 (8)	4.084 ± 0.419
BBFN	4.48 (3.88–5.08)	9.194 (8)	3.99 ± 0.414
BBAI	4.41 (3.67–5.14)	14.25 (8)	4.085 ± 0.419
HSAL	4.65 (3.83–5.54)	13.498 (7)	4.070 ± 0.457
HSBL	5.21 (4.72–5.74)	2.075 (7)	4.434 ± 0.526
MASA	3.69 (3.01–4.38)	9.280 (6)	4.021 ± 0.470
MAAI	4.91 (4.03–5.87)	17.220 (7)	3.723 ± 0.410
MAAL	4.14 (3.64–4.64)	1.887 (8)	3.386 ± 0.366

Note: calculations were carried out by POLO-Plus software.

3.3. Hydrophobin

The highest amounts of hydrophobin recorded in BBAL1, BBLD5, BBLD1, BBSI, BBBL1, BBLN1, BBLN2, HSAL and MASA, respectively (Table 4), while the least amounts of hydrophobin were in TSRT, ASAI and TSAH (Table 4). Entomopathogenic fungi achieve the nutrients at host bodies through the cuticle, so the first step of pathogenesis is adhesion to the integument. Therefore, the external surface of conidia has a fundamental protein with a hydrophobic rodlet layer that connects to the insect epicuticle [6]. Hydrophobins are a class of unique fungal proteins important in sporulation, pathogenesis, thermotolerant, growth and development of fungi [43,55,56]. Some studies reported that inhibition of hydrophobin gene expression negatively affected pigmentation, conidiation, hydrophobicity and virulence of entomopathogenic fungi [24,55,57]. Our findings revealed that the lesser amount of hydrophobin could be one of the reasons for no mortality of some isolates against the larvae of *C. suppressalis*. In fact, the proper attachment of the conidia to the host cuticle and subsequent germinations are the primarily important steps to effective infection by entomopathogenic fungi. The higher amounts of hydrophobin were obtained in the isolates with the more virulence-like BBLN1 and BBLN2. Although the higher amounts of hydrophobin were also found in HSAL with the least virulence, it should be noted that hydrophobin is not necessarily the main factor in the virulence of a fungus, but it only shows the better interaction with the host cuticle.

Table 4. Amount of the hydrophobin (mg/mL) in the collected entomopathogenic fungi from the larvae of *Chilo suppressalis*.

Isolates	Amount of Hydrophobin (mg/mL)
ALRR	0.0603 ± 0.007^{fghi}
ALFN	0.0628 ± 006^{efghi}
ALAL	0.0535 ± 0.005^{hi}
ALRT	0.0586 ± 0.002^{ghi}
ALLN	0.0627 ± 0.003^{efghi}
AMRT	$0.0687 \pm 0.003^{cdefgh}$
AMAI	$0.0663 \pm 0.005^{cdefgh}$
AMAL	0.0656 ± 0.001^{defgh}
AMBL	$0.0679 \pm 0.002^{cdefgh}$
ASAI	0.038 ± 0.005^{j}
BBAL1	0.0953 ± 0.001^{a}
BBAL2	$0.0745 \pm 0.004^{bcdefgh}$
BBAL3	$0.0765 \pm 0.002^{abcdefg}$
BBAL4	$0.0749 \pm 0.002^{bcdefgh}$
BBBL1	0.0846 ± 0.002^{abcd}
BBBL2	$0.0780 \pm 0.001^{abcdefgh}$
BBLN1	0.822 ± 0.003^{abcde}
BBLN2	0.0803 ± 0.003^{abcde}
BBLN3	$0.0756 \pm 0.003^{abcdefgh}$
BBLD1	0.0854 ± 0.004^{abc}
BBLD2	$0.0796 \pm 0.006^{abcdefg}$
BBLD3	$0.0704 \pm 0.003^{bcdefgh}$
BBLD4	$0.0782 \pm 0.002^{abcdefg}$
BBLD5	0.0897 ± 0.002^{ab}
BBRT1	$0.0704 \pm 0.004^{bcdefgh}$
BBRT2	$0.0774 \pm 0.002^{abcdefg}$
BBRR1	$0.0767 \pm 0.002^{abcdefg}$
BBRR2	$0.0677 \pm 0.003^{cdefgh}$
BBSI	$0.0762 \pm 0.001^{abcdefg}$
BBFN	$0.0729 \pm 0.001^{bcdefgh}$
BBAI	0.0832 ± 0.002^{abcd}
HSAL	0.0816 ± 0.001^{abcde}
HSBL	$0.0684 \pm 0.003^{cdefgh}$
MASA	0.0805 ± 0.002^{abcde}
MAAI	0.0631 ± 0.002^{ifghi}
MAAL	$0.0714 \pm 0.001^{bcdefgh}$
TSRT	0.0036 ± 0.002^{j}
TSAH	0.043 ± 0.002^{ij}

Note: Statistical differences are shown by different letters (Tukey's test, $p \leq 0.05$).

3.4. Extracellular Enzymes

The conidia of entomopathogenic fungi attach to the cuticle of host insects, germinate and penetrate to the hemocoel with the assistance of extracellular enzymes, such as chitinases, proteases and lipases [58]. Trypsin (Pr1) and subtilisin-like (Pr2) proteases are the primitive synthesized enzymes to simplify penetration of the hyphae into the host body. Then, synthesis of the chitinases increases the penetration efficiency [59], and finally, lipases involved in hydrolyzing lipid derivatives within the cuticle and facilitating the infection of host cells [60]. Our results revealed differences in the activities of extracellular enzymes between the fungal isolates. Isolates BBAL1, BBRR1, BBLN2, BBLN1 and BBLD2 demonstrated the highest activity of Pr1 while the least activity was observed in ASAI, TSRT and TSAH isolates (Figure 2). In the case of Pr2, BBRR1, BBLN2, BBLD2 and BBLN1, isolates showed the highest activity (Figure 2). The highest activity of lipase was recorded in BBLD4, BBSI, BBLN2 and BBRR1 isolates (Figure 3). BBRR1, BBLN2,

BBAL1 and BBLN1 isolates showed the highest activity of exochitinase (Figure 4). In the case of endochitinase, the highest activity was obtained in BBRR1, BBLN2, BBBL1, BBAL1 and BBLN1 isolates (Figure 4). The higher Pr1 activity in the given isolates indicates the capability of protein digestion by these isolates in the initial stages of infection, so the efficiency of this enzyme may ensure the success of other enzymes to feasible penetration through insect cuticle. Charnley and St. Leger [61] believe in facilitating the cuticle infiltration by the proteases produced during invasion prior to chitinases during later steps. They concluded the major role of proteases in cuticle penetration compared to chitinases. Ramzi and Zibaee [12] demonstrated the different levels of proteinases, chitinase and lipase produced by *B. bassiana*, *M. anisopliae*, *L. lecanii* and *I. fumosoroseus* in the larvae *C. suppressalis* in which the isolates with the highest enzymatic activity led to the higher mortality Lu et al. [1] showed, the higher levels of protease and chitinase produced by ZJLSP09 isolate of *Lecanicilium* sp. in comparison with ZJLA07 and ZJLP08 isolates which were related to mortality in *Diaphorina citri* Kuwayana (Hemiptera: Psyllidae). Maqsoudi et al. [62] reported that the isolate of *B. bassiana* with the higher activity of proteases and chitinases led to the lower LC50 and LT50 values against *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae). In our study, no clear correlation was obtained between lipase production and virulence of isolates, similar to earlier studies [12,23,63,64]. This conclusion on lipase may be more obvious in the case of BBLN2, which is the only isolate with higher virulence and lipase activity. Other isolates with higher virulence showed lower lipase activity. It seems that lipases are more important in the utilization of integument lipids for fungal development, not necessarily penetration. In contrast, the isolates with the higher virulence demonstrated the higher activity of proteases and chitinases, mainly BBLN1, BBLN2 and BBRR1. These findings apparently disclosure the correlation between efficiency of extracellular enzymes and higher virulence of the entomopathogenic fungi. Such isolates properly or rapidly penetrate through host cuticle with efficient cleavage of polypeptide and carbohydrate bonds then achieve hemocoel to continue the latter steps of infection. It should be mentioned that this process is accompanied by better production of blastospores and secondary metabolites within the host hemocoel to impose virulence on infected individuals.

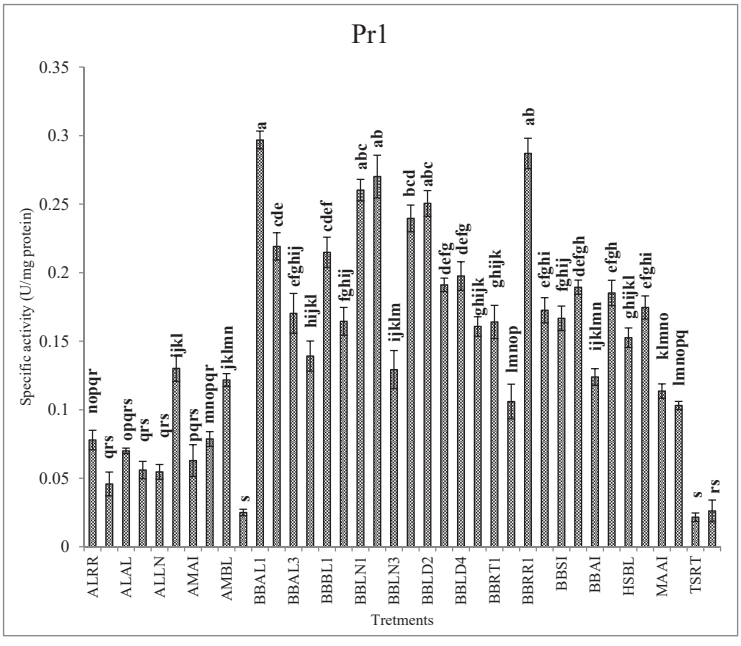

Figure 2. *Cont.*

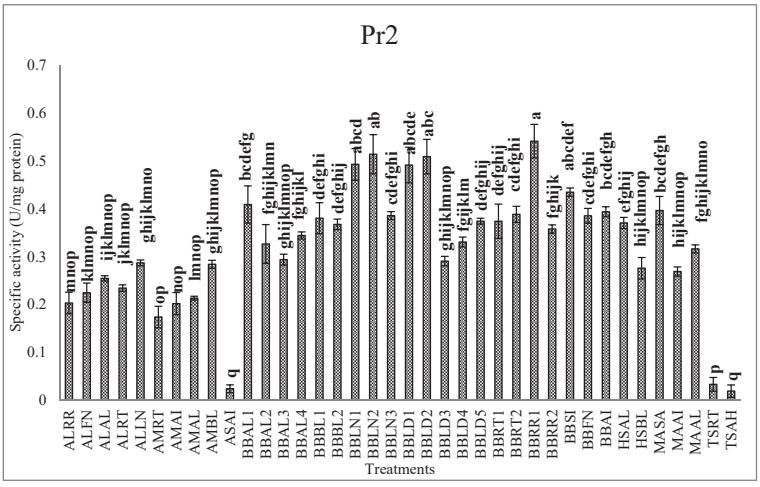

Figure 2. Activities of the proteases (U/mg protein, Mean ± SE) in the liquid culture media of the entomopathogenic fungi in the presence of *C. suppressalis* cuticle. Statistical differences are shown by different letters (Tukey's test, $p \leq 0.05$).

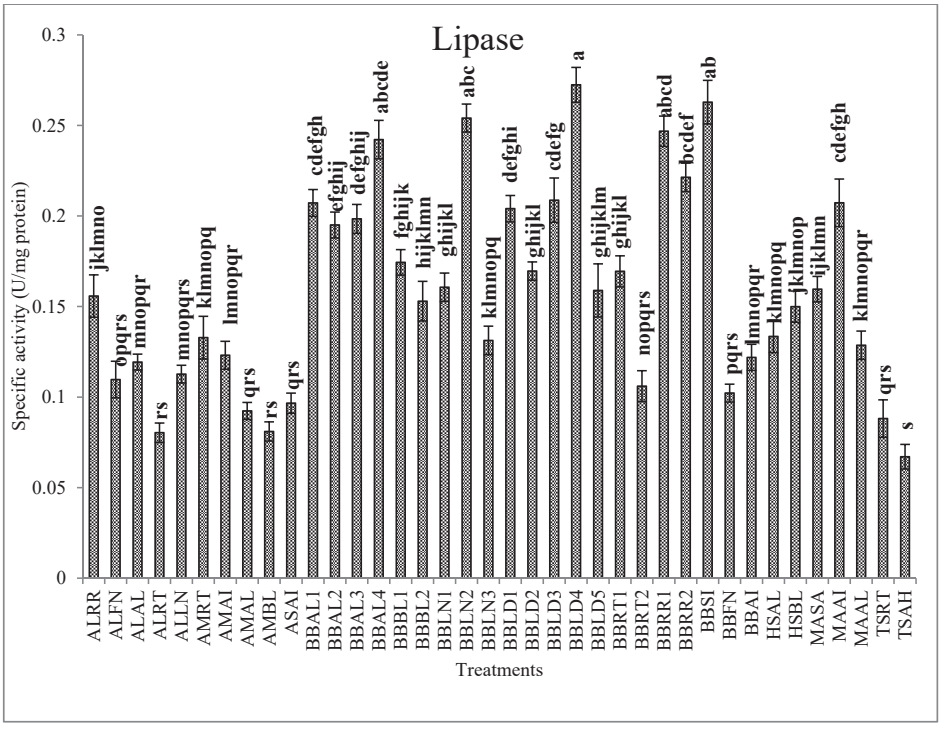

Figure 3. Activity of the lipase (U/mg protein, Mean ± SE) in the liquid culture media of the entomopathogenic fungi in the presence of *C. suppressalis* cuticle. Statistical differences are shown by different letters (Tukey's test, $p \leq 0.05$).

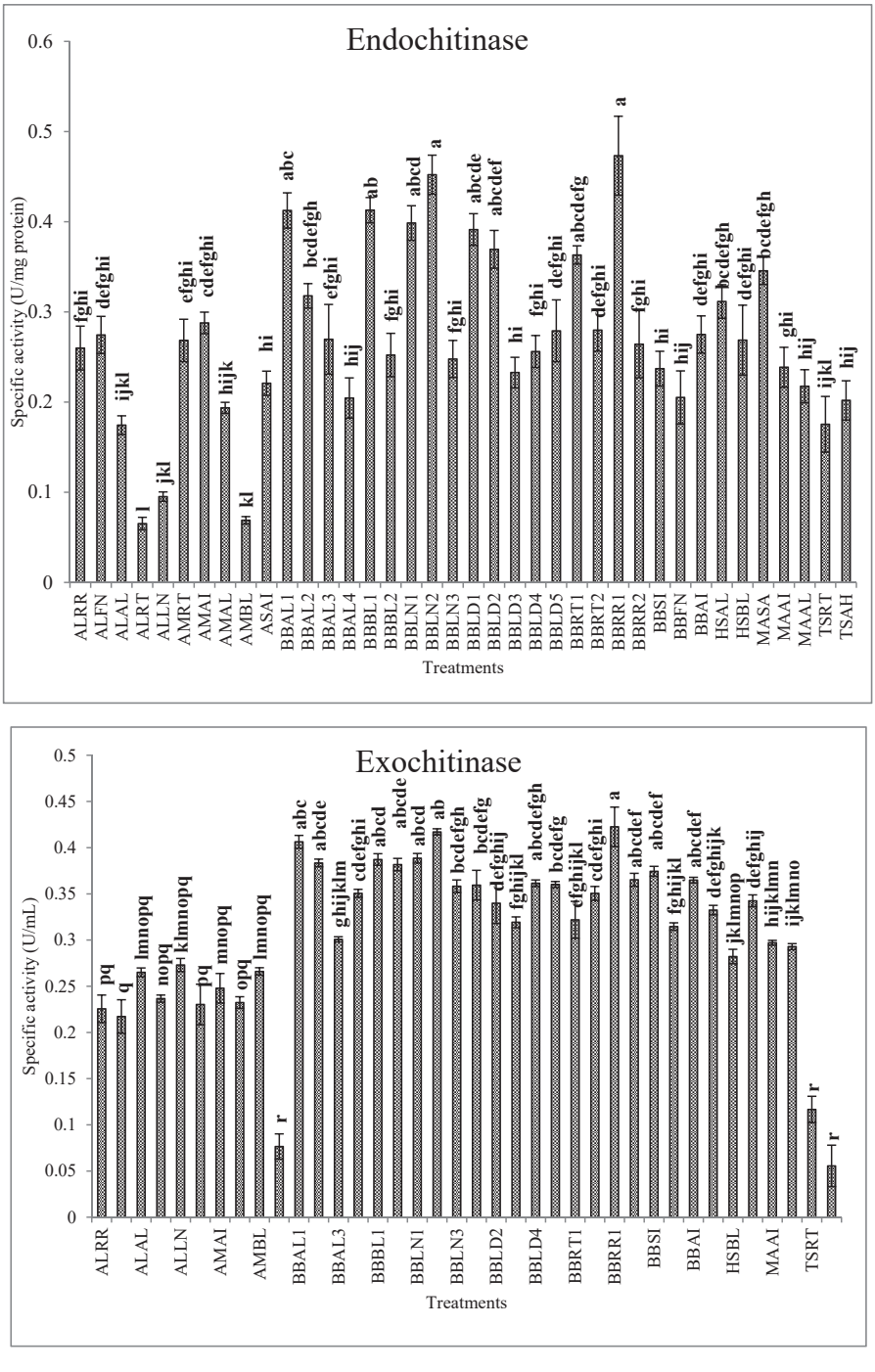

Figure 4. Activities of the chitinases (U/mg protein, Mean ± SE) in the liquid culture media of the entomopathogenic fungi in the presence of *C. suppressalis* cuticle. Statistical differences are shown by different letters (Tukey's test, $p \leq 0.05$).

3.5. Effects of Thermotolerance and Cold Activity on Conidial Germination

The inactivation and delay of conidia germination caused by heat and cold as the most important environmental factors reduce the efficiency of the entomopathogenic fungi as the biocontrol agents from both the virulence against host and persistence in ecosystems. Selection of the entomopathogenic fungi that tolerate thermal fluctuations te is necessary before field application [21,27,65]. The effect of thermotolerance on the germination rate of the conidia of the collected isolates in the current study have shown in Table 5. Fifteen isolates demonstrated a germination rate of more than 50% after 1 h exposure to 45 °C, while only one isolate exhibited a high tolerance after 2 h (Table 5). After 2 h of exposure, the isolate thermotolerance could be divided into three classes: low (below 30%), moderate (between 30% and 60%) and high tolerance (above 60%). Among the isolates, *Aspergillus* sp. (ASAI) showed high tolerance. Moderate tolerances were observed in four isolates of *B. bassiana* (BBLN1, BBAL1, BBLN2 and BBLD2), three isolates of *M. anisopliae* (MASA, MAAI and MAAL) and two isolates of *Trichoderma* sp. (TSAH and TSRT). The other isolates were low tolerance to the heat of 45 °C. Similar results have been reported by Lee et al. [27] as the rate of the conidial germination in *B. bassiana*, *M. anisopliae* and *Lecanicillium attenuatum* significantly reduced after 2 h at 45 °C. Rivas et al. [66] demonstrated the significant lower conidial germination of *Lecanicillium* isolates after incubation at 32 °C. The susceptibility of *Metarhizium* isolates to high temperatures (45 °C) was demonstrated by Rangel et al. [65]. Exposure to 35 °C for 10 min harmed the conidial germination of *B. bassiana*, but the *M. anisopliae* isolate germinated readily at this temperature [67]. Generally, the optimal temperature for conidia germination and growth of entomopathogenic fungi is between 23 and 28 °C. The growth was reduced above 30 °C, and it was totally inhibited above 34 °C [20,27,65–67]. Our results imply only *Aspergillus* sp. isolates as the highly thermotolerant isolate has although it had no virulence against the larvae of *C. suppressalis*. Finally, the cold activity of fungal isolates was examined through the treatment of the conidia at 5 °C for one and two weeks. All the isolates showed high activity (above 80%) at 5 °C for both time intervals except for *Aspergillus* sp. (ASAI) (Table 5). Lee et al. [27] reported the high cold activity at 7–14 days for almost all collected isolates. Based on earlier reports, most entomopathogenic fungi have high cold activity, although germination and sporulation may be delayed or stopped at a cold temperature [21,27,68,69]. Such a property may be important in the survival of entomopathogenic fungi in cold periods of the year.

Table 5. Thermotolerance and cold activity of the entomopathogenic fungi collected from the larvae of *Chilo suppressalis*.

Isolates	Conidial Germination (%)			
	Exposure to 45 °C		Incubating at 4 °C	
	1 h	2 h	7 Day	14 Day
ALRR	29.86 ± 1.2 ^{mnopq}	10.90 ± 0.6 ^{op}	84.36 ± 1.6 ^{ghijk}	90.53 ± 1.5 ^{abcdef}
ALFN	27.78 ± 1 ^{nopq}	12.21 ± 0.3 ^{mnop}	94.20 ± 0.9 ^{abcde}	95.85 ± 0.9 ^{abcd}
ALAL	37.75 ± 2.1 ^{jklmn}	16.39 ± 0.5 ^{ijklmno}	88.58 ± 1. ^{abcdefghij}	93.98 ± 0.9 ^{abcde}
ALRT	29.54 ± 1.5 ^{mnopq}	11.98 ± 0.9 ^{nop}	95.66 ± 1.6 ^{abc}	96.28 ± 0.8 ^{abc}
ALLN	35.46 ± 1.3 ^{klmnop}	12.98 ± 0.9 ^{klmnop}	86.39 ± 1.7 ^{efghij}	90.72 ± 0.9 ^{abcdef}
AMRT	22.67 ± 1.6 ^{pq}	9.07 ± 0.6 ^{op}	91.14 ± 1.4 ^{abcdefgh}	96.49 ± 1.3 ^{abc}
AMAI	27.13 ± 1.8 ^{pq}	10.64 ± 0.6 ^{pq}	94.98 ± 0.7 ^{abcd}	97.07 ± 0.9 ^{ab}
AMAL	29.95 ± 1.9 ^{mnopq}	12.76 ± 0.7 ^{klmnop}	87.13 ± 1.6 ^{defghij}	94.30 ± 0.9 ^{abcde}
AMBL	21.91 ± 1.7 ^{pq}	8.93 ± 0.5 ^{pq}	95.95 ± 1.3 ^{a}	98.08 ± 0.6 ^{a}
ASAI	97.33 ± 0.8 ^{a}	92.41 ± 0.8 ^{a}	0 ^{l}	0 ^{g}
BBAL1	75.25 ± 1.6 ^{bc}	38.55 ± 1.6 ^{bcd}	93.19 ± 0.7 ^{abcdef}	97.52 ± 0.7 ^{ab}
BBAL2	57.24 ± 1.4 ^{fg}	23.55 ± 1.1 ^{gh}	92.14 ± 0.8 ^{abcdefg}	94.42 ± 0.5 ^{abcde}
BBAL3	38.14 ± 1.6 ^{klm}	14.43 ± 0.9 ^{klmnop}	88.45 ± 1.6 ^{abcdefghij}	93.40 ± 0.9 ^{abcde}
BBAL4	36.36 ± 1.9 ^{jklmno}	12.60 ± 1.0 ^{lmnop}	87.80 ± 1.0 ^{cdefghij}	91.94 ± 0.7 ^{abcdef}
BBBL1	50.41 ± 1.3 ^{ghi}	18.93 ± 1.1 ^{hijkl}	89.30 ± 1.1 ^{abcdefghij}	94.65 ± 0.7 ^{abcd}
BBBL2	51.03 ± 1.8 ^{ghi}	20.95 ± 1.0 ^{ghij}	87.96 ± 1.4 ^{bcdefghij}	91.07 ± 1 ^{abcdef}

Table 5. *Cont.*

Isolates	Conidial Germination (%)			
	Exposure to 45 °C		Incubating at 4 °C	
	1 h	2 h	7 Day	14 Day
BBLN1	76.29 ± 1.9 ^{b}	41.58 ± 1.3 ^{bc}	95.84 ± 0.7 ^{ab}	97.29 ± 0.9 ^{ab}
BBLN2	69.56 ± 1.2 ^{bcd}	35.83 ± 1.8 ^{cde}	86.54 ± 0.9 ^{efghij}	92.33 ± 0.8 ^{abcde}
BBLN3	42.47 ± 1.7 ^{ijkl}	21.85 ± 1.2 ^{ghi}	88.24 ± 1.6 ^{abcdefghij}	93.19 ± 0.9 ^{abcde}
BBLD1	43.06 ± 1.5 ^{hijk}	26.70 ± 1.1 ^{fg}	93.78 ± 0.9 ^{abcdef}	91.51 ± 6.1 ^{abcde}
BBLD2	57.79 ± 1.3 ^{efg}	30.97 ± 1.1 ^{ef}	86.48 ± 1.1 ^{efghij}	91.68 ± 0.8 ^{abcde}
BBLD3	26.51 ± 1.4 ^{pq}	11.27 ± 0.7 ^{op}	94.98 ± 0.9 ^{abcd}	96.86 ± 0.7 ^{ab}
BBLD4	37.00 ± 1.1 ^{jklmno}	14.55 ± 1.1jkl ^{mnop}	85.03 ± 1.1 ^{fghijk}	90.64 ± 0.9 ^{abcdef}
BBLD5	33.88 ± 2.3 ^{lmnop}	12.19 ± 1.1 ^{mnop}	85.95 ± 1.2 ^{fghijk}	91.11 ± 1 ^{abcdef}
BBRT1	51.95 ± 1.4 ^{gh}	19.08 ± 1.1 ^{hijk}	93.83 ± 0.9 ^{abcdef}	96.50 ± 0.7 ^{abc}
BBRT2	35.18 ± 1.7 ^{klmnop}	18.59 ± 0.8 ^{hijklm}	90.53 ± 0.8 ^{abcdefghi}	95.47 ± 0.8 ^{abcd}
BBRR1	72.63 ± 1.2 ^{bc}	33.95 ± 1.6 ^{de}	94.23 ± 0.9 ^{abcde}	98.14 ± 0.5 ^{a}
BBRR2	28.27 ± 1.2 ^{opq}	12.29 ± 1.6 ^{mnop}	84.83 ± 1.2 ^{fghijk}	93.03 ± 0.9 ^{abcde}
BBSI	42.16 ± 1.7 ^{ijkl}	18.04 ± 1.1 ^{hijklmn}	82.78 ± 1.3 ^{ijk}	90.66 ± 1.2 ^{abcdef}
BBFN	44.60 ± 2.1 ^{hij}	19.08 ± 1.1 ^{hijk}	87.55 ± 1.2 ^{defghij}	91.90 ± 1.2 ^{abcde}
BBAI	42.76 ± 2.3 ^{ijkl}	18.59 ± 0.8 ^{hijklm}	87.60 ± 1.4 ^{defghij}	93.38 ± 1.1 ^{abcde}
HSAL	11.38 ± 0.9 ^{r}	2.90 ± 0.7 ^{q}	83.48 ± 1.6 ^{hijk}	88.83 ± 1.5 ^{cdef}
HSBL	9.77 ± 0.6 ^{r}	1.59 ± 0.5 ^{q}	82.95 ± 3.3 ^{ijk}	86.87 ± 1.4 ^{ef}
MASA	73.41 ± 1.2 ^{bc}	36.91 ± 2.1 ^{bcde}	89.87 ± 2.1 ^{abcdefgij}	94.93 ± 1.7 ^{abcd}
MAAI	67.65 ± 1.6 ^{bcd}	33.82 ± 1.5 ^{de}	83.93 ± 1.4 ^{hijk}	89.85 ± 1.3 ^{bcdef}
MAAL	63.04 ± 1.4 ^{def}	31.31 ± 1.2 ^{ef}	82.04 ± 1.5 ^{jk}	88.30 ± 0.7 ^{def}
TSRT	69.8 ± 1.7 ^{bcd}	40.57 ± 1.7 ^{bc}	88.11 ± 1.2 ^{abcdefghij}	93.64 ± 0.8 ^{abcde}
TSAH	75.05 ± 1.7 ^{bc}	43.29 ± 1.2 ^{b}	84.74 ± 2.3 ^{ghijk}	89.89 ± 0.8 ^{bcdef}

Note: Statistical differences are shown by different letters.

4. Conclusions

Despite there are several commercial mycoinsecticide against major insect pests of agricultural products, exploration to native entomopathogenic fungi in each region may contribute to represent isolates or species with environmental and host adaptations. These adaptations may ensure virulence, environmental persistence and the least non-target effects of native isolates when they are used in field scale. The overall results of our study revealed the isolates of *B. bassiana,* including BBRR1, BBAL1 and BBLN1, were the most virulent and environmental adaptive isolates among the fungi associated with *C. suppressalis* based on bioassay, biochemical traits and thermal experiments. These isolates should undergo further studies considering field trials on the target pest and some predators and parasitoids of rice fields to better elucidate their role in pathogenesis.

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Insights into the *Gryllus bimaculatus* Immune-Related Transcriptomic Profiling to Combat Naturally Invading Pathogens

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Abstract: Natural pathogen pressure is an important factor that shapes the host immune defense mechanism. The current study primarily aimed to explore the molecular basis of the natural immune defense mechanism of a sporadic pest, *Gryllus bimaculatus*, during swarming by constructing cDNA libraries of the female mid-gut, male mid-gut, testes, and ovaries. The Illumina HiSeq platform generated an average of 7.9 G, 11.77 G, 10.07 G, and 10.07 G bases of outputs from the male mid-gut, female mid-gut, testes, and ovaries and libraries, respectively. The transcriptome of two-spotted field crickets was assembled into 233,172 UniGenes, which yielded approximately 163.58 million reads. On the other hand, there were 43,055 genes in common that were shared among all the biological samples. Gene Ontology analysis successfully annotated 492 immune-related genes, which comprised mainly Pattern Recognition Receptors (62 genes), Signal modulators (57 genes), Signal transduction (214 genes), Effectors (36 genes), and another immune-related 123 genes. In summary, the identified wide range of immune-related genes from *G. bimaculatus* indicates the existence of a sophisticated and specialized broad spectrum immune mechanism against invading pathogens, which provides, for the first time, insights into the molecular mechanism of disease resistance among two-spotted field crickets.

Keywords: antimicrobial peptides; genomics; host defense; immunity; next generation sequencing; transcriptome; two-spotted field crickets

1. Introduction

The two-spotted field cricket, *Gryllus bimaculatus* (Orthoptera, Gryllidae, Gryllinae), is becoming a popular model insect in order to explore behavioral adaptations [1,2], evolutionary biology [3], and physiological [4] and developmental mechanisms [5,6]. The acceptance of two-spotted field cricket for research has mainly occurred because of its widespread abundance in different geographical regions, especially in Asia, Africa, and Europe, in addition to its ease of rearing under laboratory conditions. Furthermore, two-spotted field crickets are currently praised for their possible contribution to global food security by providing an alternate source of protein due to their intrinsic ability to efficiently utilize water and feed compared to traditional livestock [7]. In this regard, a recent study has successfully grown two-spotted field crickets on the by-products of the food industry in order to achieve the targets for the circular economy [7].

The *G. bimaculatus* is categorized as a sporadic pest due to its occasional outbreak under special circumstances at a specific time of the year. In the Kingdom of Saudi Arabia, the outbreak of two-spotted field crickets (black crickets) made its way to such western regions of the country as Makkah and Madinah, where it invaded human dwellings, including places of worship, in which chemical control approaches are not feasible. The residents of the regions were perplexed during their swarming in the last quarter of 2018 till the first quarter of 2019. The latest surveys (unpublished) revealed changing climatic conditions and vegetation covers providing an enormous breeding opportunity for their reproductive success to build sporadic outbreaks of two-spotted field crickets, especially in the Kingdom of Saudi Arabia.

The management of such sporadic outbreaks has become very challenging in urban areas, as synthetic chemical pesticides cannot be applied due to potential threats to public health. Therefore, the use of effective biocontrol agents, which can quickly overcome the host immune defense mechanism, are gaining special attention [8,9]. In the meantime, the host has evolved a highly specialized immune response mechanism to combat the invading pathogens in their surroundings. Therefore, it is very important to document how sporadic pests that occasionally appear in swarms overcome surrounding natural fungal pathogens by fully exploiting their immune defense mechanisms. In the past, the transcriptome of immune response mechanisms of different pest species that appeared in the form of swarms have been well explored in insects such as ants [10,11], fall armyworms [12], hemipteran stinkbugs [13,14], potato leafhoppers [15], and termites [16,17]. The current study was aimed to fully explore the immune-related gene expression patterns evolved in the testes, ovaries, and mid-guts of the adults (males and females) of two-spotted field crickets during swarming to document for the first time a profound understanding of the highly specialized immune responsive combating strategy against natural pathogens that will be useful to develop high-quality reference transcriptomes of two-spotted field crickets for future research into the host–pathogen interactions.

2. Materials and Methods

2.1. Collection, Maintenance, and Tissue Extraction of Two-Spotted Field Crickets

The populations of two-spotted field crickets were directly collected from their outbreak areas located in the western part of the Kingdom of Saudi Arabia. The males and females of the adults of collected populations of two-spotted field crickets were separately kept at a photoperiod of 16 h light, 8 h dark under controlled temperature conditions (30 ± 0.50 °C). Male and female adults of two-spotted field crickets were separately dissected in saline in order to separate different tissues including male mid-gut, female mid-gut, male testes, and female ovaries. These target tissues were stored in 2 mL Eppendorf tubes pre-chilled with liquid nitrogen at −80 °C. Three biological replicates for each target tissue were prepared by separately extracting them from different two-spotted field crickets.

2.2. Construction of cDNA Libraries of Two-Spotted Field Crickets

The frozen tissues were separately ground using liquid nitrogen in a mortar and pestle. The cDNA libraries were constructed by following the previous methodology [16]. In brief, total RNA from each sample was separately extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). The quality of the extracted total RNA was evaluated by electrophoresis (1% agarose gel).

The mRNA molecules were purified using an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), which were used as templates to synthesize the first-strand cDNA using random hexamer primers, and ultimately to synthesize second-strand cDNA libraries. In this study, twelve cDNA libraries including three males mid-guts, three females mid-guts, three males testes, and three females ovaries were prepared to construct four cDNA libraries, each with three independent biological replicates that were separately prepared by sequencing through an Illumina HiSeq^{TM} 4000 platform at MicroAnaly Gene Technologies Co., Ltd. (Wuhan, Hubei, China). These data have been made available at the NCBI Sequence Read Archive (SRA BioProject Acc. No. PRJNA647692).

2.3. Sequence Assembly of the cDNA Libraries of Two-Spotted Field Crickets

After filtering, the resulting clean reads were mapped to the *G. bimaculatus* genome on NCBI (Accession: PRJNA647692 submitted by the investigators) using the Hierarchical Indexing for Spliced Alignment of Transcripts, HISAT program. All reads were assembled by the Trinity assembly program (v2.8.6) in its genome-guided mode, and biological sequences were clustered to remove sequence redundancy in order to obtain the UniGene sequence set for subsequent analysis using the CD-HIT program [18].

2.4. Analysis of Differential Expression of Genes

The DEGs between the paired comparisons (male mid-gut versus female mid-gut; male testes versus female ovaries) were analyzed with the Cuffdiff method. DEGs were considered between four libraries when the screening threshold of the p-value for False Discovery Rate (FDR) was less than 0.05, and an absolute value of log2Ratio FC (Fold Change) for a gene was greater than 1, using R language package DESeq2 (the screening threshold is FDR (false discovery rate) < 0.05, $\log_2$FC (fold change) for a gene > 1 or $\log_2 FC < -1$) [19].

2.5. Functional Annotation of the cDNA Libraries of Two-Spotted Field Crickets

The new transcripts were annotated via BLAST searches against the NCBI non-redundant protein database. All identified genes were quantified in terms of the expected number of fragments per kb of transcript sequence per million base pairs sequenced (FPKM) with the software program Cufflinks. The UniGenes were explored by performing annotations through the Kyoto Encyclopedia of Genes and Genomes (KEGG) [20], through a web-based interface. Immunity-related genes were accordingly classified into their main categories.

3. Results

3.1. Samples Correlation Analysis

The correlation of gene expression levels between samples performed in this study revealed a higher similarity of the expression patterns between samples. Among all the experimental units, biological samples collected from male mid-gut tissues were found to have the highest similarity (Figure 1). On the other hand, ovary samples have shown variability. The samples of testes have also shown similar expression patterns but remained between mid-gut and ovary samples.

3.2. Sequence Assembly Characteristics of Two-Spotted Field Crickets

In this study, the Illumina HiSeq platform was used to perform next-generation sequencing of two-spotted field crickets from different organs including the ovaries, testes, and male and female mid-gut samples. The RNA-Seq led to an average of 50.04 million, 74.88 million, 63.90 million, and 64.63 million clean reads for the constructed full-length cDNA libraries of male mid-gut and female mid-gut, testes, and ovaries of two-spotted field crickets, respectively (Table 1). The Illumina platform generated an average of 7.9 G, 11.77 G, 10.07 G, and 10.07 G bases of outputs of the male mid-gut, female mid-gut, testes, and ovaries libraries, respectively (Table 1). The reads were deposited on the NCBI SRA under accession SRX8826426, SRX8826427, and SRX8826430 (mid-gut of Male two-spotted field crickets); SRX8826431, SRX8826432, and SRX8826433 (mid-gut of female two-spotted field crickets); SRX8826434, SRX8826435, and SRX8826436 (testes of male two-spotted field crickets); SRX8826437, SRX8826428, and SRX8826429 (ovaries of female two-spotted field crickets).

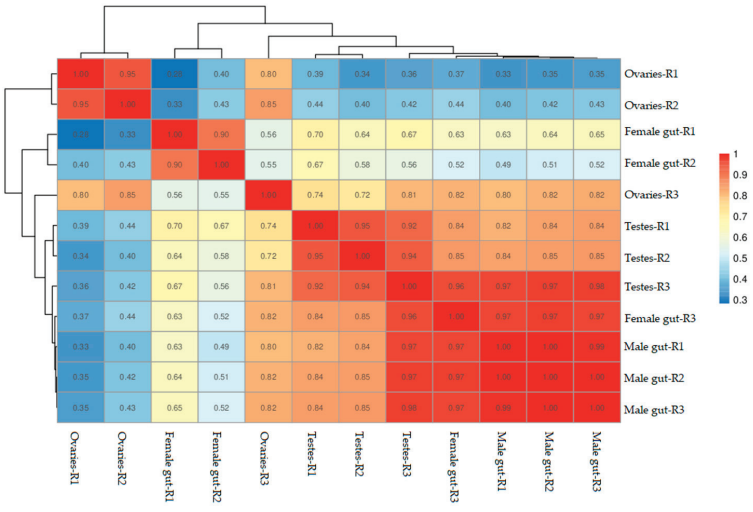

Figure 1. Correlations between cDNA libraries constructed from different parts including the ovaries, testes, female mid-gut, and male mid-gut of two-spotted field crickets. The correlation coefficient close to 1 represents the higher similarity of the expression patterns between samples.

Table 1. Summary of the Illumina Platform RNA-Seq Metrics for two-spotted field crickets.

Sample	NCBI SRA Accession	Biosample Accession	Clean Reads Number	No of Bases	Q20 Content (%)	Q30 Content (%)	GC Content (%)
Mid-Gut of Male Two-Spotted Field Crickets							
R1	SRX8826426	SAMN15594732 (SRS7091132)	42,341,558	6.7 G	93	83	38.1
R2	SRX8826427	SAMN15594733 (SRS7091133)	52,441,310	8.3 G	93	84	39.0
R3	SRX8826430	SAMN15594734 (SRS7091136)	55,325,614	8.7 G	93	84	39.2
Mid-Gut of Female Two-Spotted Field Crickets							
R1	SRX8826431	SAMN15594735 (SRS7091137)	88,936,348	14.0 G	94	84	49.9
R2	SRX8826432	SAMN15594736 (SRS7091138)	80,383,866	12.6 G	94	84	47.2
R3	SRX8826433	SAMN15594737 (SRS7091139)	55,325,614	8.7 G	94	85	44.3
Testes of Male Two-Spotted Field Crickets							
R1	SRX8826434	SAMN15594738 (SRS7091140)	67,447,566	10.6 G	94	84	49.3
R2	SRX8826435	SAMN15594739 (SRS7091141)	59,198,274	9.4 G	93	84	49.0
R3	SRX8826436	SAMN15594740 (SRS7091142)	65,058,620	10.2 G	94	85	45.2
Ovaries of Female Two-Spotted Field Crickets							
R1	SRX8826437	SAMN15594741 (SRS7091143)	70,968,356	11.1 G	95	86	43.6
R2	SRX8826428	SAMN15594742 (SRS7091134)	58,530,318	9.1 G	95	87	41.9
R3	SRX8826429	SAMN15594743 (SRS7091135)	64,386,128	10.0 G	95	87	42.5

3.3. Classification and Functional Annotation of Two-Spotted Field Cricket Transcriptomes

Each organ transcript assembly was completed by assembling all the three replicates of the reads and were archived into 158,658 UniGenes from the female mid-gut, 121,025 UniGenes from the male mid-gut, 157,036 UniGenes from the ovaries, and 72,216 UniGenes from the testes of two-spotted field crickets. Overall, the generated transcript sequence resources of all the studied organs were assembled into 233,172 UniGenes, which yielded approximately 163.58 million reads. Furthermore, the assembly comprised of 316.91 million reads of 325,568 transcripts. The length of the sequence reads in each case ranged between 201 to 32,390 base pairs.

The relationship of the genes assembled in this study was visualized by drawing a Venn diagram (Figure 2). Overall, 43,055 genes were common genes that were shared by all the biological samples. On the other hand, there were 15,787 unique genes from the male mid-gut, 22,369 unique genes from the female mid-gut, 31,418 unique genes from the ovaries, and 3261 unique genes from the testes that did not show any relationship.

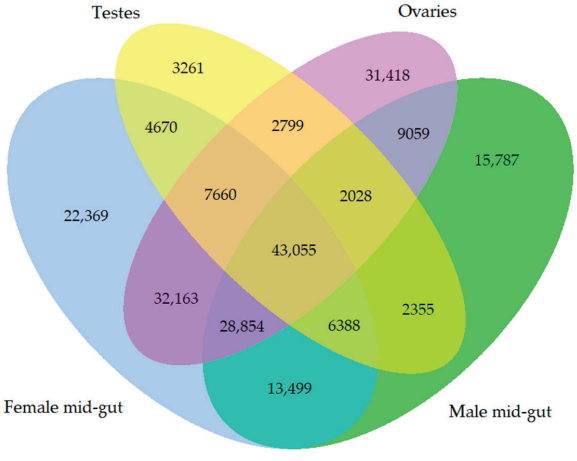

Figure 2. Venn diagram showing the number of overlapping genes among all the libraries of two-spotted field crickets.

The assembly of cDNA libraries showed variations in identified and unidentified reads among different organs. Among all the libraries, the highest reads (average of three male mid-gut libraries 22.52%) from the transcriptome of the male mid-gut were successfully identified, while the least reads (average of three Ovaries libraries 3.51%) were identified from the cDNA libraries constructed from the ovaries of two-spotted field crickets (Table 2). On the other hand, 10.28% (average of three female mid-gut libraries), and 12.40% (average of three testes libraries) assembled reads from the cDNA libraries of the female mid-gut and testes were identified, respectively. Interestingly, a major proportion of the identified reads matched with the Arthropoda, as can be seen in Table 2.

The transcriptomes were successfully mapped onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in order to reveal biochemical pathways operating in two-spotted field crickets. Overall, the KEGG analysis revealed the seven different main functional processes (level 1), which were further composed of 48 GO terms as shown in Figure 3. Based on the analysis, a great number of genes mapped in this analysis were involved in the regulation of host defense mechanisms through signal transduction and immune system GO terms among all the cDNA libraries.

Table 2. Assembly metrics for two-spotted field crickets transcriptome sequencing.

Assembled Reads Information	Male Mid-Gut			Female Mid-Gut			Ovaries			Testes		
	R1 (%)	R2 (%)	R3 (%)	R1 (%)	R2 (%)	R3 (%)	R1 (%)	R2 (%)	R3 (%)	R1 (%)	R2 (%)	R3 (%)
Unidentified Reads	68.68	79.08	84.68	95.38	91.87	81.92	96.3	97.28	95.9	87.76	89.21	85.83
Identified Reads	31.32	20.92	15.32	4.62	8.13	18.08	3.7	2.72	4.10	12.24	10.79	14.17
Matched with Arthropoda	23.71	15.32	11.69	2.76	5.61	11.47	1.56	1.13	2.05	5.96	5.24	8.84

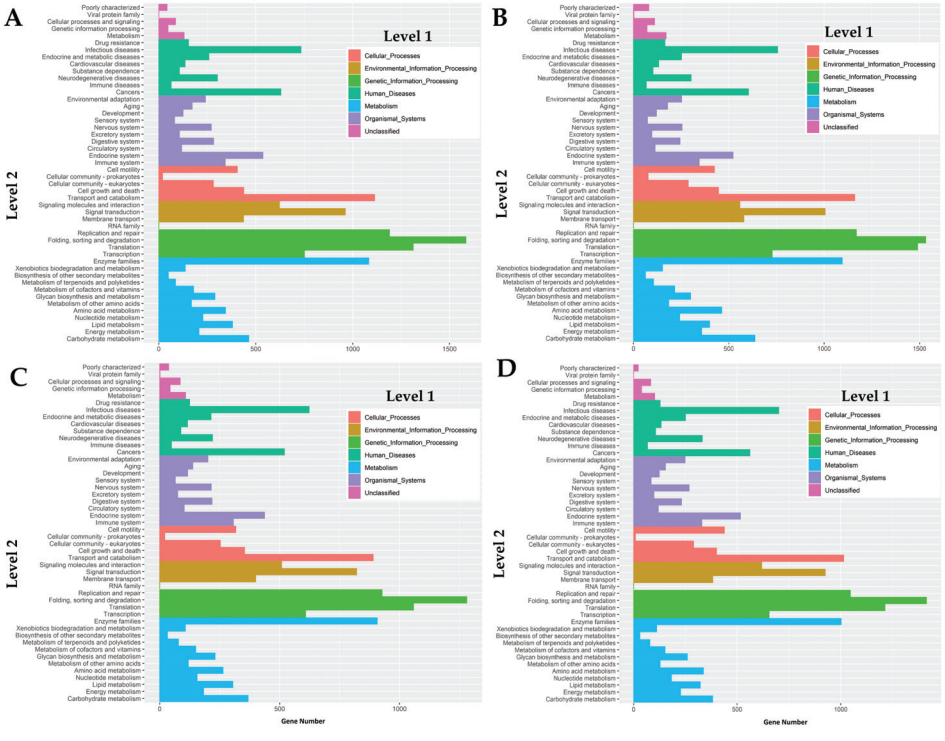

Figure 3. GO mapping of the cDNA libraries of (**A**) female mid-gut, (**B**) male mid-gut, (**C**) testes, and (**D**) ovaries of two-spotted field crickets by the Kyoto Encyclopedia of Genes and Genomes, KEGG classification.

3.4. Patterns of Differential Gene Expression Levels

Cluster analysis of gene expression patterns can intuitively reflect the level of gene expression and expression patterns in multiple samples. Overall, 2242 UniGenes were up-regulated, while 1805 were down-regulated between the male and female mid-gut samples. On the other hand, a comparatively a higher number of UniGenes were up-related (8921), and concurrently a higher number of genes were also down-regulated (8716) between teste and ovary samples of the two-spotted field crickets.

