Identification and Pathogenicity of a New Entomopathogenic Fungus, *Mucor hiemalis* (Mucorales: Mucorales), on the Root Maggot, *Bradysia odoriphaga* (Diptera: Sciaridae)

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Abstract

*Bradysia odoriphaga* Yang and Zhang (Diptera: Sciaridae), the Chinese chive root maggot, is a destructive pest of *Allium* vegetables and flowers that causes severe losses in northern China. Novel biological control technologies are needed for controlling this pest. We identified a new entomopathogenic fungus isolated from infected *B. odoriphaga* larvae and evaluated the susceptibility of the biological stages of *B. odoriphaga* and the effects of temperature on fungus growth and pathogenicity. Based on morphological characteristics and molecular phylogeny, the fungus was identified as *Mucor hiemalis* BO-1 (Mucorales: Mucorales). This fungus had the strongest virulence to *B. odoriphaga* larvae followed by eggs and