3.5. Immune-Related Transcriptome of Two-Spotted Field Crickets

The transcriptome of two-spotted field crickets assembled as a result of the current study revealed the identification of 492 different types of genes regulating the immune response mechanism under

natural conditions (Supplementary Tables S1–S5). The immunity-related transcript analysis successfully identified a wide range of Pattern Recognition Receptors (62 genes), most importantly *PRPs*, *CTLs*, *GALE*, *GNBP*, *Immulectin*, *Beta-1*, *3-glucan-binding protein*, and *DSCAM*, which actually initiate the host immune defense mechanism (Table 3).

Table 3. Immune-related transcriptome of two-spotted field crickets.

Functional Categories	Characterized Annotations
Pattern Recognition Receptors	Apolipophorin, Ataxin, Beta-1,3-glucan-binding protein, C-type lectin (CTL), Down syndrome cell adhesion molecule-like protein (DSCAM), Galectin (GALE), Gram negative binding protein (GNBP), Hemolymph lipopolysaccharide-binding protein, Hemolymph juvenile hormone binding protein, Immulectin, Lectin, Peptidoglycan-recognition protein (PRP), Regenectin, Septin, Spondin, Techylectin
Signal Modulators	Allatotropin (ATs), Allatostatin (Ast), Angiopoietin (Ang), Chymotrypsin, Kazal domain-containing peptide, Kunitz-type protease inhibitor, Porin, proPO, Prostaglandin, Serine protease (SPs), Serpin, Tetraspanin
Signal Transductors	Adiponectin receptor protein, Allatostatin A prohormone, Ankyrin repeat and fibronectin type-III domain-containing protein 1, Angiomotin, Beta-arrestin2, Bursicon-beta, C2 domain-containing protein, CSN5 cop9 signalosome subunit 5, C-Jun-amino-terminal kinase-interacting protein 3, Calmodulin, Cactin, Calpain, Chimaerin, Contactin, COP9 signalosome complex, EF-hand domain-containing protein, Folliculin, Four-and-a-half LIM domain protein 1 isoform B, Frizzled, Hippo, Hippocampus abundant transcript 1 protein, IMD-like protein, JNK-interacting protein, Kruppel-like protein, Leucine-rich repeat-containing protein, Malectin-B, NACHT and WD repeat domain-containing protein 1, NACHT and Ankyrin domain protein, Nesprin, Notch, Octopamine, Pelle, Rab, Ras, Rho, Serine/threonine-protein kinase, Spaetzle, Striatin, TATA-box binding protein, Toll-like receptors, Transgelin, Transducin beta-like protein, Target of rapamycin complex 2 subunit MAPKAP1, Tubulin beta chain, WD repeat-containing protein, Wnt, WW domin protein, Zinc finger protein, 14-3-3 family protein
Effectors	Attacin, Bacteriocin, Carboxypeptidase, Cathepsin, Caspase, Lysozyme, Pyocin, C-type lysozyme, I-type lysozyme, Pyocin, Thaumatin-like protein 1

We identified 57 genes amplifying the pathogen invasive signals through signal modulation. The representatives of these genes including *ATs*, *Ast*, *Ang*, *Chymotrypsin*, *Kazal domain-containing peptide*, *Kunitz-type protease inhibitor*, *Porin*, *proPO*, *Prostaglandin*, *SPs*, *Serpin*, and *Tetraspanin*, modulate a wide range of signaling pathways to combat pathogen invasion (Table 3 and Table S2). The activated signaling pathways including the JAK-STAT signaling Pathway, JNK pathway, Toll-like receptor (TLR) signaling pathways, Wnt signaling pathway, Notch signaling pathway, Hedgehog signaling pathway, Hippo signaling pathway, Immune Deficiency (Imd) pathway, and MAPK (Mitogen-activated protein kinases) signaling pathways are regulated through the identification of 214 genes (Table S3), involved in signal transduction (Table 3). The effectors expressed as a result of the immune mechanism among two-spotted field crickets revealed the identification of 36 genes (Table S4), encoding antimicrobial peptides and proteins. However, *Attacin*, *Bacteriocin*, *Carboxypeptidase*, *Cathepsin*, *Caspase*, *Lysozymes*, *Pyocin*, and *Thaumatin-like protein* were the most prominent genes identified in this study from two-spotted field crickets (Table 3). On the other hand, our transcriptome exploration study also revealed the identification of 123 genes categorized as other immunity-related genes because their role in immune mechanism is not yet well categorized (Table S5).

4. Discussion

Our study to explore the molecular mechanisms of the immune system of two-spotted field crickets by next-generation sequencing of male mid-gut, female mid-gut, ovaries, and testes revealed the identification of novel genes contributing in the regulation of host defence. The characterized annotated

transcriptome as a result of this study enabled us to suggest that *G. bimaculatus* displayed a strong antimicrobial response in the form of effectors to defend naturally occurring pathogens. The current findings greatly help us to understand the molecular mechanisms of host–pathogen relationships.

The transcriptome sequencing of two-spotted field crickets performed in this study from tissues mainly aimed to explore for the first time the molecular mechanism of immunity in *G. bimaculatus*. Our methodology was successfully able to assemble the genome into 233,172 UniGenes, which yielded approximately 163.58 million reads. Furthermore, the assembly yielded 316.91 million reads of 325,568 transcripts. However, results revealed the variability, especially ovaries R3, and female mid-gut R3 biological replicates. The variations among few biological replicates evidenced in the current study and strengthened by previous investigation are quite obvious due to environmental and genetic differences because each sample was prepared by separately extracting from different two-spotted field crickets [21]. Interestingly, the annotated sequences identified a huge number of genes (492) regulating the host defence mechanisms. The Pattern Recognition Receptors (PRRs) initiate the host immune defence system by recognising the receptors for pathogen-associated molecular patterns (PAMPs) [22]. Upon recognition of PAMPs, these PRRs can either mediate pathogen killing directly through phagocytosis and encapsulation or indirectly through intra-cellular signal transduction pathways. These pathways ultimately lead to the transcription of effector genes [23–26]. Our transcriptomic analysis revealed the identification of several different classes of PRRs, and most importantly *GNBP1*, *beta-1,3-glucan-binding protein*, multiple isoforms of *apolipophorin*, *Ataxin*, *CTL*, *DSCAM*, *GALE*, and *Immulectin*. These recognition receptors are bounded with the components of the microbial cell wall, and it ultimately started a tug-of-war between the host and the invading pathogen [27,28].

Our two-spotted field cricket transcriptome analysis revealed the identification of five different isoforms of the *apolipophorin family (apoLp).* The members of the *apoLp* family are known to be involved in multiple functions, especially in activating the immune response through binding with β-1,3-glucans of fungi, LPSs of Gram-negative bacteria, and lipoteichoic acid of Gram-positive bacteria [29,30]. The previous findings of *Galleria mellonella* already showed that *apoLp* not only binds to fungal conidia and beta-1,3-glucan, but also stimulates cellular encapsulation [31]. The *apoLp* of *G. mellonella* affects the fungal cell wall components and exhibits antibacterial activity against selected gram-positive and gram-negative bacteria *in vitro* [32,33]. Our results showed five *apoLp*, which might play an important role in microbial infection. The transcriptome analysis also revealed the identification of *C-type lectins*, *Immunolectin*, *Lectin-related*, and *Galectin*, which are known to be involved in the innate immune response. They recognize the chains of polysaccharide present on the surface of pathogens [34,35]. Another class of PRRs, such as *GNBP1*, which triggered the protease cascades by recognizing gram-positive bacteria, was identified in this study and ultimately causes the cleavage of Spaetzle [36]. Such a wide range of transcripts of PRRs revealed for the first time as a result of this study from two-spotted field crickets enabled us to suggest that the host has well-developed weaponry mechanisms to recognize the invading natural pathogens prevailing in their surroundings.

Once the invading pathogen has been recognized, PRRs triggers the initiation of signal modulation genes that amplify the signals of pathogen invasion. These signals ultimately activate various lines of defence against the invasion of pathogens. In this transcriptome analysis, we identified 57 genes encoding proteins potentially involved in signal modulation. From our database, a number of signaling modulation genes were observed such as CLIP domain (CLIPs), *serine*, and *serpins*. Signal modulation genes, especially *serine proteases* (*SPs*), regulate several invertebrate defense responses, including hemolymph coagulation, antimicrobial peptide synthesis after toll signal-transduction pathway and activation of phenoloxidases (POs) [37]. Serine-type protease inhibitors (*Serpins*) and *Kazal* play an important role in inhibiting the protease cascades that activate toll and melanization reaction in *Drosophila* [23,38]. Serpin-like proteins have already been reported in many insects such as *H. cunea*, *A. melifera*, *A. gambiae*, *B. mori*, and *D. melanogaster* [39–41]. Interestingly, our transcriptome analysis successfully annotates genes modulating the immune mechanism.

Various pathways regulate the immune response of invertebrates against invading pathogens by transmitting signals from recognition receptors to the synthesis of AMPs and other effectors. The current exploration annotated 214 genes involved in various types of immune signaling pathways including JAK-STAT, JNK, Toll-like receptor (TLR), Wnt, Notch, Hedgehog, Hippo, Immune Deficiency (Imd), and MAPK (Mitogen-activated protein kinases) signaling pathways.

Members of the Ras superfamily identified in this transcriptome exploration are reported to be involved in the complex signaling pathways of *Drosophila* [42]. The Ras superfamily is the protein of small guanosine triphosphatases (GTPases) comprised of five major families, including *Arf/Sar*, *Rab*, *Ran*, *Ras*, and *Rho* [43,44]. The exact role of these genes in *G. bimaculatus* is unknown. However, *Ras* genes are known to be involved in the cellular immune response of beet armyworm, *S. exigua* [45]. Furthermore, Rojas., et al. [44] reviewed the role of *Ras* superfamily member genes and explained that they act as signaling nodes that regulate apoptosis, cell proliferation, and differentiation. Another important gene was the *Four-and-a-half LIM domain protein 1 isoform B.* These cysteine-rich LIM domain-containing genes are previously found to mediate signal transduction cascades. Their deficiency in mice resulted in delayed wound healing [46]. The presence of *Serine/threonine protein kinase (STK)* from the transcriptome of two-spotted field crickets is an important finding because these genes are well known to function as an important defence gene by mediating signal transduction pathways in plants [47]. Their role in the insect immune signaling pathway has been explored by Belvin and Anderson [48]. They suggested that *STK* is an important component of the Toll-Dorsal pathway responsible for the degradation of cactus proteins in *Drosophila*. The various isoforms of genes encoding *Zinc finger proteins* characterized in the current study promote the *Toll-like receptors* as depicted in the current study to trigger innate immune responses by pressing IκBα gene transcription as previously reported from human beings [49]. On the other hand, *Kruppel-like protein* characterized here is a transcriptional repressor associated with signal transduction and activator of transcription (STATs) in the immune response [50].

The transcriptome analysis showed the presence of *14-3-3* genes from the two-spotted field crickets. These signal transduction regulatory genes interact in a phospho-serine dependent manner. The members of this protein are involved in cellular and physiological processes. The recent findings suggested that these genes are important phagocytosis mediators that play a pivotal role in defending *S. aureus* attack on *Drosophila* and zebrafish [51]. Furthermore, they illustrated that the depletion of *14-3-3* expression reduced the ability to fight against microbial infection. However, this depletion does not compromise the production of antimicrobial peptides [51]. In addition to gene encoding *14-3-3* proteins, an important multifunctional group of genes encoding *COP9 signalosome complex subunits* was identified from two-spotted field crickets. These *COP9 signalosome complex subunits* are known to function as an important defence protein and dispensable for Toll/IL-1 activation in the fat body by mediating signal transduction pathways in *Drosophila* [52]. They suggested that *CSN5* is an important component of signaling pathways involved in Cactus and Dorsal regulation to mediate immune response in *Drosophila*.

The genes encoding *EF-hand domain-containing proteins* were also identified in this study. The members of this domain, including *calmodulins*, are known to regulate Ca^{2+} channel in vertebrates and invertebrates [53]. These genes are known to play an important role in immune responses by maintaining calcium levels. In addition to the above mentioned signal transduction genes, *Tubulin beta chain*, *Transgelin*, *Hedgehog protein*, *Hippo*, *Hippocampus abundant transcript 1 protein*, *IMD-like protein*, *JNK-interacting protein 1*, *Leucine-rich repeat-containing protein 68*, *Notch*, *Pelle*, *Relish*, *Renin*, *Spaetzle*, and *Wnt* identified in this study might help to understand their role in signal transduction cascades.

Our study revealed the identification of effectors among which *Attacin*, *Bacteriocin*, *thaumatin-like protein*, *C-type Lysozyme*, *I-type Lysozyme*, *Cathepsin D*, *Carboxypeptidase*, *Caspase*, and *Pyocin* were prominent. These molecules play an important direct role in combating pathogenic microorganisms. Generally, *thaumatin-like proteins* possessed 16 conserved cysteine residues that form eight disulfide bonds [54]. The antifungal activities of more than 20 isoforms of *thaumatin-like proteins* have been reported [55,56]. These proteins were first identified from the West African shrub *Thaumatococcus*

daniellii and is known to synthesize in plants against stress and microbial infection [57]. Later, these proteins were further identified from animals [58], and fungi [59]. More recently, these proteins have also been identified from insects including *Acyrthosiphon pisum*, *Coptotermes formosanus* Shiraki, and *Tribolium castaneum* [16,60].

The transcriptome analysis of two-spotted field crickets showed multiple isoforms of several genes encoding *c-type lysozymes*, *i-type-lysozymes*, and *lysozymes*. Generally, insect lysozymes are known to play a pivotal role in insect immunity [61–63]. In addition, *lysozymes* isolated from the eggs and salivary glands of *Reticulitermes speratus* showed a strong novel egg recognition activity [64]. Furthermore, they observed that the newly laid eggs are frequently coated with the saliva of the worker caste containing lysozyme through egg grooming. Along with multiple isoforms of *lysozymes*, numerous genes encoding various *cathepsins* were identified. These genes have shown to regulate multiple proteins facilitating bacterial killing [65]. These proteins are known to play an important role against infections because they are believed to be highly expressed in immune-related organs [66].

Glycine-rich proteins such as *Attacin* belong to an important antimicrobial peptide group, which was for the first time discovered from *Hyalophora cecropia* [67]. The latest review article revealed the identification of different isoforms of *attacin* from different insects including silkworms, tse-tse fly, housefly, tobacco budworm, cabbage looper, and wild silkworm [68]. Our transcriptome study for the first time identified *attacin* from two-spotted field crickets. These findings enabled us to suggest the highly specialized broad spectrum host–antimicrobial response against invading pathogens.

5. Conclusions

In conclusion, the current study successfully constructed cDNA libraries from different tissues of the male mid-gut, female mid-gut, ovaries, and testes of two-spotted field crickets. The Illumina HiSeq platform generated an average of 7.9 G, 11.77 G, 10.07 G, and 10.07 G bases of outputs, which assembled into 121,025, 158,658, 72,216, and 157,036 UniGenes from the male mid-gut, female mid-gut, testes, and ovaries, respectively. The transcriptome analysis of the generated libraries revealed for the first time the identification of 492 different types of genes under the categories including Pattern Recognition Receptors (62 genes), Signal modulators (57 genes), Signal transduction (214), effectors (36 genes), and others (123 genes) to regulate the immune mechanism under natural conditions among two-spotted field crickets. In summary, *G. bimaculatus* transcriptome analysis provides preliminary evidence for their survival through their sophisticated and a specialized broad spectrum host immune defence mechanism against natural pathogens. It will open new avenues of research to develop molecular insecticides, and drug development by targeting genes regulating host immune defence mechanisms.

Supplementary Materials: The following are available online at http://www.mdpi.com/2309-608X/6/4/232/s1, Table S1: Immune-related Pattern Recognition Receptors from the transcriptome of two-spotted field crickets, Table S2: Immune-related Signal Modulators from the transcriptome of two-spotted field crickets, Table S3: Immune-related Signal Transductors from the transcriptome of two-spotted field crickets, Table S4: Immune-related Effectors from the transcriptome of two-spotted field crickets, Table S5: Other Immune-related sequences from the transcriptome of two-spotted field crickets.

Author Contributions: A.H., A.M.A. and S.N.A.-K. conceived the idea. A.H., M.W.A., A.M.A. and S.N.A.-K. designed the experiments. A.M.A. provided the resources. A.H. and A.M.A. performed the experiments. A.H., M.W.A., A.M.A. and S.N.A.-K. analyzed the data. A.H. wrote the original draft. A.H., M.W.A., A.M.A. and S.N.A.-K. revised and edited the manuscript. A.H., M.W.A., A.M.A. and S.N.A.-K. approved the manuscript for publication. All authors have read and agreed to the published version of the manuscript.

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Low Efficacy of *Isaria fumosorosea* against Box Tree Moth *Cydalima perspectalis*: Are Host Plant Phytochemicals Involved in Herbivore Defence against Fungal Pathogens?

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Abstract: *Buxus* sp. is an important native and ornamental tree in Europe threatened by a serious invasive pest *Cydalima perspectalis*. The larvae of this moth are able to defoliate box trees and cause their death. The development of novel biopesticides targeting this pest might help protect *Buxus* trees grown wildly or in city parks. Laboratory experiments were conducted to assess the efficacy of entomopathogenic fungus *Isaria fumosorosea* strain CCM 8367 against *C. perspectalis*. The last-instar larvae of the box tree moth were treated by the suspension of fungus conidia at concentrations ranging from 1×10^4 to 1×10^8 spores per 1 mL. Fungus infection was observed mostly in pupae, but the maximum mortality did not exceed 60%, indicating a very low susceptibility of *C. perspectalis* to *I. fumosorosea*. Furthermore, a number of ungerminated fungal conidia were found on larval cuticles using a low-temperature scanning electron microscopy. Our data also reveal that the hydroalcoholic extract from *B. sempervirens* leaves significantly inhibits both the germination of *I. fumosorosea* conidia and fungus growth. It can be speculated that the strain CCM 8367 of *I. fumosorosea* is not a potent biocontrol agent against *C. perspectalis* and low virulence of the fungus might be due to the accumulation of host plant phytochemicals having antimicrobial activity in larval cuticle of the pest.

Keywords: *Buxus*; entomopathogenic fungi; invasive pests; virulence; alkaloids; antimicrobial activity

1. Introduction

Box trees, *Buxus* sp., are important evergreen shrubs occurring in natural *Buxus* forests [1] or grown as ornamental trees in city parks in Europe. They are now endangered by the box tree moth (BTM), *Cydalima perspectalis* (Walker) (Lepidoptera: Crambidae), which is a serious invasive pest native to Asia that was first detected in Germany in 2007 and has since invaded a large area causing significant damage [2,3]. This pest overwinters at the larval stage [4,5] and can have two to four generations per year in Europe depending on abiotic conditions [6]. Natural enemies do not suppress the *C. perspectalis* population, which is probably because this exotic species does not seem to be a good host for native parasitoids [4,7,8]. Thus, the pest is able to destroy *Buxus* tree completely in one season [9]. Some synthetic chemical insecticides are effective in *C. perspectalis* control [10]. Still,

their application in natural habitats is problematic because of their adverse side effects on non-target species. Their frequent application possibly leads to the risk of resistance development in the pest.

The use of microbial biopesticides against BTM offers a unique alternate solution to broad-spectrum chemical insecticides. The best results have been obtained by using products based on *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) while entomopathogenic nematode *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) was less successful [11]. Entomopathogenic fungi (EPFs) represent other promising biocontrol agents. Their advantages are that they do need to be ingested as they are able to penetrate the host cuticle and can be relatively easily produced [12]. A number of mycoinsecticides, most commonly based on *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* (Metsch.) Sorokin, (Hypocreales: Clavicipitaceae), *Isaria fumosorosea* (WIZE) Brown & Smith (Hypocreales: Cordycipitaceae), and *B. brongniartii* (Saccardo) (Hypocreales: Cordycipitaceae) have been developed in the world [13]. To our knowledge, only *B. bassiana* strain GY1-17 was tested against BTM in Korea, but larvae were not affected significantly [14].

The present study aimed to assess the possibility of fungal biocontrol of *C. perspectalis* by *I. fumosorosea*, which is known to be virulent to many insect species including a wide variety of butterflies and moths [15–17] and has received significant attention as a possible biological control agent for several economically important pests [18]. The obtained results showed low efficacy of the fungus against this pest. Therefore, additional experiments were conducted to test the hypothesis that the low effectiveness might be due to the antifungal activity of some host plant phytochemicals consumed by the moth larvae. Low-temperature scanning electron microscopy revealed that the number of *I. fumosorosea* conidia did not germinate. In vitro experiments confirmed that the hydroalcoholic extract of *Buxus* leaves suppressed spore germination and fungus growth.

2. Materials and Methods

2.1. Insects

Last-instar larvae of *C. perspectalis* were collected from unsprayed *Buxus sempervirens* trees located in a private garden in Staré Hodějovice (South Bohemia, Czech Republic, 49° N) and maintained in net cages at a room temperature with 16L:8D photoperiod for a few days before they were used in bioassays. Young twigs of untreated box trees collected in the vicinity of the Biology Centre, České Budějovice were provided as food and replaced with fresh ones when needed.

2.2. Entomopathogenic Fungus

Isaria fumosorosea strain CCM 8367 was used in this study. The strain was isolated from the pupa of the horse chestnut leaf miner, *Cameraria ohridella*, Deschka & Dimić (Lepidoptera: Gracillariidae) collected in the Czech Republic [19] and deposited in the Czech Collection of Microorganisms in Brno as a patent culture [20,21].

The fungus was grown on PDA medium (Sigma-Aldrich, Darmstadt, Germany) at 25 ± 1 °C and a 16L:8D photoperiod. After 10 days of incubation, the spore suspensions were prepared from each strain by scraping off conidia into the sterile solution of 0.05% (*v*/*v*) Tween® 80 (Sigma-Aldrich, Darmstadt, Germany). The suspension was filtered through sterile gauze to separate the mycelium and clusters of spores. In uniform suspension, the spores were counted with a Neubauer improved counting chamber (Sigma-Aldrich, Darmstadt, Germany), and subsequently, the suspension was adjusted to the required concentration.

The viability of spores was verified using a standard germination test [22]. Ten drops from suspension were applied using a 1 µL inoculation loop on the surface of 2% water agar, which was poured in a thin layer onto the surface of a sterile slide. After the drops had dried, the slides were moved into a wet chamber and incubated at temperature 25 ± 1 °C for 24 h. The percentage of germinating spores was determined using an Olympus CH20 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan); bright field, 400× magnification. The spore germination in all tests was 100%.

2.3. Bioassays

2.3.1. The Efficacy of *I. fumosorosea* against *C. perspectalis*

Five concentrations of *I. fumosorosea* ranging from 1×10^4 to 1×10^8 spores per 1 mL were used in this experiment. The last-instar larvae of BTM in treated groups were individually immersed in the suspension of conidiospores of the fungus for five seconds (dip-test). All specimens in a control group were immersed in sterile solution of 0.05% Tween® 80 only. Then, the larvae were placed into polystyrene Petri dishes (vented, inner diameter 90 mm, height 15 mm, Gosselin™, Borre, France) lined with moist filter paper (KA 0, Papírna Perštejn, Ltd., Perštejn, Czech Republic) and kept under constant conditions (25 ± 1 °C and 16L:8D photoperiod). Larvae were fed with *B. sempervirens* leaves, which were replaced daily until larva developed into pupa or died. The filter paper was also daily moistened by distilled water to maintain optimal humidity inside the Petri dishes. The insects were monitored daily for a period of three weeks to record insect development, mortality, and the development of mycosis on cadavers until all individuals died or adults emerged.

All bioassays described above were repeated twice; each replication tested 15 insect individuals. Mycosis on cadavers and emerged adults were documented by digital cameras Olympus SP-510 (Olympus Optical Co., Ltd., Tokyo, Japan) and Nikon Coolpix 4500 (Nikon Corporation, Tokyo, Japan) mounted on a tripod and using macro mode.

2.3.2. Scanning Electron Miscroscopy of *I. fumosorosea* Conidia Germination on Cuticle of *C. perspectalis* Larvae

In vivo germination of fungal conidia on the insect cuticle was examined by low-temperature scanning electron microscopy (LT-SEM). BTM larvae were treated by immersing in suspension of *I. fumosorosea* conidia (concentration 5×10^7 spores mL^{-1}) and incubated for 0, 24, and 48 h at the temperature of 25 °C. The larvae were mounted on an aluminum stub using Tissue-Tek (C.C.T.D. Compound, The Netherlands). The samples were extremely fast ($<10^{-3}$ K/s) frozen in vapor of liquid nitrogen. After freezing, the samples were transfered into a GATAN ALTO-2500 high vacuum cryo-preparation chamber (Gatan Inc., Abingdon, UK). The surface of the sample was sublimated (freeze-etched) for 5 min at the temperature of −95 °C and at −130 °C. After sublimation, the samples were sputter-coated with gold at the temperature of −130 °C. Coated samples were inserted into the chamber of a JEOL JSM-7401F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan). Images were obtained by the secondary electron signal at an accelerating voltage of 4 kV and current 10 μA using an Everhart–Thornley Detector (ETD).

2.3.3. The Effect of *B. sempervirens* Extract on *I. fumosorosea* Germination and Growth

Plant material was collected from untreated *B. sempervirens* trees grown in the Biology Centre garden. The extract used for the study was prepared at the concentration of 20% (*w/v*) by grinding 2 g of fresh leaves in 10 mL of solvent (water–ethanol 1:1 mixture). Analytic grade ethanol (Penta Ltd., Czech Republic) and distilled water were used. The mixture was filtered through filter paper (KA 0, Papírna Perštejn, Ltd., Czech Republic) to remove particulate materials, and one milliliter of fresh extract was spread on the surface of 2% water agar in Petri dish and left to dry for 24 h. Then, a suspension of *I. fumosorosea* conidia was applied using an inoculation loop on the surface. Germination was evaluated in 100 spores after 24 h of incubation at 25 ± 1 °C as described above. The control plate was treated with solvent only. The experiment was conducted in three replicates. Spore germination was documented by NIS-Elements Imaging Software and a Nikon Eclipse E200 microscope equipped with Nikon DS-Fi3 color camera (Nikon Corporation, Tokyo, Japan).

The effect *B. sempervirens* extract on fungus growth was measured by a modified inhibition zone assay [23]. A half mL of conidia suspension in 0.05% Tween® 80 at a concentration 1×10^4 spores mL^{-1} was spread evenly on potato dextrose agar (PDA) medium in a plastic Petri dish (diameter 90 mm). A hydroalcoholic extract of *B. sempervirens* leaves prepared as described above was applied on filter

paper discs (diameter 14 mm) in a dose 150 µL per disc. Control discs were treated with 150 µL of the pure solvent. The solvent was allowed to evaporate, and paper discs were placed carefully in the center of PDA plates. After 7 days incubation at 25 °C, the plates were photographed by a digital camera Olympus SP-510 (Olympus Optical Co., Ltd., Tokyo, Japan) mounted on a tripod to document differences in fungus growth. Then, the area of plate in the center not covered by *I. fumosorosea* mycelium was measured using ImageJ, a Java-based image analysis software [24]. The assay was conducted using 10 dishes (replications) for both treatment and control.

2.4. Data Analysis

To analyze the effect of treatment on developmental time of *C. perspectalis* larvae and pupae, we fitted generalized linear models (GLM) with a Poisson error distribution and log link function. Mortality as well as germination data were analyzed using GLM with a binomial distribution and logit link. Treatment and replication were set as fixed effects. The analyses were performed in SAS® Studio for Linux [25] using the GLM procedure (PROC GENMOD) of SAS/STAT® module [26]. Means were separated by the least-square means (LSMEANS) statement of SAS with Tukey–Kramer adjustment for multiple comparisons. p-values <0.05 were considered statistically significant. Lethal concentrations (LC_{50} and LC_{90}) were estimated using Probit analysis (PROC PROBIT). Differences in area not covered by *I. fumosorosea* mycelium were compared by an exact Wilcoxon two-sample test (PROC NPAR1WAY) of the SAS/STAT® module.

3. Results and Discussion

Most BTM larvae successfully passed to pupa regardless of treatment (Table 1), and no statistically significant effect of treatment on the duration of the larval stage was observed ($\chi^2 = 10.08$, df = 5, $p = 0.0730$). Similarly, treatments had no significant effect on the duration of the pupal stage ($\chi^2 = 0.19$, df = 5, $p = 0.9992$), but higher mortality was observed in all treatments; the maximum mortality of 46.4% pupae was found with the highest concentration of fungal treatment.

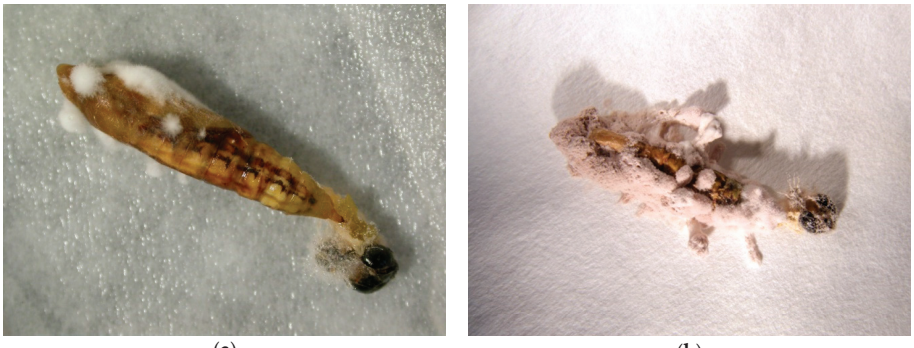
Table 1. The effects of *Isaria fumosorosea* on the development of *Cydalima perspectalis*.

Treatment [1]	Last-Instar Larvae			Pupae			Malformed Adults
	Duration		Died/Mycosed	Duration		Died/Mycosed	
	Mean ± SE	*n*	%	Mean ± SE	*n*	%	%
Control	4.24 ± 0.41	29	3.3/0	10.10 ± 0.15	21	27.6/0	0
1×10^4	5.04 ± 0.58	28	6.7/0	10.33 ± 0.11	18	35.7/0	0
1×10^5	5.53 ± 0.54	30	0/0	10.17 ± 0.13	24	20.0/0	0
1×10^6	4.87 ± 0.43	30	0/0	10.46 ± 0.10	26	13.3/0	0
1×10^7	5.00 ± 0.31	30	0/0	10.33 ± 0.11	21	30.0/10.0	0
1×10^8	3.96 ± 0.32	28	6.7/3.3	10.27 ± 0.25	15	46.4/28.6	20.0

[1] Concentration of conidia per milliliter of suspension.

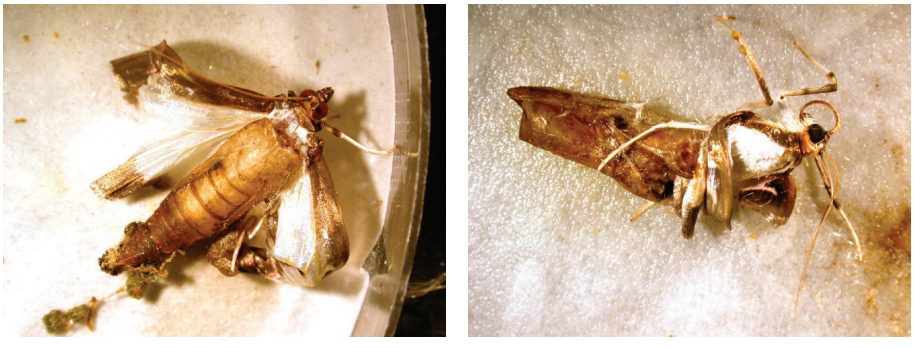
Mycosis was observed only in treatments of 1×10^7 and 1×10^8 conidia per 1 mL when 10% and 28.6% of pupae cadavers, respectively, were obviously infected by the fungus (Table 1). Infection by *I. fumosorosea* was later confirmed when fungus sporulated (Figure 1).

Interestingly, several adults that emerged from larvae treated by the highest conidia concentration were malformed (Table 1, Figure 2) and died in 1–2 days. A similar effect was observed when *I. fumosorosea* was applied to *Spodoptera littoralis* (Boisd.) [27].



(**a**) (**b**)

Figure 1. (**a**) Early mycosis of *Isaria fumosorosea* on *Cydalima perspectalis* pupa; (**b**) Cadaver of *C. perspectalis* covered with sporulating fungus.



(**a**) (**b**)

Figure 2. (**a**,**b**) Malformed adults of *Cydalima perspectalis* emerged in a group of larvae treated by *Isaria fumosorosea* at a concentration 1×10^8 conidia/mL.

The cumulative mean mortality, including mortality in malformed adults, varied among treatments and reached a maximum value of 60% when larvae were treated by a suspension of 1×10^8 conidia per 1 mL (Figure 3a). Thus, the highest mortality corrected for mortality in the control group [28] was only 42.9%. Although the effect of treatment on mortality was significant ($\chi^2 = 18.67$, df = 5, $p = 0.0022$) and no significant differences were found between replications ($\chi^2 = 0.45$, df = 1, $p = 0.5004$), the results indicate the very low susceptibility of *C. perspectalis* to *I. fumosorosea*.

The low efficacy is rather surprising, because the strain CCM 8367 of *I. fumosorosea* used in this study was previously found to be highly virulent against several pest species. For example, the mortality of pupae of *C. ohridella*, an invasive pest of *Aesculus hippocastanum* in Europe [29], treated by blastospores or conidia suspension of concentration 5×10^7 spores per 1 mL reached 100% over a few days [20]. Later, the high efficacy of this strain was confirmed against *Spodoptera littoralis* (Boisd.) in which an application of CCM 8367 blastospores at a concentration of 5×10^7 per 1 mL caused larval mortality >90% [27]. The high efficacy of CCM 8367 under laboratory conditions similar to that used in the present study was reported also against Colorado potato beetle, *Leptinotarsa decemlineata*, (Say) (Coleoptera: Chrysomelidae) larvae [30]. This indicated that the strain could be a prospective biocontrol agent, although some side effects against non-target natural enemies were also reported [31].

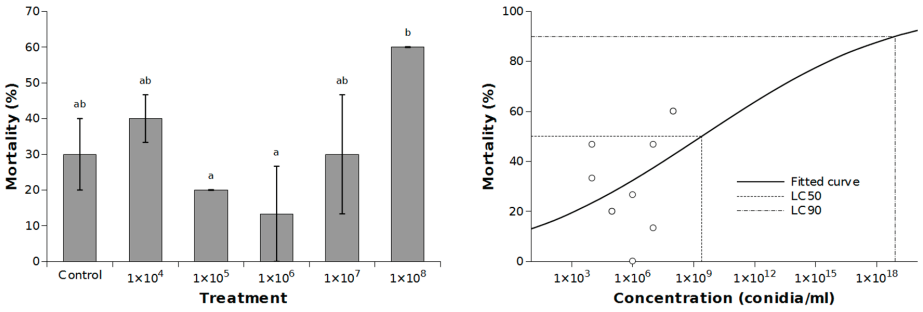


Figure 3. (**a**) Mean cumulative mortality (±SE) of *Cydalima perspectalis* (including mortality of malformed adults) treated with various concentrations of *Isaria fumosorosea* conidia. A generalized linear model was fitted and pairwise between treatment differences were tested using the least-square means. Different letters indicate significant differences between columns ($p < 0.05$); (**b**) Log-probit regression line of concentration-mortality response of *C. perspectalis* to *I. fumosorosea*.

The log-probit regression line describing the relationship between concentration and mortality has a form $y = -1.264 + 0.135x$ (Figure 3b), but the slope was not statistically significant ($\chi^2 = 3.27$, df = 1, $p = 0.071$). Thus, the extrapolated values of $LC_{50} = 2.42 \times 10^9$ and $LC_{90} = 7.88 \times 10^{18}$ were very high. For example, this contrasts with the LC_{50} and LC_{90} values of 1.03×10^6 and 8.67×10^7, respectively, reported for *L. decemlineata* [30].

LT-SEM imaging of *I. fumosorosea* conidia on the cuticle of BTM larvae revealed a high number of spores immediately after treatment (Figure 4a,b), but after 24 and 48 h of incubation, the number of attached spores seemed to be much lower, as we found them only in some places of larvae, usually as small groups or individual conidia. This indicates low conidial attachment to the larvae cuticle. Examination further showed that the number of spores did not germinate (Figure 4d–f). This finding might explain the low virulence of the fungus against *C. perspectalis* because the successful germination of fungus conidia on the host cuticle has been considered to be necessary for infection [32,33]. Several studies documented that the cuticle of some arthropods repress the germination of EPF spores or further development of germlings and appressoria formation [34,35]. One of the reasons might be the presence of antifungal compounds on the cuticle [36,37], which might be also case of *C. perspectalis*.

Results of in vitro experiments using *B. sempervirens* hydro-alcoholic extract revealed that this extract has a negative effect on the germination of *I. fumosorosea* conidia (Figure 5). In the control treated by solvent, the mean germination was 100% (SE = 0, $n = 3$), while on extract-treated agar, the mean germination was only 92.67% (SE = 0.88, $n = 3$) in average. The difference was statistically highly significant ($\chi^2 = 31.36$, df = 1, $p < 0.0001$).

The inhibition zone assay showed the suppression of *I. fumosorosea* mycelium growth on filter paper discs treated by *B. sempervirens* extract. The mean area not covered by mycelium was 0.02 ± 0.01 mm^2 and 53.83 ± 19.86 mm^2 in control and treated discs, respectively. The differences were statistically significant ($Z = -2.184$, $p = 0.028$).

Our findings indicate the presence of phytochemicals in box tree leaves having some activity against entomopathogenic fungi. Several secondary plant compounds were found to have a negative effect on the germination of *I. fumosorosea* blastospores, indicating that the presence of allelochemicals on a substrate (e.g., insect cuticle or leaf) may be an additional constraint to the survival of entomopathogenic fungi [38]. The *Buxus* trees contain a lot of alkaloids, some of which are sequestered by *C. perspectalis* larvae, while some are metabolized and/or excreted [39]. The antimicrobial activity of substances extracted from *B. sempervirens* by 65% ethanol were found earlier [40], and similar effects of box tree extracts were confirmed by other authors [41]. Thus, it is thus likely that BTM larvae use

phytochemicals obtained from the host plant for their own defense against the invasion of microbial pathogens. This might explain the low efficacy of two strains of entomopathogenic fungi, *I. fumosorosea* (present study), and *B. bassiana* [14] against BTM.

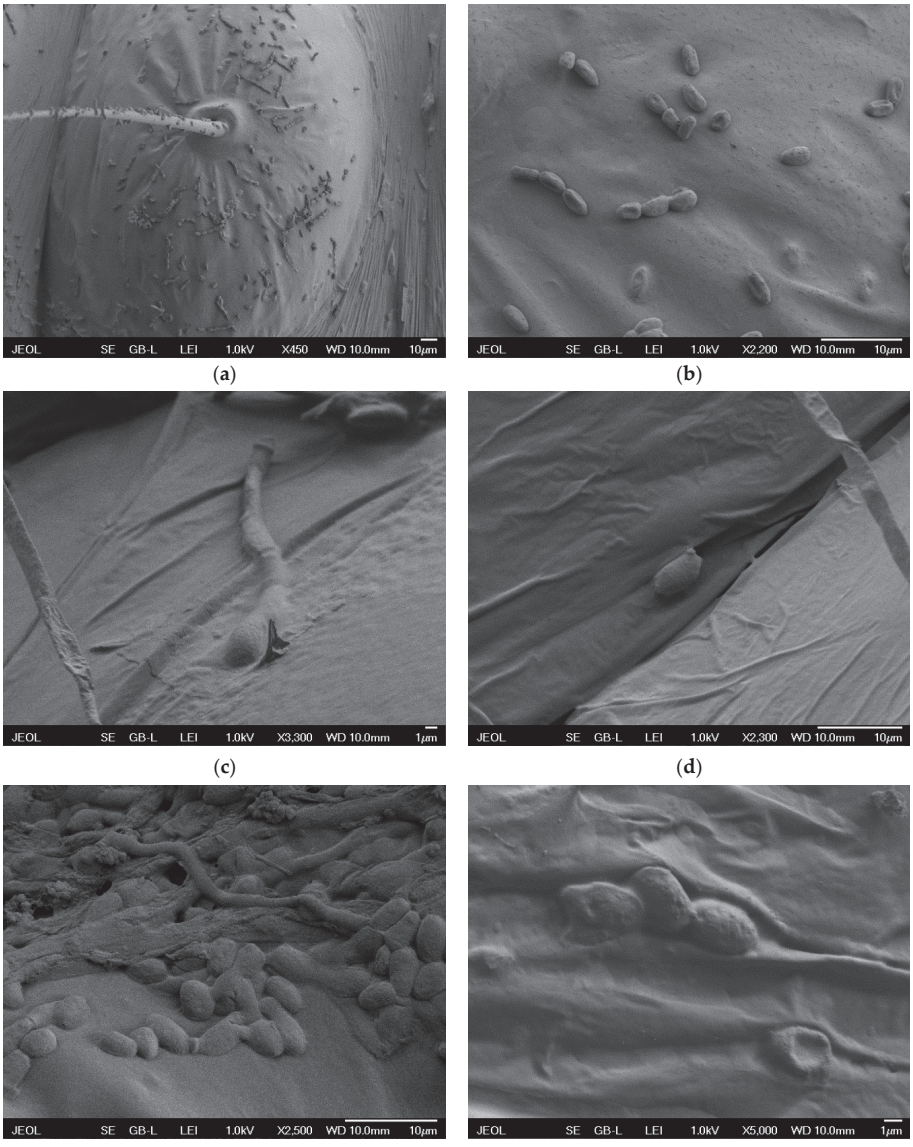
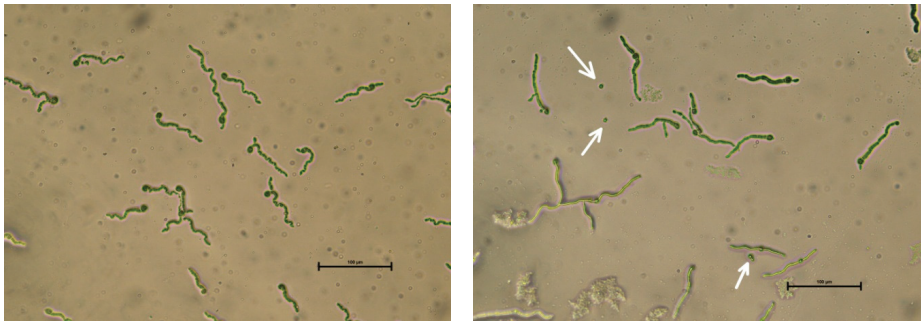


Figure 4. Low-temperature scanning electron microscope (LT-SEM) image of *Isaria fumosorosea* conidia on the cuticle of *Cydalima perspectalis* larva. (**a**,**b**) Conidia immediately after the fungus application; (**c**) Conidium with germ tube after 24-h incubation; (**d**) Ungerminated conidium after 24-h incubation; (**e**) Group of ungerminated conidia after 24-h incubation; (**f**) Ungerminated conidia after 48-h incubation.



(**a**) (**b**)

Figure 5. Germination of *Isaria fumosorosea* conidia on: (**a**) a control agar plate treated with solvent only; (**b**) agar plate treated with *Buxus sempervirens* extract. Arrows indicate spores with no or little germination peg. Traces of plant extract are visible on the agar surface.

It may be concluded that the strain CCM 8367 of *I. fumosorosea* is not a potent biocontrol agent against *C. perspectalis* and that the reason for the low efficacy of the fungus might be the accumulation of host plant phytochemicals with antimicrobial activity in the fifth-instar larvae cuticle of the pest.

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Isolation of *Beauveria bassiana* from the Chagas Disease Vector *Triatoma infestans* in the Gran Chaco Region of Argentina: Assessment of Gene Expression during Host–Pathogen Interaction

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Abstract: A native strain of the entomopathogenic fungus *Beauveria bassiana* (Bb-C001) was isolated from a naturally infected *Triatoma infestans*, Klug (Hemiptera: Reduviidae) adult cadaver in the Gran Chaco region, Salta province, Argentina. The isolate was both phenotypic and molecularly characterized in a context of fungus-insect interaction, by measuring the expression pattern of toxin genes during infection and immune response of *T. infestans*. The commercial strain GHA of *B. bassiana*, which was previously used in field interventions to control these vectors, was used as reference in this study. The phylogenetic trees based on both ribosomal internal transcribed spacer (ITS) and elongation factor 1-alpha (EF1-α) indicated that Bb-C001 fits into a *B. bassiana* cluster, and the sequence-characterized amplified regions (SCAR) showed that Bb-C001 is different from the GHA strain. There were no differences between both strains regarding viability, radial growth, and conidia production, either in the median survival time or insect mortality. However, Bb-C001 showed a higher expression than GHA of the bassianolide synthetase gene (*BbbslS*) during infection, and similar levels of the beauvericin synthetase gene (*BbbeaS*). Immune-related genes of *T. infestans* nymphs (*limpet-2* and *defensin-1, -2,* and *-6*) were later expressed and thus insects failed to stop the infection process. These results showed that *B. bassiana* Bb-C001 is a promised fungal strain to be incorporated in the current biological control programs of *T. infestans* in Salta province, Argentina.

Keywords: entomopathogenic fungi; resistant triatomines; biological control; bassianolide; beauvericin; limpet; dual gene expression

1. Introduction

The blood-sucking insect *Triatoma infestans*, Klug (Hemiptera: Reduviidae), the main Chagas disease vector in the Southern Cone of South America, has been the target of continuous control programs to reduce the disease transmission risk. The control strategy used has been the indoor application of pyrethroid insecticides [1]. However, it has been recognized that this strategy has limited efficacy, mainly confined to the Gran Chaco area shared by Argentina, Bolivia, and Paraguay [2]. Moreover, in the last years several foci of pyrethroid-resistant *T. infestans* have been documented in

wide regions of Bolivia and Argentina [3,4]. These difficulties make it necessary to search for alternative tools to insect control. The entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales) commercial strain GHA has shown to be useful in field interventions, showing promising results in houses infested with pyrethroid-resistant *T. infestans* [5,6].

Fungal pathogenesis depends on many factors that are related to germination capacity, growth rate, and spore yield and production, among other factors [7,8]. But the way in which fungus and host interact is key to knowing the final result of the infection process [9]. *B. bassiana* secretes toxic or immunosuppressive compounds (often referred to as secondary metabolites) during hemocoel invasion, such as the cyclooligomer nonribosomal peptides beauvericin and bassianolide, the diketomorpholine bassiatin, the cyclic peptides beauverolides, the dibenzoquinone oosporein, and the 2-pyridone tenellin [10–12]. Even though the genes involved in some of these secondary metabolites biosynthetic pathways have been studied, most of their biological roles remain to be uncovered [11]. In this regard, their expression pattern when the fungus grows within its insect host might help to better understand their role in pathogenesis. Our research group has developed a methodology based on absolute quantification by qPCR to follow it, reporting an induction of the genes encoding for the synthetase enzymes of the secondary metabolites beauvericin (*BbbeaS*), bassianolide (*BbbslS*), and tenellin (*BbtenS*) during the first days of infection, perhaps to be used as virulence factors, and then in moribund insects and/or cadavers to protect them from competitive microorganisms [13]. On the other hand, insects possess both innate cellular and humoral defense strategies to defend from microbe infections. Humoral response comprises the production of many different antimicrobial peptides, including defensins [13]. *T. infestans* contains six genes encoding for defensins, and their expression is regulated at least by a pair of limpet transcription factors [14].

All these properties allow selecting strains with optimal characteristics to achieve effective control results; however, the fungus also need to be host-specific, virulent, and adapted to a regional environment [15]. In this regard, exploration for local isolates is crucial to establish long-term, effective, and sustainable biological control programs. There are only two reports on the isolation of entomopathogenic fungi naturally infecting *T. infestans* in Argentina; i.e., isolates of *B. bassiana* [16] and *Paecilomyces lilacinus* [17]. However, there is abundant information about *T.* infestans-*B. bassiana* interaction [13,18–22], even from a coevolutive perspective [23].

In the present study, we reported the isolation of a native *B. bassiana* strain from a *T. infestans* cadaver. We characterized its phylogeny, growth, virulence, and toxin expression during infection of both pyrethroid-susceptible and pyrethroid-resistant *T. infestans*, and also evaluated the host immune response within the context of a fungus–insect interaction.

2. Materials and Methods

2.1. Fungus Isolation

As part of periodical entomological interventions performed by members of the Ministry of Public Health of Salta Province, Argentina, a cadaver of *T. infestans* adult with apparent signs of mycosis was isolated in August 2011 at the "Misión Aborigen el Cañaveral", Santa Victoria Este municipality, Rivadavia department, Salta province (22°16′52.78″ S; 62°42′5.60″ W). This place belongs to the Gran Chaco region and is located at the edge of the Pilcomayo River. The cadaver of the infected insect was sterilized with 0.5% sodium hypochlorite and was sowed in a Petri dish with potato dextrose agar (PDA) with chloramphenicol and incubated at 27 °C for 7 d to isolate the fungus. The colony obtained was subcultured several times to obtain a pure culture, which was preliminary identified on the basis of macromorphological aspects [24] as *Beauveria* sp. and conserved at the Mycological Culture Collection of the School of Natural Sciences, National University of Salta, Argentina, under the code Bb-C001. The strain GHA *of B. bassiana* (obtained from Laverlam International, Butte, MT, USA) was used as reference for the entire characterization. In order to regain the optimal virulence parameters, both strains were inoculated on *T. infestans* nymphs and recovered in PDA prior to use [8].

2.2. Molecular Identification

For the molecular and phylogenetic characterization, fungal mycelia were disrupted using a minibead beater homogenizer (BioSpec, Bartlesville, OK, USA) with glass beads (0.5 mm diameter) as previously described [25]. Genomic DNA was extracted with Tri Reagent® (Molecular Reagent Centre, Cincinnati, OH, USA) and used as template in PCR amplifications to detect sequence-characterized amplified regions (SCAR), ribosomal internal transcribed spacer (ITS), and elongation factor 1-α (EF1-α). Primers used are shown in Table 1. A SCAR marker specific for *B. bassiana* strain GHA was amplified with the thermal profile described by Castrillo et al. [26]. The ITS fragment (~600 bp) and EF1-α amplicon (~1200 bp) were amplified employing the touchdown PCR procedure described by Don et al. [27] and modified by Rehner and Buckley [28]. PCR products were visualized in 1% agarose gels stained with ethidium bromide. A 100-bp ladder standard (Productos Bio-Logicos, Quilmes, Argentina) was also used. Amplicons were ligated and cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* JM109. Ampicillin-resistant colonies were isolated and their plasmid purified (Qiagen, Hilden, Germany). Inserts were sequenced (Macrogen, Seoul, Korea) to confirm their identity and use in phylogenetic analysis. Both ITS and EF1-α sequences corresponding to Bb-C001 were aligned along with sequences from several ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) isolates (including GHA) belonging to the genus *Beauveria* [28]. The sequences from *Cordyceps* cf. *scarabaeicola* (ITS, GenBank AY532058; EF1-α, GenBank AY531967) were selected as out-group. Alignments were generated using ClustalW [29]. Phylogenetic analysis was carried out, and maximum parsimony tree was constructed with the program Mega 6.0 [30]. Gaps were excluded from the analysis. A bootstrap analysis with 1000 replicates was used to infer branch support.

Table 1. Oligonucleotides used in this study.

Name	Forward (5′-3′)	Reverse (5′-3′)	Amplicon (bp)	Target	Assay
OPA14F/OPA14R	TCTGTGCTGGCCCTTATCG	TCTGTGCTGGGTACTGACGTG	455	Fungus	SCAR
BbITS4/BbITS5	TCCTCCGCTTATTGATATGC	GGAAGTAAAAGTCGTAACAAGG	~600	Fungus	ITS
BbEF1T/Bb1567R	ATGGGTAAGGARGACAAGAC	ACHGTRCCRATACCACCSATCTT	~1200	Fungus	EF1-α
qBbtenS	ACTGTCCGCATTGGCAGCTAAG	TGTCCTTTGGTGGTGGTGATGG	113	Fungus	qRT-PCR
qBbbeaS	GTTCTTCCTCCGCATTCCGTTC	TAGAGCGCAACGTCTTTCGGTC	97	Fungus	qRT-PCR
qBbbslS	CAATCGACTGAGACGCCATTCC	TTTGACCTGCGAATCCATACGG	156	Fungus	qRT-PCR
qTi18S	GGCGGGGGCATTCGTATTG	ATCGCTGGCTGGCATCGTTTAT	123	Insect	qRT-PCR
qTiEF1	AAAGTGCGACCGTCGTACAGG	TCACGAACGGCAAAGCGA	100	Insect	qRT-PCR
qTiLp2	GCATTTCTGCCAAGAAGAGG	ATGGAATCAAAGCTGGCCTA	110	Insect	qRT-PCR
qTiDef1	TGACTTAGCCCAGCAACCAT	GCACAGGCTGCATGATTAGG	150	Insect	qRT-PCR
qTiDef2	CTTCTTAGTAGCCGCCCTCG	GGTGCCACCATCGTATCCAT	210	Insect	qRT-PCR
qTiDef6	TGAAGTGTGCACTCTCTTTGGT	GGCTCCCATAGGGCTTCATC	106	Insect	qRT-PCR

2.3. Phenotypic Characterization

Total conidia were collected from PDA cultures at different time periods (see below) and suspended in distilled water containing 0.01% Tween 80. Conidia concentration of each initial suspension was estimated with a Neubauer haemocytometer using serial dilutions up to a factor of 10^{-7}. For viability assays, three aliquots (5 µL each) were taken from this mother solution, plated on PDA and incubated at 27 °C. Twenty-four hours later, they were observed under a microscope using a 100× objective. Germination percentage was calculated as the number of germinated conidia/total number of conidia × 100. Both procedures (conidia concentration and viability) were repeated in the different postsowing times (10, 20, and 30 days) to evaluate whether both parameters were time-affected. For radial growth, each strain was inoculated in the center of the 80 mm diameter Petri dish with PDA medium. Photographs were taken every 48 h, and the radial growth was measured using the software Image Tool 3.0, considering four perpendicular diameters. Observations were recorded for 30 days after inoculation. The slope was calculated by lineal regression and expressed as radial growth (mm/h) as described in [8]. For each strain, 12 replicates were done.

2.4. Insects

Both pyrethroid-susceptible (Py-S) and pyrethroid-resistant (Py-R) *T. infestans* used in this study come from a well-established colony at the insectary of the School of Natural Sciences, National University of Salta, Argentina. Insects were fed on ketamine-anesthetized rat blood once per each development stage. All animal care and laboratory experimental protocols were carried out following the Regulation of the Institutional Committee for Care and Use of Laboratory Animals and Field Studies (CICUALEC), School of Natural Sciences, National University of Salta, Argentina. The colony was periodically renewed by incorporating first generation, field-collected insects.

2.5. Mortality Bioassays

Fungal suspensions were prepared in sunflower oil at a concentration of 1×10^{12} conidia mL^{-1}, determined with a hemocytometer. Groups of 15 Py-R insects were used for each fungal strain, which included first (NI) and third stage (NIII) nymphs, and adults (A). Each group was placed in Petri dishes (10 cm diameter) containing 1 mL of each fungal formulation homogenously dispersed with a silicone brush (1.3×10^{10} conidia/cm^2). The insects were allowed to be in contact with it for ten minutes, and then transferred individually to acrylic containers and incubated at 27 °C with a photoperiod of 12:12 h without feeding. As control treatments, Petri dishes sprayed with sunflower oil were used. Mortality was registered daily and dead insects were put in a humid chamber in order to confirm that death was caused by fungal infection. Medium survival time (MST), Maximum survival reached (S%), and Kaplan and Meier survival curves were performed nine days after fungal infection.

2.6. Gene Expression by qRT-PCR

We used the dual gene expression approach for studying the molecular interaction between fungi and insects, according to the protocol described by Lobo et al. [13]. For this, two-week-old fourth-instar nymphs, either Py-S or Py-R, were used one week after a blood meal. Individual insects were immersed for 6 s in aqueous (0.01% Tween 80) conidial suspensions of either 0 (control), 1×10^2, or 1×10^4 conidia mL^{-1}. Insects were returned and maintained at the rearing conditions described above. At different time periods (three, six, and nine days after treatment), three live insects were separated, and their total RNA was extracted from both fungus-treated and control insects by employing the Tri Reagent® (Molecular Reagent Centre, Cincinnati, OH, USA) technique, according to manufacturer's instructions. RNA was quantified by a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its integrity was assessed on a 1% (*w*/*v*) agarose gel. Two-step real-time polymerase chain reaction (RT-PCR) was carried out with iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Amplification was performed on an AriaMx Real-Time PCR (qPCR) Instrument (Agilent Technologies, Santa Clara, CA, USA) employing 40 ng reverse-transcribed total RNA for each sample. Targeted fungal genes were those encoding for enzymes involved in the biosynthesis of some secondary metabolites [13]; i.e., tenellin synthetase (*BbtenS*), beauvericin synthetase (*BbbeaS*) and bassianolide synthetase (*BbbslS*). Insect genes assayed were a limpet transcription factor (*Tilimpet-2*) and three defensin genes (*Tidef-1*, *Tidef-2*, and *Tidef-6*) regulated by limpet [23]. Primers used are listed in Table 1. The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 30 s at 55 °C for annealing, and 30 s at 72 °C for DNA chain elongation). In order to confirm that only single products were amplified, a temperature-melting step was then performed. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The assay was performed in duplicate, and three independent biological replicates were done. To analyze the expression profiles, we applied the NRQ model, consisting of the conversion of quantification cycle values (Cq) into normalized relative quantities (NRQs),

the adjustment for differences in PCR efficiency between the amplicons [31], and the normalization with reference genes [32].

2.7. Statistical Analyses

Concentration data were analyzed with the nonparametric Mann–Whitney test. In order to assess viability and radial growth speed, time was used as a regression variable. Survival data were compared with the Kaplan Meier test. For gene expression analysis, statistical significance was assessed using a one-way analysis of variance followed by Tukey's multiple comparison test or an unpaired Student's *t*-test when it corresponded, depending on the number of experimental groups under analysis. Graph Pad Prism 8.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses.

3. Results and Discussion

3.1. Molecular and Phenotypic Characterization

We reported the isolation of a native fungal strain from a cadaver of an adult specimen of the Chagas disease vector, *T. infestans*. After a first morphological characterization suggesting that the fungus belongs to *Beauveria* sp., we characterized the isolate by molecular tools in order to confirm the identity at species level. The alignment of both ITS and EF1-α fragment sequences showed high similarity (99.5 and 99%, respectively) with *B. bassiana sensu lato* genes (Figure 1A,B) and was named and deposited in local mycological repository (see Material and Methods Section) as Bb-C001.

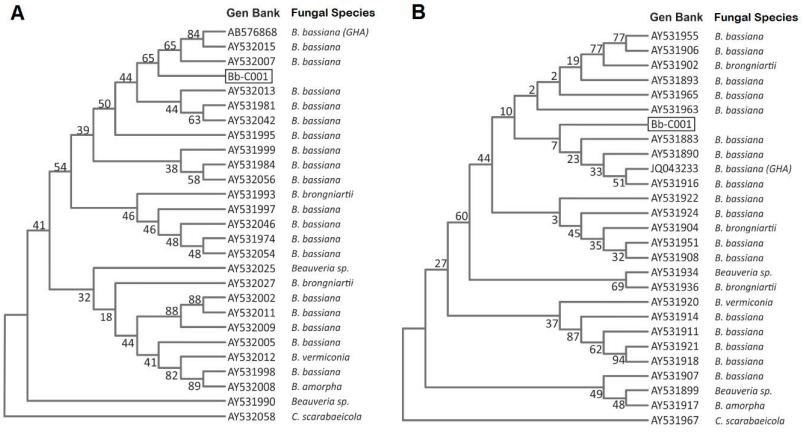

Figure 1. Phylogenetic tree generated by the maximum likelihood analysis of the ribosomal internal transcribed spacer (ITS) (**A**), and elongation factor 1-α (EF1-α) (**B**) sequences of *Beauveria bassiana* Bb-C001 (boxed) and related isolates. The fungal isolates, origin and access numbers to the GenBank are detailed. Numbers on branches are bootstrap support from 1000 replicates.

Then, as *B. bassiana* strain GHA was previously used in field trials in 2008 and 2009 [5,6] in Salvador Mazza municipality, distant ~130 km from the site of Bb-C001 collection, we assayed a molecular identification using a SCAR marker in order to discard a potential reisolation of naturally dispersed GHA strain from Salvador Mazza to Santa Victoria Este. Sequence-characterized amplified region (SCAR) is a very useful PCR-based technique to differentiate between fungal isolates since it can be developed to be strain-specific. In this sense, Castrillo and coworkers have set a SCAR marker specific for GHA, sensitive enough to differentiate this strain from others *B. bassiana* isolates [26]. Thus, the absence of the SCAR amplicon corresponding to 455 bp in Bb-C001 (Figure 2) confirms that this isolate is different from Bb GHA.

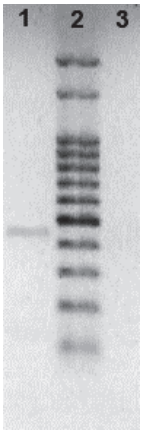

Figure 2. PCR amplification of the sequence-characterized amplified region (SCAR) marker (455 bp) specific for *B. bassiana* strain GHA (lane 1) which is absent in the Bb-C001 isolate (lane 3). Lane 2, molecular weight marker.

Conidia production did not show any statistically significant differences between both strains according to the Mann–Whitney test (U = 40.00; p = 0.999), with mean values of 4.5 ± 1.6 × 10^{12} conidia mL^{-1} for GHA and 4.0 ± 2.4 × 10^{12} conidia mL^{-1} for Bb-C001 (Figure 3A). Germination percentage did not exhibit statistically significant differences between strains (p = 0.59) during the time period studied (0, 10, 20, and 30 days). Regression analysis showed that there is significant loss of viability with time for both stains, GHA (b = −0.4% viability day-1; p = 0.02) and Bb-C001 (b = −0.4% viability day-1; p = 0.01) (Figure 3B). Radial growth did not exhibit statistically significant differences between the assessed strains (p = 0.48), showing an increment in diameter with time. Values obtained were 0.074 ± 0.003 mm h^{-1} (GHA) and 0.071 ± 0.002 mm h^{-1} (Bb-C001) (Figure 3C).

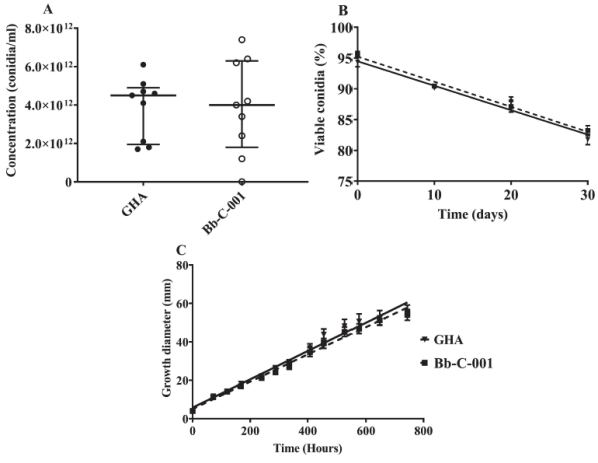

Figure 3. Comparative physiological characteristics of the Bb-C001 and GHA isolates of *Beauveria bassiana*. (**A**) Production of conidia. (**B**) Germination of conidia collected from PDA cultures at different time periods. (**C**) Growth diameter of fungi grown in PDA at different time periods.

3.2. Fungal Virulence Assessment

Nine days after infection with fungal isolates, no survivors were observed in all *T. infestans* development stages studied (Figure 4). Mean lethal time (MLT) observed for Bb-C001 were 4.6 ± 0.5 days (first instar nymph), 7.2 ± 0.2 days (third instar nymph), and 6.7 ± 0.5 days (adults), whereas MLT obtained for GHA was 5.5 ± 0.4 days (first instar nymph), 7.4 ± 0.4 days (third instar nymph), and 7.9 ± 0.3 (adults). In relation to the survival rate, no statistically significant differences were observed according to the Kaplan and Meier tests between both strains. The isolation and use of native strains of entomopathogenic fungi generate a greater specificity in the infection process, since they are better adapted to the local natural conditions and their host [22,33–35].

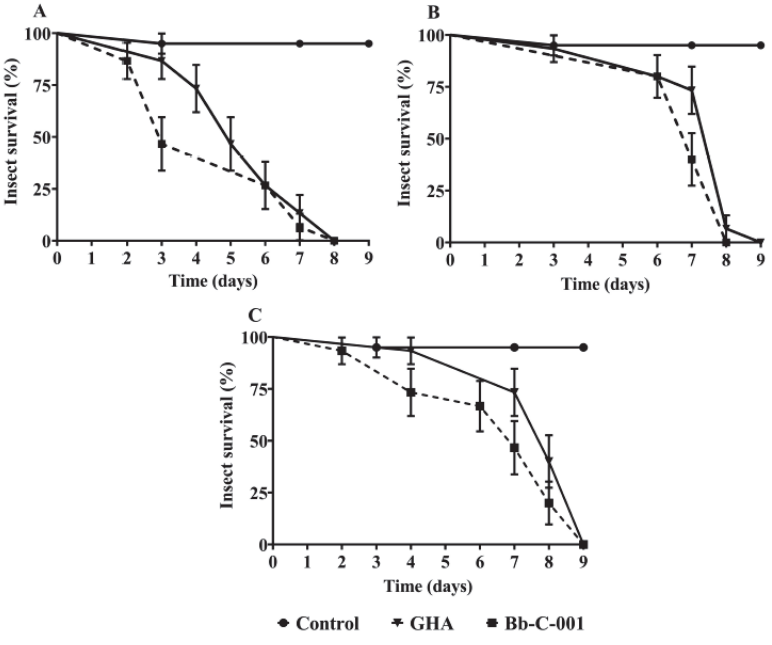

Figure 4. Survival curves of *Triatoma infestans* exposed to both Bb-C001 and GHA isolates of *Beauveria bassiana*, and controls (healthy insects). (**A**) First instar nymphs. (**B**) Third instar nymphs. (**C**) Adults.

3.3. Fungal Toxin Expression

The expression pattern of Bb-C001 genes encoding for nonribosomal peptides was dependent on the type of nymphs inoculated (Py-S or Py-R), the conidial concentration used (1×10^2 or 1×10^5 conidia mL^{-1}), and the time period assayed (three, six, or nine days after fungal inoculation). Even at low values, all three genes (*BbbslS*, *BbbeaS*, and *BbtenS*) were detected inside both Py-S and Py-R nymphs three days after inoculation. In Py-S insects treated with 1×10^2 conidia mL^{-1}, the *BbbslS* gene peaked at day six, showing significant higher values than three and nine days post inoculation. *BbbeaS* also peaked at day six, but with lower values than *BbbslS*. *BbtenS* showed the lowest values at the three times assayed (Figure 4A). In Py-S treated with 1×10^5 conidia mL^{-1}, *BbbslS* and *BbbeaS* also exhibited the highest values at day six post inoculation, with not significantly different values as on day nine after inoculation (Figure 5A). With respect to Py-R, also higher values were detected at day six post inoculation, *BbtenS* peaked with 1×10^2 conidia mL^{-1}, and *BbbslS* with 1×10^5 conidia mL^{-1} (Figure 5B). Comparing both Py-S and Py-R, *BbbslS* and *BbbeaS* genes were higher expressed inside Py-S than in Py-R nymphs at day six post immersion in 1×10^2 conidia mL^{-1} (*BbbslS*, $p < 0.001$; *BbbeaS*,

$p < 0.01$) and in 1×10^5 conidia mL^{-1} (*BbbeaS*, $p < 0.001$), and also at day nine post inoculation with 1×10^5 conidia mL^{-1} (*BbbslS*, $p < 0.05$; *BbbeaS*, $p < 0.01$).

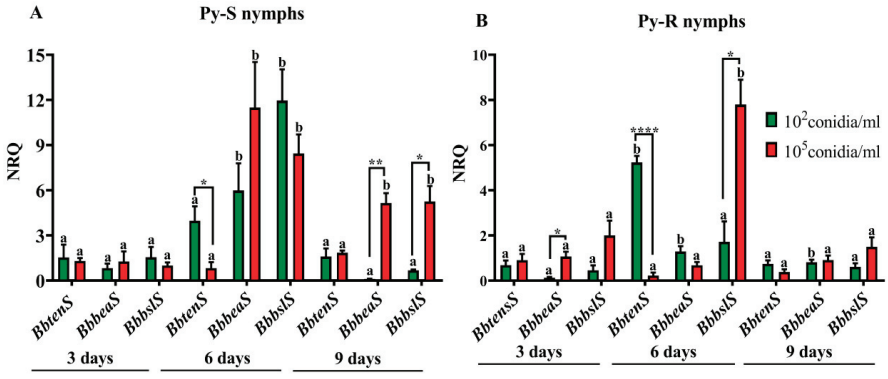

Figure 5. Normalized relative quantities (NRQ) of *Beauveria bassiana* transcripts encoding tenellin (*BbtenS*), beauvericin (*BbbeaS*) and bassianolide (*BbbslS*) synthetases into fourth instar nymphs of pyrethroid-susceptible (**A**) and pyrethroid-resistant (**B**) *Triatoma infestans* at different time periods after insect immersion in conidial suspensions. Values are means of three replicates ± SEM. Different letters indicate significant differences for a single gene through time. Asterisks indicate significant differences in gene expression at each time point. * $p < 0.05$; ** $p < 0.005$; **** $p < 0.00005$.

The same three genes were measured in GHA strain during infection of the same host [13]. Although both strains exhibited a peak of expression at day six post inoculation, only *BbbeaS* was highly expressed in by both *B. bassiana* strains into Py-S insects. The accompanying gene with high expression was different, namely *BbtenS* in GHA [13], and *BbbslS* in Bb-C001 (this study). Although this result is bounded to a small piece of research, it might be possible that the expression of the plethora of secondary metabolites described in entomopathogenic fungi is being isolate-dependent but also is likely to depend upon the exposure level (i.e., the inoculum) to the pathogen and the physiological state or host condition (e.g., the pyrethroid-related behavior). In this regard, Pedrini et al. [5] have previously studied the susceptibility of both Py-S and Py-R insects to *B. bassiana*. They found no differences in fungal virulence towards different nymphal stages from both insect populations [5]. The same study demonstrates that although a cuticle thickening and higher surface hydrocarbon content of Py-R bugs (compared with Py-S insects) might be related to a reduced penetration of the pyrethroid, and thus contribute to decrease the effective dose of insecticide, these differences do not seem to affect the fungal contact and penetration through the cuticle [5]. However, the current study found some differences in fungal toxin production either inside Py-S or Py-R insects. We can speculate that these different barriers might be responsible for differences of the fungal inoculum starting the infection, and thus, ultimately, provoke differential expression of secondary metabolites within the insect body invasion process.

3.4. Insect Immune Response

The immune-related gene expression was also different between Py-S and Py-R insects, and the inductions were observed mostly at day nine post inoculation. Comparing with controls, Py-S nymphs treated with 1×10^2 conidia mL^{-1} showed induction in the all three defensin genes at day nine post inoculation, and *Tidef-6* was induced also at day six post inoculation. At the higher dose assayed (1×10^5 conidia mL^{-1}), the genes induced were *Tidef-1* at day nine and *Tidef-6* at day six post inoculation (Figure 6). *Tilimpet-2* was not induced either with time period or dose used.

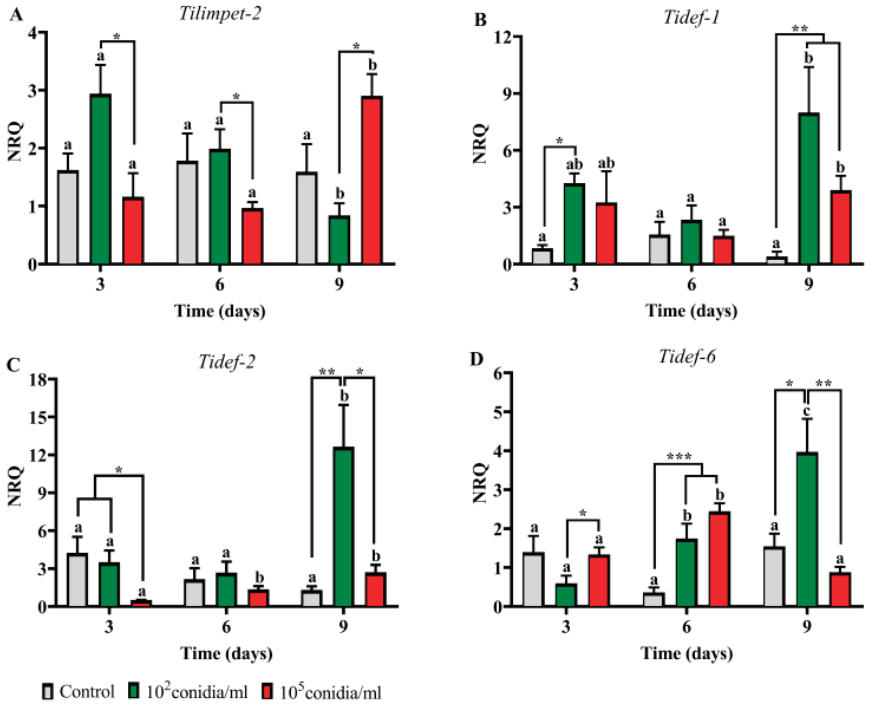

Figure 6. Expression pattern of limpet (**A**) and defensin (**B**–**D**) genes in *Beauveria bassiana*-infected pyrethroid-susceptible nymphs of *Triatoma infestans*. Normalized relative quantities (NRQ) are shown at different time periods after insect immersion in conidial suspensions. Values are means of three replicates ± SEM. For each gene, different letters indicate significant differences for each treatment through time. Asterisks indicate significant differences in gene expression at each time point. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

In Py-R insects, *Tilimpet-2* was induced at day three after treatment with 1×10^2 conidia mL^{-1} and at day nine with 1×10^5 conidia mL^{-1}, as same as the three defensin genes. *Tidef-2* was also induced at day six after treatment with 1×10^2 conidia mL^{-1} (Figure 7). Comparing the immune response in both Py-S and Py-R at day nine post inoculation with 1×10^2 conidia mL^{-1}, *Tidef-1*, *-2* and *-6* were more expressed in Py-S than in Py-R nymphs ($p < 0.001$, $p < 0.001$, $p < 0.05$, respectively). On the contrary, in insects inoculated with 1×10^5 conidia mL^{-1} at same time period, Py-R showed higher expression in *Tidef-2* ($p < 0.001$) and *Tidef-6* ($p < 0.001$).

Two variants of the limpet transcription factor were previously characterized in *T. infestans*, and their function have been linked with the humoral innate immune response [14]. As *Tilimpet-2* showed to be more involved than *Tilimpet-1* in defensin regulation, and the most affected defensin genes after limpet silencing were *Tidef-1*, *Tidef-2* and *Tidef-6* [14], we selected these genes to follow the host response in the current study. Although we found similar results as those in the previous work, the role of *Tilimpet-2* in this process (i.e., peaking before defensing genes) was observed only in Py-R insects (day three and day six for limpet and defensins, respectively), but no differences in its expression were detected in Py-S insects. As the defensin genes were more expressed in Py-S nymphs, it might be possible that the induction of *Tilimpet-2* has taken place before day three and thus it has not been detected by this study design.

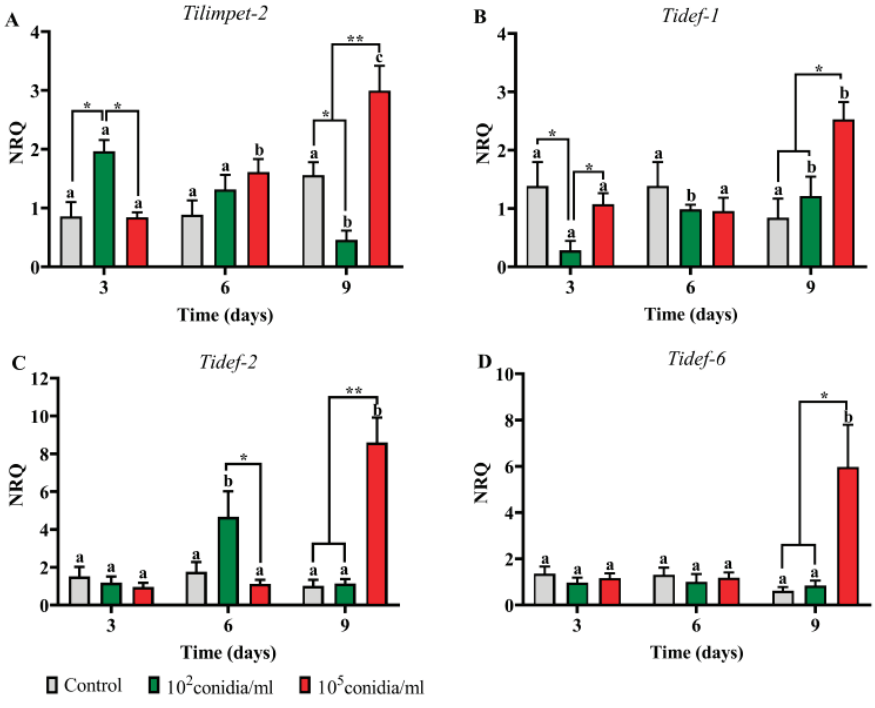

Figure 7. Expression pattern of limpet (**A**) and defensin (**B**–**D**) genes in *Beauveria bassiana*-infected pyrethroid-resistant nymphs of *Triatoma infestans*. Normalized relative quantities (NRQ) are shown at different time periods after insect immersion in conidial suspensions. Values are means of three replicates ± SEM. For each gene, different letters indicate significant differences for each treatment through time. Asterisks indicate significant differences in gene expression at each time point. * $p < 0.05$; ** $p < 0.005$.

4. Conclusions

In the current study, we compared several aspects of the interaction of two *B. bassiana* strains (GHA and Bb-C001) with the Chagas disease vector *T. infestans*. Both fungal strains did not show any differences on physiological parameters evaluated; i.e., conidial yield, viability, and radial growth, the same as virulence against nymphs and adults. However, the expression of genes encoding nonribosomal peptides was quite different, being the bassianolide syntethase gene the most expressed in Bb-C001, and beauvericin and tenellin synthesase genes the most expressed ones in GHA [13], in all cases peaking at day six post infection. The immune response by measuring the expression of antimicrobial peptides was late and peaked at day nine post inoculation, when the infection process seems to be irreversible. However, differences in the expression of these immune-related genes were observed between pyrethroid-susceptible and pyrethoid-resistant insects. In summary, the infection with both strains develops with similar virulence but the expression of antimicrobial peptides inside insects is different between strains. This finding is an important conclusion from this work, because it confirms that once the fungus reaches the hemocoel, the insect has very little chance to survive the fungal infection despite the activation of the immune response as a last-ditch effort to overcome the fungus. Thus, the main battle in this fungus-insect interaction is to try enter the host by any of the different routes available [36], which determines either a successful infection and the death of the host or an effective defense by the host. Overall, we conclude that the strain coming from of the Gran Chaco region (Bb-C001) is a promissory candidate for the development of a fungal formulation to control both

pyrethroid-susceptible and pyrethroid-resistant *T. infestans*. Further studies are underway to evaluate massive production and formulation methods for this strain.

Author Contributions: Investigation, analysis and interpretation of data, critical review of the draft, L.V.B.; conceptualization, analysis and interpretation of data, writing the manuscript, N.P. and R.M.C.; analysis and interpretation of phylogenetic data, M.S. and N.P.; analysis and interpretation of dual qPCR data, M.C.M. and N.P.; funding acquisition, critical review of the draft, L.B.N., A.G. and R.M.C.; supervision, N.P. and R.M.C. All authors have read and agreed to the published version of the manuscript.

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MrHex1 is Required for Woronin Body Formation, Fungal Development and Virulence in *Metarhizium robertsii*

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Abstract: The Woronin body (WB) is a peroxisome-derived dense-core vesicle, a self-assembling hexagonal crystal of a single protein Hex1. This organelle is specific to the ascomycete fungi belonging to the Pezizomycotina subphylum by functioning in sealing septal pores in response to mycelium damage and the control of cell heterogeneity. We retrieved all available Hex1-domain containing proteins of different fungi from the GenBank database and found considerable length variations among 460 obtained Hex1 proteins. However, a highly conserved Hex1 domain containing 75 amino acid residues with a specific S/A-R/S-L consensus motif for targeting peroxisome is present at the carboxy-terminus of each protein. A homologous *Hex1* gene, named *MrHex1*, was deleted in the entomopathogenic fungus *Metarhizium robertsii*. It was found that MrHex1 was responsible for WB formation in *M. robertsii* and involved in sealing septal pores to maintain cell integrity and heterogeneity. Different assays indicated that, relative to the wild-type (WT) strain, Δ*Mrhex1* demonstrated a growth defect on a solid medium and substantial reductions of conidiation, appressorium formation and topical infectivity against insect hosts. However, there was no obvious virulence difference between WT and mutants during injection of insects. We also found that Δ*MrHex1* could tolerate different stress conditions like the WT and the gene-rescued mutant of *M. robertsii*, which is in contrast to the reports of the stress-response defects of the *Hex1* null mutants of other fungal species. In addition to revealing the phenotypic/functional alterations of the *Hex1* deletion mutants between different pathotype fungi, the results of this study may benefit the understanding of the evolution and WB-control of fungal entomopathogenicity.

Keywords: Woronin body; conidiation; stress response; appressorium formation; virulence; *Metarhizium robertsii*

1. Introduction

The filamentous ascomycete *Metarhizium robertsii* is an omnipresent and soil-dwelling pathogen of insects, ticks and mites [1,2]. It has been developed as a promising mycoinsecticide to control different insect pests and investigated as a genetically tractable system for studying fungus–insect interactions [3–5]. Similar to plant pathogens like the rice blast fungus *Magnaporthe oryzae*, *M. robertsii* infects insect hosts by penetrating host cuticles through the differentiation of the infection structure appressoria and build-up of the turgor pressure within appressorial cells [6,7]. The generation of the turgor pressure requires the accumulation of high concentrations of glycerol and or solutes within

appressorial cells [8–10], which are separated from conidial mother cells by the formation of septa with the central septum pores sealed [11,12]. The mechanism of septal pore sealing in insect pathogens like *M. robertsii* is still unclear.

Fungal hyphal cells are separated by perforate septa, and filamentous fungi evolved with finely tuned strategies to balance the inter-cellular exchanges and the need for compartmentalization [13]. For ascomycete fungi belonging to the Pezizomycotina subphylum, Woronin bodies (WBs) are formed for plugging/unplugging the septal pores to regulate organelle exchanges between compartments, maintain hyphal cell heterogeneity and prevent excessive cytoplasmic bleeding in the event of hyphal damage [13–15]. WB is a peroxisome-type and hexagonal crystal-like organelle, which is membrane-bound and contains a dense core developed from the self-assembly of a single protein Hex-1, which has been first characterized in the model fungus *Neurospora crassa* in a very close association with septa [16,17]. The Hex-1-like proteins (either called HexA or Hex1) have then been identified and characterized in a few fungal species such as *Aspergillus oryzae* [15], *A. fumigatus* [18] and the plant pathogenic fungi like *M. oryzae* [12] and *Fusarium graminearum* [19]. The deletion of the *Hex1* gene resulted in the disappearance of WBs in fungal hyphae and the null mutants demonstrated impaired stress resistance abilities against the osmotic and cell-wall integrity interference agents, a dramatically reduced ability to survive wounding and or a reduced capacity in the infection of hosts [18,19]. For example, the *Hex-1* null mutant of *N. crassa* had reduced growth on a minimal medium and was impaired in sporulation [20]. After the deletion of *Aohex1* in *A. oryzae*, septal plugging was abolished and hyphal heterogeneity also affected [15]. The HexA of *A. fumigatus* was verified to be important for stress resistance and virulence [18]. For plant pathogenic fungi, it has been revealed that the formation of the Hex1-associated WB was required in *M. oryzae* for the development and function of the infection structures appressoria and therefore host colonization [12]. The disruption of the *Hex1* gene in *F. graminearum* reduced fungal asexual reproduction, infectivity and virus RNA accumulation in the infected cells when compared with the wild-type strain [19]. Likewise, the homologous *Hex1* gene was found to be required for WB formation, conidiation and the formation of the capturing trap in the nematophagous fungus *Arthrobotrys oligospora* [21]. The gene(s) responsible for WB formation and function in ascomycete entomopathogenic fungi has yet to be investigated.

In this study, it is intriguing to find the substantial length variation amongst the Hex1-domain-containing proteins from different fungi. We then performed the loss-of-function investigation of a homologous *Hex1* gene (MAA_00782, designated as *Mrhex1*) in the insect pathogenic fungus *M. robertsii*. It was found that *Mrhex1* was required in *M. robertsii* for WB formation, asexual growth and sporulation, appressorium differentiation and the topical infection of insect hosts. In contrast to the findings in other fungi, however, the null mutant of *MrHex1* could tolerate different stress conditions like the wild-type strain.

2. Materials and Methods

2.1. Strains and Culture Conditions

The wild-type (WT) strain and mutants of *M. robertsii* strain ARSEF 2575 were routinely cultured on potato dextrose agar (PDA; BD Difco, Franklin Lakes, USA) at 25 °C. Spore germination and appressorium induction assays were conducted using locust (*Locusta migratoria manilensis*) hind wings or the minimal medium (MM) ($NaNO_3$, 6 g/L; KCl, 0.52 g/L; $MgSO_4{\cdot}7H_2O$, 0.52 g/L; KH_2PO_4, 0.25 g/L) amended with 1% glycerol as the sole carbon resource (MM-Gly) [22]. For genomic DNA, RNA extractions and hyphae staining, fungal spores were cultured in Sabouraud dextrose broth (SDB; BD Difco, Franklin Lakes, USA) for three days at 25 °C and incubated at 200 rpm in a rotary shaker.

2.2. Protein Feature Characterization and Phylogenetic Analysis

Homologous Hex1 proteins were retrieved from GenBank for those containing the conserved S1_Hex1 domain (Table S1). The conservation analysis of the S1_Hex1 domains of 460 proteins obtained

from different fungal species/strains was characterized with the program WebLogo (ver. 2.8.2) [23]. For phylogenetic analysis, 21 proteins selected from representative fungal species were aligned with the program Clustal X ver. 2.0 [24], and a bootstrapped (1000 replicates) neighbor-joining (NJ) tree was constructed with the program MEGA X [25] using the pairwise deletion of the alignment gaps and a Dayhoff substitution model.

2.3. Gene Deletion and Complementation

To determine the function of *MrHex1*, targeted deletion was performed by homologous recombination via the *Agrobacterium*-mediated transformation of the WT strain of *M. robertsii* as described before [26]. In brief, the 5′- and 3′- flanking sequences were amplified using the genomic DNA as a template with the primer pairs hex1UF (CGGAATTCGTACGGACCGATAAAACGTG) and hex1UR (CGGAATTCGAATGTCCTCCTTGATGTC), hex1DF (GCTCTAGACTGTCGACTGC-TTTCGAGTC) and hex1DR (GCTCTAGATAAGACACCCCATGTCAGC), respectively. The products were digested with the restriction enzymes *Eco*RI and *Xba*I, and then inserted into the same enzyme-treated binary vector pDHt-bar (conferring resistance against ammonium glufosinate) to produce the plasmid pBarhex1-KO for fungal transformation. For null mutant complementation, the full open reading frame (ORF) of the *Mrhex1* gene was amplified together with its promoter and terminator regions using the primer pairs hex1U (GGACTAGTGCACAGAGGACAAAACATGG) and hex1L (GGACTAGTTTACAGGCGAGAGCCGTGAA). The product was digested with *Spe*I, and then inserted into the binary vector pDHt-ben to produce the plasmid pBenhex1 (conferring resistance against benomyl) [27]. The drug-resistant mutants were isolated and verified by PCR and reverse transcription-PCR (RT-PCR) analyses with the primers hex1F (CACCACCACCATGACCAC) and hex1R (GAGAGCCGTGAATGACCTT). The β-tubulin gene (MAA_02081) was used as the control and amplified using the primers TubF and TubR [28].

2.4. Phenotyping, Cell Integrity and Stress Response Assays

To determine the effect of *MrHex1* deletion on fungal growth and conidiation, fungal cultures were grown on PDA and the colony diameters were measured at different times post inoculation. After growth for 18 days, conidial production was assayed and compared between the WT and mutants by two-tailed Student's *t*-tests [29]. To determine cell integrity after gene deletion, the level of cellular content leakage was determined via the detection of free amino acids in liquid culture filtrates by reaction with ninhydrin [30]. Thus, the spores of the WT and mutants were collected from 14 day-old PDA plates and inoculated in SDB at 25 °C and 200 rpm for 4 days. Fungal mycelia were collected by filtration, washed twice with sterile distilled water and transferred to MM–N (i.e., without the addition of $NaNO_3$ in MM) liquid medium for 24 h. The supernatants were collected by filtration and transferred (4 mL each) into the test tubes followed by the individual addition of 1 mL of 2% (*w*/*v*) of ninhydrin reagent and 1 mL phosphate buffer (pH, 8.0). The samples were mixed by vortexing prior to being treated in a boiling water bath for 15 min. After cooling at room temperature, the absorbance of each sample was recorded at 570 nm (A570) using a Biophotometer (Eppendorf). The reaction solutions were also transferred into 1.5 mL centrifuge tubes for photographing. The corresponding mycelia of each strain were dried in an oven at 50 °C overnight and weighed. The unit of A570 was then normalized with the mycelium dry weight of each sample. There were three replicates for each strain and the experiments were repeated twice. The two-tailed Student's *t*-tests were conducted to compare the differences between strains.

For stress challenges, fungi were grown on PDA or PDA amended with the final concentrations of 0.01% sodium dodecyl sulphate (SDS), 200 μg/mL Calcofluor white and 250 μg/mL Congo red for cell wall integrity challenges; 50 μM farnesol for antifungal resistance, and 1.5 M KCl and 1 M Sorbitol for osmotic challenges [18,26], respectively. For inoculation, 2 μL of the 10-fold diluted spore suspensions (2×10^7 conidia/mL) were spotted onto the plates and incubated at 25 °C for three days.

2.5. Microscopy Observations

To determine the effect of *MrHex1* deletion on WB formation, a transmission electron microscope (TEM) analysis was conducted as described before [27]. The spores of the WT and mutants were inoculated in SDB for three days and the mycelia were harvested by filtration. After washing twice with distilled water, fungal samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS; pH, 7.2) at 4 °C for 12 h, rinsed three times in the phosphate buffer, and fixed overnight in 1% osmium tetroxide buffered in 0.1 M cacodylate (pH, 7.0) at 4 °C. After rinsing three times with the phosphate buffer, samples were dehydrated in an ethanol gradients, infiltrated with a gradient series of epoxy propane, and then embedded in Epon resin for sectioning [27]. The ultrathin samples were treated in 2% uranium acetate and then lead citrate prior to the observations under a TEM (H-7650; Hitachi).

The mycelia collected from SDB were also used for fluorescent staining. After washing with PBS, the mycelia of each strain were jointly stained with DAPI (4'6-diamidino-2-phenylindole, Sigma-Aldrich, St. Louis, USA) and Calcofluor white (CW, Sigma-Aldrich) to detect nuclei and cell septa, respectively. A stock solution of DAPI (100 μg/mL) was prepared in water and diluted to 1–2 μg/mL in PBS for staining for 30 min. After washing with PBS three times, the samples were then treated with CW solution (4 μg/mL) buffered in 10% potassium hydroxide for 1 min prior to the observations with an Olympus microscope (BX51-33P, Tokyo, Japan).

2.6. Appressorium Induction and Insect Bioassays

Appressorium formation of the WT and mutants were induced on both a hydrophobic surface and locust hind wings [28]. Briefly, the spores of each strain were inoculated into individual polystyrene petri dishes (6 cm in diameter) containing 2 mL MM-Gly at a final concentration of 2×10^5 conidia/mL. After incubation for 24 h, the appressorium differentiation rates were recorded for > 300 conidia under a microscope. The locust hind wings were surface sterilized in 37% H_2O_2 for 5 min, washed twice with sterile water and immersed in conidial suspensions (2×10^7 spores/mL) for 20 s. The inoculated wings were lined on 0.8% water agar at 25 °C for 16 h. The Student's *t*-tests were conducted to compare the differences between strains.

Insect bioassays for the WT and mutants were conducted using the newly emerged last instar larvae of the mealworm *Tenebrio molitor* and silkworm *Bombyx mori*. Conidia were harvested from the two-week old PDA plates and suspended in 0.05% Tween-20 at the concentration of 1×10^7 conidia/mL. Insects were chilled on ice before immersion in spore suspensions for 30 s. In addition, injection assays were performed using the silkworm larvae. Each insect was injected at the second proleg with 10 μL of the suspensions each containing 1×10^6 conidia/mL. The mortality was recorded every 12 h and the median lethal time (LT_{50}) was calculated by Kaplan–Meier analysis [31]. The control insects were treated with 0.05% Tween-20. Each treatment had three replicates with 15 insects each and the experiments were repeated twice.

3. Results and Discussions

3.1. Length Variation of the Hex1 Proteins with Conserved C-termini

The single copy and complete ORF of *Mrhex1* (MAA_00782) encodes a protein possessing 392 amino acid (aa) residues and containing a carboxyl-terminal S1_Hex1 domain (75 aa) like other proteins such as Hex-1 of *N. crassa* and HexA of *A. fumigatus* [18,20], however, with substantial total length variations between each other (Figure 1A). Further survey of the S1_Hex1 domain proteins catalogued in GenBank obtained 460 proteins (single copy within each genome) from those fungal species belonging to the clade Sordaromyceta of the subphylum Pezizomycotina (Ascomycota) (Table S1). Unexpectedly, the substantial length variation was further evident for the Hex1 proteins from different fungal species, ranging from 79 aa (EPQ66756, *Blumeria graminis f. sp. tritici*) to 2958 aa (ERF74742, *Endocarpon pusillum*) (Table S1). The misannotation of some of these proteins could not be

precluded. Statistically, the major distribution of Hex1 protein length is within the regions 470–534 aa (26.7%, 123/460), 405–469 aa (24.3%, 112/460) and 145–209 aa (13.9%, 64/460) (Figure 1B). The last group includes those characterized in *N. crassa* (Hex-1, NCU08332, 176 aa) and *A. nidulans* (AnHex1, AN4965, 221 aa). Length variations were also evident in different species from the same genus. For example, the Hex1 homologues from *Metarhizium* genus vary from 392 aa (MAA_00782 and MAN_09889, *M. anisopliae*) to 423 aa (MAC_08379, *M. acridum*) and 454 aa (NOR_02601, *M. rileyi*). Likewise, the proteins from the *Aspergillus* and other fungal genera are also highly variable in total length (Table S1). Similar to this finding, length differences have also been observed between other proteins belonging to the same family. Some protein domains are functionally permissive to length variation (termed length-deviant domains) while some others are less tolerant to length alteration (termed length-rigid domains) [32]. Considering the conserved function of Hex1 in WB formation in different fungi [13], it is therefore length-deviant for Hex1 proteins in term of their full lengths. It was found that the Hex-1 cleavage occurred in *N. crassa* [20]. The mature and functional length of Hex1 proteins remains to be determined in different fungi.

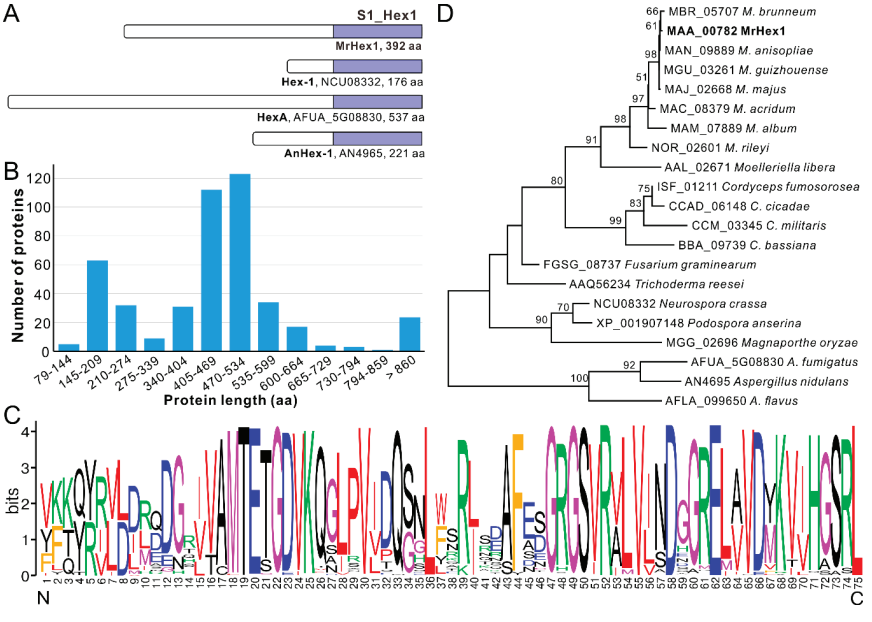

Figure 1. Schematic structuring and phylogenetic analysis of the selected Hex1 proteins. (**A**) Schematic structuring of MrHex1 and the selected homologues. Selected proteins are: Hex-1 from *Neurospora crassa*, HexA from *Aspergillus fumigatus* and AnHex-1 from *A. nidulans*. (**B**) Length variations of the Hex1 proteins from different fungi. *X* axis represents the amino acid (aa) length region of proteins. *Y* axis represents the number of proteins belonging to different length regions. (**C**) Conservation analysis of the Hex1-domain sequences. The sequences (75 aa each) were extracted from 460 Hex1 proteins from different fungal species. The N and C letters labeled at the bottom represent the N- and C-termini of the Hex1 domains. (**D**) Phylogenetic analysis of the selected Hex1 proteins. Protein sequences were retrieved from the selected fungal species and aligned to generate a neighbor joining tree with a Dayhoff substitution model and 1000 bootstrap replicates.

Irrespective of clear length variations among Hex1 proteins, a highly conserved C-terminus S1_Hex1 domain with 75 aa residues is evident in each Hex1 protein, a typical feature of the length-rigid domain (Figure 1A; Table S1). In particular, the characteristic and specific peroxisome-targeting signal 1 (PTS1) tripeptide S/A-R/S-L [17] is present at the C-terminal of MrHex1 and other proteins (Figure 1C;

Table S1), which is different from the consensus PTS1 motif S/A/C-K/R/H-L/M reported before [13,17]. In particular, the PTS1 motif A-S-L is found from the putative Hex1 proteins of the plant pathogen *Monosporascus* genus and an S-S-L pattern from the Hex1 proteins of the *Valsa* genus (Table S1), where the second residue of serine (S) has not been suspected before. A phylogenetic NJ tree generated with 21 selected Hex1 proteins revealed that the clustering pattern of these proteins largely correlated with fungal speciation relationships (Figure 1D). For example, consistent with previous analyses [33,34], the Hex1 proteins from *Metarhizium* species evolved following the trajectory from the specialists (*M. rileyi* and *M. album*) to the generalist species (e.g., *M. robertsii* and *M. brunneum*) with a broad host range. In this respect, *Hex1* might have evolved by following fungal divergence and speciation after its birth in the ancestor of the Pezizomycotina fungi.

3.2. MrHex1 Effecting on Fungal Growth, Sporulation and Stress Responses

By checking the previous RNA-seq transcriptome data, relative to the conidial sample, *MrHex1* was found to be highly transcribed by the fungus during the formation of appressoria on locust wings [35]. To determine the function of *MrHex1* in *M. robertsii*, the gene was deleted and the obtained null mutant was also complemented by the verification of RT-PCR analysis (Figure 2A). Phenotypic growth assays showed that the deletion of *MrHex1* substantially reduced the fungal growth rate when compared with the WT and complemented (Comp) strains (Figure 2B,C). In addition, we found that the sporulation ability of Δ*MrHex1* was severely ($P = 3.94 \times 10^{-4}$) impaired when compared with that of the WT (Figure 2D). Unexpectedly, the gene-rescued mutant Comp also had a reduced level of conidiation when compared with the WT ($P = 2.64 \times 10^{-6}$). Otherwise, relative to the WT, both the null and rescued mutants did not show obvious defects in their stress responses against the challenges with the detergent SDS, osmotic stressors KCl and sorbitol, antifungal agent farnesol or cell wall biosynthesis inhibitors CW and Congo red (Figure 3).

The requirement of Hex1 for asexual growth and sporulation has also been found in a few fungal species like *N. crassa* [17], *A. oligospora* [21], *F. graminearum* [19] and *M. oryzae* [12]. However, in contrast, the Δ*HexA* of *A. fumigutas* showed normal growth and sporulation like the WT strain [18]. Thus, similar to the observation of functional divergence between the conserved transcription factors in different fungi [36,37], Hex1 also shows functional alterations in different fungi. The fact that no obvious differences were observed in the stress responses between WT and Δ*MrHex1* provided further supports of species-dependent functional variations of Hex1 in different fungi. For example, it has been found that, in contrast to Δ*MrHex1* and Δ*HexA* of *A. fumigatus* [18], *Hex1* null mutant of *A. oligospora* was sensitive to osmotic stress [21]. However, relative to the WT of *A. fumigatus*, Δ*HexA* became sensitive to SDS, farnesol, CW and Congo red [18], which was not the case for Δ*MrHex1* as we showed.

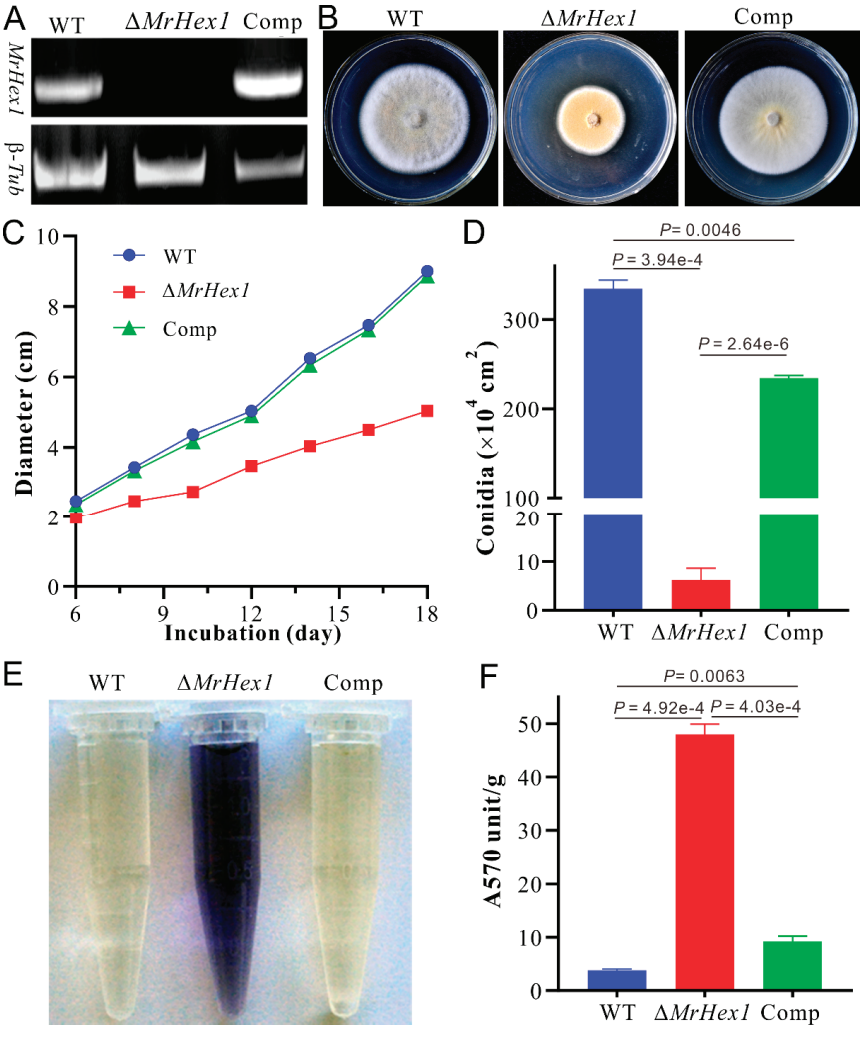

Figure 2. Gene deletion and phenotypic characterizations: (**A**) RT-PCR verification of gene deletion and complementation. Comp, the complemented mutant; β-*Tub*, the β-tubulin gene used as a control. (**B**) Phenotypic characterization of the wild-type (WT) and mutants after growth on potato dextrose agar (PDA) for 14 days. (**C**) Time-scale growth assays by measuring colony diameters. (**D**) The quantification of conidial production by WT and mutants after growth on PDA for 18 days. (**E**) Culture filtrates of different strains after reaction with ninhydrin. (**F**) Photometric estimation of the leaked amino acids after reaction with ninhydrin. The unit absorbance of A570 was normalized to the mycelium dry weight. Error bar on top of each column represents standard deviation.

3.3. Requirement of Mrhex1 for Woronin Body Formation and Maintaining Cell Integrity

Hex1 is the major WB protein in Pezizomycontina fungi [13,16,20]. To determine the function of MrHex1 in WB formation in *M. robertsii*, mycelial samples of the WT and mutant strains were subject to TEM analysis. As a result, the dense and characteristic WBs were evident on both sides of the WT cell septa but absent in Δ*MrHex1*. For Comp, after the examination of multiple section samples, the WT-like distribution of WBs was not observed but the WBs were found to be plugged or anchored

in proximity to the septum pore (Figure 4A). Thus, MrHex1 is similarly required for WB formation in *M. robertsii*. This kind of WB number and positioning differences between WT and the complemented mutant has also been found in *F. graminearum* [19] and *A. oligospora* [21]. It is noteworthy that WB positioning and localization are associated with the WB enveloping protein (i.e., the Woronin sorting complex protein, WSC) and a tethering protein Leashin (Lah) [38]. The *N. crassa* WSC-like protein (NCU07842 vs. MAA_02499, 71% identity at amino acid level) is present in *M. robertsii*. However, in contrast to the finding in *Aspergillus* fungi [39], the large and nonconserved Lah-like protein remains elusive in *M. robertsii*. In addition, it has been known that the proper function of some genes requires their positions preferentially located in genomes [40]. The importance of the *Hex1* gene positioning remains to be determined for function. It could not be precluded at this stage that the imperfect issue of gene rescue might be due to the non-original position insertion.

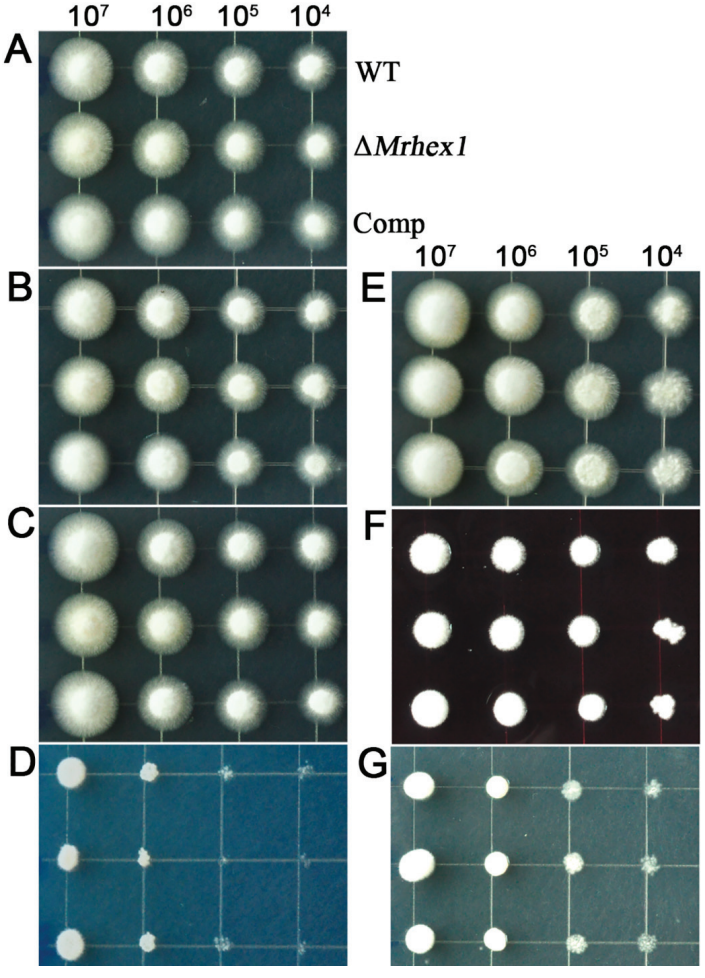

Figure 3. Stress response assays. The spores of the WT and mutants were inoculated on PDA (**A**), PDA amended with farnesol at 50 μM (**B**), PDA plus Calcofluor white at 200 μg/mL (**C**), PDA plus KCl at 1.5 M (**D**), PDA plus sodium dodecyl sulphate (SDS) at 0.01% (**E**), PDA plus Congo red at 250 μg/mL (**F**) and PDA plus sorbitol at 1 M (**G**). The phenotypes were photographed after inoculation with 2 μL of spore suspensions (started at 2×10^7 conidia/mL) diluted 10-fold for three days.

The anchoring of WBs to the septum pore in fungal cells is essential for preventing cytoplasmic leakage after cell damage [11], and maintaining cell integrity and heterogeneity [13]. We first performed ninhydrin reaction assays to determine if any difference between WT and mutants in terms of the amino acid leakage in culture filtrates. The results indicated that a deep purple color, the result of amino acid reaction with ninhydrin, was evident for the Δ*MrHex1* sample but not for the WT and Comp strains (Figure 2E). Consistently, the photometric assays indicated that the A570 value of the Δ*MrHex1* sample was significantly higher than those of the WT ($P = 4.92 \times 10^{-4}$) and Comp ($P = 4.03 \times 10^{-4}$) (Figure 2F). It was also found that the A570 value of Comp was higher than that of the WT ($P = 0.0063$) for an unclear reason. We also performed the joint fluorescent staining of different strains for detecting the distribution pattern of the nuclei within each hyphal cell. The results showed that only one nucleus was observed within one hyphal cell of the WT and Comp whereas more than one nucleus were frequently evident in Δ*Mrhex1* cells, especially within the cells close to the injured end (Figure 4B). MrHex1 is therefore functionally important in maintaining cell integrity and heterogeneity in *M. robertsii*. Likewise, it has been shown that the hyphal heterogeneity of *A. oryzae Hex1* null mutant was affected [15]. It has also been shown that the peroxisome-related WB formation affects fungal secondary metabolisms [13], which remains to be determined in *M. robertsii*.

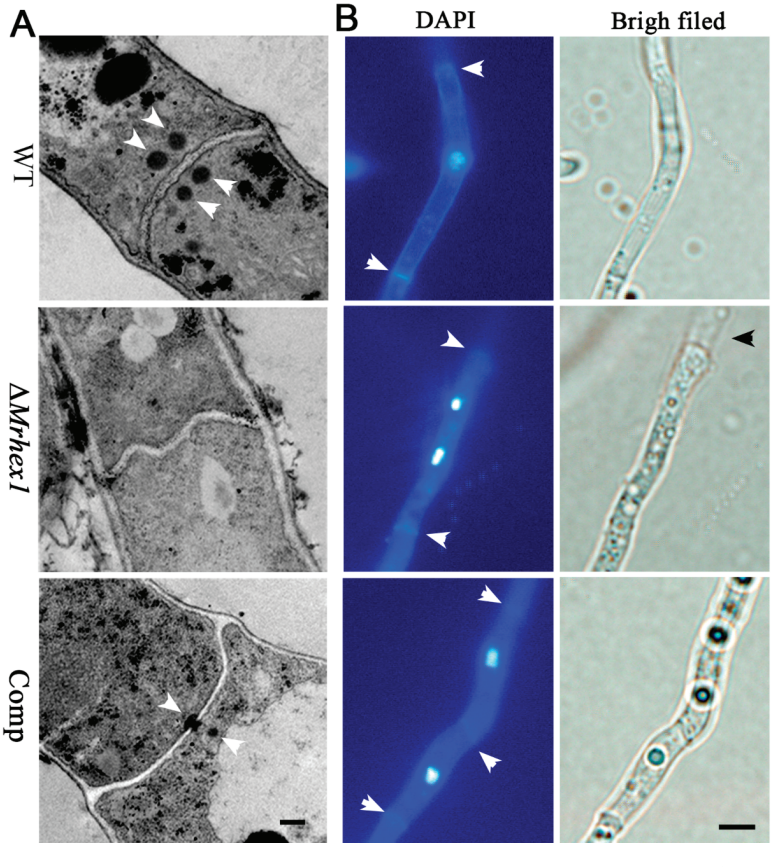

Figure 4. Microscopic observations: (**A**) transmission electron microscope observation showing the presence or absence of Woronin bodies (arrowed) in the WT and mutant cells. Bar, 0.5 μm; (**B**) the co-staining of the mycelium cells for detecting nuclei and septa (arrowed). The broken end of the Δ*MrHex1* mycelium is arrowed for its bright field image. Bar, 5 μm.

3.4. Defects of Mrhex1 Null Mutant in Appressorium Formation and Topical Infection of Insects

We then performed infection structure induction and insect bioassays with the WT and mutant strains. Appressorium formation was induced on both the hydrophobic surfaces and locust hind wings. As a result, we found that appressorium production was considerably impaired for Δ*MrHex1* when compared with the WT and Comp under both conditions (Figure 5A). Statistically, the rate of appressorium production by Δ*Mrhex1* (23.3% ± 2.53) declined significantly ($P < 0.001$) when compared with those formed by WT (83.6% ± 5.69) and Comp (82.9% ± 4.38) on a hydrophobic surface. The failure of septal pore sealing might lead to the defects in building up turgor pressure within appressorium cells. Considering that the mutants of *M. robertsii* with impaired abilities in generating cellular turgor pressure could still form appressoria [10,27,41], the defect of Δ*MrHex1* in appressorium formation might not be due to the turgor generation failure of the mutant. The exact mechanism between WB and infection structure formations requires further investigation.

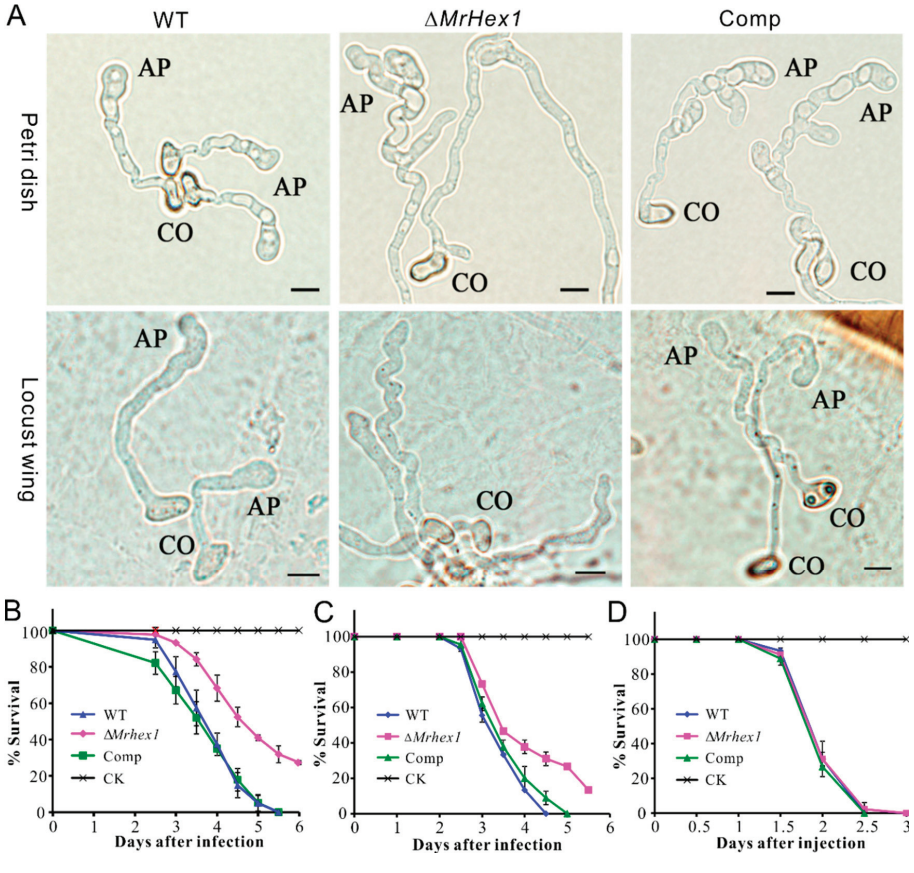

Figure 5. Appressorium induction and insect survival assays. (**A**) Microscopic examination of appressorium formation by the WT and mutants on hydrophobic surface (upper panels) and locust hind wings (lower panels). CO, conidium; AP, appressorium. Bar, 5 μm. (**B**) Survival of the mealworm larvae after topical infection. (**C**) Survival of the silkworm larvae after topical infection. (**D**) Survival of the silkworm larvae after injection.

Consistent with the mutant defect in appressorium formation, the topical infection of the mealworm and silkworm larvae revealed that the virulence reduction of Δ*MrHex1* was evident (Figure 5B,C).

Thus, the LT_{50} value of Δ*MrHex1* (4.98 ± 0.18 days) was significantly higher than those of the WT (3.94 ± 0.12 days; $\chi^2 = 25.12$, $P < 0.0001$) and Comp (3.80 ± 0.15 days; $\chi^2 = 22.24$, $P < 0.0001$) during the topical infection of *T. molitor* larvae. For the topical infection of silkworm larvae, the LT_{50} value of Δ*MrHex1* (4.02 ± 0.14 days) was also higher than those of the WT (3.48 ± 0.09 days; $\chi^2 = 11.04$, $P < 0.001$) and Comp (3.62 ± 0.10 days; $\chi^2 = 7.0$, $P < 0.01$). However, survival dynamics were similar between the WT and mutant strains during the injection assays ($\chi^2 < 2.0$, $P > 0.1$) of the silkworm larvae (Figure 5D). These results confirmed that the deletion of *MrHex1* impaired the fungal ability to penetrate host cuticles due to the null mutant defect in appressorium formation and or the generation of turgor pressure. Considering that the sporulation ability of Δ*MrHex1* was impaired, the mycosis of insect cadavers killed by either topical infection or injection might also be negatively affected for Δ*MrHex1* when compared with the WT and Comp strains.

Similar to our observations, the defects in appressorium formation and therefore virulence reduction were also observed in the Δ*Hex1* of *M. oryzae* [12]. Likewise, the failure of trap formation was evident for the Δ*AoHex1* of *A. oligospora* and the mutant lost its ability to capture nematodes [21]. Both the deletion and overexpression of *FgHex1* in *F. graminearum* reduced fungal infectivity [19]. However, intriguingly, the deletion of *CoHex1* in the cucumber anthracnose fungus *Colletotrichum orbiculare* did not produce any detectable defects in appressorium formation and infectivity [42]. This kind of species-dependent phenotypic diversity of *Hex1* deletion mutants indicates again the functional alterations of this conserved gene in different fungi.

4. Conclusions

In this study, the WB-formation protein MrHex1 was characterized in the insect pathogenic fungus *M. robertsii*. Unexpectedly, we first found the substantial length variation among Hex1 proteins from different fungi but each with a highly conserved C-terminal tail and the characteristic PTS1 sorting signature. Taken together with the finding that MrHex1 is similarly required for WB formation in *M. robertsii*, the data suggest that the length variation of Hex1 proteins might have no hindrance for their similar functions in WB formation in different fungi. However, phenotypic alterations were clearly evident between Δ*MrHex1* and *Hex1* null mutants of other fungi. In particular, unlike other fungal mutants [18,19], Δ*Mrhex1* demonstrated an equal tolerance to different stress conditions like the WT and Comp of *M. robertsii*. This kind of phenotypic and functional divergence of *Hex1* genes implies the necessity of investigating evolutionarily conserved genes in different fungal pathotypes. The finding that MrHex1 is required in *M. robertsii* for infection structure formation and the topical infection of insect hosts advances our understanding of the control and evolution of fungal entomopathogenicity. Future efforts are still required to investigate the mature type of Hex1 within fungal cells, the relationship between gene positioning and function, and the feasibility of functional complementation of the length-varied Hex1 among different fungi.

Supplementary Materials: The following are available online at http://www.mdpi.com/2309-608X/6/3/172/s1, Table S1: Characteristics of the Hex1 proteins retrieved from different fungal species.

Author Contributions: G.T. and Y.S. performed the experiments and data analysis; S.L. performed insect bioassays; G.T. and Y.S. drafted the manuscript; C.W. designed the experiments, performed bioinformatic analysis, managed the project and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Effect of Juvenile Hormone on Resistance against Entomopathogenic Fungus *Metarhizium robertsii* Differs between Sexes

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Abstract: Juvenile hormone has been suggested to be a potential mediator in the trade-off between mating and insects' immunity. Studies on various insect taxons have found that juvenile hormone interferes with humoral and cellular immunity. Although this was shown experimentally, studies using highly virulent parasites or pathogens are lacking so far. In this study, we tested if juvenile hormone administration affected resistance against entomopathogenic fungi, *Metarhizium robertsii*, in the mealworm beetle, *Tenebrio molitor*. In previous studies with *T. molitor,* juvenile hormone has been found to reduce a major humoral immune effector-system (phenoloxidase) in both sexes and decrease the encapsulation response in males. Here, we found that juvenile hormone administration prolonged survival time after infection with *M. robertsii* in males but reduced survival time in females. This study indicates that the effects of juvenile hormone on insect immunity might be more complicated than previously considered. We also suggest that there might be a trade-off between specific and non-specific immunity since, in males, juvenile hormone enhances specific immunity but corrupts non-specific immunity. Our study highlights the importance of using real parasites and pathogens in immuno-ecological studies.

Keywords: immune defense; immunocompetence; *Metarhizium robertsii*; pathogens; sex; *Tenebrio molitor*

1. Introduction

The immunocompetence handicap hypothesis [1] suggests that the expression of secondary sexual traits honestly signals male quality because testosterone—needed to develop these traits—has immunosuppressive effects. However, many studies in vertebrates have failed to reveal a clear relationship between the expression of secondary sexual ornamentation and immune defense. This might be because the physiological relationship between these traits is not as simple as originally thought and because of the alterations of stress hormones, which may involve trade-offs between

sexual ornamentation and immunity [2]. However, many studies in insects and spiders have found that the expression of males' secondary sexual characteristics correlates positively with their immune defense [3–7]. Since insects lack male-specific hormones such as testosterone, it has been suggested that in these animals, the immunocompetence handicap mechanism would be mediated by juvenile hormone (JH) [8].

Juvenile hormone is synthesized in the corpora allata, and it is known to play a crucial role in many aspects of development, reproduction, aging, and behavior in insects. For example, it seems that juvenile hormone type III is associated with sex pheromone production in cockroaches [9]. Similarly, it was found that the administration of juvenile hormone increased the attractiveness of male pheromones in the mealworm beetle, *Tenebrio molitor*. Still, it reduced the strength of the encapsulation response and phenoloxidase activity of hemolymph [8]. Interestingly, it has been found that while JH increases male attractiveness, it reduces the size of the testis and sperm viability, suggesting another cost of high juvenile hormone levels [10]. In the territorial damselfly, *Calopteryx virgo*, it has been found that the administration of methoprene acid (an analog of JH) increased aggression and occupation time in territories but decreased phenoloxidase activity of hemolymph [11]. Juvenile hormone has also been shown to affect genes related to antibacterial peptide expression [12,13]. In the diamondback moth, *Plutella xylostella*, it was shown that juvenile hormone inhibited hemocyte-spreading behavior [13], suggesting that JH induces immune suppression because effective hemocyte-spreading is important for phagocytosis, nodulation, and encapsulation [14]. Furthermore, they found that pyriproxyfen (a JH analog) enhanced the pathogenicity of *Bacillus thuringiensis* subsp. *kurstaki* [13].

Juvenile hormone may also be associated with a trade-off between reproduction and immunity in insects. For example, mating reduced the activity of phenoloxidase enzyme in the hemolymph of both sexes of *T. molitor* [15], but there was no effect on the hemocyte load. It has been found that the observed decrease in phenoloxidase levels was caused by an increase in juvenile hormone levels due to mating, which indicates that juvenile hormone might indeed mediate the trade-off between mating and immunity [15]. Previously, it was shown that mating enhances resistance against entomopathogenic fungi, *Beauveria bassiana,* infection and that the effect was stronger in males than among females of *T. molitor* [16]. This shows that the effect of juvenile hormone on the immune system may be more complicated than previously thought. Overall, these results suggest that studies testing the effect of juvenile hormone on the resistance against highly virulent parasites and pathogens are needed.

Entomopathogenic fungi (EPF), including entomophthoralean fungi, offer environmentally friendly alternatives to conventional synthetic chemicals for arthropod pest control. There are over 750 different species of EPF identified so far [17]. Although entomophthoralean fungi are highly efficacious, much attention has focused on researching species belonging to the order Hypocreales because they are more amenable for mass production and have a relatively wide host range. Approximately 80% of the commercially available EPF products are based on the *Metarhizium robertsii, M. anisopliae, M. brunneum,* and *B. bassiana*.

The study aimed to test whether juvenile hormone affects the resistance of *T. molitor* against a real pathogen and whether there is a sex difference in the pathogen resistance. We tested juvenile hormone-related effects on the immunity of *T. molitor* against *M. robertsii*.

2. Materials and Methods

2.1. Study Animals

T. molitor beetles used in the experiment originated from a natural population. They were collected from several barns in southeastern Latvia in 2007 [18] and maintained at the University of Turku. They were reared in plastic boxes (5 l) and fed with wheat bran and apple at a constant temperature of 28 °C under a 14 L:10 D photoperiod and constant humidity of 70%. We collected pupae daily and determined the sex of each pupa by examining the developing genitalia on the eighth abdominal segment. Shortly after emergence, the beetles were placed individually in plastic film roll canisters with

an excess of fresh apple. Sexes were physically isolated to ensure virginity. We excluded individuals that had visible developmental abnormalities or whose size deviated strongly from the population mean. Beetles of each sex were randomly allocated to the treatments when aged between 10 and 14 days. Before the experiments, we weighed the fresh body mass of each beetle to the nearest 0.1 mg.

2.2. Experimental Treatments

In total, we had 242 females and 171 males. The insects were randomly allocated to each treatment in which beetles were injected ventrally either 5 or 10 μg of JH type III (Sigma, St Quentin Fallavier, France) in 5 μL of Ringer: acetone (9:1) solution between the 2nd and 3rd sternite region using a 10 μL Hamilton syringe (30 G) (Hamilton Company, Switzerland). Control males and females received 5 μL of Ringer: acetone solution only, but otherwise, both groups were treated identically. Before injection, the beetles were anesthetized with carbon dioxide. The inoculated beetles were placed individually into plastic film roll canisters kept in an environmental chamber at 24 °C under a 16 L:8 D photoperiod and fed with fresh apple.

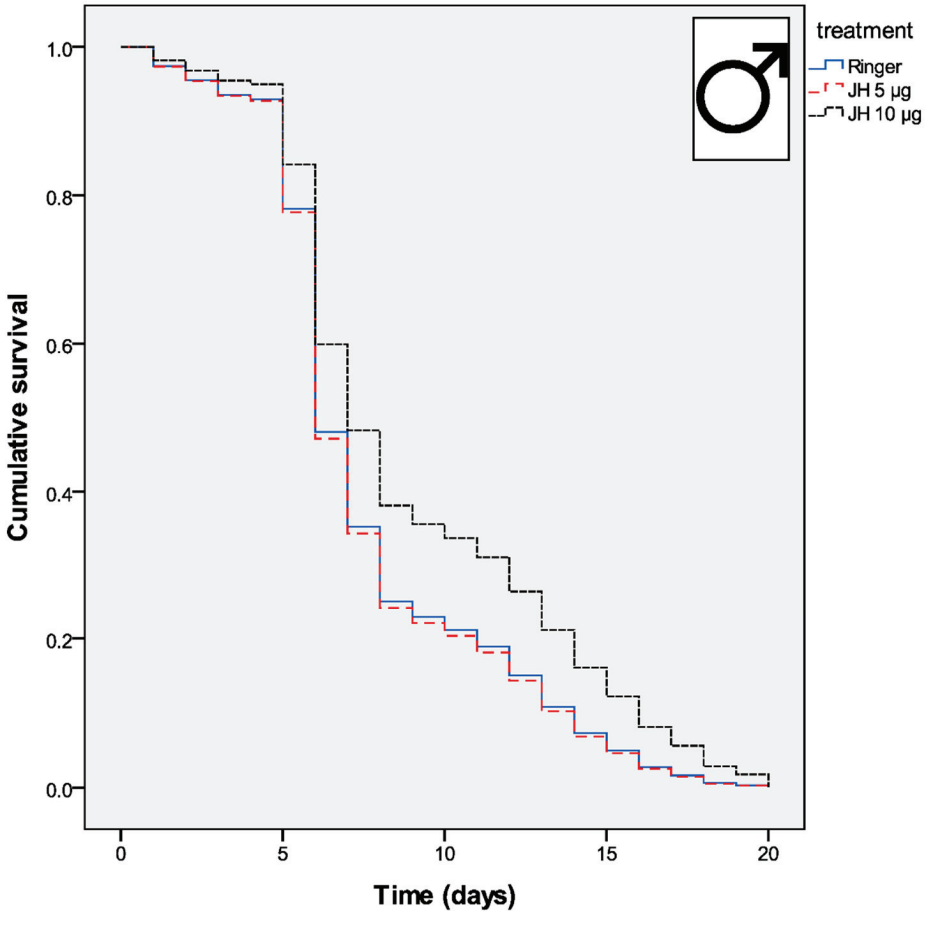
During the following day, we infected the beetles with the conidia of entomopathogenic fungi, *M. robertsii* [19]. To infect the insects with the fungi, we anesthetized beetles with carbon dioxide. We dropped dorsally 5 μL of LD50 solution containing conidium (5 × 106 conidium/mL) with a pipette on the abdomen under the beetles' wings. LD50 doses were determined in preliminary experiments by infecting a separate group of insects with different doses of conidium and selecting the dose that closest to kill 50% of the treated animals. Unfortunately, the mortality caused by the fungi was much higher in the experiment than in our preliminary studies (probably because wounding by the needle made it easier for the fungi to penetrate the cuticula). In our previous studies on *T. molitor*, we found that juvenile hormone administration did not influence on beetles' survival [8]. There was no mortality among beetles when dipped in the control solution. Thus, we left the control solution out from this experiment to double the sample size in the fungal treatment groups. After the infection with fungi, beetles were placed individually back to the plastic film roll canisters in an environmental chamber at 24 °C under a 16 L:8 D photoperiod and fed with fresh apple for 21 days to check daily for mortality rates of experimental individuals.

2.3. Statistical Analyses

We used Cox proportional hazards regression (survival analysis) to examine survival differences between the different treatments after infection with the entomopathogenic fungi. In the model, we presented sex and JH hormone treatment as categorical covariates, mass as a continuous covariate, and survival time as a dependent variable. We initiated a model fitting with a model that included all the main effects and the two-way interaction terms that best address the subject of interest. We searched for the best model using a backward stepwise method (backward LR). We conducted all statistical analyses by using PASW Statistic 18.0 for Windows.

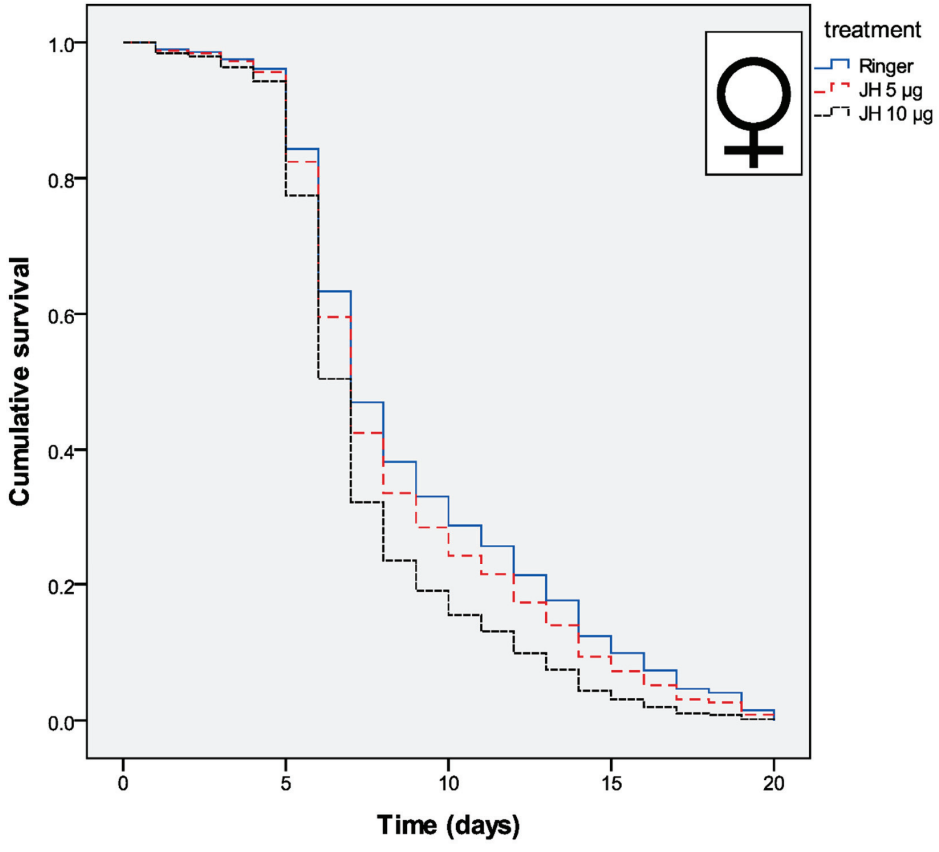
3. Results

The best model predicting survival after the fungal infection contained treatment (Wald = 5.520, df = 2, $p = 0.063$), 5 μg of JH treatment (OR = 1.030, Wald = 0.025, df = 1, $p = 0.875$), 10 μg of JH treatment (OR = 0.693, Wald = 3.848, df = 1, $p = 0.050$), sex × treatment (Wald = 10,706, df = 2, $p = 0.005$), sex × 5 μg of JH treatment (OR = 1.099, Wald = 0.025, df = 1, $p = 0.875$) and sex × 10 μg of JH treatment (OR = 2135, Wald = 9136, df = 1, $p = 0.003$). The females had stronger resistance against the fungi than males in the control treatment group (Wald = 3.897, $p = 0.048$). However, administration of the small dose (5 μg) of juvenile hormone did not have any effect on the survival after the fungal infection in either of the sexes. Instead, administration of the larger juvenile hormone dose (10 μg) increased male survival but decreased females survival after the fungal infection (Figure 1a,b). Thus, it seems that juvenile hormone enhances male's immunity but corrupts female immunity.



(a)

Figure 1. *Cont.*



(**b**)

Figure 1. Cumulative survival of (**a**) male and (**b**) female beetles after topical application with the entomopathogenic fungi *M. robertsii*. Curves represent the survival functions calculated by the Cox regression survival analysis.

4. Discussion

In this study, we found that juvenile hormone enhances resistance against the entomopathogenic fungi in the males of *T. molitor*, which contradicts the results by some previous studies [8,11,15], which have found that juvenile hormone corrupts immunity. However, we found that juvenile hormone reduced resistance against the entomopathogenic fungi in females. Thus, the effects of juvenile hormone on the immune system are much more complicated than previously thought. The reason why juvenile hormone had a different effect on the immune system in males and females in our study remains unclear. One possibility is that instead of corrupting the immunocompetence, juvenile hormone may rather cause a reallocation of the resources to those parts of the immune defense which need them the most (as has been proposed in vertebrates for testosterone and immunity [20]). Since optimal life history strategies differ between the sexes, the optimal reallocation of the resources between different immunity arms may also differ.

Interestingly, there were sex differences in resistance in the control treatment group: females having stronger resistance against the fungi than males. It has been suggested that the ultimate mechanism for the observed sex differences in immune function could be a differential selection favoring different

investment levels in the immune defense system [21,22]. Because female fitness is limited by the number of offspring produced, whereas male fitness is limited by the number of females fertilized, males are expected to invest more in sexual competition and current reproduction at the expense of their immune defense compared to females (the Bateman Principle) [21]. However, an experimental study found no sex differences in parasite infections among arthropod hosts [23]. Likewise, efforts to examine the sex differences in innate immune function in insects have been met with mixed results [24]. Thus, more studies testing the sex differences in insects using real parasites and pathogens would be needed to test the Bateman principle in insects. On the other hand, it was shown that sex-specific responses to experimental manipulation of fitness-limiting resources affects both the magnitude and direction of sex differences in immune function [22,25]. This suggests that for species similarly limited in their reproduction, phenotypic plasticity would be an important determinant of sex differences in immune function and other life-history traits. Likewise, immunological sex differences were found in the autumnal moth, *Epirrita autumnata*, which varied in populations differing in their degree of inbreeding [26]. Thus, it seems that there are plausible explanations for sexual dimorphism in immunity other than just the Bateman principle, which is traditionally used to explain the observed sex difference in immunity [21].

Since our previous studies with *T. molitor* found that the administration of juvenile hormone reduced phenoloxidase activity and the encapsulation response against a nylon monofilament [8], the results of this study suggest that the effect of juvenile hormone differs between specific and non-specific immunity in *T. molitor*. Interestingly, in the autumnal moth, *E. autumnata*, the encapsulation response against a nylon monofilament was positively associated with the resistance against *B. bassiana* [26]. However, it has been shown that cellular antifungal reactions, such as phagocytosis and multicellular encapsulations, are suppressed during the development of fungal diseases [27]. Thus, encapsulation or phenoloxidase activities may not mirror the resistance against fungal pathogens, being indirectly correlated via the individuals' general condition. Thus, it seems that the association between the specific and non-specific parts of immunity appears to be very complicated. Our study highlights the importance of using real parasites and pathogens in immuno-ecological studies.

Author Contributions: I.M.D., M.P., T.K., M.J.R. and I.A.K. conceived the idea. I.M.D., M.P., M.J.R. and J.C.-G., designed the experiments. I.M.D., T.K. and I.A.K. provided the resources. I.M.D. and M.P. performed the experiments. M.J.R., M.P., I.M.D., T.K. and J.C.-G. analyzed the data. M.J.R. and I.M.D. wrote the original draft. M.J.R., I.M.D., M.P., T.K., J.C.-G. and I.A.K. revised and edited the manuscript. M.J.R., I.M.D., M.P., T.K., J.C.-G. and I.A.K. approved the manuscript for publication. All authors have read and agreed to the published version of the manuscript.

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Effectiveness of the Entomopathogenic Fungal Species *Metarhizium anisopliae* Strain NCAIM 362 Treatments against Soil Inhabiting *Melolontha melolontha* Larvae in Sweet Potato (*Ipomoea batatas* L.)

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Abstract: The effect of fungal entomopathogen *M. anisopliae* strain NCAIM 362 against *M. melolontha* larvae in sweet potato was tested under open field conditions when crop management included compost supply and soil cover (agro-foil or agro-textile). Additionally, the effect of *M. anisopliae* same strain against *M. melolontha* was compared with the effect of α-cypermethrin under greenhouse conditions. Soil microbial community using Illumina sequencing and soil biological activity were tested as possible parameter influencing *M. anisopliae* effect. According to the results, compost supply and textile cover may enhance the effectiveness of *M. anisopliae* under open field conditions, while no effect of fungal treatment was detected under greenhouse conditions. Even if soil parameters (chemical composition, bacterial, and biological activity) were identical, the effect of α-cypermethrin against *M. melolontha* larvae was significant: lower ratio of larval survival and less damaged tubers were detected after the chemical treatment. Our results suggest that *M. anisopliae* strain NCAIM 362 is not effective to control *M. melolontha* larvae, further pieces of research are needed to test other species of the *Metarhizium* genus to find an effective agent for sustainable pest control in sweet potato.

Keywords: field production; sustainable management; pest control; soil properties; microbial community; biological activity; soil DNA analyses; α-cypermethrin

1. Introduction

Microbial pesticide, and especially mycoinsecticides, products based on living fungi to control arthropod pests, were given valuable research efforts in the past decades [1–5]. *Metarhizium* strains are soil-dwelling organisms detected extensively all over the world, regardless of climatic and soil limitations [6,7]. Members of the genus are facultative saprophytes and may either live freely within the topsoil or in the presence of a suitable arthropod host act as parasites [3,8]. The number and scope

of research on *Metarhizium* species suggest that strains and isolates of *M. anisopliae* have been given the highest scientific attention within the genus, and also, they are the most widely used organisms in microbial pest control [9,10]. The first scientific recognition of *Metarhizium anisopliae* dates to Russia in 1879, when E. Metchnikoff discovered a fungus that not only covered the cadaver of a chafer, but was evidently the cause of death of the arthropod [6]. It was then named *Entomophthora anisopliae*, referring to the chafer, *Anisoplia austriaca*. Later, N.V. Sorokin repositioned this species to the genus *Metarhizium* [9,11]. When the species finds an arthropod to parasite, its conidial growth is predominantly green, giving the reason for the original name of "green muscardine" to the condition induced by the fungus [7,12].

Strains and isolates of *M. anisopliae* have long been recognized as entomopathogens, with a wide range of targeted (host) arthropods including mites, ticks, and members of the following insect orders: Diptera, Coleoptera, Hemiptera, Lepidoptera, Isoptera, Orthoptera, Thysanoptera, Homoptera, Sternorrhyncha, Heteroptera. Ongoing research of the past two decades, however, has shown that the position and effect of *M. anisopliae* is more complex. The fungus was found to colonize plants within rhizosphere, have a symbiotic relationship with plants, promote plant growth, and may act as an antagonist to plant diseases [13–18]. Commercialized products based on strains and isolates of *M. anisopliae* dominate the selection of mycoinsecticides worldwide. Formulation, application methods, targeted environment (arable or protected production), targeted crops, targeted pests, and strategies of use (inundative and non-inundative, or conservative way) are varying [4,5,10,19]. The potential of *M. anisopliae* isolates against pests of sweet potato (*Ipomoea batatas*) has been tested for more than three decades. One of the earliest virulence tests was performed in 1984, where the efficacy of three *M. anisopliae* strains were investigated in laboratory conditions against adult individuals of the sweet potato weevil (*Cylas formicarius*) [20]. A subsequent study compared 12 isolates of three fungal entomopathogens including *M. anisopliae*, also on adults of the same pest [21]. This resulted in one of the *M. anisopliae* isolates giving the lowest LD_{50} values. In another laboratory experiment, *M. anisopliae* isolates were found not only to infect and destroy coleopterans, but to have an effect on the feeding and reproduction characteristics of *Cylas puncticollis* as well [20]. It was only in 2014, when the pathogenicity of *M. anisopliae* against *C. formicarius* was evaluated not only as a standalone treatment, but in a combination with *Beauveria bassiana* [22]. The possible ways of transmitting the fungal disease in sweet potato beetle was investigated when fecundity, expressed in the number of eggs and the rate of viable eggs was significantly hindered even when the eggs themselves had no contact with the fungus. It appeared that the presence of *M. anisopliae* altered the behavior of the pest, resulting in less eggs being positioned appropriately [23].

One of the earliest accounts of testing the efficacy of the fungus in field conditions dates to 1998, when damage by the Banded Cucumber Beetle (*Diabrotica balteata*) and White grub (larvae of *Phyllophaga* spp.) were evaluated using *M. anisopliae* [24]. Although a single application before planting was found to have promising results against *D. balteata*, the effects on the other pest (i.e., Melolontha larvae) were uncertain, which may suggest that more research should be focusing on finding the conditions to enhance the efficacy of *M. anisopliae* on *M. melolontha* larvae [24].

Laboratory essays and open field experiments together suggests that, there are many abiotic and biotic factors contributing to the success and failure of using *M. anisopliae* in pest control. Among them several factors need further attention, such us soil chemical composition, soil microbiota, and biological activity [3,25]. Soil properties are governed by a complexity of factors, so in order to obtain helpful suggestions that can be used in the practice of Integrated Pest Management (IPM) or organic production, complex studies are required, with a set-up of complex models, and their viability must be trialed in realistic situations as well [26]. Altogether, more information is needed on what mechanisms endophytes establish and interact within a plant, the *M. anisopliae* on circumstances that favor the establishment of endophytism, so as to utilize its benefits [18,27,28]. Since the effect of *M. anisopliae* against *M. melolontha* larvae in sweet potato has not been a widely researched topic, we set up the present study to find answers to the following questions. (1) Can the fungal entomopathogen

M. anisopliae strain NCAIM 362 (commercialized against coleopteran larvae) serve as an effective biological control agent against *M. melolontha* larvae in sweet potato?; (2) In sweet potato production, which soil parameters can significantly influence the efficacy of *M. anisopliae*?; (3) Is *M. anisopliae* more effective in sweet potato than the chemical insecticide?

2. Material and Methods

2.1. Experimental Set-up under Open Field and Greenhouse Conditions

Open field experiments were conducted between 2018 and 2019. Sweet potato plants Beauregard variety were obtained in 4-leaf stage from the Lajosmizse Sweet Potato Company (Lajosmizse, Hungary), and planted in eight rows/block, each row containing 22 plants. The soil was chernozem (6.5 pH). The field was chosen for our experiment because the soil inhabiting pests was dominated by *M. melolontha* larvae. This was determined before the experiment, with an average of one 3rd instar larvae/m^2 detected. *M. melolontha* larvae infection was also influenced by the nearby (200 m distance) oak forests and orchards (100 m distance, mostly apple, pear, and plum trees at a 1.4 ha area). Since open field sweet potato production in the temperate zone usually involves the application of compost and soil cover systems (using agro-foil or textile), we followed and tested this routine. The eight rows and 22 plants within each row were also treated or not with compost and covered by agro-foil or textile (Figure 1, Figures S1 and S2). From each row, half of the plants were treated with *M. anisopliae* strain NCAIM 362 and the other half served as control (no *M. anisopliae*). There were 4 replications to each treatment, resulting in a total of eight replicates for each type. The presence of compost was marked K^+ or K^-; the presence of agro-foil and textile; and the presence or absence of *M. anisopliae* (M^+ or M^-) (Figure 1A, Figures S1 and S2). The whole system was set up at the end of May, 2018, connected to automatic irrigation system (Irritrol junior max, placed below the soil cover systems, so each plant got the same amount of water), while the *M. anisopliae* treatment in Wettable Powder (WP) formulation (as it was commercially recommended) was added on 27 June, after all plants were carefully checked. No plant pathogen symptoms or pest damages were detected on plants, and all plants were in the phenophase when the fungal entomopathogen treatment was added. This was done by preparing a 10% fungal solution (1400 g *M. anisopliae* to 12.6 L of water) transferred to all 700 plants. Treatment was added to each plant separately using a 20-mL syringe. The whole system was daily controlled until harvest. Crop harvest started on 1 October, with leaves and stems harvested first. Next, all soil covers were removed, and tubers were mechanically harvested. Each tuber from each treatment and cover system were separately collected, and tuber weights for each plant were measured and assigned to cover systems and treatments (M^+ or M^-). Because synthetic pesticides (i.e., α-cypermethrin) against soil inhabiting insects' larvae are not allowed in open field sweet potato control in Europe, this treatment was only used under well controlled conditions in a greenhouse experiment. Next, the damage made by soil inhabiting insects' larvae were evaluated using the following classification system: 0—no damage, 1—superficial damage, found only on the epidermal surface of tubers, 2—deep damage, found in deeper tissues (Figure 2). As no severe damages were detected, there was no reason to set up more levels in our classification system. The weight of missing tuber parts at level 2 damages were assessed by the following method: using gelatinized plastic with the same density as that of sweet potato tubers. Each hole was filled with this plastic. After drying (24 h), the plastic was removed and its weight (g) was measured (Figure 2). Yield was also measured at the end of the experiment by measuring every tuber under each plant. Weight results were averaged per compost use, soil cover systems, treatments and blocks. The whole experiment was replicated again in the next year, using the same cover systems, treatments, and methods.

Experimental set-up under greenhouse conditions was conducted in 2019, starting from May, parallel with the second-year field experiments. Soil properties, its chemical and microbial compositions and biological activities, were monitored under standardized and controlled conditions. The same sweet potato variety was obtained and used from the same company. For one experimental plot, there were

210 plants in three treatments (control 70 plants, fungal treatment 70 plants, and α-cypermethrin 70 plants); each divided in two sections (35 plants for each treatment) with (P^+) and without (P^-) *M. melolontha* larvae, all treatments replicated seven times again. Plants first were potted in 30 L plastic containers using 2:1 universal substrate/peat ensuring the same soil pH as under open field conditions. Pots were then organized in rows (Figure 1B, Figures S3 and S4). The whole system was connected to an automatic irrigation system, controlled by Irritrol junior max. Temperature inside the greenhouses were controlled and kept around 35 °C during the vegetation period. Micro and macro elements were added twice, first after potting and later, in mid-July, to each plant using automatized Dosatron® systems. During the course of the whole experiment soil moisture, pH and EC were tested every three days. *M. melolontha* larvae were collected from natural environment (forest soil) from about 100 km from the experimental site and placed into the sweet potato containers when tubers were already developed, on 2 September. Two third-instar larvae were placed into each *M. melolontha*-treated container. The soil insecticide α-cypermethrin and *M. anisopliae* in a same WP formulation were added on 13 September. The insecticide was added in a concentration of 10 mL/10 L to each treated plant. The fungus was applied the same way and in the same concentration as described for the open field experiment. Tuber damage and yield weight were evaluated, as described above, too. The ratio of survived, dead, and infected *M. melolontha* larvae were counted at the end of the experiment by manually searching for larvae from containers after the plants were removed during harvest.

For soil chemical analyses, microbial assay and biological activity measurements from soil samples were collected twice: one month after planting (first week in June) and again, a month later. The same soil samples were divided and used for chemical assay, microbial analyses, and biological activity. From the soil of each treated and control plants (6 plants soil sample/treatments) 100 g soil was put into sterile pots and deposited at −70 °C until analyses. Damages on sweet potato tubers were assessed in the same way as under open field conditions.

2.2. Chemical Composition Assay of Sweet Potato Soil

EDX measurements were used to identify the elemental composition of the soil. Soil samples were dried in a drying cabinet at 80 °C to constant weight. Dried samples were powdered in a mortar using an electric grinder and were stored in airtight boxes. Samples were evaluated in homogenized powder form using a JEOL (Peabody, MA, USA) JSM 5510 LV scanning electron microscopy at various magnifications. The same samples were further analyzed with Scanning Jeol JEM 5510 JV and Oxford Instruments EDS Analysis System Inca 300 (UK) to determine the elemental composition of samples (*Wt*%). Values are the means of five measurements from each soil samples and replicates [29,30].

2.3. 16S rRNA Gene Amplicon Sequencing of Soil Bacterial Community and Biological Activity Assay

The soil bacterial community analysis was performed based on amplicon sequencing of the 16S rRNA gene as in our previous work [31]. Briefly, total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen), a part of the 16S rRNA gene was amplified with primers containing the Bacteria-specific sequences Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′; [32]) and Bakt_805NR (5′-GACTACNVGGGTATCTAATCC-3′; [33]), and DNA sequencing was performed on an Illumina MiSeq platform using MiSeq standard v2 chemistry as a service provided by the Genomics Core Facility RTSF, Michigan State University (East Lansing, MI, USA). There, Illumine-compatible, dual indexed adapters were added by PCR with primers targeting the CS1 and CS2 sites. PCR products were then batch normalized using SequalPrep DNA Normalization plates and all product recovered from the normalization plate was pooled. Subsequently, a clean-up of this pool was performed with Agencourt AMPure XP magnetic beads. Quality control and quantification was carried out using a combination of Qubit dsDNA HS (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Fragment Analyzer High Sensitivity DNA (Advanced Analytical) and Kapa Illumina Library Quantification qPCR (Kapa Biosystems, Wilmington, MA, USA) assays. The pool was then loaded onto a standard MiSeq v2 flow cell Illumina. Sequencing was performed in a 2 × 250 bp paired end format using a v2, 500 cycle

MiSeq reagent cartridge. Custom sequencing and index primers complementary to the CS1/CS2 oligomers were added to appropriate wells of the reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1. Raw sequence data were submitted to NCBI under BioProject ID PRJNA632727.

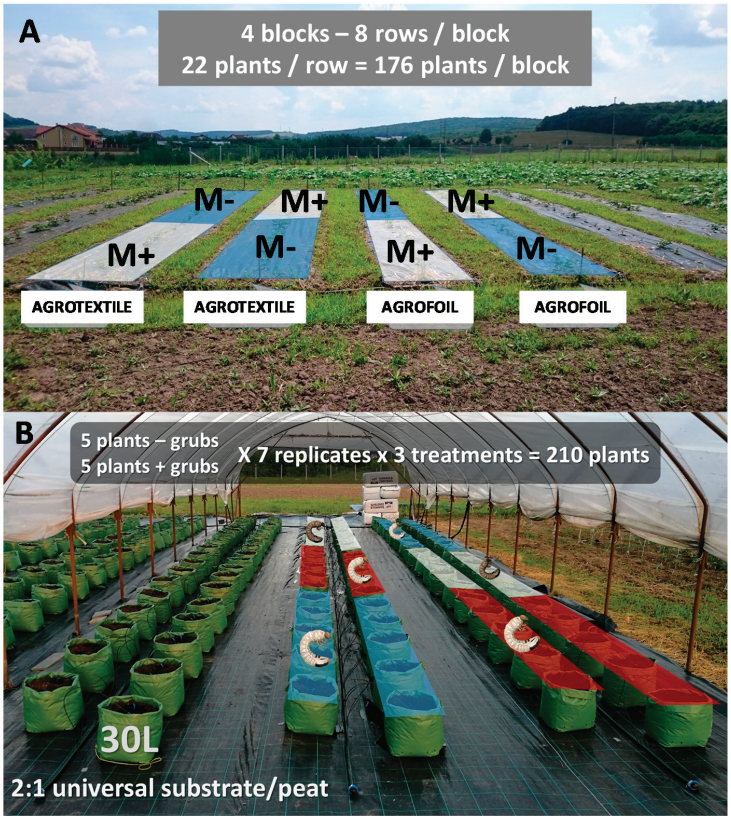

Figure 1. (**A**,**B**). Field (**A**) and greenhouse (**B**) experiment. Field experiment was replicated 4 times, having eight replicates for each cover. Greenhouse experiment was replicated seven times. Abbreviations: *M. anisopliae* present (M^+) or absent (M^-). Blue represents control, red represents insecticide, white represents fungal treatment.

For biological activity assays homogenized soil samples, sieved through a 1.6 mm sieve to remove stones and plant debris were used. For the FDA hydrolysis, 1 g of soil was measured, placed in a 500-mL conical flask, 50 mL of 100 mM potassium phosphate buffer (pH 7.6) and 0.l5 mL 12.01 µM FDA was added to start the reaction. Blank was prepared without the FDA substrate along with a control probe without soil sample. Time was monitored, and the hydrolysis took place at 37 °C for 1 h, hand-stirring every 5 min. After the hydrolysis, 2 mL of acetone were added to each probe to stop the reaction. Then the probes were centrifuged on 4000 rpm for 10 min and sieved through Whatman nr. 1 filter papers. Fluorescein concentrations were determined with spectrophotometer (PG Instruments T60 UV/VIS Spectrophotometer) on 490 nm. The obtained absorbance values were placed in the equation of calibration graph obtained by 0.03–10 µg/mL fluorescein standards, from where we obtained the

FDA enzyme activities of the soil probes in μg/g soil/h. Determination was replicated three times for each sample and treatment.

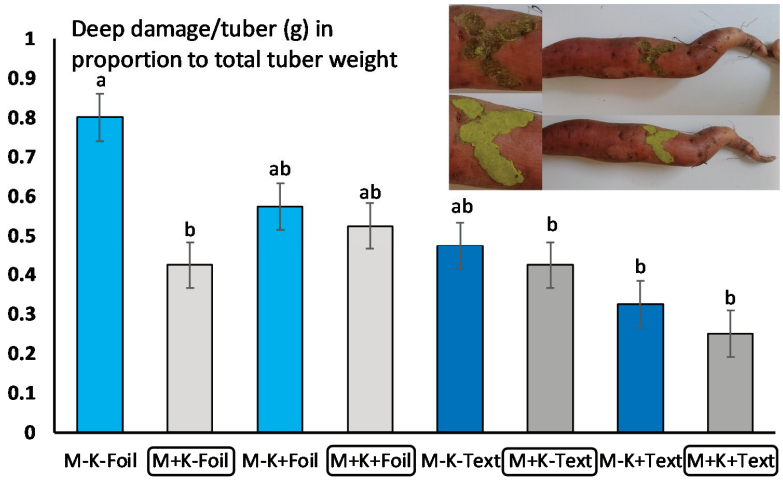

Figure 2. Sweet potato deep damage (defined in gram/tuber) on tubers with compost and fungal treatment and different soil cover systems. Analysis of variance (ANOVA) was used, followed by Tukey's HSD test to compare the effect of *M. anisopliae* on tuber damages using average damage/tuber/plant/compost application/soil cover/block (n = 22). Grey bars represent *M. anisopliae*, blue bars represent control (no fungus). Bars represent standard errors. Upper figure presents damage assessment using gelatinized plastic. Different letters (a, b) means statistical significant differences.

2.4. Data Analyses

Sweet potato damage data from the field experiment were first tested for the normality of errors and homogeneity of variances. Because data were normally distributed, analysis of variance (ANOVA) was used, followed by Tukey's HSD (Honestly Significant Difference) test to compare the effect of *M. anisopliae* on tuber damages (deep damages only) using average damage/tuber/plant/compost application/soil cover/block/treatment (n = 11). Data were first compared between years, then block and side effects were tested using multivariate ANOVA; MANOVA). Because no significant differences were detected between years, and no blocks and side effects detected, pooled and averaged data between years were used for further analyses. Next, crop yield (average tuber weight/plant/compost application/soil cover/fungal treatment/block (n = 11) were compared between control and fungal treatment using the same method (data were normally distributed).

Data from greenhouse experiment were again tested for the normality of errors and homogeneity of variances. Here, only the crop weight data was normally distributed, therefore analysis of variance (ANOVA) was used, followed by Tukey's HSD test to compare the effect of treatments (fungal, insecticidal treatment and control) using average tuber weight (g)/plant/treatment/block (n = 35). Tuber damage data and *M. melolontha* larval survival and infection data did not meet the assumption of normality, therefore the nonparametric Kruskal-Wallis test was used, followed by a Mann-Whitney U test to compare damages (averaged on plants/treatments/blocks (n = 35)) and average survived and dead larvae (average number/plant/treatment/block (n = 35)). All analyses were made using R version 3.0.1 [34] and values below $p \leq 0.01$ were considered as statistically significantly different.

Chemical composition values of the soil were compared between collection dates and between treatments using analysis of variance (ANOVA), followed by Tukey's HSD test (data of five measurements/treatments and control).

Statistical analyses of soil bacterial communities were described in Benedek et al. [31], the differences were that the resulting sequence reads were processed using the mothur v1.41 software ([35]; based on the MiSeq standard operating procedure, downloaded on 03/04/2020) and the removal of chimeric sequences was performed using VSEARCH [36]. OTUs (operational taxonomic units) were defined at a 97% nucleotide sequence similarity level. For the statistical analysis of amplicon sequencing data, the subsampling of reads was performed to the read number of the smallest dataset (n = 19,791). Microbial α diversity (estimated using the Shannon-Wiener and Inverse Simpsons's (1/D) diversity indices) and species richness values (using the Chao1 and the ACE richness metrics) were calculated using mother v1.38.1. Linear regression was used to assess the variation in total bacterial diversity indices (Shannon and Simpson) under different treatments and control, R^2 values computed using PAST. Variation in bacterial community composition was also compared between genera for each treatment and control with ANOVA followed by Welch F test using mean percentages of DNA from total samples.

Data of soil biological activity was again normally distributed, thus, analysis of variance (ANOVA) was used, followed by Welch F test to compare the biological activity under different treatments and control using average data/plant/block (n = 6). Analyses were made using R version 3.0.1 [34].

3. Results

3.1. Field Experiment

The presence and development of *M. anisopliae* was detected both under compost and soil cover management systems. While no differences in crop weight (an average of 1600 gr/plant) were detected between treatments (Table 1), there were differences in tuber damage, and significantly higher damage (only at level 2—deep damage) was detected when the crop was covered with agro-foil, and not treated with compost and fungus. These damage figures, however, were not different from those obtained by agro-foil and compost cover, with or without fungal treatment, and textile cover without compost and *M. anisopliae* (Figure 2, Table 2). Altogether, a tendency of lower damage of Melolontha larvae was detected when sweet potato was treated with *M. anisopliae* strain NCAIM 362 and covered by agro-textile (Figure 2, Table 2).

Table 1. Statistical analyses of tuber weight in the open field experiment (F values below and p values above line), data were compared using average tuber weight/plant/compost application/soil cover/block (n = 22).

Treatments	M-K-Foil	M + K-Foil	M-K + Foil	M + K + Foil	M-K-Text	M + K-Text	M-K + Text	M + K + Text
M-K-Foil	-	0.984	0.992	0.999	1	1	1	0.995
M + K-Foil	1.27	-	1	0.999	0.999	0.963	0.906	1
M-K + Foil	1.132	0.137	-	0.999	0.999	0.978	0.936	1
M + K + Foil	0.538	0.731	0.593	-	1	0.999	0.994	1
M-K-Text	0.513	0.756	0.618	0.024	-	0.999	0.995	0.999
M + K-Text	0.221	1.491	1.354	0.76	0.735	-	1	0.984
M-K + Text	0.520	1.790	1.653	1.059	1.034	0.298	-	0.950
M + K + Text	1.058	0.212	0.074	0.519	0.544	1.279	1.578	-

Table 2. Statistical analyses of tuber damages in the open field experiment (*F* values below and *p* values above line), the effect of *M. anisopliae* on deep tuber damages (level 2) were compared using average damage/tuber/plant/compost application/soil cover/block (n = 22). Bold numbers represent statistically significant *p* values.

Treatments	M-K-Foil	M + K-Foil	M-K + Foil	M + K + Foil	M-K-Text	M + K-Text	M-K + Text	M + K + Text
M-K-Foil	-	**0.013**	0.153	0.113	0.060	**0.010**	**0.004**	**0.0008**
M + K-Foil	4.453	-	0.483	0.422	0.593	0.896	0.661	0.333
M-K + Foil	0.134	0.101	-	0.986	0.815	0.420	0.248	0.099
M + K + Foil	0.121	0.211	0.431	-	0.774	0.359	0.190	0.063
M-K-Text	0.145	0.322	0.561	0.981	-	0.511	0.302	0.113
M + K-Text	5.061	0.111	0.431	0.789	0.891	-	0.778	0.426
M-K + Text	6.275	0.321	0.671	0.671	0.791	0.991	-	0.580
M + K + Text	8.334	0.451	0.451	0.961	0.954	0.781	0.871	-

3.2. Greenhouse Experiment

Again, the presence and development of *M. anisopliae* was detected in all containers M^+. While no differences in crop weight (an average of 1700 gr/plants) were observed (Table 3, Figure 3A), variations in *M. melolontha* larvae survival and damage were detected between treatments (Figure 3B,C). Significantly lower numbers of survived larvae were detected in plots treated with α-cypermethrin (CipermP + ControlP$^+$ $U = 3.2$, $p < 0.01$; CipermP + MetarhP$^+$ $U = 3.0$, $p < 0.01$) and no differences were detected between *Metarhizium* treatment and control (MetarhP + ControlP$^+$ $U = 0.67$, $p < 0.23$), where generally half of the larvae died before the end of the experiment. The numbers of dead larvae were higher in plots treated with α-cypermethrin (CipermP + ControlP$^+$ $U = 4.1$, $p < 0.01$; CipermP + MetarhP$^+$ $U = 3.9$, $p < 0.01$) and again no differences were detected between *Metarhizium* treatment and control (MetarhP + ControlP$^+$ $U = 0.88$, $p < 0.56$) (Figure 3B). Signs of fungal infection among larvae were hardly observed at all (an average of one infected larva was found in 10 *Metarhizium* treated pots) at the end of the experiment in M^+ treatments, and this did not make statistical analysis possible (Figure 3B). The damage rate of tubers also varied between treatments. Significantly lower damage rates were detected in pots with α-cypermethrin (CipermP + ControlP$^+$ $U = 5.2$, $p < 0.01$; CipermP + MetarhP$^+$ $U = 3.9$, $p < 0.01$), while no differences between *Metarhizium* treatment and control were observed (MetarhP + ControlP$^+$ $U = 0.66$, $p < 0.45$) (Figure 3C).

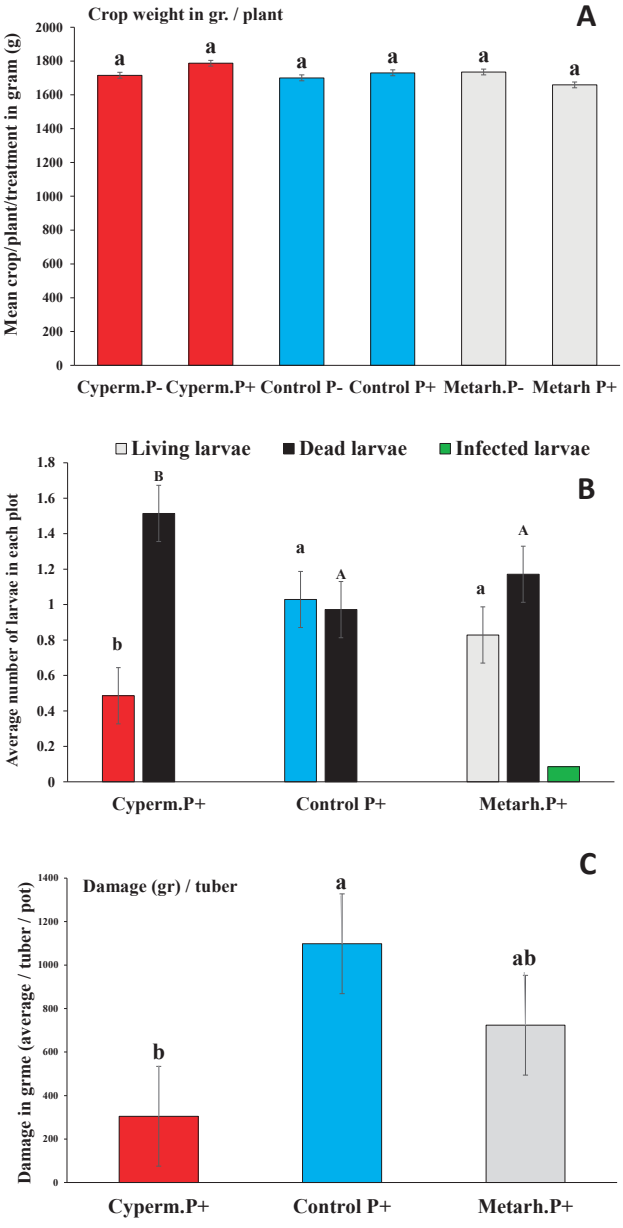

Figure 3. (**A**–**C**) Crop weight (g) with α-cypermethrin (red bars), control (blue bars), and fungal treatment (grey bars) (**A**); average number of alive, dead, and infected *Melolontha* larvae (**B**); and damages under different treatments and control (**C**). Crop weight data was analyzed using ANOVA, followed by Tukey's HSD test using average tuber weight (g)/plant/treatment/block (n = 35). For bulb damage and *M. melolontha* larval survival data Kruskal-Wallis test was used, followed by a Mann-Whitney using average values for plants/treatments/blocks (n = 35)) and average values of alive, dead, and infected larvae (average number/plant/treatment/block (n = 35)). Bars represent standard errors. Different letters (a,b) means statistical significant differences.

Table 3. Statistical analyses of tuber weight in the greenhouse. * Significance: same letters indicate no significant differences (Tukey HSD test).

Treatments	Tuber Weight/Plant (g)	*
Cypermethrin P^-	1716.19	a
Cypermethrin P^+	1787.46	a
Control P^-	1700.85	a
Control P^+	1730.68	a
Metarhizium P^-	1735.36	a
Metarhizium P^+	1659.32	a

3.3. Chemical Composition Assay of Sweet Potato Soil

Representative elemental composition of sweet potato soil results was averaged out from five measurement each. Since a small amount of sample was used, results below 0.5 are considered as qualitative information, given that these elements only appear in trace amount. No differences in the chemical composition of sweet potato soil were detected between treatments (Table 4).

Table 4. Representative elemental composition of sweet potato soil. Results were obtained by calculating the average of five measurements. * Significance: same letters indicate no significant differences (Tukey HSD test).

Elements	ControlP$^+$	ControlP$^-$	Metarh.P$^+$	Metarh.P$^-$	Cyperm.P$^+$	Cyperm.P$^-$	*
C	47.379	41.403	47.043	44.720	51.307	47.773	a
Na	0.122	0.108	0.109	0.079	0.191	0.111	a
Mg	0.328	0.334	0.330	0.414	0.354	0.265	a
Al	1.715	1.668	1.606	2.133	1.261	1.501	a
Si	4.648	7.114	4.184	5.816	3.131	6.456	a
P	0.131	0.220	0.132	0.158	0.133	0.104	a
S	0.251	0.150	0.210	0.348	0.220	0.148	a
Cl	0.022	0.010	0.016	0.011	0.019	0.019	a
K	0.623	0.395	0.364	0.540	0.587	0.242	a
Ca	3.255	4.570	3.706	2.198	1.968	1.908	a
Ti	0.087	0.100	0.077	0.140	0.070	0.048	a
Mn	0.040	0.068	0.041	0.072	0.010	0.020	a
Fe	1.362	2.337	1.122	1.294	0.876	0.722	a
Cu	0.216	0.202	0.253	0.348	0.246	0.310	a
Zn	0.186	0.152	0.222	0.272	0.188	0.253	a
Mo	0.038	0.029	0.030	0.098	0.092	0.086	a

3.4. Microbial Community and Biological Activity in Sweet Potato Soil

A total of 697,221 high-quality bacterial 16S rRNA gene sequences were obtained from the samples (38,734 ± 9 336 reads per sample). Good's coverage values were higher than 0.94 in all cases, whic indicated that sequencing depth was sufficient to recover all major bacterial taxa (Figures S5–S7, and Table S1). The average length of sequences was ~450 nt, which allowed genus-level taxon identification. No significant differences between bacterial community were detected when the soil was treated with insecticide or the fungus, and control with and without *M. melolontha* larvae (Welch F test $F = 0.0006$, $df = 22.37$, $p < 0.9$) (Figure 4). Also, no significant differences in soil biological

activity were detected when treatments and control were compared Welch F test $F = 0.03$, $df = 6$, $p < 0.76$ (Figure 5).

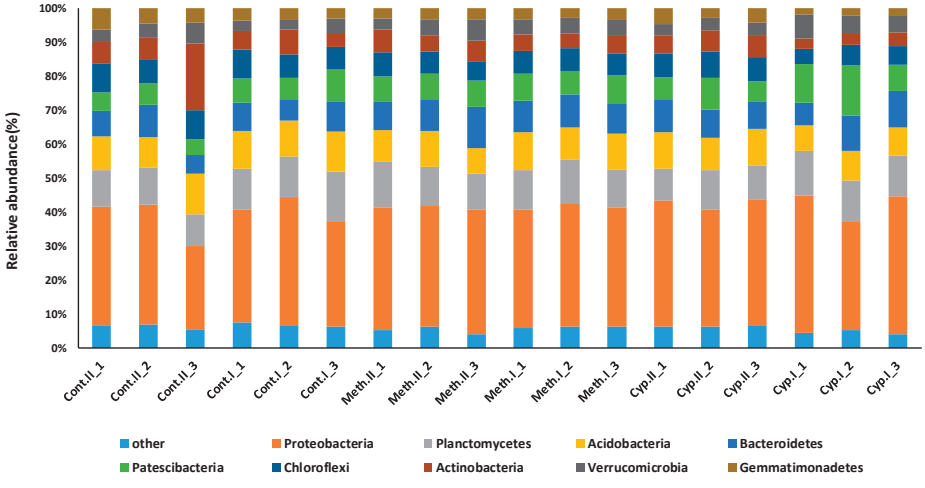

Figure 4. Soil bacterial community analysis performed on amplicon sequencing of the 16S rRNA gene. Total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen), a part of the 16S rRNA gene was amplified with primers containing the Bacteria-specific sequences Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′; Herlemann et al., 2011) and Bakt_805NR (5′-GACTACNVGGGTATCTAATCC-3′). DNA sequencing was performed on an Illumina MiSeq platform using MiSeq standard v2 chemistry as a service provided by the Genomics Core Facility RTSF, Michigan State University, USA. Cont.II represents control P^-, Cont.I represents control P^+, Meth.II represents Metarhizium P^-, Meth.I represents Metarhizium P+, Cyp.II represents insecticide P^- treatments, Cyp.I represents insecticide P+. Only data of 3 sample/treatment are presented.

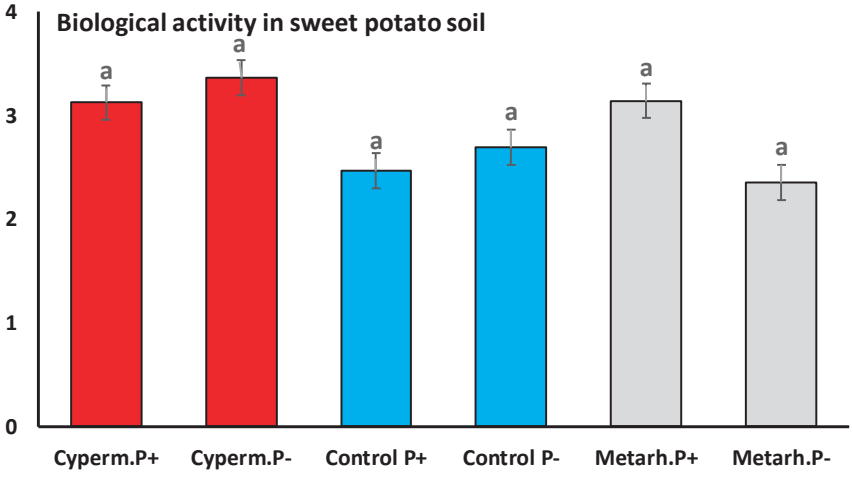

Figure 5. Soil biological activity with insecticide, *M. anisopliae* treatment, and control. The concentration of fluorescein was determined with spectrophotometer (*PG Instruments T60 UV/VIS Spectrophotometer*) on 490 nm. The FDA enzyme activities of the soil probes performed in µg/g soil/h. Same letters indicate no significant differences (Tukey HSD test).

4. Discussion

The number of studies investigating the effect of *M. anisopliae* on *M. melolontha* larvae is low, although Horaczek and Vierstein (2004) mention that *Beauveria bassiana* and *M. anisopliae* have significance in controlling soil-inhabiting pests of various genera, including *Melolontha* [37]. A still earlier study reported that while a single application of *M. anisopliae* before planting was found to have promising results against *D. balteata*, the effects on *Melolontha* larvae, however, was not significant [24]. Our study showed that under open field conditions, with and without compost and different soil cover (agro-foil or textile), damage by *Melolontha* larvae was lower when sweet potato was treated with *M. anisopliae* strain NCAIM 362 in WP formulation and covered by textile (Figure 2, Table 2). A similar effect was detected by other authors on the larvae of *Polyphylla fullo* (Coleoptera: Scarabaeidae) when the highest mortality rates of young and older larvae caused by a *M. anisopliae* product were 74.1 and 67.6% for the granular formulation [38]. By comparing the effect of *M. anisopliae* with soil insecticide α-cypermethrin, a significantly lower number of survived larvae were detected with α-cypermethrin, and no differences were detected between *Metarhizium* treatment and control (Figure 3B). Even when *Metarhizium* concentrations were tripled (1400 g *M. anisopliae* to 12.6 l. of water) compared to the commercially suggested dosage, only 50% of the larvae died on average by the end of the experiment in M^+ treatments. Also, the number of dead larvae were higher with α-cypermethrin, and again no differences were detected between *Metarhizium* and control (Figure 3B). Altogether, this suggests that *Metarhizium* strain NCAIM 362 in WP formulation (recommended and commercialized against coleopteran larvae) is less effective than α-cypermethrin against *Melolontha* larvae, making its future application in sweet potato control uncertain. This can also be supported by the fact that only a low amount of *Melolontha* larvae were observed to have signs of fungal infection (an average of one infected larvae in 10 *Metarhizium* treated pots) at the end of the experiment in M^+ treated pots (Figure 3B). The damage rate of sweet potato tubers had a strong connection with larval survived rate. Significantly lower damages were observed with α-cypermethrin, and no differences were detected between *Metarhizium* and control detected. Recent studies also reported the effect of *Beauveria brongniartii* (three isolates) and *M. anisopliae* (three isolates) on *M. melolontha, Amphimallon solstitiale,* and *Anoxia villosa* under laboratory conditions. The highest mortality rates were caused by *B. brongniartii* isolates (100%) on *M. melolontha* larvae, and 60% mortality on *A. villosa*. In comparison, *A. villosa* was found the most susceptible to *M. anisopliae* (35.5% mortality rate), and the fungus had little to no significant effect on *A. solstitiale* or on *M. melolontha* [39]. In our experiment, no differences were detected in the chemical composition of the soil, in the microbial community, and biological activity of the soil either between treatments and control. The fungal effects on *Melolontha* larvae were completed under very similar conditions, yet no effects on larval mortality and thereby on tuber damage were detected, suggesting that *M. anisopliae* strain NCAIM 362 cannot effectively control *M. melolontha* in sweet potato. This can be explained in different ways. The apparent resistance of *Melolontha* larvae against *M. anisopliae* is hard to be explained without further research. One possible reason can be the fact that a long evolutionary interrelation exists between this soil inhabiting larva and the fungus, meaning that a genetic resistance may have evolved. The effects of formulation may also result in different characteristics of the fungus including conidial growth, viability, potential to cause mortality to target organisms, persistence, and resistance to certain environmental factors. One may also notice that while the impact of formulation on fungal performance was intensively researched in the 1990s, the number of research pieces conducted in this area has been lower ever since. One inevitable challenge formulation faces when trying to enhance the efficiency of the fungal entomopathogen is the presence of ultra-violet light among unprotected field conditions that has a significant negative effect on *M. flavoviride* germination [40]. The frequency of bacterial genera were similar for both treatment and control. Most bacterial taxons identified as dominant (Proteobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, Patescibacteria, Chloroflexi), are known to have a significant role in litter biodegradation and mineralization processes [31]. While no high variation in bacterial community were detected between control and treatment, means that no soil inhabiting microorganisms with inhibitory effect on

M. flavoviride were detected. These further demonstrate that, *M. flavoviride's* effects were tested under ideal conditions.

These environmental effects on *M. anisopliae* need further, more detailed tests. When looking for a successful pest control species, other species of the Metarhizium genus may have more potential against Melolontha larvae. In 2015, a genetic characterization studies performed on fungal isolates obtained from fungus-infected larvae of the Coleopteran *Amphimallon solstitiale* collected from roots of various plants in north eastern Turkey revealed that the hosts were infected by *M. flavoviride* [41]. Finally, a series of experiments, including this present one indicate that the effect of *M. anisopliae* on *Melolontha* larvae is non-significant, therefore the effect of other *Metarhizium* species (e.g., *M. flavoviride*) as an effective control agent in sustainable management needs to be investigated.

5. Conclusions

According to the results, the effect of fungal entomopathogen *M. anisopliae* strain NCAIM 362 in WP formulation against *M. melolontha* larvae in sweet potato is not an effective biological control method. Even if the soil parameters are identical, the effect of α-cypermethrin against *Melolontha* larvae is more significant, and less survived larvae and damaged tubers can be detected after the insecticidal treatment. Under open field conditions, some soil management methods such as compost supply and textile cover may enhance the effect of *M. anisopliae*, but further research is needed to test other species of the *Metarhizium* genus to find if they are an effective agent in sweet potato sustainable pest control.

Supplementary Materials: The following are available online at http://www.mdpi.com/2309-608X/6/3/116/s1, Figure S1. The scheme of the field experiment, Figure S2. Plots of the field experiment with or without foil cover, Figure S3. Experimental greenhouse at the beginning of the season, Figure S4. Experimental greenhouse in the middle of the season, Figure S5. Genus-level of the bacterial composition of sweet potato soil, Figure S6. NMDS ordination of soil samples based on the Bray-Curtis distance of the bacterial OTUs, Figure S7. Rarefaction curves of OTUs based on 16S rRNA gene amplicon sequencing data, Table S1. Bacterial species numbers (sobs, ACE and Chao-1) and diversity indices (inverse Simpson and Shannon-Weaver) calculated from 16S rRNA gene amplicon sequencing data. KI—represents control P+, KII—represents control P-, MI – represents Metarhizium P+, MII—represents Metarhizium P-, VI- represents insecticide P+, VII- represents insecticide P- treatment.

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Abbreviations

Compost present	K^+
No compost	K^-
M. anisopliae present	M^+
No *M. anisopliae*	M^-
M. melolontha grubs present	P^+
No *M. melolontha*	P^-

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Target Activity of *Isaria tenuipes* (Hypocreales: Clavicipitaceae) Fungal Strains against Dengue Vector *Aedes aegypti* (Linn.) and Its Non-Target Activity Against Aquatic Predators

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Abstract: The present investigation aimed to determine the fungal toxicity of *Isaria tenuipes* (My-It) against the dengue mosquito vector *Aedes aegypti* L. and its non-target impact against the aquatic predator *Toxorhynchites splendens*. Lethal concentrations (LC_{50} and LC_{90}) of My-It were observed in 2.27 and 2.93 log ppm dosages, respectively. The sub-lethal dosage (My-It-1×10^4 conidia/mL) displayed a significant oviposition deterrence index and also blocked the fecundity rate of dengue mosquitos in a dose-dependent manner. The level of major detoxifying enzymes, such as carboxylesterase (α-and β-) and SOD, significantly declined in both third and fourth instar larvae at the maximum dosage of My-It 1×10^5 conidia/mL. However, the level of glutathione S-transferase (GST) and cytochrome P-450 (CYP450) declined steadily when the sub-lethal dosage was increased and attained maximum reduction in the enzyme level at the dosage of My-It (1×10^5 conidia/mL). Correspondingly, the gut-histology and photomicrography results made evident that My-It (1×10^5 conidia/mL) heavily damaged the internal gut cells and external physiology of the dengue larvae compared to the control. Moreover, the non-target toxicity against the beneficial predator revealed that My-It at the maximum dosage (1×10^{20} conidia/mL) was found to be less toxic with <45% larval toxicity against *Tx. splendens*. Thus, the present toxicological research on *Isaria tenuipes* showed that it is target-specific and a potential agent for managing medically threatening arthropods.

Keywords: mycotoxins; entomopathogen; arthropods; CYP450; gut-histology; non-toxicity

1. Introduction

Parasites have been a major threat for millions of humans and animals since ancient times, bringing about chronic debilitating and disabling diseases [1]. Mosquitoes (Diptera: Culicidae) are vectors for serious parasites and pathogens, including malaria, filariasis, and important arboviruses, such as dengue virus, yellow fever, chikungunya, West Nile virus, and Zika virus [2,3]. Aedes spp.is a vector transmitting the previously mentioned arboviruses, whose dispersion is wide-reaching [3,4]. Almost 40% of the global population live under hazard of dengue, and yearly, 24,000 deaths are reported. The incidence of dengue viruses has grown intensely around the world in the current scenario. The actual numbers of dengue cases are underreported and many cases are misclassified [4]. Dengue is considered to be an endemic disease prominent in more than a hundred nations, including the Americas, Africa, the Western Pacific, and, more importantly, Southeast Asian countries [5]. Managing this disease is mainly achieved through decreasing mosquito populations [6,7]. Overuse and misuse of synthetic insecticides led to the development of resistance, environmental contamination, toxicity to non-target organisms, and adverse effects on animal and human health; accordingly, there is an urgent need to use eco-smart, bio-rational insecticides including cultural and biological ways which could be integrated into mosquito control strategies [8–11].

Such alternative approaches through biological ways have been widely recognized for decreasing the selective pressure made by chemical pesticide-resistance against arthropods [11,12]. Among them, microbial toxins can target different life-cycle stages of mosquitoes, and, more importantly, they are harmless to non-targets [13]. Entomopathogenic fungi (EPF) are an active substitute for synthetic chemicals due to their degradability [14,15]. Presently, the mode of activity of fungal strains has revealed several avenues for effective arthropod management [15–17]. EPF are a promising agent for arthropod management and the operative fungal strain selection is well established according to its virulence against targeted mosquitoes in the applied settings [18].

Isaria tenuipes (formerly *Paecilomyces tenuipes*) is a common fungal species that frequently affects major agricultural pests usually belonging to the group lepidopteran [18], and we refer to it as "My-It". Moreover, it has been found that the *Isaria* fungi hold a diversified blend of chemical derivatives delivered chiefly through non-ribosomal peptide synthetase (NRPS), terpenoid synthetase (TS), polyketide synthase (PKS), etc. [18,19]. All of these active metabolites deliver potential anti-viral, anti-bacterial, and anti-cancer agents [20,21]. There is also other previous research on their biological activity against humans and other beings [18]. Active metabolites derived from *I. tenuipes*, such as cephalosporolides B and F, deliver inhibitory activity against the *Panagrellus redivivus* nematode [22]. Despite such benefits, there is no active research on the biological activity of *I. tenuipes* against mosquito vectors of medical importance. Moreover, their mode of action against mosquito reproduction was also unclear.

Thus, the present investigation aimed (i) to determine the effective lethal larvicidal dosage of active My-It against the dengue vector *Aedes aegypti;* (ii) to detect the sub-lethal dosage activity of My-It on the reproductive potential in dengue vector; (iii) to detect enzyme regulations in the dengue larvae treated with the sub-lethal dosage of My-It; (iv) to determine the non-target impact of My-It against aquatic mosquito predators sharing the same ecological niche as the dengue vector.

2. Methodology

2.1. Mosquito Culture

The *Ae. aegypti* larval culture was maintained at the insect toxicology laboratory in St. Peters Institute of Higher Education and Research, Avadi, Chennai from 2019, without disclosure to any prior chemicals, and it was preserved at 26 ± 2 °C at 75–80% relative humidity (RH) under a photoperiod of 14L: 10D. Brewer's yeast, ChooStix Biskies-branded dog biscuits, and algae collected from pools

in a ratio of 3:2 were fed as a diet to the dengue larvae. The first-generation larvae were used for conducting experiments.

2.2. Isaria Tenuipes

Isolation and maintenance of an *Isaria tenuipes* fungal strain were adapted from our previous research (Vasantha-Srinivasan et al. [15], originally obtained from MTCC (Institute of Microbial Technology (IMT), CSIR, Chandigarh, India). The culture was preserved in a potato dextrose agar (PDA) medium for 14 days at 27 °C. The obtained conidia from the suspension media were prepared using 0.1% Tween 80 diluted using double sterilized distilled water and the conidia were well spun for 20 min to avoid any clumpiness. The number of conidia was counted and we determined their active dosage using fluorescent microscopy (Optika Fluoroscence series B-600TiFL, Italy) at 10× using a Neubauer hemocytometer chamber. Several concentrations were prepared of 1×10^2, 1×10^4, 1×10^6, and 1×10^8 conidia/mL through dilution into double distilled water.

2.3. Larvicidal Bioassay

Larvicidal bioassays were adapted based on the methodology of the World Health Organization [4] with slight modifications. The second, third, and fourth instar larvae were transferred into 250 mL sterile plastic containers covering 25 mL of dosage treatments with different discriminate concentration of My-It (1×10^2, 1×10^4, 1×10^6, and 1×10^8 conidia/mL) with the blend of 24 mL de-chlorinated sterile water, along with 1 mL of mycotoxin dosage, and kept at 27 °C. This procedure was replicated three times and one control was kept for each replication, i.e., 20 larvae were used without any chemicals. The mortality of the larvae was documented 24 h post-treatments. The percentage of mortality was deliberate and mortality corrections, wherever required, were analyzed using Abbott's formula [23]. To determine population growth, water was treated with My-It 1×10^3 and newly emerged larvae were controlled. Each treatment was replicated five times. Percentage of mortality was recorded daily until death.

2.4. Oviposition Deterrence Index

Sub-lethal dosages of My-It (1×10^1, 1×10^2, 1×10^3, and 1×10^4 conidia/mL) were mixed thoroughly with 200 mL of rearing food in 300 mL glass jars to obtain the desired dosage for the experiments. The gravid females (20 nos.) were alienated equally between treated and control containers. Throughout the experiments, the female groups were kept isolated for 48 h in mosquito cages ($25 \times 25 \times 30$ cm). After counting eggs, the oviposition deterrence index (ODI) was calculated using the formula adapted from Hwang et al. [24].

2.5. Fecundity Assay

An identical number of unfed male and female (20 nos. each) dengue mosquitoes were used for fecundity experiments. They were introduced into cages for matting measuring ($30 \times 30 \times 30$ cm) at different sub-lethal dosages of My-It (1×10^1, 1×10^2, 1×10^3 and 1×10^4 conidia/mL). After receiving a blood meal, the eggs were collected daily using the small plastic containers containing water as an ovitrap in the cages. Three number of eggs laid in the ovitraps by the female was calculated.

The mean number of eggs laid in the ovitraps by the female (fecundity) was calculated by the number of the eggs laid in the ovitraps divided by the number of females. Adult death during the procedure was also measured.

2.6. Enzyme Assays

The third and fourth instars of *Ae. aegypti* previously used in the larval bioassays were thoroughly washed with dechlorinated distilled water then rinsed using sterile tissue paper [25]. The prepared enzyme homogenates were prepared based on our previous research [15] and kept on ice for

further enzyme assays. Furthermore, enzyme estimations of carboxylesterase (α and β), SOD, glutathione S-transferase (GST), and CYP450 were analyzed based on the adapted methodology of Thanigaivel et al. [26].

2.7. Gut Histological and External Physiological Assay

The My-It-treated (1×10^5 conidia/mL) and control larvae were fixed overnight in Bouin's solution and then de-hydrated and fixed in blocks using paraffin wax. Microtome (Model: Leica, Germany) larval tissue blocks were fixed on sterile microscopic glass slides and stained using hematoxylin and eosin for examination under a bright field microscope and images were captured under an Optika vision lite microscope (2.0 ML). The captured midgut images of both My-It-treated and control larvae were further compared for toxicological screening.

The photomicrography assay was performed with the previous adapted protocol of Coelho et al. [27] with slight modifications. The My-It-treated and control fourth instar larvae were sequentially stabilized in an ethanol dehydration range from 35–70% for 25 min at 27 °C and fixed on microscopic glass slides. Finally, the sections were observed at 40× magnification under a light microscope (Optika vision lite 2.0 ML).

2.8. Non-Target Toxicity Assay

The non-target toxicity assay of My-It against beneficial aquatic organisms was performed according to our previous procedure [15]. The non-target beneficial organism *Toxorhynchites splendens* Wiedemann (Culicidae: Diptera) was tested with lethal dosage of My-It (1×10^5, 1×10^{10}, 1×10^{15} and 1×10^{20} conidia/mL) with three replications and each replication contained twenty larvae. Dechlorinated sterile water without the addition of My-It was kept as the negative control. For individual assay, ten replications followed. Finally, the mortality rate was recorded 24 h post-treatment.

2.9. Data Analysis

Data from the mortality experiments were analyzed by analysis of variance (ANOVA) of arcsine, logarithmic, and square root transformed percentages, and data were expressed as the means of three replicates. Significant differences between treatment groups were analyzed by Tukey's multiple range test (significance at $p < 0.05$) using the Minitab®17 program. For enzyme activity, Microcal Software (Sigma plot 11) was used. The lethal concentrations required to kill 50% (LC_{50}) of larvae in 24 h were calculated by Probit analysis with a dependability interval of 95% using the Minitab®17 program.

Mosquito longevity was analyzed using a log-rank χ^2 test of equality over strata (PROC LIFE Table) along with Formula (1) (Allison [28], 1995) with Minitab® 17 statistical software package (Minitab, State College, PA, USA).

$$\text{Growth index} = \frac{\text{Percent survival of mosquito}}{\text{Duration of larvae/adult larvae/adult nymph/adult}} \quad (1)$$

3. Results

3.1. Effect of My-It on Mosquito Survival

The larvicidal activity of My-It with its discriminating dosage (1×10^2, 1×10^4, 1×10^6, and 1×10^8 conidia/mL) against the second instar larvae displayed that the maximum mortality rate of 96% was obtained at the maximum dosage of 1×10^8 conidia/mL and it was more significant than that of the other treatments—1×10^6 (86.32%-$F_{4,20} = 16.66$, $p \leq 0.001$), 1×10^4 (63.21%-$F_{4,20} = 16.66$, $p \leq 0.001$), 1×10^2 (35.45%-$F_{4,20} = 16.66$, $p \leq 0.001$) conidia/mL—and the control (5.140%-$F_{4,20} = 16.66$, $p \leq 0.001$) (Figure 1).

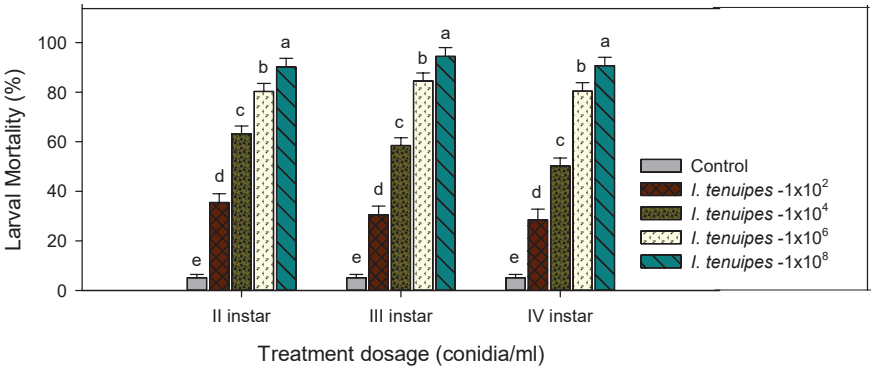

Figure 1. Percentage mortality of second, third and fourth instar larvae of *Ae. aegypti* after treatment with *Isaria tenuipes* conidial spores (My-It). Means (±SE) followed by the same letters above bars indicate no significant difference ($p \leq 0.05$) using Probit analysis. The different letters (a–e) indicate significant differences between the control and treatments.

Similarly, the larval toxicity of My-It against the third instar larvae was also uplifted in a dose dependent manner. At the maximum dosage (1×10^8 conidia/mL), the larval mortality increased significantly to 94.44% ($F_{4,20} = 18.22$, $p \leq 0.001$) when compared to those of the other treatments (further total mosquito survival significantly declined when compared with that of the control) (Figure 2).

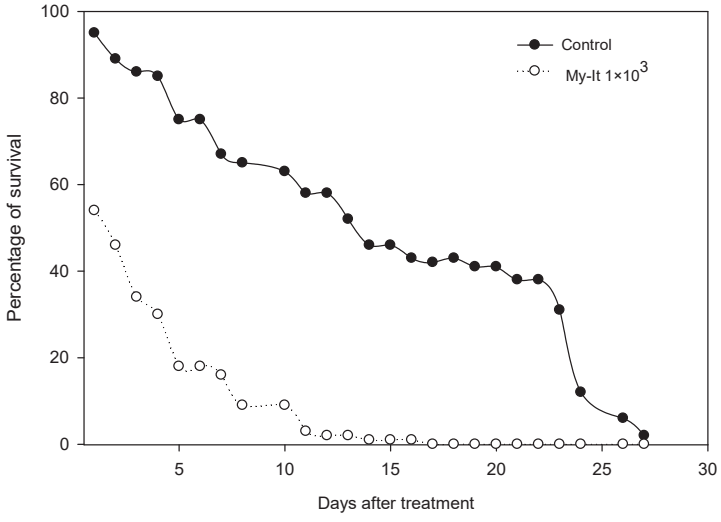

Figure 2. Survival rate of *Ae. aegypti* after treatment with My-It (1×10^3) conidial spores and the control. Survivorship curves differ at the $\alpha = 0.05$ confidence interval according to log-rank statistics.

Likewise, the larvicidal activity of My-It against the fourth instar larvae was increased to 90.67% at the prominent dosage of 1×10^8 conidia/mL and it was significant at 1×10^6 (80.41%-$F_{4,20} = 12.33$, $p \leq 0.001$), 1×10^4 (50.18%-$F_{4,20} = 12.33$, $p \leq 0.001$), 1×10^2 (28.45%-$F_{4,20} = 12.33$, $p \leq 0.001$) conidia/mL and with the control (5.110%-$F_{4,20} = 12.33$, $p \leq 0.001$) (Figure 1). The lethal concentrations (LC_{50} and LC_{90}) of My-It were 2.27 and 2.93 log ppm, respectively (Figure 3).

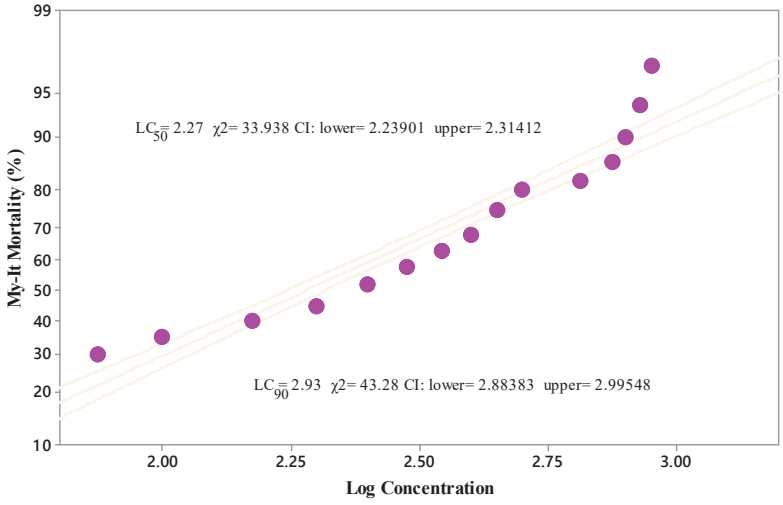

Figure 3. Lethal concentrations (LC_{50} and LC_{90}) of My-It conidial spores against fourth instars of *Ae. Aegypti*, obtained using Probit analysis. Dot represents the lethal concentration of *My-It* conidial spores against fourth instar larvae of *Ae. aegypti*.

3.2. Oviposition Deterrence Index

The sub-lethal dosage of My-It statistically reduced the oviposition deterrence index of the dengue mosquito at 1×10^4 conidia/mL with maximum deterrence index of 83.4% ($F_{4,20} = 25.88$, $p \leq 0.001$) and it is more significant than those of the other treatments—1×10^4 (57.6%-$F_{4,20} = 25.88$, $p \leq 0.001$), 1×10^3 (57.6%-$F_{4,20} = 25.88$, $p \leq 0.001$), 1×10^2 (28.45%-$F_{4,20} = 25.88$, $p \leq 0.001$), 1×10^1 (10.2%-$F_{4,20} = 25.88$, $p \leq 0.001$)—as well as the control (7.50%-$F_{4,20} = 25.88$, $p \leq 0.001$) (Figure 4).

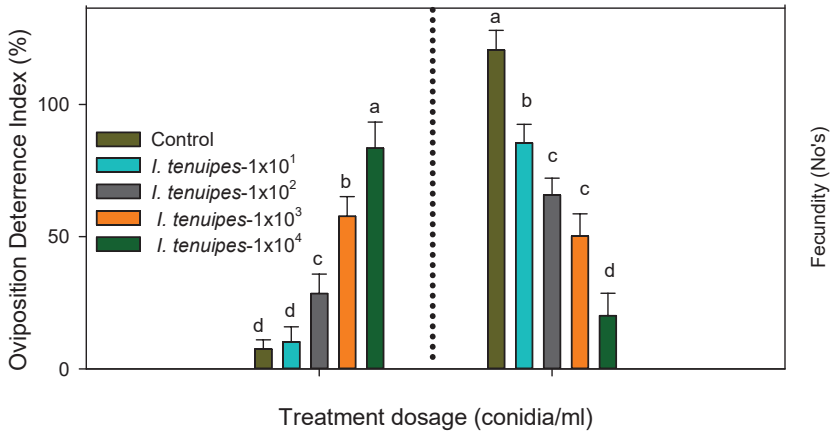

Figure 4. Oviposition deterrent index and fecundity evaluation of My-It against dengue mosquitoes. Means (± SE) followed by the same letters above bars indicated no significant difference ($p \leq 0.05$) using ANOVA analysis. The different letters (a–d) indicate significant differences between the control and treatments.

3.3. Fecundity of My-It

The sub-lethal dosage significantly reduces the mean number of eggs laid by the female dengue mosquito in a dose dependent manner. At the maximum dosage of 1×10^4, My-It showed maximum reduction in fecundity rate (20.1-$F_{4,20}$ = 14.22, $p \leq 0.001$), followed by 1×10^3 (50.11-$F_{4,20}$ = 14.22, $p \leq 0.001$), 1×10^2 (66.1-$F_{4,20}$ = 14.22, $p \leq 0.001$), 1×10^1 (85.45-$F_{4,20}$ = 14.22, $p \leq 0.001$), and the control (120.45-$F_{4,20}$ = 14.22, $p \leq 0.001$) mean number of eggs (Figure 4).

3.4. Enzyme Inhibition of My-It

A sub-lethal dosage of My-It statistically regulates the major enzymes of both third and fourth instars of dengue larvae. The level of α- carboxylesterase was significantly reduced in a concentration dependent manner on both the larval instars. The level of α- carboxylesterase in the third instar reduced at the maximum rate of 0.3451 mg/protein at the maximum dosage of 1×10^5 ($F_{4,20}$ = 18.99, $p \leq 0.001$), and it was not significant with 1×10^4 (0.312 mg/protein-$F_{4,20}$ = 18.99, $p \leq 0.001$) and 1×10^3 (0.351 mg/protein-$F_{4,20}$ = 18.99, $p \leq 0.001$). However, there is no significant difference between 1×10^2 (0.5110 mg/protein-$F_{4,20}$ = 18.99, $p \leq 0.001$) and 1×10^1 (0.6543 mg/protein-$F_{4,20}$ = 18.99, $p \leq 0.001$) (Figure 5A). Similar trends were observed in the α- carboxylesterase level in fourth instars with the maximum reduction rate observed in My-It 1×10^5 (0.4514 mg/protein-$F_{4,20}$ = 20.12, $p \leq 0.001$) which is significant, as is the case with with 1×10^2 and 1×10^1 dosages. However, there was no statistical significance observed with the 1×10^4 and 1×10^3 dosages ($F_{4,20}$ = 20.12, $p \leq 0.001$) (Figure 5A).

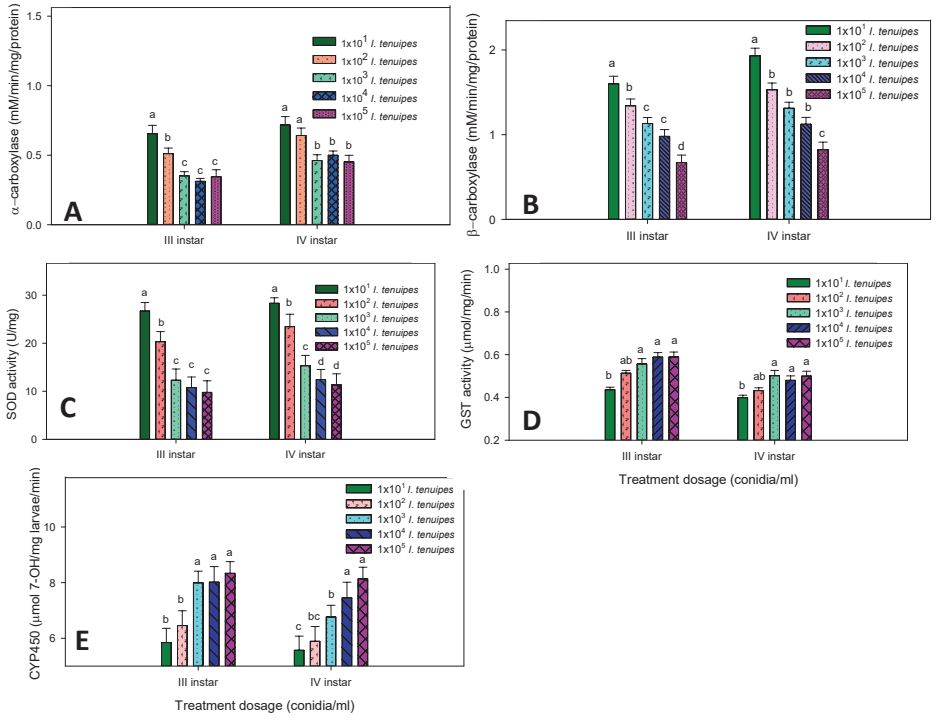

Figure 5. (**A**) α-carboxylestrase; (**B**) β-carboxylestrase; (**C**) SOD; (**D**) GST; (**E**) CYP450 enzyme activity of third and fourth instar larvae of *Ae. aegypti* after treatment with My-It. The data were fitted on a polynomial (regression) model. Letters (a–d) mean (± SE) followed by the same letters above bars indicated no significant difference ($p \leq 0.05$) using ANOVA analysis.

The level of β- carboxylesterase statistically declined at the maximum sub-lethal dosage of My-It of 1×10^5 (0.6700 mg/protein-$F_{4,20} = 18.25$, $p \leq 0.001$) and (0.823 mg/protein-$F_{4,20} = 16.66$, $p \leq 0.001$) in third and fourth instars, respectively (Figure 5B). However, the level of β- carboxylesterase was 1.600 mg/protein and 1.9320 mg/protein in the third and fourth instars, respectively, at the minimal dosage of My-It (1×10^1) (Figure 5B).

Correspondingly, the level of SOD also declined in a concentration dependent manner with the maximum enzyme reduction observed in the My-It dosage of 1×10^5 conidia/mL with 9.76 U/mg ($F_{4,20} = 12.45$, $p \leq 0.001$) and 11.32 U/mg ($F_{4,20} = 17.77$, $p \leq 0.001$) in the third and fourth instars, respectively (Figure 5C). However, the level of SOD increased to 26.70 U/mg and 28.32 U/mg in the third and fourth instars, respectively.

The level of glutathione S-transferase uplifted steadily in both third and fourth instar larvae treated with My-It. The level increased to 0.589 mg/min ($F_{4,20} = 18.27$, $p \leq 0.001$) and 0.4995 mg/min ($F_{4,20} = 12.44$, $p \leq 0.001$) in the third and fourth instar larvae, respectively, treated with My-It 1×10^5 conidia/mL (Figure 5D). However, there is no significant difference between My-It 1×10^5 conidia/mL, My-It 1×10^4 conidia/mL, and My-It 1×10^3 conidia/mL in both of the treated larvae.

The enzyme activity of CYP450 increased in a dose dependent manner with the maximum enzyme rate in My-It 1×10^5 conidia/mL in third (8.3341 µmol 7-OH/mg larvae/min-$F_{4,20} = 25.22$, $p \leq 0.001$) and fourth (8.1320 µmol 7-OH/mg larvae/min-$F_{4,20} = 18.88$, $p \leq 0.001$) instar larvae, respectively (Figure 5E). In all the treatments, third instar larvae were slightly sensitive in the enzyme regulations of the sub-lethal dosage of My-It (Figure 5).

3.5. The Efficacy of of My-It on Gut-Histology

Treatment with a sublethal dosage of My-It significantly induced adverse effects on the gut though uniformity in the epithelial layer (Epi), gut lumen (Lu), and peritrophic membrane (pM) were detected in the control larva (Figure 6A), whereas the cellular organelles were severely affected and cranked in the treatment with My-It (1×10^5 conidia/mL) (Figure 6B).

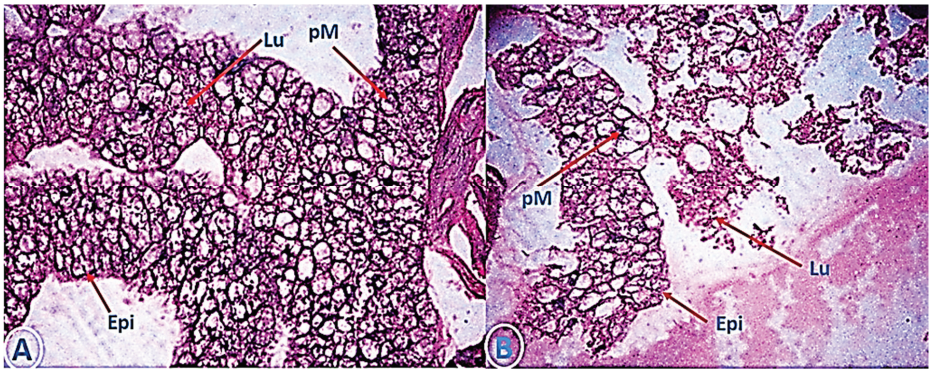

Figure 6. Cross-section through mid-gut of fourth instar *Ae. aegypti* treated with My-It. (**A**), control, compared with (**B**), treated. (Epi) vacuolated gut epithelium; (Lu) gut lumen; (pM) peritrophic membrane.

3.6. The Efficacy of My-It on the External Physiology of Ae. aegypti Larvae

The external physiological analysis of the fourth instar larvae showed that the sub-lethal dosage of My-It (1×10^5 conidia/mL) drastically affected the gut lumen (GL), segments (S), epithelial layer (EL), and anal segments (AS) (Figure 7B), whereas in the control larvae, the gut cells, including EL, AS, and GL, appeared to be normal (Figure 7A).

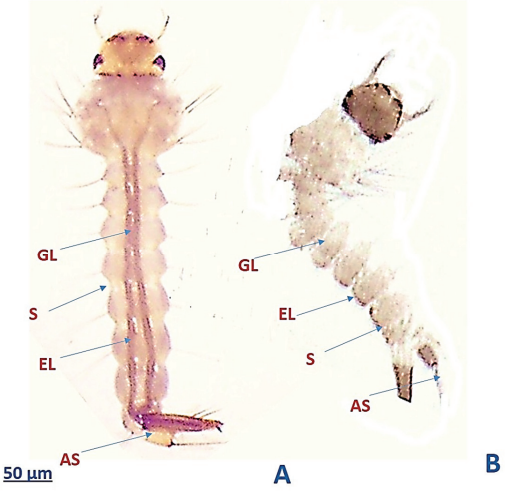

Figure 7. Photo-micrographic analysis of fourth instar larvae of *Ae. aegypti* (**A**) control larvae and (**B**) My-It-treated larvae. (GL)—gut lumen; (S)—segments; (AS)—anal segments; (EL)—epithelial layer.

3.7. Non-Target Toxicity of My-It

The non-target toxicity of the aquatic predator *Tx. Splendens*-discriminating dosage of My-It 1×10^{20} (3 to 4 fold higher dosages used in larvicidal assay) displayed a lower mortality rate (45.43%-$F_{4,20} = 25.66$, $p \leq 0.001$), followed by My-It 1×10^{15} (32.14%-$F_{4,20} = 25.66$, $p \leq 0.001$), My-It 1×10^{10} (25.40%-$F_{4,20} = 25.66$, $p \leq 0.001$), My-It 1×10^{5} (14.43%-$F_{4,20} = 25.66$, $p \leq 0.001$), and the control (3.20%-$F_{4,20} = 25.66$, $p \leq 0.001$). There is significant difference between the My-It treatments and the control (Figure 8).

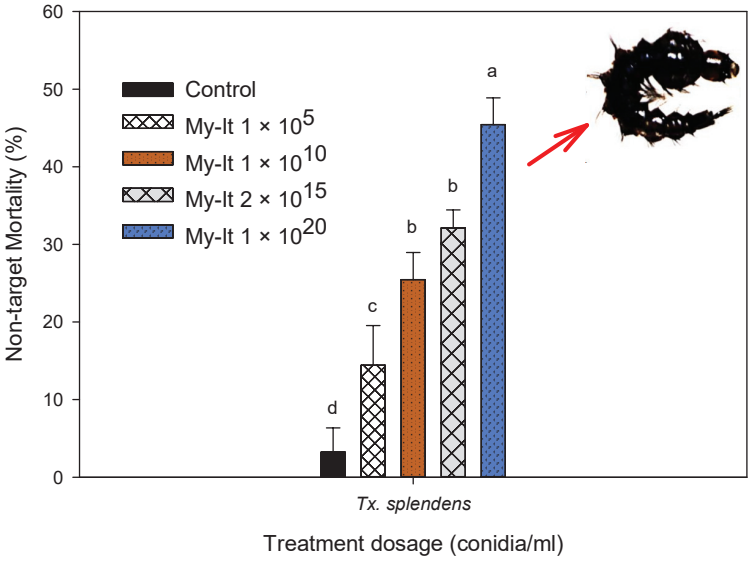

Figure 8. Impact of My-It on the non-target organism *Tx. splendens*. Letters (a–d) mean (± SE) followed by the same letters above bars indicate no significant difference ($p \leq 0.05$) using Probit analysis.

4. Discussion

Determining the mosquito resistance pattern against different groups of synthetic chemicals displays a significant role in managing arthropod vectors [29]. Due to up-surging resistance observed in synthetic chemicals, there is an urgent need for novel substitutes to managing blood-sucking pests [30,31]. Novel discovery of natural materials with diversified blends of active ingredients with potential insecticidal properties may provide a suitable remedy for synthetic chemical resistance [32–34]. Among different biological insecticides, fungal strains have unique modes of action by penetrating the cuticle and blocking the development of pests [35]. Fungal strains generate a vast range of chemicals with an extensive band of actions against insect pests [36]. Amid fungi, the genus Isaria is an important fungal strain composing >100 different species playing a significant role in conserving biodiversity and that are widely used in agriculture and medical treatment [18,37,38]. *I. tenuipes* is most common species of "*Isaria*" with a wide range of insecticidal activity, especially against agriculture pests belonging to the lepidopteran group [39].

The present study revealed that larval toxicity of My-It displayed a significant mortality rate at the maximum dosage of 1×10^8 conidia/mL, with a larval mortality rate of more than 94% recorded in all the treated larval instars. Similar to our results, mycotoxins derived from *Aspergillus flavus* also displayed prominent mortality rates of more than 90% at the maximum lethal dosage of 2×10^8 conidia/mL [15]. Generally, fungal strains enter the body of a mosquito and create a way to the hemocoel and deliver humoral and cellular immune defensive mechanism straddling by the host of the mosquito species as it tries to overawe the mycotoxin infections [40,41]. Similar to the above statements, My-It delivers acute toxicity to the different instars of dengue mosquito vector.

In general, oviposition represents the vital position in the life cycle of any arthropods, as oviposition inhibition directly signifies the reduction rate in the growth and development of pest populations [42]. Similar to the larvicidal activity, the sub-lethal dosage of My-It significantly affects the reproduction stage of dengue mosquito in dose dependent manner. The sub-lethal dosage of My-It (1×10^4) delivered significant ODI percentage as compared to the control; likewise, fungal strains can significantly inhibit the oviposition of other agriculture pests [36]. Previously, the phyto-pathogenic fungal strains derived from *Botrytis cinerea* blocked the oviposition of a major European insect pest (grapevine moth), *Lobesia botrana* [43]. Likewise, the mean number of eggs laid by the gravid female mosquito (fecundity rate) was also reduced considerably due to the maximum sub-lethal dosage of My-It (1×10^4). The volatile and non-volatile metabolites of fungal strains play a significant role in blocking the fecundity rate of arthropods, especially blood-sucking pests [44]. Similar to the above statement, the major allelochemicals in My-It might cause the blockage of the egg-laying capability of female dengue vectors. Similar to our report, a previous in vitro assay revealed that fungal toxins marginally declined the fecundity rate [45]. Similarly, a previous review by Ondiaka et al. [46] stated that the entomo-toxin derived from *Metarhizium anisoplia* displayed a significant fecundity rate against different insect pests.

Generally, insect resistance against any chemical toxins can primarily be accessed through investigating the level of key biomarker enzymes, including detoxifying and digestive enzymes, such as carboxylesterase, SOD, glutathione S-transferase, and, more importantly, the chief detoxifying enzyme cytochrome P-450 [47]. In the present investigation, the sub-lethal dosage of My-It (1×10^5 conidia/mL) heavily reduced carboxylesterase (both α and β) enzyme regulation ratios in a dose dependent manner. In support of our findings, the sub-lethal dosage of *A. flavus* heavily inhibited the level of both α-β-carboxylesterase and SOD activity [15]. Generally, upregulation of esterase activity will deliver substantial insect resistance against the specific chemicals tested. Resistance developed in esterase-related protein delivered regulatory alterations in the structural genes by modifying the loci of specific genes in insects and also amplified the DNA methylation genes in insect pests [48]. Likewise, Hemingway and Ranson [49] reported that the enzyme families esterase, CYP450s, GST, and SOD are the major four enzymes responsible for pest resistance against the chemical toxins. Backing the above statement, the mycotoxins derived from *I. tenuipes* delivered significant reduction in

the carboxylesterase and SOD activity and also delivered a substantial increase in the rate of GST and CYP450 levels.

The gut-histology and physiological alterations results clearly evident that My-It heavily damaged the internal gut cells and external physiology of dengue larvae compared to the control. Similarly, the sub-lethal dosage of *A. flavus* considerably injured the gut epithelial and lumen cells of *Ae. aegypti* larvae [15]. Comparably, a previous review by Rudin and Hecker [50] stated that the pM (peritrophic membrane) stimulates parasite growth in mosquitoes by developing barriers. The above statement was well supported by our present study which shows that the sub-lethal dosage of My-It affected the gut cells of dengue larvae.

It is essential to gage the primary and secondary impact of any forms of pesticides upon non-target species [26,31,51]. *Toxorhynchites* are an excellent predator against the dengue larvae '*Aedes*' and are measured to be not hurtful to their well-beings and well-fixed as they are non-blood feeders and considered to be a good biological predator for reducing the populations of blood-sucking mosquitoes [52]. Since they share the same ecological regions as dengue larvae, it is essential to investigate the non-toxicity screening of same chemicals tested against dengue larvae. The non-target screening of My-It against the giant mosquito (*Tx. splendens*) showed they are less at-risk (maximum 45% mortality rate), even if they are treated with the maximum dosage of My-It (1×10^{20}) which is the highest dosage used in the larvicidal assays. It is evident that biologically-derived pesticides, especially mycotoxins and their related compounds, were target specific and harmless or less toxic to the beneficial species. Thus, the present toxicological investigation of My-It recommends that it is a highly favorable biological agent in managing medically-challenging arthropods, especially the dengue mosquito, and its non-toxic activity against aquatic predators will add on to its biologically safe insecticides. Further investigation on the active allelochemicals of My-It and its specific mode of action against the dengue mosquito vector's biological activity needs to be intensely inspected.

Author Contributions: S.K., P.V.-S., S.S.-N. and P.K. designed the research plan and drafted the manuscript. S.K., P.V.-S., S.S.-N., V.R. and P.K. performed the experimental works and data compilation. R.G., V.R., H.F.K., N.R., K.A., T.-J.K., M.A.E.-S., P.K. and S.K. coordinated the work and discussed the results. S.K., S.S.-N. and P.K. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Interplay between Fungal Infection and Bacterial Associates in the Wax Moth *Galleria mellonella* under Different Temperature Conditions

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Abstract: Various insect bacterial associates are involved in pathogeneses caused by entomopathogenic fungi. The outcome of infection (fungal growth or decomposition) may depend on environmental factors such as temperature. The aim of this study was to analyze the bacterial communities and immune response of *Galleria mellonella* larvae injected with *Cordyceps militaris* and incubated at 15 °C and 25 °C. We examined changes in the bacterial CFUs, bacterial communities (Illumina MiSeq 16S rRNA gene sequencing) and expression of immune, apoptosis, ROS and stress-related genes (qPCR) in larval tissues in response to fungal infection at the mentioned temperatures. Increased survival of larvae after *C. militaris* injection was observed at 25 °C, although more frequent episodes of spontaneous bacteriosis were observed at this temperature compared to 15 °C. We revealed an increase in the abundance of enterococci and enterobacteria in the midgut and hemolymph in response to infection at 25 °C, which was not observed at 15 °C. Antifungal peptide genes showed the highest expression at 25 °C, while antibacterial peptides and inhibitor of apoptosis genes were strongly expressed at 15 °C. Cultivable bacteria significantly suppressed the growth of *C. militaris*. We suggest that fungi such as *C. militaris* may need low temperatures to avoid competition with host bacterial associates.

Keywords: insects; mycoses; spontaneous bacterioses; fungal–bacteria interactions; *Cordyceps militaris*; antimicrobial peptides

1. Introduction

The development of infectious diseases in animals is often accompanied by the proliferation of complex concomitant microorganisms in addition to the development of the main pathogen. In particular, mycoses of insects may develop as mixed infections when opportunistic bacteria are actively involved in the pathogenesis. This occurs due to tissue damage [1] and deregulation of host immune reactions in response to the pathogenic fungi [2,3]. Direct and indirect interactions between fungi and bacteria may lead to both antagonistic and synergistic effects on survival [2,4–6]. In addition, interrelations between fungi and bacteria in insect hosts may be mediated by complex environmental factors, such as temperature, chemicals, or parasitoids, that have an influence on the outcome of the disease [7,8]. However, these immune-ecological studies are just beginning to develop.

Temperature is one of the crucial factors that influences the development of mycoses and bacterioses in insects. Temperature acts on both microorganism growth and on insect immune and

behavioral reactions [9–14]. Entomopathogenic ascomycetes usually have optimal growth between 20–30 °C [15]. In contrast, many bacteria that are associated with terrestrial insects exhibit more active growth between 28–37 °C [16,17]. In many cases, host cellular and humoral antifungal reactions and resistance to fungi are increased with a short or prolonged elevation of temperature [18–24]; however, cold stresses may also activate antifungal systems [18]. Insect antibacterial responses are also dependent on environmental temperatures [25–27] and elevated temperature often promotes bacterial infection [16,17]. There are examples of increased antibacterial responses in insects under short-term or prolonged exposure to low temperatures [16,26,28,29]. Importantly, in a state of cold diapause, cellular immunity continues to work [30] and changes in microbiome composition, immune response and susceptibility to fungal and bacterial infections may also occur [27,31]. It is likely that the outcome of complex infections may be shifted toward mycoses under low temperatures and toward bacterioses under high temperatures. However, the changes in immune response and microbiota composition during complex infections under different temperature conditions are insufficiently understood.

Various antimicrobial peptides (AMPs) of insects have key roles in both antibacterial and antifungal responses [32]. Some AMPs, such as gallerimycin and galiomycin, which are regulated by the Toll immune signaling pathway, exhibit activities against filamentous fungi, but not against bacteria [33,34]. Many AMPs (e.g., cecropins, gloverins, lysozymes) synthesized via the IMD and Toll pathways have a broad spectrum of activities predominantly against gram-positive and gram-negative bacteria as well as against fungi [35,36]. It is likely that AMPs control the proliferation of bacteria during the development of mycoses. In fact, the level of AMP gene expression clearly responds to changes in the microbial community during fungal infections [3]. Moreover, AMP gene expression is dependent on temperature [23,29].

The expression of apoptosis, reactive oxygen species (ROS) and stress-related genes may be crucial in the development of infections caused by entomopathogenic fungi and concomitant bacteria. In particular, a key regulator of programmed cell death, inhibitor of apoptosis (IAP), has paramount physiological importance, including in the antifungal response as was recently shown by Zhang and coworkers [37]. RNAi-mediated knockdown of the IAP homologue in locusts led to a decrease in the total hemocyte count, a degeneration of the gut, a shift in the microbiota, and increased susceptibility to fungal infection. In addition, IAP is involved in immunity to bacterial infections, as shown for *Drosophila* [38]. The generation of ROS has a large impact in reactions against different pathogens [39], as well as in maintaining microbial homeostasis, especially in the insect gut [40–42]. The main source of ROS in insect hemolymph is the prophenoloxidase cascade. As a result of its activation, ROS (primarily semi-quinone radicals and H_2O_2) are formed [43–45]. In the gut, fat body and in other tissues, the formation of ROS occurs with the participation of members of the NADPH oxidase (NOX) family, such as dual oxidases (DUOX). The enzyme generates superoxide and H_2O_2, which are powerful oxidants that exhibit microbicidal activity [40]. Both fungal and bacterial infections led to changes in DUOX activity in the gut and hemocoel tissues [2,3,42]. RNAi knockdown of the DUOX system caused a decrease in ROS and uncontrolled proliferation of bacteria [42]. Heat shock proteins (HSPs) have functions in protein folding and unfolding, and participate in immune signaling pathways and other processes [46]. HSPs are important stress markers, which sense different thermal actions, diapause formation [47] and infections [12].

It is important to note that entomopathogenic fungi produce various secondary metabolites and enzymes (oosporeins, destruxins, different proteinases, AMPs) for inhibiting both host immune responses and competitive microorganisms [36,48,49]. The set of enzymes and secondary metabolites present is significantly different between fungal species and depends on host and habitat specificity. As a rule, generalist species have a broader spectrum of metabolites compared to species with restricted host ranges [50–52].

The ascomycete *Cordyceps militaris* is characterized by a restricted host and habitat range and has a highly reduced number of genes involved in secondary metabolism and the synthesis of proteases compared to generalists such as *Metarhizium robertsii* and *Beauveria bassiana* [50,53]. This fungus mainly

infects forest lepidopterans (Lepidoptera, Macroheterocera) in the larval and pupal stages which are located in the soil, forest flour and fallen wood [54–56]. Previously, natural infections of insects with *C. militaris* were studied insufficiently. It is known that larvae and pupae could be infected by topical application with ascospores or conidia in a laboratory [22,57]. However this method is difficult to reproduce, and the outcomes strongly depend on the physiological state of the host [22]. In contrast, injection of lepidopteran larvae and pupae with blastospores or conidia has led to more stable development of the mycosis [55,58,59]. Importantly, spontaneous bacterial infections have been constantly documented after infection of *C. militaris* with insects in laboratory conditions [22,58,59]. Therefore, *C. militaris* is a convenient model to study fungal–bacteria interactions in insects. The optimal temperature for mycelial growth of *C. militaris* palearctic isolates is approximately 20 °C [22,60]. In a previous study [22], we showed in a model insect, the wax moth *Galleria mellonella*, that larvae in a state of facultative diapause induced by a low temperature (15 °C) are most susceptible to the fungus. Mycosis successfully developed after injection with *C. militaris* blastospores at 15 °C. By contrast, at 25 °C (active state), larvae were able to overcome the infection and complete metamorphosis, although, the infection may persist in pupae and adults and could still be activated by a low temperature. Activation of the antifungal response (encapsulation and phenoloxidase activity) in response to *C. militaris* infection was observed in wax moth larvae at 25 °C, while inhibition of these parameters occurred at 15 °C. We suggested that *C. militaris* uses fewer universal tools for evasion and inhibition of host immunity compared to generalists fungi, such as *Metarhizium* and *Beauveria*, that induce prolonged mycosis development, persist in hosts and have a specialization in killing dormant insects with reduced immune activity [22,59]. Moreover, because we registered spontaneous bacterioses in the wax moths post injection of *C. militaris* blastospores and conidia, we hypothesized that *C. militaris* has poorly developed mechanisms for manipulating the host microbiota and requires a low temperature for its normal development.

In the present study we investigated the microbial communities of the wax moth larvae hemolymph and midgut, as well as the expression of AMP, apoptosis, ROS and stress-related genes in the wax moth midgut and fat body after injection with *C. militaris* and incubation under two temperatures, 15 °C (state of facultative diapause) and 25 °C (active state). We found significant changes in these parameters in response to infection at different temperatures, which support the hypothesis mentioned above.

2. Material and Methods

2.1. Fungi and Insects

C. militaris isolate CNAp (GenBank No MF073255.1), from the microorganism collection of the Institute of Systematics and Ecology of Animals SB RAS, was used in this work. Conidia had been stored at −80 °C since 2015. For infections, conidia were cultivated on Sabouraud dextrose agar with yeast extract (2.5 g/L) (SDAY) for 22 days at 23 °C and a photoperiod of 8:16 (light:dark). Conidia were suspended in saline (0.9% NaCl) without any detergents and filtered through a sterile cloth to remove mycelial clumps. Concentrations of conidia were determined using a Neubauer hemocytometer. A Siberian line of wax moth larvae was maintained on artificial media as described previously [61]. Larvae of the sixth instar were used in experiments.

2.2. Procedures for Infection and Bioassays

Larvae were injected with 4 μL of a suspension containing 1250, 2500 or 5000 conidia. Control larvae were injected with saline. Punctures were made between the sixth and seventh abdominal segments using a microinjector with an insulin syringe. The needle was sterilized with 96% ethanol before each injection. Infected and control larvae were placed at two constant temperatures (25 °C and 15 °C) immediately after injection. Larvae were maintained in 90 mm glass Petri dishes (12 larvae per dish) with artificial media (3 g per one Petri dish) in the dark. Temperature in the Petri dishes at such an insect density corresponded with environmental temperatures. Ventilation of the Petri dishes and

registration of mortality was conducted every day over 10 days. To determine the causes of death, cadavers were placed on moist filter paper in the Petri dishes and maintained at the temperatures indicated above. Three replicates (one replicate = 12 larvae) were used to assay mortality after injection with each dose, and the whole experiment was repeated twice.

For detection of hyphal bodies and bacteria in the hemolymph of infected larvae, we used gradient centrifugation of the hemolymph in Percol followed by electron transmission microscopy as described previously [59]. To determine the yield of conidia on the larvae, the cadavers were incubated for 30 days in moist chambers at 25 °C and 15 °C. Then, each cadaver was placed in a tube with 20 mL of a 0.1% water-Tween 20 solution and vortexed for 3 min until the mycelia and conidia were completely washed off. Conidia were counted using a hemocytometer and the concentrations were calculated for each cadaver.

2.3. Bacterial Colony Forming Unit (CFU) Counts

At 96 h after injection with a dose of 2500 conidia per larvae, control and infected insects were surface sterilized by 3% H_2O_2 and 70% ethanol. Forty five μL of hemolymph from three larvae were placed in 100 μL of 150 mM cool NaCl and immediately homogenized using an ultrasonic homogenizer (Sonopuls, Bandelin electronic GmbH & Co. KG, Berlin, Germany). Midguts were pooled in the same NaCl (one sample = three larvae) and homogenized by the same technique. Samples were diluted with the same NaCl by 10, 100, and 1000-fold and a 100 μL aliquot was plated on media (Bile esculin azide agar for enterococci and Endo agar for enterobacteria) in 90 mm Petri dishes. The cultures were incubated for 2 days at 35 °C and then CFUs were counted. CFU counts were calculated for each midgut or 10 μL of hemolymph. A total of 5–6 samples from each treatment were used for analysis.

2.4. Analysis of Bacterial Communities

At 96 h post-treatment, infected (2500 conidia) and control larvae were surface-sterilized by 3% H_2O_2 and 70% ethanol and dissected. Midguts with content were isolated and frozen in liquid nitrogen (one sample = 5 midguts). In addition, decomposed cadavers (6–7 d post infection) were analyzed. Whole cadavers were frozen in liquid nitrogen (one sample = 3 whole bodies). Three biological replicates from each treatment were used.

DNA was isolated using a DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 16S rRNA region was amplified with the primer pair V3–V4 combined with Illumina adapter sequences [62]. PCR amplification was performed as described previously [63]. A total of 200 ng of PCR product from each sample was pooled together and purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The obtained libraries were sequenced with 2 × 300 bp paired-end reagents on a MiSeq (Illumina Inc., San-Diego, California, USA) in the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia). The sequencing data reported in this study were submitted to GenBank under the study accession PRJNA650299.

Raw sequences were analyzed with the UPARSE pipeline [64] using Usearch v11.0. The UPARSE pipeline included a merging of paired reads, read quality filtering, length trimming, merging of identical reads (dereplication), discarding singleton reads, removing chimeras, and operational taxonomic unit (OTU) clustering using the UPARSE-OTU algorithm. The OTU sequences were assigned a taxonomy using the SINTAX [65] and 16S RDP training set v.16 [66]. The final dataset included 384,875 reads (mean ± SE = 20,532 ± 630 per midgut sample and 46,162 ± 1453 per cadaver sample, see Dataset). All rarefaction curves showed a trend of approaching the saturation plateau (Figure S1), which indicated a reasonable volume of the sequenced reads.

2.5. Gene Expression

Wax moth larvae at 96 h after injection (2500 conidia) were dissected on ice cold PBS and midguts without contents and fat bodies were collected. Midguts content were removed by eye forceps. Midguts from ten larvae or fat bodies from five larvae were pooled in each sample. A total of 5–6 samples

(biological replicates) from each treatment were used for analysis. The tissues were frozen in liquid nitrogen and stored at −80 °C. Samples were lyophilized at −65 °C, 400 mtorr for 15 h and disrupted in liquid nitrogen using micropestles just before RNA isolation. The tissues were homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and RNA was isolated according to the manufacturer's instructions. Quantity and quality of the total DNA were estimated by NanoDrop NanoVue Plus (GE Healthcare, Chicago, Illinois, USA). Each sample was normalized to a concentration of 1.5 μg/μL and treated by RQ1 RNase-free DNase (Promega, Madison, WI, USA). RNA was converted to cDNA using 6 μg DNA-free RNA, 3 μL 100 nM random nanomers and 4 μL RevertedAid^{TM} M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania).

qPCR was carried out using HS-qPCR SYBR Blue (2×) mix (BioLabMix, Novosibirsk, Russia) with a CFX96 Touch (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed in triplicate under the following conditions: 95 °C for 3 min, and 40 cycles of 15 s at 94 °C and 30 s at 60/62/64 °C (depending on the primer Tm), followed by melt curves (70–90 °C). Gene expression was estimated by the ΔΔCq protocol with Bio-Rad CFX Manager (Bio-Rad, Laboratories, Inc., Hercules, CA, USA). The following *G. mellonella* genes were used as references: translation elongation factor 1-alpha 1 (eEF1α1) and the subunit of DNA-directed RNA polymerase II. The expression dynamics of the following ten genes of interest were studied: antimicrobial peptides gallerimycin, galiomycin, gloverin, cecropin-like and lysozyme-like, apoptosis-related IAP, the ROS-related NOX-DUOX domain and heat shock proteins Hsp70 and Hsp90. These genes and primer sequences were from the work of Lange and coauthors [67] and Melo and coauthors [68] or designed by us (Table S1). Primer properties were estimated by IDT OligoAnalyser 3.1 (http://eu.idtdna.com/calc/analyzer). Primers were synthesized by Biosintez, Koltsovo, Russia.

2.6. In Vitro Interaction between Fungi and Bacteria

For the interaction studies, we used the predominant cultivable bacteria previously isolated from *G. mellonella* midgut, *Enterococcus faecalis* and *Enterobacter* sp. [7] In addition to *C. militaris*, the fungi *M. robertsii* (strain MB-1) and *B. bassiana* (strain Sar-31) from the microorganism collection of the Institute of Systematics and Ecology of Animals SB RAS were used as positive controls. Bacteria were cultivated on nutrient agar (Himedia, Mumbai, India) and fungi were cultivated on SDAY media. One-day-old plugs of bacteria (8 mm) or plugs of nutrient agar (control) were placed on freshly plated cultures of fungi in 90 mm Petri dishes. Zones of mycelial growth inhibition were measured at 4 days of incubation at 25 °C. Similarly, four-day-old plugs of fungi were placed on freshly plated bacterial cultures. Zones of growth inhibition were estimated on the first and second day of incubation at 25 °C. Radial mycelial growth on cultures of bacteria was recorded over 24 days. As a control, measurements of mycelial growth on bacteria-free nutrient agar were conducted. Three replicates were used in each treatment.

2.7. Statistics

Differences in mortality dynamics were analyzed by a log rank test followed by Holm–Sidak adjustment. The χ^2 criterion was applied in estimating the portion of sporulated and decomposed larvae. Other data were checked for normality of distribution using the Shapiro–Wilk W test. Conidia yields from cadavers had a normal distribution and were analyzed by a Student *t*-test. Data from CFU counts, OTU abundances, diversity indexes and gene expression had abnormal distributions and were analyzed by a nonparametric analogue of the two-way ANOVA, namely, the Scheirer–Ray–Hare test [69], followed by Dunn's post hoc test. Data from the antagonistic interactions between fungi and bacteria in vitro were analyzed by the Kruskal–Wallis test with Dunn's post hoc test.

3. Results

3.1. Bioassays

Mortality of larvae injected with *C. militaris* conidia began at 5–7 days post injection and reached 80–100% after 7–10 days, depending on the dose and temperature (Figure 1A–C). More rapid mortality of larvae at 15 °C compared to 25 °C was observed following injection of all doses (log rank test, $\chi^2 > 9.6$, df = 1, $p < 0.002$). No mortality was registered for larvae injected with saline. Notably, 9–20% of insects infected with low and intermediate doses and maintained at 25 °C were able to survive and complete metamorphosis.

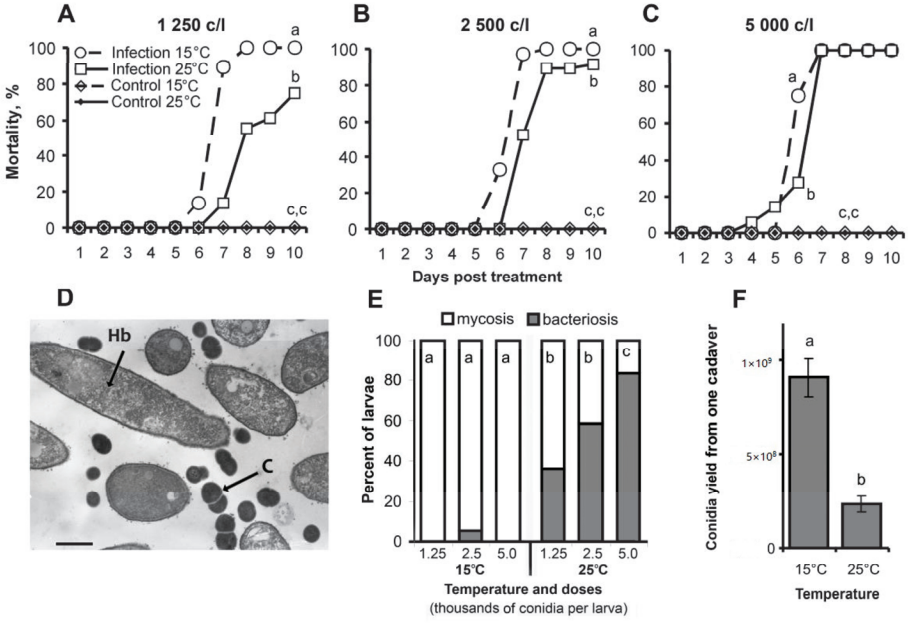

Figure 1. Mortality dynamics and outcome of infection in wax moth larvae after injection with *C. militaris* conidia and subsequent incubation at 15 °C and 25 °C. (**A**–**C**)—mortality dynamics after injection of larvae with 1250, 2500 and 5000 conidia per larva (c/L). Different letters indicate significant differences determined by log rank test ($\chi^2 > 9.6$, df = 1, $p < 0.002$). (**D**)—*C. militaris* hyphal bodies (Hb) and cocci (C) in wax moth hemolymph at 5 days after injection with the fungus. Scale bar, 1 μm. (**E**)—portion of mummified and decomposed larvae during the development of mycoses at different temperatures. Different letters indicate significant differences ($\chi^2 > 8.7$, df = 1, $p < 0.003$). (**F**)—*C. militaris* conidial yield on mummified cadavers at 15 °C and 25 °C. Different letters indicate significant differences determined by *t*-tests ($t = 6.1$, df = 8, $p < 0.001$).

Microscopy observations showed the simultaneous presence of hyphal bodies and cocci in the hemolymph of infected insects maintained at 25 °C (Figure 1D), however, these cocci were not observed in the hemolymph at 15 °C. At 15 °C, mycosis led to the formation of mummified cadavers (94–100%) after all treatment doses (Figures 1E and 2A). However, at 25 °C, we documented a large number of bacterially decomposed insects ($\chi^2 > 8.7$, df = 1, $p < 0.003$ compared to 15 °C). The bacterioses were identified by symptoms of darkening and liquefaction of the larvae for several hours after death (Figure 2C). The increase in frequency of bacterioses at 25 °C was dose-dependent and increased from 36% after injection with the lowest dose and to 83% after injection with the highest dose (Figure 1E). Notably, we registered the formation of abnormally dark mummies in these experiments (Figure 2B).

The percent of abnormal mummies was 52% at 25 °C and only 12% at 15 °C (χ^2 = 8.3, df = 1, p = 0.004). Moreover, the production of conidia on mummified cadavers at 25 °C decreased 2.5-fold compared to 15 °C (t = 6.1, df = 8, $p < 0.001$, Figures 1F and 2D,E). Thus, insects were less susceptible to *C. militaris* infection at 25 °C, but they were more predisposed to spontaneous bacterial infections compared to those incubated at 15 °C.

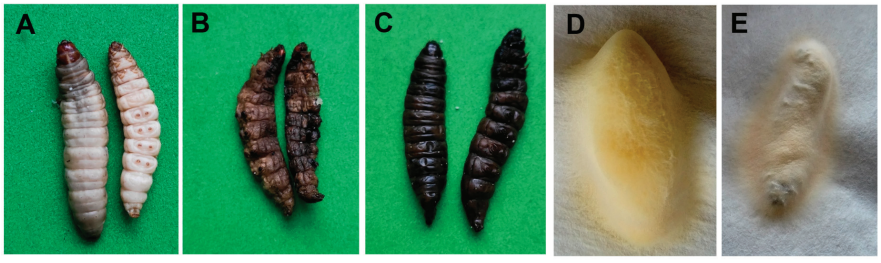

Figure 2. Phenotypes of larvae that died after injection with *C. militaris* conidia. (**A**)—mummification, (**B**)—defective mummies, (**C**)—bacterial decomposition, (**D**)—conidiation at 15 °C, (**E**)—conidiation at 25 °C.

3.2. CFU Counts in the Hemolymph and Midgut

In the hemolymph of control larvae, we registered single colonies of enterococci and enterobacteria at both temperatures (Figure 3A). At 15 °C, fungal infection did not lead to significant changes in the CFU count (Dunn's test, $p > 0.17$ compared to controls). In contrast, CFU counts of both enterobacteria and enterococci increased in the hemolymph by 39,000–54,000-fold at 25 °C in response to *C. militaris* infection ($p < 0.002$ compared to controls). A significant interaction between factors (mycosis × temperature) was found for enterobacteria ($H_{1,19}$ = 9.4, p = 0.002). However, this interaction was not found for enterococci ($H_{1,19}$ = 1.5, p = 0.23) because there was still a slight increase in these bacteria at 15 °C in response to *C. militaris* infection.

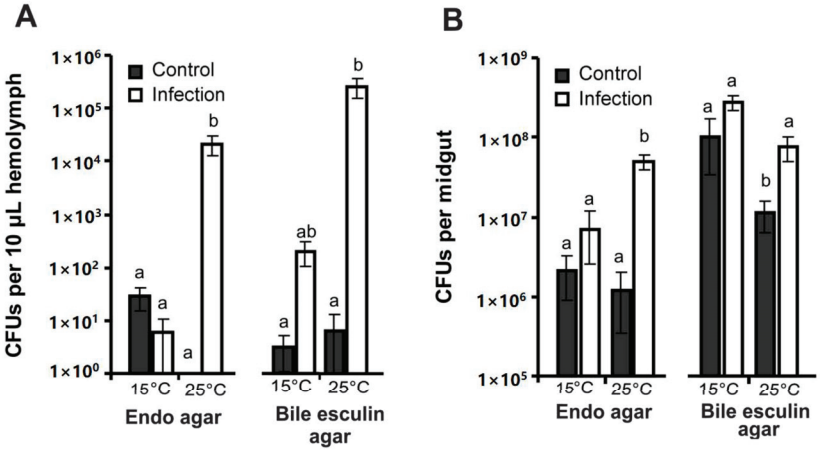

Figure 3. CFU counts in the hemolymph (**A**) and midgut (**B**) of wax moth larvae at 96 h post injection of *C. militaris* (2500 conidia per larva) with subsequent incubation at 15 °C and 25 °C. Selective media for enterobacteria (Endo agar) and enterococci (Bile esculin agar) was used. Different letters show significant differences within the specified media and tissue (Dunn's test, $p < 0.05$).

In the midgut, we observed an elevation in the enterobacteria and enterococci CFU counts in response to fungal infection at both temperatures (enterobacteria, $H_{1,19} = 4.2$, $p = 0.04$; enterococci, $H_{1,19} = 8.5$, $p = 0.004$). However, the post hoc tests showed significant elevation only at 25 °C (4–7-fold relative to controls, Dunn's test, $p < 0.04$, Figure 3B). Effects of temperature on CFU counts were not significant, however, a trend toward increased enterococci was observed at 15 °C compared to 25 °C ($H_{1,23} = 2.7$, $p = 0.09$). Notably, uninfected larvae maintained at 15 °C were characterized by the highest enterococci CFU counts compared to larvae maintained at 25 °C ($p = 0.03$).

3.3. Bacterial Communities in Midguts and Cadavers

In the midgut, we registered 168 OTUs (37 ± 5.2 OTUs per sample) with a predominance of two *Enterococcus* OTUs (Figure 4). A BLAST search against sequences in GenBank showed strong similarity with *Enterococcus faecalis* (OTU 1, 100% similarity) and *E. lemanii* (OTU 100, 99.53% similarity). Temperature did not have a significant impact on the relative abundance of different groups and diversity indexes ($H_{1,11} < 0.4$, $p > 0.52$, Figure S2). However, trends toward increased diversity indexes in warm conditions were observed for uninfected larvae (Dunn's test, $p > 0.08$, Figure S2). Fungal infection led to a significant decrease in OTU counts and the Chao1 index ($H_{1,11} > 4.7$, $p < 0.03$), as well as to shifts in community structure. Under both temperatures, *C. militaris* infection caused a partial displacement of *E. faecalis* by *E. lemanii* (effect of the infection: $H_{1,11} = 8.3$, $p = 0.004$). In addition, a decrease in the abundance of the subdominant bacteria *Acinetobacter*, *Melaminivora*, *Comamonas*, and *Diaphorobacter* was revealed under the influence of the fungal infection ($H_{1,11} > 5.0$, $p < 0.024$). These effects were more evident at 25 °C (Dunn's test, $p < 0.013$) compared to 15 °C (Dunn's test, $p > 0.17$).

In bacterially decomposed cadavers, we registered the lowest bacterial diversity (OTU count, 10 ± 1.9; Chao1, 13 ± 2.6; Shannon, 0.47 ± 0.14). In the cadavers, either the enterococci *E. faecalis* or *Enterobacter* sp. prevailed (Figure 4). Enterobacteriaceae were represented by two predominant OTUs that were also detected in the midgut. One of them, OTU 2, was close in identity to *Enterobacter* sp. (99.53% similarity) which was previously isolated from the midgut of same line of *G. mellonella* [7]. The other, OTU 144, was close to *Cronobacter sakazakii* (99.77% similarity).

3.4. AMP Gene Expression

We observed a significant upregulation in the expression of the studied AMP genes (except for cecropin) in both the fat body and the midgut under the influence of fungal infection (Figure 5, Table 1). Temperature had a significant impact on the expression of cecropin and lysozyme only. Overall, we observed a stronger expression of antifungal peptide genes in response to infection at 25 °C compared to 15 °C. In contrast, antibacterial peptide genes trended toward higher expression at 15 °C compared to 25 °C. For example, expression of the antifungal peptide gene gallerimycin in the fat body was increased by 77-fold at 25 °C, but only by 12-fold at 15 °C compared to uninfected insects (Dunn's test, $p < 0.0005$ and $p = 0.10$, respectively). The galiomycin gene in the fat body was upregulated by 18-fold at 25 °C but only 8-fold at 15 °C ($p = 0.001$ and $p = 0.04$ compared to controls, respectively). Gallerimycin and galiomycin gene expression followed the same pattern in the midgut (Figure 5, Table 1).

Unlike the antifungal peptides, expression of the antibacterial peptide gloverin in the fat body in response to fungal infection increased by 55-fold at 15 °C ($p = 0.005$ compared to control) and 17-fold at 25 °C ($p = 0.01$ compared to control). For the cecropin and lysozyme genes, we observed increased expression in the fat body at 15 °C compared to 25 °C (effect of temperature, $H_{1,19} = 3.9$, $p = 0.05$ and $H_{1,23} = 3.2$, $p = 0.07$, respectively), and more active expression in response to fungal infection was also observed at low temperature (Figure 5). Changes in the expression of the gloverin, cecropin and lysozyme peptide genes in the midgut were less than in the fat body. The gloverin gene was upregulated by 2.8-3-fold in the midgut in response to fungal infection ($H_{1.19} = 6.2$, $p = 0.01$), independent of temperature. Expression of cecropin in the midgut was not significantly changed in

response temperature or fungal infection. Expression of the lysozyme gene in the midgut was decreased at 15 °C compared to 25 °C ($H_{1,23}$ = 4.6, p = 0.03); however, there was a significant upregulation in response to *C. militaris* infection, which occurred only at 15 °C (6-fold, p = 0.004 compared to control) and not at 25 °C (2-fold, p = 0.15 compared to control).

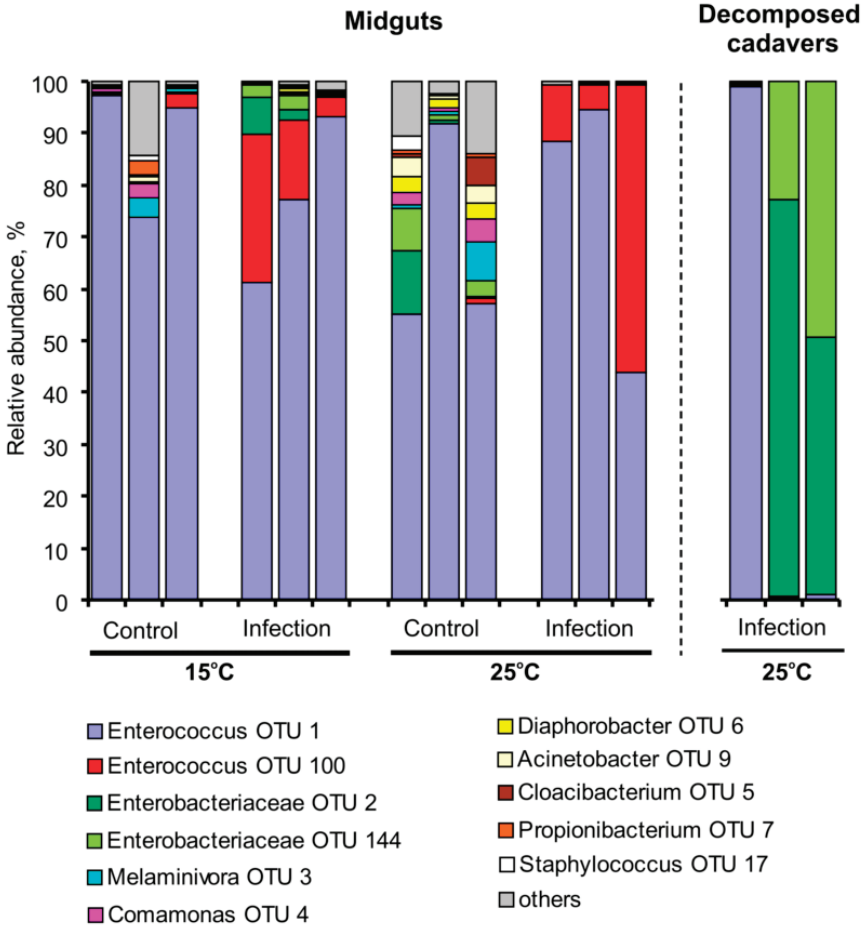

Figure 4. Bacterial communities (16S rRNA) in the midguts of wax moth larvae during the development of *C. militaris* infection at different temperatures and the communities in the cadavers that decomposed after the infection. Midgut communities were analyzed at 96 h after injection with a dose of 2500 conidia per larva. Decomposed cadavers were analyzed at 6–7 days post injection. Each treatment represents 3 replicates.

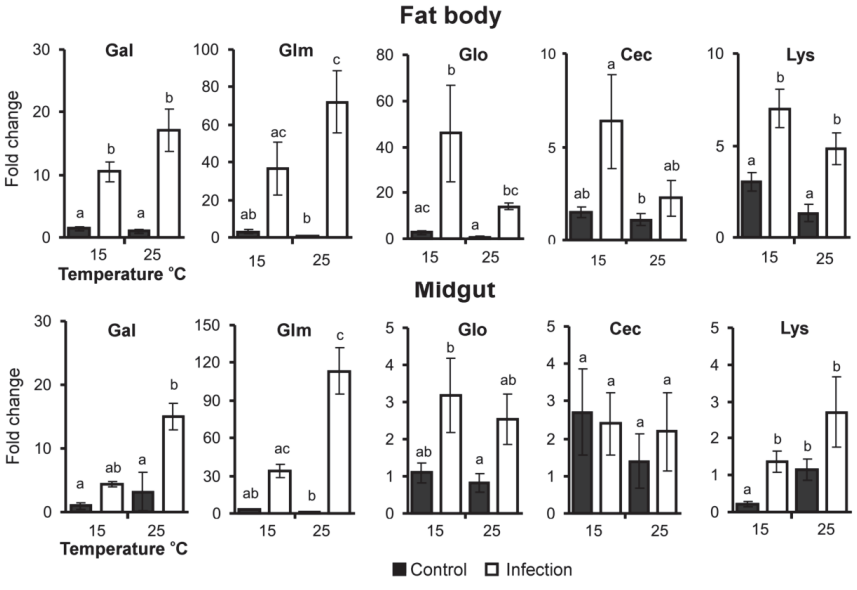

Figure 5. Relative expression of AMP genes in the fat body and midgut of wax moth larvae at 96 h after injection with *C. militaris* (2500 conidia per larva) and subsequent incubation at 15 °C and 25 °C. Data were normalized to the expression of two reference genes, eEF1a and RBP11. The *Y*-axis shows the fold change relative to uninfected larvae maintained at 25 °C. **Gal**—galiomycin, **Glm**—gallerimycin, **Glo**—gloverin, **Cec**—cecropin-like, **Lys**—lysozyme-like. Different letters indicate significant differences between treatments (Dunn's test, $p < 0.05$).

Table 1. Two-way effects of *C. militaris* infection and temperature on the expression of AMP genes. Significant effects are highlighted in bold. Arrows show up- or downregulation of genes in response to infection and in response to cooling to 15 degrees. Arrows are shown only for significant ($p < 0.05$) and marginal ($p = 0.05$–0.10) effects.

	Effects		
	Infection	**Temperature**	**Infection × Temperature**
Fat body			
Galiomycin	**↑$H_{1,19} = 14.3$ $p < 0.001$**	$H_{1,19} = 0.0$ $p = 1.00$	$H_{1,19} = 0.7$ $p = 0.40$
Gallerimycin	**↑$H_{1,19} = 14.3$ $p < 0.001$**	$H_{1,19} = 0.0$ $p = 0.88$	$H_{1,19} = 2.1$ $p = 0.15$
Gloverin	**↑$H_{1,19} = 14.3$ $p < 0.001$**	$H_{1,19} = 1.5$ $p = 0.23$	$H_{1,19} = 0.1$ $p = 0.82$
Cecropin	↑$H_{1,19} = 3.3$ $p = 0.08$	**↑$H_{1,19} = 3.9$ $p = 0.05$**	$H_{1,19} = 0.1$ $p = 0.76$
Lysozyme	**↑$H_{1,23} = 12.8$ $p < 0.001$**	↑$H_{1,23} = 3.2$ $p = 0.07$	$H_{1,23} = 0.1$ $p = 0.77$
Midgut			
Galiomycin	**↑$H_{1,19} = 10.1$ $p = 0.002$**	$H_{1,19} = 1.2$ $p = 0.29$	$H_{1,19} = 1.3$ $p = 0.27$
Gallerimycin	**↑$H_{1,19} = 14.3$ $p < 0.001$**	$H_{1,19} = 0.1$ $p = 0.76$	$H_{1,19} = 2.5$ $p = 0.11$
Gloverin	**↑$H_{1,19} = 6.2$ $p = 0.01$**	$H_{1,19} = 0.2$ $p = 0.65$	$H_{1,19} = 0.0$ $p = 0.88$
Cecropin	$H_{1,23} = 1.2$ $p = 0.27$	$H_{1,23} = 1.0$ $p = 0.33$	$H_{1,23} = 0.5$ $p = 0.49$
Lysozyme	**↑$H_{1,23} = 9.4$ $p = 0.002$**	**↓$H_{1,23} = 4.6$ $p = 0.03$**	$H_{1,23} = 1.1$ $p = 0.30$

3.5. Apoptosis, ROS and Stress-Related Gene Expression

Expression of the IAP gene in the fat body was temperature dependent (Figure 6, Table 2). The gene was upregulated in the fat body in response to fungal infection only at low temperature (Dunn's test, $p = 0.07$ compared to control at 15 °C and $p < 0.01$ compared to other treatments). At 25 °C, expression of this gene in response to infection was not changed compared to the control ($p = 0.50$). In the midgut,

regulation of the IAP gene was not caused by temperature (Table 2), but only by the infection (effect of fungus: $H_{1,23} = 7.7$, $p = 0.006$). Significant upregulation in response to *C. militaris* was also registered only at 15 °C ($p = 0.04$ compared to control).

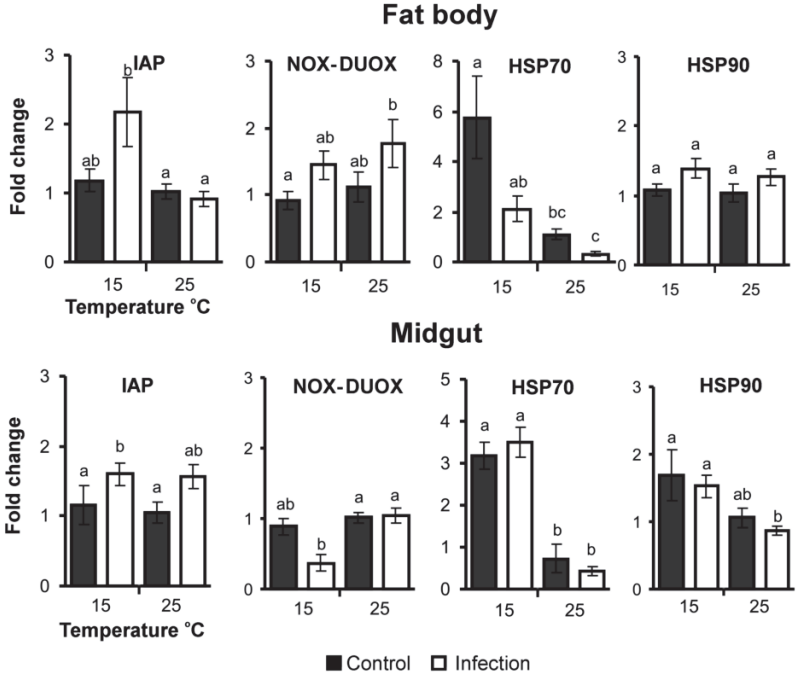

Figure 6. Relative expression of apoptosis, ROS and stress-related genes in the fat body and midgut of wax moth larvae at 96 h after injection with *C. militaris* (2500 conidia per larva) and subsequent incubation at 15 °C and 25 °C. Data were normalized to the expression of two reference genes, eEF1a and RBP11. The *Y*-axis shows the fold change relative to uninfected larvae maintained at 25 °C. Different letters indicate significant differences between treatments (Dunn's test, $p < 0.05$).

NOX-DUOX domain gene expression was slightly (1.6-fold) upregulated in the fat body in response to fungal infection at both temperatures (effect of infection: $H_{1,23} = 4.2$, $p = 0.04$), but the effect of temperature was not significant (Table 2). Scheirer–Ray–Hare test showed a downregulation of this gene in the midgut under the influence of a low temperature ($H_{1,23} = 6.16$, $p = 0.01$), however strong downregulation (>2.4-fold) was observed during mycosis development at 15 °C only ($p = 0.08$ compared to control at 15 °C and $p < 0.01$ compared to other treatments).

HSP70 gene expression in the fat body was increased at a low temperature (Figure 6, Table 2) and fungal infection downregulated its expression at both temperatures (effect of fungus: $H_{1,23} = 4.6$, $p = 0.03$). In the midgut, upregulation of HSP70 was also observed at 15 °C ($H_{1,23} = 13.7$, $p < 0.001$) but fungal infection had no significant effect. The HSP90 gene was slightly and insignificantly upregulated in the fat body in response to infection and independent of temperature. Its expression in the midgut was increased under the influence of low temperature ($H_{1,23} = 8.7$, $p = 0.003$) but fungal infection had no significant effect.

Table 2. Two-way effects of *C. militaris* infection and temperature on the expression of apoptosis, ROS and stress-related genes. Significant effects highlighted in bold. Arrows show up- or downregulation of genes in response to infection and in response to cooling to 15 degrees. Arrows are shown only for significant ($p < 0.05$) and marginal ($p = 0.05$–0.10) effects.

	Effects		
	Infection	**Temperature**	**Infection × Temperature**
Fat body			
IAP	$H_{1,23} = 0.8$ $p = 0.39$	**↑$H_{1,23} = 7.4$ $p = 0.007$**	$H_{1,23} = 3.0$ $p = 0.08$
NOX-DUOX	**↑$H_{1,23} = 4.3$ $p = 0.04$**	$H_{1,23} = 0.6$ $p = 0.45$	$H_{1,23} = 0.0$ $p = 0.86$
Hsp70	**↓$H_{1,23} = 4.6$ $p = 0.03$**	**↑$H_{1,23} = 14.5$ $p < 0.001$**	$H_{1,23} = 0.0$ $p = 0.95$
Hsp90	↑$H_{1,23} = 2.8$ $p = 0.09$	$H_{1,23} = 0.3$ $p = 0.56$	$H_{1,23} = 0.1$ $p = 0.77$
Midgut			
IAP	**↑$H_{1,23} = 7.7$ $p = 0.006$**	$H_{1,23} = 0.0$ $p = 0.91$	$H_{1,23} = 0.0$ $p = 0.91$
NOX-DUOX	$H_{1,23} = 1.8$ $p = 0.18$	**↓$H_{1,23} = 6.2$ $p = 0.01$**	$H_{1,23} = 1.3$ $p = 0.25$
Hsp70	$H_{1,23} = 0.0$ $p = 0.93$	**↑$H_{1,23} = 13.7$ $p < 0.001$**	$H_{1,23} = 0.1$ $p = 0.71$
Hsp90	$H_{1,23} = 0.2$ $p = 0.64$	**↑$H_{1,23} = 8.7$ $p = 0.003$**	$H_{1,23} = 0.8$ $p = 0.39$

3.6. Interaction between Fungi and Bacteria In Vitro

We showed that *E. faecalis* and *Enterobacter* inhibited *C. militaris* more strongly than *M. robertsii* and *B. bassiana*. In particular, *E. faecalis* inhibited *C. militaris* mycelial growth on SDAY medium by 2–2.2-fold more than *M. robertsii* or *B. bassiana* growth (Dunn's test, $p < 0.012$, Figure 7). *Enterobacter* sp. also inhibited *C. militaris* growth more strongly than *M. robertsii* and *B. bassiana*, but the differences were only marginally significant ($p = 0.06$–0.10). None of these fungi inhibited bacterial growth on nutrient agar. However, *M. robertsii* and *B. bassiana* were able to grow on cultures of both bacteria (Figure 7). In contrast, *C. militaris* was not able to grow on cultures of *E. faecalis* or *Enterobacter* sp.

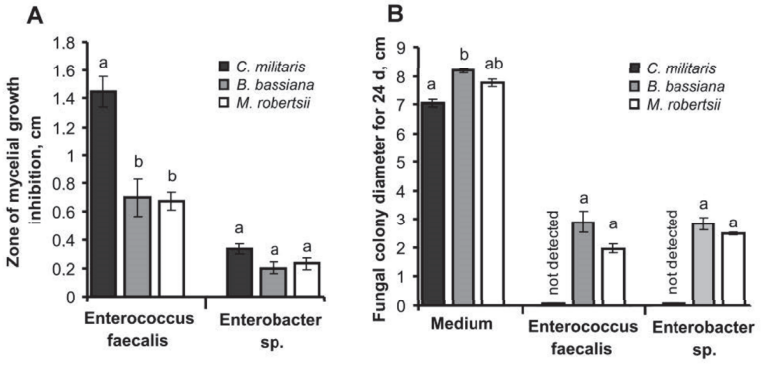

Figure 7. Inhibition of fungi by *Enterococcus faecalis* and *Enterobacter sp.* in vitro. (**A**)—zone of mycelial growth inhibition by bacteria on SDAY medium. (**B**)—radial growth of fungi on nutrient agar and this medium with lawns of the bacteria. Different letters indicate significant differences between treatments (Dunn's test, $p < 0.05$).

4. Discussion

The development of mycoses in insects is not restricted to fungal monoinfections, and bacterial commensals and pathogens are also involved in this process [1,2,5]. We show that bacterial involvement in fungal pathogenesis and its outcome is dependent on environmental conditions, particularly temperature (Figure 8). The development of *C. militaris* in wax moth larvae was faster and more successful at 15 °C compared to 25 °C. At 25 °C, fungal virulence was decreased however a high frequency of spontaneous bacteriosis was observed, which was caused by the proliferation of enterococci

and enterobacteria in the hemolymph. We also showed that *C. militaris* is a weak competitor of bacteria compared to generalist fungi such as *M. robertsii* and *B. bassiana*. This is consistent with the more specific conditions for cultivation required by *C. militaris* in vivo or in vitro [70]. Occurrence of bacterioses after topical infection or injection of *C. militaris* in insects has been documented previously [22,55,58,71]. We suggest that *C. militaris* has a limited ability to suppress host commensal bacteria, as the fungus is associated with narrow environmental requirements, including a specific temperature range [22]. Low temperatures (~15 °C) limit the active influence of bacteria on fungal pathogenesis. This may be explained by the fact that low temperatures are suboptimal for the proliferation of many bacteria. In addition, we showed a stronger antibacterial response in the host during *C. militaris* development under low temperature conditions.

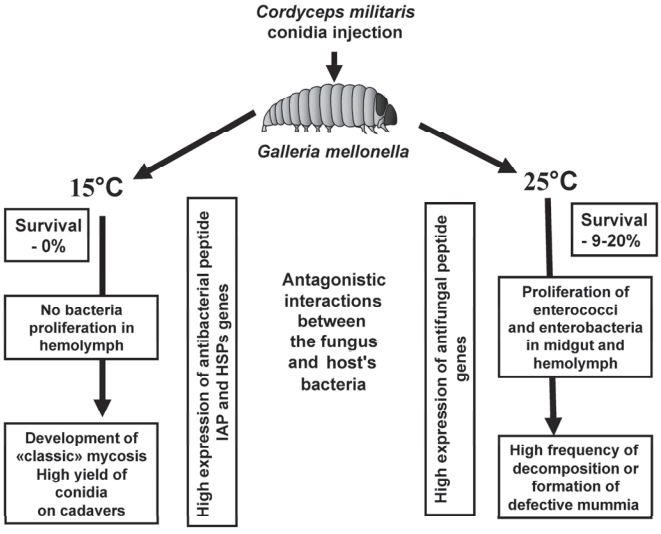

Figure 8. Outline of the interactions between *C. militaris* and bacterial associates in wax moth larvae under different temperature conditions.

Consistent with our study of the midgut microbiome, the predominant bacteria in the gut of healthy wax moths are *Enterococcus* species [72,73]. In different pathological states (e.g., toxicosis caused by *Bacillus thuringiensis* or envenomation with parasitoids), a shift in the microbiome structure toward Enterobacteriaceae prevalence occurred in the wax moth gut [7,74]. However, we observed another effect in the present study, the replacement of one *Enterococcus* species with another under the influence of a fungal infection. Change in the dominance between different *Enterococcus* species was also documented previously after injection of wax moth larvae with *C. militaris* blastospores (unpublished [75]). The mechanism of this restructuring is not clear and is likely associated with the selective action of fungal metabolites on different species of enterococci. For example, significant changes in the mouse gut microbiome were observed after feeding mice a major metabolite of *C. militaris* cordycepin [76]. Gamage and coworkers [77] showed that *C. militaris* water extracts exhibited different levels of inhibition of various gram-positive and gram-negative bacteria.

We observed an increase in bacterial CFU counts in the midgut during the development of *C. militaris* infection. This confirmed previous work performed on adult mosquitos following topical infection with *Beauveria* and *Isaria* species [2,3], as well as work on Colorado potato beetle larvae after topical treatment with *Metarhiziun robertsii* [78]. These enhancements may be caused by a disturbance in feeding, gut peristalsis or by a deregulation in immune reactions during mycosis development.

It should be noted that significant elevations in enterococci and enterobacteria loads in response to *C. militaris* infection were observed only in warm (25 °C) conditions and not in cold (15 °C) conditions.

In the hemolymph of uninfected larvae, we observed single colonies of enterococci and enterobacteria. Dramatic (39–54-thousand-fold) elevations in the CFU counts of these bacteria in the hemolymph during fungal infection were observed only in warm conditions (25 °C). It should be noted that the enterococci are a prevalent group of bacteria in wax moth integuments and enterobacteria are also present in these tissues [73,79]. However, it is hardly possible that the observed septicemia was the result of an influx through a cuticle puncture since bacterial-induced death began at five days post injection and occurred simultaneously with death due to mycosis. Moreover, the frequency of spontaneous bacterioses at 25 °C was positively correlated with the dose of *C. militaris* conidia. The source of bacterial penetration into the hemolymph could be the gut or other organs such as the trachea or excretory organs, the biome of which has not been studied in the wax moth. It is interesting to note that the occurrence of septicemia was less common after injection of wax moth larvae with conidia of the generalist fungi *Metarhizium* or *Beauveria*. For example, injection of the larvae with *B. bassiana* and *M. robertsii* at doses of 2500 conidia per larvae and subsequent incubation at 25 °C did not lead to bacterial decomposition and all cadavers were mummified and overgrown with these fungi (Figure S3). Fan and coauthors [48] showed that at the final stages of mycoses, *B. bassiana* suppresses the proliferation of bacteria in the host through the production of secondary metabolites such as oosporeins. However, compared to *Beauveria* and *Metarhizium* species, *C. militaris* has fewer genes involved in secondary metabolism [50,53]. It is likely that the combination of less developed mechanisms for the suppression of bacteria and harsher tools for host tissue destruction caused the septicemia during *C. militaris* infection. In particular, we recently showed that *C. militaris* infection led to necrotic death of hemocytes and a strong elevation in dopamine and ROS in wax moth larvae, which were not observed after *M. robertsii* infection [59].

The development and outcome of the fungal infections can also be mediated by differences in host immune responses at 15 °C and 25 °C. Antifungal peptides (gallerimycin and galiomycin) more actively responded to *C. militaris* infection at a higher temperature. This is consistent with previous investigations in which we showed a stronger elevation in phenoloxidase and encapsulation levels in wax moths in response to *C. militaris* infection at 25 °C compared to 15 °C [22], and this correlated with a greater survival of the infected insects at 25 °C. It is interesting that the antifungal peptide genes were actively expressed at 25 °C, not only in the fat body but also in the midgut. This may be due to a systemic immune response or an attack of lateral midgut tissues by the fungus. Unlike the antifungal response, the expression of antibacterial peptide genes (gloverin, cecropin, lysozyme) was more active in the fat body at 15 °C, which correlated with the absence of elevated CFUs and bacterial decomposition at this temperature. It was previously shown that a short exposure of *G. mellonella* to low temperatures led to an increase in AMP expression in response to *B. thuringiensis* infection [28]. Similar exposure led to enhanced AMP expression in *Ostrinia furnacalis* in the absence of infection [29]. Elevated antibacterial responses were also observed under prolonged cooling. For example, Ferguson and Sinclair [27] showed that overwintering *Eurosta solidagnis* larvae were characterized by an increased clearance of the gram-positive bacteria *Bacillus subtilis* in the hemolymph compared to autumn and spring larvae. According the present study, under cold conditions, insects may exhibit increased antibacterial responses during fungal infections.

We observed an increase in the expression of the gloverin and lysozyme genes in the midgut in response to fungal infection. This elevation was obviously caused by changes in the microbiota structure and the elevation in the bacterial load in the midgut during the development of mycosis. Similar changes were observed by Ramirez and coworkers [3] in the midgut of adult *Aedes aegypti* mosquitos in the acute stages of mycoses caused by *Beauveria* and *Isaria* species. However, we did not observe general temperature-dependent trends in the expression of antibacterial genes in the midgut.

The IAP gene was upregulated in the fat body at a low temperature and its upregulation in response to the infection was also observed only at a low temperature. Previous studies showed that IAP is

linked to the IMD immune signaling pathway in insects [38]. In particular, knockdown of this gene in *D. melanogaster* led to confined expression of AMPs in response to bacterial infections and increased susceptibility to gram-negative bacteria [38]. In locusts, IAP knockdown led to blocked defensin expression, which was induced by *Metarhizium acridum* infection [37]. In our experiments, a lack of IAP expression at 25 °C was associated with a lower upregulation of antibacterial peptides and an active proliferation of bacteria in the hemolymph, which is consistent with the abovementioned studies.

The NOX-DUOX domain gene displayed an interesting pattern of expression. This system functions in the regulation of bacterial homeostasis, as has been shown in *Drosophila* and mosquitoes [40,42,80]. In our study, the gene was upregulated slightly in the fat body in response to fungal infection at both temperatures. In the midgut, we observed a significant downregulation of this gene at 15 °C (Table 2). This was correlated with a trend toward increased enterococci CFU counts in the midgut at 15 °C compared to 25 °C (Figure 3). This gene was downregulated in response to *C. militaris* infection only at 15 °C. This may be caused by the high acuity of mycosis at this temperature and it may be a consequence of the prioritization in immune reactions between the hemocoel and gut, as was suggested by Wei and coworkers [2]. However, this decrease in gene expression and the elevation in the enterococci load at 15 °C did not lead to the colonization of the hemocoel by bacteria, i.e., the proliferation of bacteria occurred only in the gut lumen under this temperature. Further immunological and histopathological studies are needed to establish the mechanisms of septicemia development during fungal infections.

We observed an upregulation of HSP70 in both tissues and an upregulation of HSP90 in the midgut at a low temperature. This result was expected because an increase in the expression of these genes during cold diapause has been observed in various insect taxa [46]. We also observed a downregulation in HSP70 expression in the fat body in response to *C. militaris* infection. Previous studies reported either an increase in HSP expression in different tissues of *G. mellonella* after infection with *B. bassiana* and *Conidiobolus coronatus*, or no change compared to uninfected insects [7,61,81]. These inconstancies may be caused by differences in pathogenesis that occur after infection with different fungal species and strains. Regarding the antibacterial response, it was shown that HSP70 transcripts were highly induced in arthropods (*Penaeus monodon*) after injection with bacteria *Vibrio* [82]. In wax moths, an increase in HSP90 expression was observed in response to *Bacillus thuringiensis* infection [83] and mixed (bacteria and yeast) infections [84]. Linder and coworkers [16] suggest that HSPs may improve immune functions against bacterioses at cool temperatures in *Drosophila melanogaster*. The authors have shown elevated expression of HSP83, PGRP-LS and AMPs, and increased resistance to bacteria (*Pseudomonas aeruginosa* and *Lactococcus lactis*) in cold conditions (17 °C) compared to warm conditions (29 °C). Similarly, in our work, septicemia was observed most often with the lowest levels of HSP expression (fungal infection at 25 °C), although we did not observe any correlations between HSP and AMP expression. It is possible that increased expression of HSPs at low temperature may help maintain tissue integrity in gut and other organs and prevent penetration of bacteria into hemolymph.

5. Conclusions

Bacterial associates of insects may influence the development and outcome of fungal infections. Using a model system of *C. militaris* and *G. mellonella*, in the present study we found that these interactions are significantly dependent on temperature. At high temperatures, these relationships develop in favor of spontaneous bacterioses, while under low temperatures they develop in favor of mycoses. The explanation for these outcomes may lie in the properties of the fungus, as well as in the immune reactions of the host during mycosis development. *C. militaris* is a weak competitor of bacteria and therefore it requires low temperatures to avoid antagonism with bacterial associates of the host to complete its development successfully. In addition, we observed weakened antifungal responses along with increased antibacterial responses in wax moths at a low temperature, which should be beneficial for the development of the fungus. We confirmed the previous works that have shown that AMP expression in *G. mellonella* is temperature-dependent [18,23,28]. However, a comparison of AMP

expression in response to fungal infection at constant low (15 °C) and moderate (25 °C) temperatures was performed for the first time. Our results are consistent with previous studies in which short cooling of *G. mellonella* [28] and prolonged cooling of other lepidopterans [27] have led to an increase in antibacterial response. Moreover, we found increased expression of stress-related genes in the midgut under the constant low temperature, which may prevent the disruption of gut tissues and penetration of bacteria from the gut into the hemocoel. Further studies should focus on the interaction between bacterial growth and fungal infections using histopathological and histomolecular approaches, as well as on development of *C. militaris* in natural hosts using natural methods of infection. Our research may promote physiological and ecological studies into the interactions between pathogenic fungi, insect hosts and bacterial associates.

Supplementary Materials: The following are available online at http://www.mdpi.com/2309-608X/6/3/170/s1, Figure S1. Rarefaction curves of the OTU numbers for each sample. Table S1. List and description of genes and primer sequences used in qPCR. Figure S2. Diversity indexes of bacterial communities in the midgut of wax moth larvae at 96 h post injection of *C. militaris* (2500 conidia per larva) with incubation at 15 °C and 25 °C. Indexes were calculated for OTU levels. Different letters indicate significant differences between treatments (Dunn's test, $p < 0.05$). Figure S3. Wax moth larvae overgrown with *B. bassiana* (A) and *M. robertsii* (B) at four days after injection with 2500 conidia per larva and incubation at 25 °C.

Author Contributions: V.Y.K. and O.T. designed experiments, performed bioassays, analyzed data, prepared figures and tables and wrote the main manuscript text; E.K. and U.R. performed gene expression analysis and wrote the manuscript; O.P., M.T. and O.Y. performed bioassays and sample preparation, and approved the final draft; T.A. and M.K. performed 16S rRNA sequence analyses and wrote the manuscript; O.Y. and V.G. managed the project and approved the final draft. All authors have read and agreed to the published version of the manuscript.

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Review

Fungi–Nematode Interactions: Diversity, Ecology, and Biocontrol Prospects in Agriculture

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Abstract: Fungi and nematodes are among the most abundant organisms in soil habitats. They provide essential ecosystem services and play crucial roles for maintaining the stability of food-webs and for facilitating nutrient cycling. As two of the very abundant groups of organisms, fungi and nematodes interact with each other in multiple ways. Here in this review, we provide a broad framework of interactions between fungi and nematodes with an emphasis on those that impact crops and agriculture ecosystems. We describe the diversity and evolution of fungi that closely interact with nematodes, including food fungi for nematodes as well as fungi that feed on nematodes. Among the nematophagous fungi, those that produce specialized nematode-trapping devices are especially interesting, and a great deal is known about their diversity, evolution, and molecular mechanisms of interactions with nematodes. Some of the fungi and nematodes are significant pathogens and pests to crops. We summarize the ecological and molecular mechanisms identified so far that impact, either directly or indirectly, the interactions among phytopathogenic fungi, phytopathogenic nematodes, and crop plants. The potential applications of our understanding to controlling phytophagous nematodes and soilborne fungal pathogens in agricultural fields are discussed.

Keywords: nematophagous fungi; cross-kingdom interactions; food-web cycling; phytophagous nematodes; soilborne fungal pathogens

1. Introduction

Ecosystems consist of many types of organisms, including different types of microscopic organisms such as bacteria, archaea, protozoa, fungi, and small animals such as nematodes. Together, these organisms interact with each other and with macroscopic organisms such as plants and large animals to perform ecosystem functions. Their interactions happen in multiple ways, can be direct or indirect, involving two or more partners, and occur through different mechanisms such as predation, parasitism, mutualism, or competition. These interactions are critical for maintaining ecosystem balance [1].

Fungi and nematodes are among the most abundant organisms in the terrestrial ecosystem. The phylum Nematoda, also known as the roundworms, is the second largest phylum in the animal kingdom, encompassing an estimated 500,000 species [2]. Ninety percent of terrestrial nematodes reside in the top 15 cm of soil, and they play an important role in the nitrogen cycle by way of nitrogen mineralization. Nematodes do not decompose organic matter but instead are parasitic or

free-living organisms that feed on living materials [3]. On the other hand, fungi are the principal decomposers of dead organic matter; they perform fundamental roles in nutrient cycling in the ecosystem. Although fungi may look like plants, they are in fact evolutionarily more closely related to animals than to plants. Both fungi and nematodes (as well as all animals) are heterotrophs. They are commonly found co-existing in a diversity of natural and man-made ecosystems, especially in the rhizosphere of plants, including crops, with significant impacts on agriculture and forestry. Consequently, interactions among fungi and nematodes have attracted significant attention.

Nematode and fungi arose about 550–600 mya and 1050 mya, respectively. They likely co-existed and interacted with each other in soils before plants colonized terrestrial habitats about 450 mya [4,5]. The co-existence and interactions between nematodes and fungi, whether antagonistic or mutualistic, direct or indirect, are fundamental for understanding their ecosystem effects and their potential manipulations in agriculture. An important long-term goal in agriculture pest and pathogen management is to identify novel control strategies against phytophagous nematodes and soilborne fungal pathogens, to help increase both the quality and quantity of agricultural products. In this review, we summarize our current knowledge of the interactions between nematodes and fungi. Specifically, we focus on the interactions between these two groups of organisms that have shown both antagonistic and mutualistic interactions to each other, either directly or indirectly (Figure 1). We note that the nature of their interactions can vary greatly among the different fungal and nematode species. Furthermore, the interactions between two organisms are not static but can be impacted by environmental factors to influence both the type and magnitude of their interactions [6].

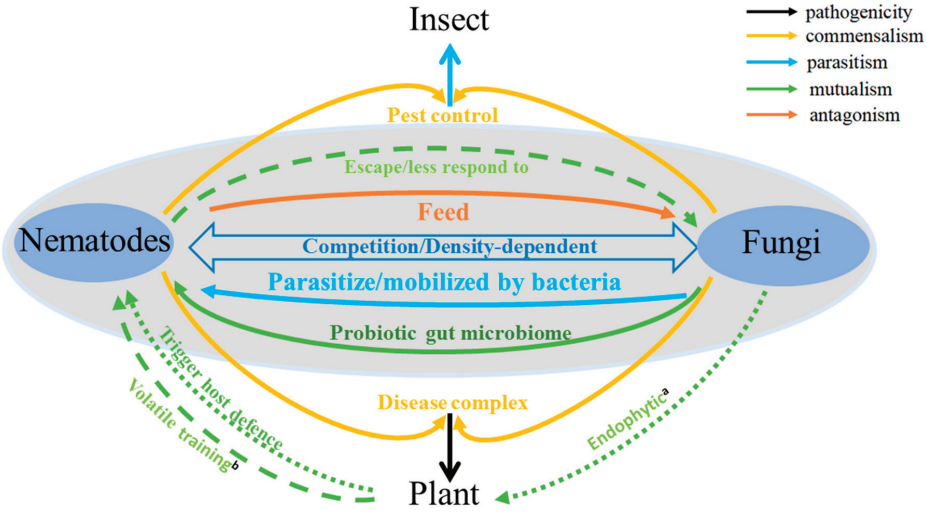

Figure 1. Fungi-nematode interactions in soil. a: Endophytic fungi trigger host plant defense against plant pathogenic nematodes (PPNs). b: Plants help nematodes escape fungal attacks through volatile training.

2. Antagonistic Interactions

Antagonistic interactions between fungi and nematodes are as numerous as they are varied. For example, many nematodes, such as *Aphelenchus avenae*, *Aphelenchoides* spp., and *Paraphelenchus acontioides*, can feed on a diversity of fungi. These are commonly referred to as fungivorous nematodes [7]. In contrast, a number of fungal species such as *Arthrobotrys oligospora* can prey on nematodes and their eggs, consuming them as food. Such fungi are known as nematophagous fungi [8].

2.1. Nematodes Feeding on and Antagonizing Fungi

Many of the nematodes have fungi in their diets or feed exclusively on fungi [9]. Thus, as a major component of the soil food web, nematodes can influence both the fungal diversity and abundance and community structure, including crop growth and tolerance to soil pollution. Fungivorous nematodes commonly exist in soil containing many different fungal species. Nematodes in the genera *Aphelenchus*, *Aphelenchoides*, *Ditylenchus*, and *Tylenchus* are among the most common fungivorous nematodes [10]. Generally, fungivorous nematodes feed on a diversity of soil fungi, including saprophytic, plant-pathogenic, and plant-beneficial (such as mycorrhizal) fungi and are known as polyphagous nematodes [11]. While the population densities of fungivorous nematodes in soil may be lower than those of phytoparasitic nematodes and bacterivorous nematodes, the population densities of fungivorous nematodes can increase rapidly in the presence of suitable fungal food [10]. Depending on the soil microbiome, nematode feeding on soil fungi could have significant impacts on soil ecology and crop productivity. For example, if fungivorous nematodes were to feed on plant-pathogenic fungi, the phytopathogen population in the soil could be suppressed. However, if mycopathogenic fungi (e.g., species in the genera *Gliocladium* and *Trichoderma*) antagonistic to plant-pathogenic fungi are found to be the food of nematodes, then the beneficial effects of these antagonist fungi to plants would be reduced due to the actions of these nematodes. All these fungi with different relationships to nematodes and to each other can be present in the same ecological niches. In addition, the food fungi for nematodes are not all identical. Different food fungi may present different attractiveness to the fungivorous nematodes and that attractiveness may vary depending on the environmental conditions. Furthermore, both fungi and nematodes are mobile, in different ways, to allow them to disperse across ecological niches [2,9].

One group of fungi that nematodes like to feed on is the mycorrhizal fungi. Indeed, the interactions between mycorrhizal fungi and fungivorous nematodes have been the subject of intensive investigations because of the potential effects of grazing on the function of the mycorrhiza in nutrient uptake and growth of the host plants. Indeed, surveys have found that fungal fruiting bodies (mushrooms) produced by ectomycorrhizal fungi often contain nematodes. Such fungal grazing by nematodes can have other effects, such as the release of nutrients immobilized in fungal biomass as resources for bacteria [12]. Aside from ectomycorrhizal fungi, endomycorrhizal fungi also interact with nematodes. For example, the reproduction of *Aphelenchoides* sp. nematodes can be triggered by the co-inoculation of arbuscular mycorrhizal (AM) fungi, which lead plants to achieve further growth and greater arsenic (As) tolerance at low As-polluted soil. [13]. This could have significant implications for changing the composition and infectivity of field assemblages of AM fungi. On the other hand, nematodes of the genus *Aphelenchus* can prevent the symbiosis between endomycorrhizal fungi of the genus *Glomus* with pine roots. In such instances, fumigant nematicides need to be used to disinfest the soil before pine seedling planting can be successful. Indeed, fumigation not only increases the endomycorrhizal infection of pine roots but also enables the pine trees to utilize the dead nematodes as an excellent pabulum [6].

Fungivorous nematodes could be multifunctional. *A. avenae* is a non-parasitic fungivorous nematode that can control plant-pathogenic fungi [7]. For example, both *A. avenae* and *Aphelenchoides* spp. suppressed *Rhizoctonia solani* and reduced the damping of disease in cauliflower seedlings [14]. In addition, *A. avenae* can suppress the propagation of the plant parasitic nematode *Ditylenchus destructor*, suggesting that it is a potential biocontrol agent against both certain plant-pathogenic fungi and plant parasitic nematodes [15]. Genetic analyses of cell wall-degrading enzymes from *A. avenae* support the roles of these enzymes in feeding on both plant pathogenic fungi and a plant parasitic nematode [16]. Interestingly, in the pinewood nematode *Bursaphelenchus xylophilus*, a cellulase similar to those in fungi and associated with the ability to parasitize living plants was identified as most likely the result of horizontal gene transfer, acquired during its evolution of plant parasitism [17].

In the fungal prey–nematode predator relationship, just like other types of prey–predator relationships, the fungal prey can develop resistance mechanisms against nematode predation.

One type of fungal prey defense is the production and secretion of toxic secondary metabolites and toxic proteins [18]. For example, the model mushroom *Coprinopsis cinerea* produces a toxic substance on its mycelial surface that can kill nematodes upon contact [19]. Indeed, upon predation by nematodes, *C. cinerea* exhibited a comprehensive set of differentially expressed genes (DEGs) and the production of a bacterial cytolysin-like toxin. Some of these DEGs in *C. cinerea* represent a novel type of fungal effector protein against nematodes [20].

2.2. Fungi Antagonizing Nematodes

The above examples show nematodes antagonizing and feeding on fungi; the opposite can also happen and is known to be quite common in nature. The interaction between nematophagous fungi and nematodes has played a crucial role in understanding broad fungi–nematode interactions. Nematophagous fungi, including those that are variously called predaceous, nematode-trapping, and nematode-destroying fungi, possess amazing abilities to capture nematodes and reduce the population size of plant-parasitic nematodes. Such abilities have significant applied interests in agriculture [21,22]. Studies of nematophagous fungi and their interactions with nematodes have revealed several mechanisms of their interactions at the molecular, cellular, organismal, and ecological levels. Indeed, such studies have propelled their interactions as models for studying inter-kingdom interactions in predator–prey coevolution, and for biocontrol applications [23–29].

2.2.1. Diversity and Evolution of Fungal Predation Structures

Nematophagous fungi have been traditionally divided into four main groups based on the mechanisms that they use to attack nematodes: (i) nematode-trapping fungi, producing extensive hyphal networks, knobs, and constricting rings as trapping devices to catch and hold live nematodes; (ii) endoparasitic fungi, as obligate parasites that exist as conidia in the environment and infect nematodes by either adhering to the surface of the prey or by directly being ingested by the nematodes followed by germination, growth, and nematode killing; (iii) egg- and cyst-parasitic fungi, as facultative parasites that grow on and parasitize the sedentary stages of nematodes such as eggs; and (iv) toxin-producing fungi, producing toxic compounds that are active against nematodes [30,31]. Except for the egg stage, most nematodes at other life stages are capable of moving through their environments, which presents a challenge for relatively slow-growing and immobile fungal parasites. However, many fungi have evolved to parasitize mobile stages of nematodes by employing complex and sophisticated predation structures, including (1) trapping structures to immobilize nematodes; (2) adhesive conidia to attach and colonize the nematodes' pseudocoeloms; (3) acanthocytes, spiny balls, and stephanocysts to damage the cuticle of nematodes and then consume them; and (4) gun cells to launch finger-like tubes directly at the target nematodes [8,21,32].

The interaction between nematophagous fungi and nematodes induces morphogenesis and virulence gene expression in these fungi, signaling a transition from their saprobic stage to phagocytic stage. Evolutionarily, nematophagous fungi are widely distributed across many phylogenetically-independent taxonomic groups, indicating that the ability to phagocytize nematodes has evolved multiple times [23,33]. Among the nematode-trapping fungi, there are also multiple types of trapping structures, including constricting rings and five types of adhesive traps (sessile adhesive knobs, stalked adhesive knobs, adhesive nets, adhesive columns, and non-constricting rings), all of which were originated from the vegetative hyphae [34]. Consistent with frequent and independent origins of nematode-trapping devices, members of the Orbiliaceae produce five types of traps, among them *Arthrobotrys dactyloides, Arthrobotrys superba, Arthrobotrys oligospora*, and *Monacrosporium gephyropagum* are capable of forming conidial traps—traps formed directly from the asexual spore, the conidia. At a low nutrient level, competition for nutrients among microorganisms can be intense; thus, the ability of fungal spores to directly germinate into the traps could be highly advantageous [35,36]. Consistent with convergent evolution in some trapping structures, two groups of fungi from two different phyla, namely *Zoophagus* species of Zygomycota and *Nematoctonus* species of Basidiomycota, can both

produce adhesive knobs [37]. However, traps based on adhesive hyphae are restricted to the fungal genera *Stylopage* and *Cystopage* of Zygomycetes [38], while the prominent fungi parasitizing cyst nematode juveniles, *Hirsutella rhossiliensis* and *Hirsutella minnesotensis*, are representatives of adhesive spores [30]. Some species of endoparasites have developed morphologically-adapted conidia that, when eaten by the nematodes, become lodged in either its buccal cavity or esophagus. These species belong almost exclusively to the genus *Harposporium* [39]. Among other nematode-trapping fungal structures, stephanocysts are restricted to the genus *Hyphoderma* of Basidiomycota [40]. Spiny balls and acanthocytes are known only by *Coprinus comatus* and *Stropharia rugosoannulata*, respectively, in Agaricales of Basidiomycota [41,42]. Finally, a very peculiar attack device called the "gun cell" is produced by endoparasitic fungi in the genus *Haptoglossa* (Oomycete fungi) [43].

Among the broad groups of nematophagous fungi, those that form specialized morphological adaptations to capture nematodes are especially interesting. These nematode-trapping fungi (NTF) can switch their lifestyle from saprophytes to predators under certain cues. Such transitions have made them good models for studying inter-kingdom communication with regard to the mechanisms of fungal pathogenesis and adaptation [23,27,44]. In recent years, -omics studies have significantly improved our understanding of host–microbe interactions, especially in those cases where the microorganisms are difficult to grow under laboratory conditions [45]. In the case of fungi–nematode interactions, sequencing of the genomes of the nematode female and egg parasite *Pochonia chlamydosporia* [46]; the nematode-trapping fungi *Arthrobotrys oligospora* [44], *Monacrosporium haptotylum* [25], and *Drechslerella stenobrocha* [47]; and the facultative nematode endoparasite *H. minnesotensis* have greatly contributed to our understanding of the evolutionarily distinct strategies of fungal pathogenesis against nematodes [48].

Using NTF in the phylum Ascomycota as models, phylogenies based on genes and genomes from both the nuclei and the mitochondria support that, within the Orbiliales, the nematode-trapping mechanisms have evolved along two major lineages. In one lineage, the species form constricting rings. In the second, the species form adhesive traps, including three-dimensional hyphal networks, adhesive hyphal branches, and adhesive knobs [23,33,49]. Furthermore, a combined five-gene phylogeny and molecular clock calibration based on two fossil records revealed that the organismic interactions between NTF and nematodes likely dates back to more than 419 million years of co-evolution, with the active carnivores (fungi with constricting rings) and passive carnivores (fungi with adhesive traps) diverged from each other around 246 Mya, shortly after the occurrence of the Permian–Triassic extinction event about 251.4 Mya [23]. However, no major carnivorous ascomycete divergence has been correlated to the Cretaceous–Tertiary extinction event. More research is needed to identify if the evolution of fungal carnivorism was a response to mass extinction events.

Despite the diverse morphogenesis, different kinds of nematode traps share two structural features that are different from vegetative hyphae. The first one is the presence of numerous cytosolic organelles, commonly known as dense bodies [50]. These dense bodies are peroxisomal in nature and only detected in nematode-trapping fungi, but not in endoparasitic nematophagous fungi that infected their host with adhesive or non-adhesive spores [51]. Their functions seemed to be involved in adhering to nematodes and supplying energy and/or structural components to the invading hyphae [51]. A recent study showed that disruption of the gene *Aoime2* caused reductions in both trap formation and electron-dense bodies in trap cells [52], with substantially fewer nematodes captured by the mutants. The second feature common to the adhesive traps (columns, networks, and knobs) is the presence of extensive layers of extracellular polymers, which are thought to be important for adhesion of the traps to the surface of nematodes [53]. Recent genome comparisons and surface structural analyses revealed evidence for expansion of adhesion genes in NTF genomes and with associated increase in trap surface adhesiveness. Both of these can enhance the ability of the fungi to penetrate and digest the nematodes and likely represent the key drivers of fungal adaptation in trapping nematodes [27].

2.2.2. Host Recognition, Adhesion, Host Specificity, and Infection Process

As a shared characteristic among all types of nematophagous fungi, recognition of the hosts and adhesion to the cuticle of the nematodes or eggshells by the fungi are the first steps in infection. The nematode cuticle is a solid exoskeleton that mainly consists of proteins. The exoskeleton acts as a barrier against environment stresses and potential pathogen attacks [54]. At present, how NTF penetrates the nematode exoskeleton has not been fully elucidated. Current research results suggest that secreted enzymes from NTF play a major role during invasion of the nematodes by the fungi. Specifically, genetic, ultrastructural, and histochemical studies showed that the presence of extracellular hydrolytic enzymes such as chitinases, collagenases, and proteases are essential for nematode cuticle penetration [55]. Indeed, phylogenetic analysis of the pathogenicity-related serine proteases from nematophagous and entomopathogenic fungi showed that they evolved from a common ancestor [56]. Penetration is typically followed by content digestion, resulting in the formation of a new fungal biomass inside and later outside the nematodes. Table 1 shows the four main steps of infection from the four main groups of nematophagous fungi, plus the producers of special attack devices (structures which mechanically damage the cuticle of nematodes, as the fifth group) [21,30].

Table 1. Infection process of nematophagous fungi.

Group	Representative Species	Host Recognition	Adhesion	Penetration	Digestion
Nematode-trapping fungi	*A. oligospora*	Mediated by lectin, proteins on the fungal surface interacting with sugar molecules on the nematode cuticle (GalNAc [57], AOL [58], AofleA [59], AoMad1 [60]), a nematode specific pheromone ascaroside [24], or olfactory mimicry that attracts nematode prey [29].	A physical contact between the trap cells and the nematodes, cell-to-cell communication [61], a group of volatile organic compounds furanone, pyrone, and maltol [62], nitrate [63] and autophagy [64] are required for switching from the saprophytic to the pathogenic stage during trap formation and adhesion.	Fungi pierce the cuticle by forming a penetration tube, with a combination of mechanical pressure and extracellular hydrolytic enzymes, such as serine proteases (PII [65], Aoz1 [55], Ac1 [66], Ds1 [67] Dv1 [68], Mlx [69], Mc1 [70]), collagenase, and chitinase [55].	Nematode content is converted to lipid droplets, these fungi obtain nutrients from the nematodes for their growth and reproduction.
Endoparasitic fungi	*Drechmeria coniospora*	Obligate parasites, using conidia that are ingested by their host, or by spores that adhere to the cuticle of the host [71].	Adhesive conidia that adhere to the nematode cuticle will form an appressorium that presses firmly against the nematode cuticle. Motile zoospores encyst on the nematode's surface and germinate to produce the injection tube, to infect nematodes by injecting a sporidium [37].	A combination of enzymatic action and mechanical force, followed by vigorous growth of the trophic hyphae, to invade nematodes [72–74].	New conidiophores develop from bulbs at the tips of trophic hyphae inside the cadaver, tightly pressed to the internal surface of the cuticle, preventing leakage of host nutrients, perturbing nematode metabolism, and causing nematode death [75].
Egg- and cyst-parasitic fungi	*Pochonia chlamydosporia*	Aurovertin D showed strong toxicity and recognition of host [76].	Glycoproteins and appressoria responsible for the adhesion of conidia and hyphae to the eggshell [77].	Proteases and chitinases, e.g., PrC from *Clonostachys rosea* and Ver112 from *Lecanicillium psalliotae* [78].	Colonizes the host tissues to obtain nutrients and uses available sugars in the egg as a carbon source [46].
Toxin-producing fungi	*Pleurotus ostreatus*	Induces paralysis via the cilia of nematode sensory neurons [79].	All developmental stages of *C. elegans* are sensitive to *P. ostreatus*. Nematodes become paralyzed upon contacting the *P. ostreatus* hyphae.	Excess calcium influx and hypercontraction of the head and pharyngeal muscle cells in nematodes.	Toxins cause rapid and systemic necrosis in multiple tissues throughout the organism.
Fungi producers of special nematode-attacking devices	*Coprinus comatus; Stropharia rugosoannulata*	Sharp projections of the special attack devices, mechanically damage the cuticle of the nematode [41,42].	A penetration peg is formed and penetrates the nematode cuticle via mechanical forces and enzymatic activities.	Hyphae colonize the interior of the nematode and project themselves from the infected nematode.	Need toxin assistance to be successful in their nematicidal role (spiny balls).

Nematode-trapping fungi (NTF) are usually not host specific and can trap many types of soil-dwelling nematodes [80]. In contrast, there is some host specificity among endoparasitic fungi. The endoparasitic fungi are obligate parasites and mostly exist as conidia in the environment. The conidial attachment to a particular nematode species does not always lead to infection, but specific recognition signals for adhesion are required, as shown by the endoparasitic fungus *Drechmeria coniospora* [71,81]. Fungi that parasitize nematodes are common soil saprophytes, attacking primarily the sedentary stages (female and egg stages) of nematodes or sedentary nematodes, such as *Heterodera*, *Globodera*, and *Meloidogyne* [30]. Nematode-toxic fungi have nematode-immobilizing activity and can kill their nematode hosts by producing toxins. The success and efficiency of nematode attacks by producers of special attacking devices are also sometimes linked to the toxins produced [41]. Special attacking devices are similar to a sharp sword or acanthocytes, spiny balls, and stephanocysts, like real medieval weapons, causing damage to the nematode cuticle, resulting in extravasation of the inner contents of the nematodes and allowing complete colonization of the nematode body by fungal hyphae.

2.2.3. Competition between Nematode-Trapping Fungi and Nematodes

Evolutionary arms races are common between pathogens and hosts. Evidence for such arms races has been found between nematodes and NTF. In these arms races, fungal predators continuously evolve predatory strategies to secure food from nematodes. In turn, the prey nematodes evolve counter measures, such as enhanced innate immunity and sophisticated nervous systems to sense and avoid their predator fungi. Many factors can influence such arms races. For example, in soil environments, the populations of NTF and their target nematodes not only interact with each other as predators and prey but also with other fungi and nematodes nearby, respectively. In addition, biotic factors such as other microbes and plants as well as abiotic factors such as nutrient levels can also influence NTF–nematode interactions. Systematic studies on the bitrophic (NTF and nematode) or multitrophic (plant, soil microorganisms, nematode, and NTF) interactions under natural conditions are required to obtain a broad understanding of the factors influencing the ecology and evolution of such interactions. Below we summarize our current understanding of the potential mechanisms involved in the arms race between NTFs and nematodes.

Innate Immune Defense Responses in Nematodes

The epidermis and the collagen-rich cuticle that surrounds the nematode provide a physical barrier to fungal pathogens. Nematodes can also sense and defend against fungal pathogens using strategies such as producing antimicrobial peptides regulated by the innate immunity system. To cope with bacterial and fungal pathogen attacks from the intestine or the cuticle, the innate immune response of *Caenorhabditis elegans* is accompanied by an increase of reactive oxygen species (ROS) [82]. The nematode genomes also contain many antimicrobial peptide (AMP)-coding genes that play important roles in their innate immunity. In one study, when *C. elegans* was infected, one of the AMPs, NLP-31, showed strong activities against several fungi, including *Drechmeria coniospora*, *Neurospora crassa*, and *Aspergillus fumigatus* [83]. The recent expansion of the AMP-encoding *nlp* genes as revealed by genome sequencing, together with the evidence for their in vivo role and the signatures of positive selection of the *nlp29* gene cluster, suggest that these genes are important for the survival of *C. elegans* when they interact with *D. coniospora* spores [84]. The FOXO transcription factor DAF-16, which lies downstream of the conserved insulin/IGF-1 signaling (IIS) pathway, is required for survival after fungal infection and wounding [85]. RNA-seq analysis further identified shared and unique signaling pathways regulated by DAF-16/FOXO and highlighted the intestinal DAF-16 regulatory components and roles of the innate immune system countering fungal pathogenesis [86].

Competition between Different Fungal Species and Nematodes

To survive and reproduce, nematodes and NTF need to successfully cope with many stressors and competing demands in soil. Competition can be among different fungal predators, among different nematodes, and between NTF and nematodes [87]. Surveys have found that multiple NTF species often coexist in the same niche, suggesting that they likely compete for the same prey in their natural environments. For example, *Arthrobotrys* species are sympatrically distributed and are generalist predators of nematodes. Two species in *Arthrobotrys*, namely *A. thaumasia* and *A. musiformis*, are sympatric with nematodes in more than 63% surveyed natural sites. In addition, the ability to sense prey among wild isolates of *Arthrobotrys oligospora* varied greatly [28]. Some nematodes are trapped/colonized by more than one NTF at the same time (e.g., colonized from opposite ends of the nematodes). In some of those cases, evidence for competition between NTFs has been found. For example, the hyphae of *A. oligospora* were often observed to be dead or degenerated when placed in close proximity to live mycelia of the endoparasitic fungus *D. coniospora*, consistent with the latter being an antagonist against *A. oligospora* under the specific conditions [88]. El-Borai et al. [89] indicated that the tested nematodes were repelled by activated *Arthrobotrys* species but were attracted to activated endoparasitic fungi from the genera *Myzocytium* and *Catenaria*.

Antagonistic interactions between NTF and nematodes have been detected in the soil environments. As expected, density-dependent parasitism has been reported, demonstrating that an increase in NTF density would lead to a decrease in nematode prey density, which subsequently would lead to a decrease in NTF density and an increase in nematode density. This negative frequency-dependent selection between NTF and nematodes regulates the densities of both groups of organisms [90]. This model successfully described changes in parasitism of the nematode *Heterodera schachtii* by the nematophagous fungus *Hirsutella rhossiliensis* as a function of host density, with the disease dynamics in soil microcosms exhibiting both a temporal density-dependent parasitism and a host threshold density [91]. Suppression of the root-knot nematode *Meloidogyne javanica* by NTFs *Monacrosporium cionopagum* and *H. rhossiliensis* was positively related to the nematode host *Steinenema glaseri* density, and the dynamics of suppression varied among different species [92]. In addition, spatial sampling of the nematophagous fungus *H. rhossiliensis* revealed a relationship between numbers of hosts (*Criconentella xenoplax*) and the degree of parasitism [93], with evidence of the two interacting partners possessing similar density-dependent dynamics among tested patches of agricultural fields [94]. Recent greenhouse trials also showed that parasitism of *H. rhossiliensis* was strongly correlated with the density of the soybean cyst nematode [30]. At broader geographic scales, in a survey of 53 citrus orchards in central ridge and flatwood ecoregions of Florida, the spatial patterns of entomopathogenic nematode species were found to be modulated by variations in their susceptibilities to nematophagous fungal species (*Catenaria* sp., *A. musiformis, Arthrobotrys dactyloides, Paecilomyces lilacinus, A. oligospora*, and *Gamsylella gephyropaga*) across habitats [95]. However, strong and diverse top-down control effects on the nematode community in coastal sand dunes were found in a recent study, where three microbial enemies of nematodes (*Catenaria* spp., *H. rhossiliensis*, and *Pasteuria penetrans*) were correlated, either positively or negatively, with plant parasitic nematode population size [96]. Together, these results are consistent with some species-specific effects for both the fungal and the nematode partners in natural and agricultural ecosystems. Aside from these individualized surveys, metagenomic methods have also been used to analyze the relationships between fungi and nematodes (as well as bacteria) in field settings [97] and revealed a diversity of spatial associations similar to those described above between plant parasitic nematodes and NTFs [98,99].

3. Synergistic Interactions between Phytophagous Nematodes and Phytopathogenic Fungi against Host Plants

3.1. Interactions between Phytophagous Nematodes and Soil-borne Fungal Pathogens

In the soil environment, opportunities exist for interactions between soil-borne pathogens and pests of plants when they occupy the same ecological niche. While antagonism can occur between them in their competitions for space and resources, synergistic interactions between them are also possible to cause greater damage to plants, including crops. For example, in the rhizosphere, nematode attacks can lower the resistance of plants to pathogens and increase their susceptibility to infection by soil-borne fungal pathogens. In these situations, the physiological states of all three interacting partners play a very important role in the outcome of such tripartite interactions.

The first example of a nematode–fungi disease complex in plants was described by Atkinson, in 1892, where he reported that the fusarium wilt of cotton (caused by *Fusarium oxysporum* f. sp. *vasinfectum*) was more severe in the presence of root-knot nematodes (*Meloidogyne* spp.) [100]. Subsequently, many other cases of synergistic interactions between nematodes and fungi have been reported, involving sedentary endoparasitic root-knot and cyst nematodes, and increasing disease severity caused by *Fusarium* or *Verticillium* wilt fungi. *Meloidogyne* spp. has been shown to interact with *Fusarium* wilt to negatively impact a number of crops, with cyst nematodes acting in a similar manner to increase wilt diseases. Meanwhile, entomopathogenic nematodes and pathogenic fungi have been shown capable of generating additive interactions to increase insect pest mortality [101]. In these cases, an initial fungal infection plays a key role in weakening the larvae and increasing the pest insects' susceptibility to nematodes by generating a stressful condition and altering the insects' behavior [102]. Table 2 summarizes recent examples of nematode–fungi pathogen disease complexes reported in crops and insects.

Table 2. Examples of nematode–pathogen disease complexes reported in crops and insects.

Nematode	Pathogen	Crop/Insect	Reference
Steinernema diaprepesi	*Fusarium solani*	Wax moth, Weevil	[103]
Heterorhabditis bacteriophora, *Steinernema feltiae*, *S. kraussei*, *H. sonorensis*	*Metarhizium anisopliae*	Black vine weevil	[104]
Meloidogyne incognita	*F. oxysporum*	Corn earworm	[105]
M. incognita	*F. oxysporium f.* sp. *phaseoli*	Bean	[106]
M. incognita	*F. oxysporium f.* sp.	Potato	[107]
M. incognita	*Rhizoctonia solani*	Green bean	[108]
M. incognita	*Phytophthora capsici*	Pepper	[109]
M. spp.	*F. oxysporum f.* sp. *lycopersici*	Tomato	[110]
M. spp.	*F. oxysporum*, *F. solani*	Tomato	[111]
M. javanica	*F. oxysporum f.* sp. *lycopersici*	Tomato	[112]
M. incognita	*F. solani*	Fig	[113]
M. incognita	*F. oxysporum f.* sp. *niveum*	Watermelon	[114,115]
M. incognita	*Ralstonia solanacearum*, *Phomopsis vexans*	Eggplant	[116]
M. incognita	*Alternaria dauci*, *Rhizoctonia solani*	Carrot	[117]
Pratylenchus spp., Trichodoridae, Heteroderidae	*Rhizoctonia solani*	Potato	[118]
S. feltiae, *S. carpocapsae*, *H. bacteriophora*,	*Aspergillus* spp., *Penicillium* spp.	Carob moth	[119]
S. diaprepesi	*F. solani*	Weevil	[120]

3.2. Factors Influencing Interactions between Phytophagous Nematodes and Phytopathogenic Fungi

As shown above, the nature of interactions between phytophagous nematodes and phytopathogenic fungi varies among the different fungal and nematode species. For example, some plant pathogenic nematodes can induce physical damage, such as small wounds, to their host plants. Such wounds may allow fungal pathogens easy access to plant tissues to cause infections. Alternatively, some nematodes may induce physiological changes in their host plants, triggering changes

in fungal pathogen populations around the host plants and making them more likely to increase their population size and/or pathogenicity [121]. In addition, other biotic and abiotic factors such as host plant genotype, organic matter and nutrient availability, and other microbes could all affect the outcome of infections by nematode pests and plant fungal pathogens [117].

In agriculture fields, the fungal species composition can vary, depending on whether the fields are infested by root-knot nematodes. *Fusarium oxysporum* (11%) followed by *Fusarium solani* (6%) were found to be the most frequent fungal species associated with the presence of *Meloidogyne* spp., and fungal diversity plays an important role in the interactions between host plants and soil microorganisms [111]. For example, inoculation of certain bacterial and fungal combinations together had an inhibitory effect on each other and reduced crop disease severity [112]. On the other hand, abiotic factors such as soil moisture and soil physicochemical properties also play important roles for infection by plant fungal pathogens and nematode pests [112].

Another interesting interaction between nematodes and fungi that could have important effects on agriculture is that between entomopathogenic nematodes and entomopathogenic fungi. Together, these entomopathogenic pests and pathogens could help control pest insect populations in agricultural fields. However, to realize their potential, it is important to understand the life cycles of both the entomopathogenic nematodes and entomopathogenic fungi, and to develop strategies to allow them to grow in the same ecological niches with minimal negative impacts on each other [102]. Indeed, a previous study has shown that the virulence of both the entomopathogenic nematode *Steinernema riobravae* and entomopathogenic fungus *Beauveria bassiana* against last larval instars of *Galleria mellonella* could be synergistic or additive, depending on environmental conditions and application strategies [122].

4. Fungi and Nematodes Interact through a Third Party

In most natural soil ecosystems, fungal species co-occur with nematodes, and both often actively interact with plants. This cross-kingdom interaction between fungi and nematodes in the plant rhizosphere is often called a tripartite interaction. Other organisms, such as bacteria, may also be involved in this network of interactions. These interactions may involve direct cell–cell contacts. Alternatively, they may interact indirectly, using chemical signals. Indeed, chemical signals such as volatile organic compounds released by organisms such as bacteria, nematodes, fungi, or plants have been detected to initiate interactions between fungi and nematodes. Due to the ubiquitous distributions of these organisms in natural environments and agricultural fields, their interactions have significant ecological and economic impacts. Thus, it is important to develop a comprehensive understanding of such interactions involving all partners in terrestrial and agricultural ecosystems.

4.1. Induced Resistance

There is a broad range of detrimental microbes and nematodes that can challenge the plant's capability for growth and survival. However, their effects on plants vary depending on other microbes in the same ecological niches. For example, colonization of plant roots by beneficial endophytic and mycorrhizal fungi can protect plants against a wide range of plant-parasitic nematodes through plant mediated mechanisms [123,124]. One example of a beneficial endophyte is the fungus *Trichoderma harzianum*. This fungus can induce jasmonic acid (JA)- and salicylic acid (SA)-regulated defense pathways in tomato (*Solanum lycopersicum*), causing resistance to the root-knot nematode *Meloidogyne incognita*. Similarly, mycorrhizal fungi represent an inextricable part of almost every plant system. Their role in suppression of plant pathogenic nematodes (PPNS) has been extensively studied and reviewed [125]. For example, plants associated with arbuscular mycorrhizal fungi (AMF) showed decreased damages caused by sedentary endoparasites than those without AMF colonization. The antagonistic action of mycorrhizal fungi against PPNS may be achieved directly, e.g., by competition for nutrients and space, or indirectly, by increasing plant tolerance, mediating induced

systemic resistance (ISR) in plants, changing rhizosphere interactions by altering root exudations, or all of the above [1].

Interestingly, some nematophagous fungi can colonize plants as endophytes. Thus, they are also capable of mediating ISR against nematodes in situ. Most studies investigating fungi–nematode interactions are conducted outside of the plant hosts; few have considered the effects of these fungi in the context of plant endophytes. The soil borne *Phialemonium inflatum* is a known nematode egg-parasite fungus, which can secrete extracellular proteases and chitinases and significantly reduce hatching of *M. javanica* juveniles [126]. A recent study revealed a novel role for this fungus. Specifically, a foliar-isolated *P. inflatum* strain was shown to be endophytic in cotton, and part of a plant-fungal defensive symbiosis in cotton. Using a seed treatment inoculation, this isolate showed significant inhibitory effects against the root-knot nematode *M. javanica* as an endophyte in cotton. This was the first study demonstrating antagonistic effects of endophytic *P. inflatum* against root-knot nematodes [127]. Similarly, compared to treatments with only the nematode *M. incognita* or with neither the nematode nor the *A. oligospora,* treatment containing endophytic and rhizospheric populations of *A. oligospora* showed reduced nematode population size and increased defense-related enzymes in tomatoes [87]. The inoculation of *Drechslerella dactyloides* and *D. brochopaga* also significantly increased plants' resistance against *M. incognita* [128].

Another example is an endophytic strain of *Pochonia chlamydosporia* that caused a moderately induced expression of genes involved in ISR in barley (*Hordeum vulgare*) [129]. However, in this study, the plants were not challenged with plant-parasitic nematodes to conclusively demonstrate priming for resistance to nematodes [129]. *Arabidopsis thaliana* root colonization by *P. chlamydosporia* showed modulated jasmonate signaling that resulted in accelerated flowering and improved yield [130]. Further studies showed that the effects were due to chitosan-mediated increases in root colonization by *P. chlamydosporia* [131,132]. Overall, these studies contribute to potential future applications of endophytes to increase plant tolerance/resistance to RKN.

4.2. Alteration of Root Exudates

Plant roots typically have a close association with mutualistic rhizosphere microorganisms. Together, they exude a wide range of both primary metabolites and secondary metabolites. Such metabolites can modify the surface properties of nematodes and affect microbial attachment to nematode surfaces [133]. In parallel, the success of the nematode infection and inhibition by the nematophagous microbes depends on how the plant roots and their associated microbes perceive the signaling molecules on the nematode surfaces. Indeed, metabolites exuded from plant roots affect not only the communication between plants and nematodes, but also the nematode–fungi interactions by modulating the surface properties of nematodes. One of the mechanisms of nematode suppression by *Fusarium* endophytes appears to be through altering root exudates [134]. A similar mechanism was proposed for AMF-mediated nematode suppression. In both tomatoes and bananas, AMF colonization of roots altered root exudates, leading to fewer nematodes penetrating AMF compared to roots with non-AMF colonization [135]. Specifically, root exudates altered by the nematophagous fungus *Pochonia indica* stimulated the hatching of mobile second-stage juveniles (J2s) that were dormant in nematode cysts [136], which subsequently caused a major inhibitory effect on the development of *Heterodera schachtii* in *Arabidopsis* roots [137]. *H. schachtii* is a plant pathogenic nematode capable of infecting more than 200 different plants including economically important crops such as sugar beets, tomatoes, bananas, cabbage, broccoli, and radish.

The modes of action of mycorrhizal fungi against PPN may be exhibited through a direct effect, by competition for nutrients and space, or indirect effect, by increasing plant tolerance, mediating ISR in plants, altering rhizosphere interactions due to changed root exudations, or all of these combined, depending on the species of both AMF and nematodes [125]. Further research on the nematode/microbial selectivity in the attachment and the influence of plants on these interactions could open up possibilities for manipulating these interactions to improve plant health.

4.3. Chemical Signals

In their natural habitats, nematodes and fungi exist as parts of complex multitrophic communities that depend on and communicate through elaborate networks of chemical signaling. A key feature of microbe–microbe interaction is the secretion of chemical mediators that can influence interactions involving both microbial partners and the co-occurring multicellular organisms. Both nematodes and fungi have developed elaborate communication systems that are based on secretion of chemicals, allowing intra- and inter-kingdom interactions.

The most common chemical signals in the evolution of predator–prey relationships are those related to recognitions of specific pathogens or food sources [138]. Recent studies based on the model nematode *Caenorhabditis elegans* identified ascarosides, a group of small molecules, as involved in inter-organismal communications. These chemicals play a central role in regulating nematode development and behavior [139,140]. Under nitrogen starvation, ascaroside-induced morphogens are required for *A. oligospora* to sense and initiate trap formation in response to the availability of nematode prey [24,141]. Recent studies have also characterized several other morphology-regulating arthrosporol metabolites from *A. oligospora* and identified them as important signaling cues for hyphal development, nematode attraction, and trap morphogenesis [29,62,142].

Ammonia is another molecule identified to influence interactions among multiple interacting partners. Specifically, it has been reported as an intracellular signal for altering fungal morphological switch and mediating interspecific interactions among bacteria, fungi, and nematodes [143]. Wang et al. [26] demonstrated that when bacteria were consumed by bacterivorous nematodes, urea production and release were enhanced by upregulating the arginase's expression. The urease within the fungi eventually catabolized urea to ammonia, which initiates formation of predatory structures. Similarly, diketopiperazines (DKPs) were shown to facilitate chemotaxis of *Stenotrophomonas* bacteria towards fungal extracts, leading to bacterial biofilm formation on fungal nematode traps and enhancing fungal trapping activity against nematodes [36].

In the relationship among entomopathogenic nematodes, plant, insects, and nematophagous fungi, behavioral plasticity of entomopathogenic nematodes in response to a plant volatile organic compound (ᴅ-limonene) affected nematode–fungi interactions. Two mechanisms were suggested for their interactions. In the first, nematodes' response to d-limonene may make them less likely to respond to other environmental stimuli, such as to attractants released by nematophagous fungi. In the second, the learned response by nematodes to plant volatiles may motivate entomopathogenic nematodes to move faster in the event of being exposed to such volatiles, potentially making it harder to catch them by fungal traps [144,145].

4.4. Microbiome

Due to technological advances in high-throughput DNA sequencing, more and more studies have examined structures of microbial communities (microbiomes), both in soil ecosystems and in nematodes' guts in order to evaluate other biotic factors potentially involved in mediating interactions between nematodes and fungi [146]. These studies have revealed that while some microbes can exert antagonistic effects on both nematode pests and plant fungal pathogens, others can form mutualistic interactions with plant disease-causing agents. For example, in the root microbiomes of many plants, nematophagous fungi in genera *Clonostachys*, *Dactylellina, Purpureocillium*, *Pochonia*, and *Rhizophydium* often co-occur. However, the nematode-suppressing efficiency in such soils is often limited. Understanding the relationships among members of native anti-nematode microbiome are required for building effective approaches to develop nematode pest-suppressing soil [147]. For example, successive monoculture of soybeans clearly affected the assembly of both bacterial and fungal communities (i.e., the genera *Pseudomonas, Purpureocillium*, and *Pochonia*,) in the rhizosphere and negatively impacted the rhizosphere microbiome in its ability to suppress the soybean cyst nematodes [148]. Crop rotation may reverse such negative effects.

On the other hand, studies have revealed that the native microbiomes of nematodes carry a species-rich bacterial community dominated by Proteobacteria such as Enterobacteriaceae and members of the genera *Pseudomonas*, *Stenotrophomonas*, *Ochrobactrum*, and *Sphingomonas*. Several studies highlighted the influence of microbiota on *C. elegans* fitness, stress resistance, and resistance to pathogen infection [149]. For example, three *Pseudomonas* isolates were identified to be able to produce an anti-fungal effect in vitro and contribute to the worm's defense against fungal pathogens in vivo [146,150]. Another study using *C. elegans* suggested that probiotic yeasts colonizing the nematode gut protected nematodes from infection with non-albicans *Candida* strains and alleviated pathogenic effects [151]. Unexpectedly, among the >5000 culturable fungal isolates obtained from the mycobiome of soybean cyst nematodes, using in vitro high-throughput screening, a large proportion of these cultured fungi showed bioactivity against nematode egg hatching or showed toxicity toward J2 stage nematodes [152]. Together, these results suggest that supplying one or a few fungi with anti-nematode activities to the soil environments are not sufficient to suppress the nematode populations.

5. Applications of Our Understanding in Fungi–Nematode Interactions in Agriculture: The Control of Phytophagous Nematodes and Soilborne Fungal Pathogens

The rhizosphere contains a complex of biological and ecological processes. A better understanding of fungi–nematode complexes could benefit the development of ecologically based management tools to control important plant pathogen and crop pests. Discoveries of antagonistic interactions between nematodes and some rhizospheric microorganisms can provide the basis for developing control strategies to enhance plant defense against soil-borne plant pathogens and root-knot nematode parasites, including *Meloidogyne* spp., etc. Below we discuss a few potential approaches.

5.1. Nematodes as Biocontrol Agents against Plant Pathogenic Fungi

Because fungal-feeding nematodes can be attracted to and actively feed on plant pathogenic fungi, these nematodes can potentially be used to reduce the load of fungal plant pathogens and minimize the effects of these fungal pathogens on crops.

Some species of the nematode genus *Aphelenchoides* feed on the cytoplasm of fungal hyphae by piercing and sucking using a strong stylet [37]. When mixed with the mycopathogenetic fungus *Trichoderma* spp., these nematodes are able to feed on two plant fungal pathogens, namely *Botrytis cinerea* and *Sclerotinia sclerotiorum* [6], and the combination of *T. harzianum* and *Aphelenchoides* nematode treatment resulted in the best disease control efficiency against fungal diseases [153]. One species in this nematode genus, *Aphelenchoides hylurgi* is able to parasitize both virulent and hypovirulent strains of the fungus *Cryphonectria parasitica*, the causal agent of chestnut blight [154]. Furthermore, the nematodes were also able to spread propagules of the hypovirulent strain, thus increasing the efficacy of biological control under field conditions. Another fungal-feeding nematode, *Aphelenchus avenae*, also showed strong abilities to reduce pathogen loads of two root-rot fungi in corn [155], *Rhizoctonia solani* and *Fusarium solani* [156,157], as well as one root-rot fungus, *Fusarium oxysporum*, in beans and peas [158]. However, fungivorous nematodes are often not discriminatory in their food fungal choice. They can also feed on fungi with potential beneficial effects to plants. For example, *Trichoderma harzianum*, an extensively studied biocontrol agent against the sclerotium-forming fungus, *Sclerotinia sclerotiorum*, is also a favorite food for the fungivorous nematode *Aphelenchoides saprophilus*. Consequently, *A. saprophilus* can reduce the biocontrol efficiency of *T. harzianum* against *S. sclerotiorum* [159].

At present, most fungivorous nematodes reported in the literature are those that are easy to propagate in large numbers and can be stored in a dormant stage (anhydrobiosis) for a relatively long time. They have so far not been extensively applied to agriculture or horticulture fields in the form of nematode applications. This is mainly due to the high costs associated with the production, storage, and distribution of fungivorous nematodes for commercial applications. One way to realize such commercial potential is to combine fungivorous nematodes with other agricultural practices

such as crop rotation and the application of other biocontrol agents to reduce the costs and maximize the benefits.

5.2. Biocontrol of Nematodes with Nematophagous Fungi

It is estimated that, worldwide, plant parasitic nematodes (PPNs) cause a combined >$150 billion worth of damages to agriculture each year [31]. From an ecological perspective, this group of nematodes is one of many components in the ecosystem that interact with other organisms, contributing to the maintenance and stability of the soil food-web. Over the last 30 years, our understanding of microbial diversity and the multitrophic interactions that are manifested in the rhizosphere, as well as biological control systems as they apply to nematodes, has improved tremendously. Indeed, several environmentally benign strategies have been developed for PPN management. Among PPNs, the root-knot nematodes (RKNs; *Meloidogyne* spp.) represent the most severe challenges to crop production. In a summary of the biocontrol methods evaluated between 2015 and April 2020, 10 microfungi and 3 mushroom species were tested for their effectiveness in controlling RKNs [160]. However, most studies were conducted in laboratories and greenhouse settings and their efficacies in the field are not known. Converting the laboratory successes into equally effective field applications represents the next step of the challenge.

5.2.1. Potential for the Discovery of Novel Candidates

It has been estimated that the number of culturable fungal species is between 2.2 and 3.8 million. On the basis of a 1:8.8 ratio between the numbers of cultured fungal species and the number of fungal operational taxonomic units estimated based on metagenome sequencing, there would be approximately 12 million fungal species on earth [161,162]. At present, only ~1.2% (140,000) of these have been described [163]. Thus, many new fungi with potential nematophagous activities await discovery. Even among the known culturable fungi, new compounds with novel mechanisms of nematode-parasite action have been continuously found. For example, in the fungus *Pleurotus ostreatus*, anthelmintic compounds were recently isolated and showed potent activity against a wide range of nematode species. It is possible that there are many novel fungi and novel fungal compounds effective at controlling parasitic nematodes of plants, animals, and humans [79].

Over the last few years, high-throughput sequencing of the universal barcode locus for fungi (18S, ITS rDNA) has revealed great potential for identifying fungi in many ecological niches. However, the fungal community comparisons between niches with different nematodes, especially those with different phytopathogenic nematodes, have been very limited. Microbiome studies of such ecological niches could help reveal the microbial diversities responsible for the differential distributions of phytopathogenic nematodes and assist in developing holistic management strategies with multi-target modes of action to control these pests. Integration of microfluidics, robotics, and machine learning technologies in interaction studies in microcosms between the microbiome and nematodes could provide novel ways to capitalize on our knowledge about the core microbiomes of pest nematodes. Such knowledge could help increase control efficiency and stress-resistance of biocontrol applications [164]. On the other hand, novel molecular markers could be developed to analyze the parasitic activities and population dynamics of nematophagous fungi. Such tools could allow us monitoring these fungi and their activities in agricultural fields.

5.2.2. Development and Integration of New Methods

To achieve successful and reproducible biological control, we must understand the ecological interactions affecting the control agent and the target. Modern technologies can help us achieve such goals. For example, real-time quantitative PCR provides an effective way to quantify and track biocontrol agents after they are applied to soil [165]. Similarly, genetic modifications of the biocontrol agents could be used to help the organisms overexpress traits involved in pathogenicity or nematocidal activity [8,166].

Several studies have demonstrated the effectiveness of using combinations of treatments, including various cultural practices (like soil solarization and soil amendment), chemical nematicides, and biological agents in controlling PPN populations under various conditions [80]. These studies have revealed that soil physical chemical properties can have a significant influence on their control efficacies. Thus, attention should be paid to develop biocontrol protocols that are specific for targeting ecological niches.

The use of nematophagous fungi as endophytes, i.e., rhizosphere colonization by biocontrol agents, is a promising strategy for implementing biocontrol of plant-parasitic nematodes. Endophytes should be relatively easy to apply as inoculants to seeds or seedlings and could therefore be established in the root system before nematodes are attracted to roots [167].

Finally, the unpredictability and relatively low efficacy of nematode antagonists against PPN in field conditions are major obstacles for the application of biocontrol agents for managing plant-parasitic nematodes. Part of the reasons for the differences between laboratory-based and field-based trial results may be related to the intrinsic mechanisms regulating ecosystem stability in field conditions. Application of a large number of a specific organism would disturb the balance of interactions among organisms in native niches, with the target interaction between the applied biocontrol agent and PPN in the field not realized. Thus, understanding how organismal interactions in native niches are regulated could help us develop better applications that take into account native agricultural ecosystems to ultimately produce sustainable methods of crop protection while maintaining biodiversity. Studies that evaluate the effects of coadministration of multiple partners such as nematophagous fungi, mycoparasites of plant pathogens, and plant growth promotors could help generate significant data to allow a systems approach in developing biocontrol measures to minimize the effects of nematode pests and fungal pathogens on agricultural crops [168,169].

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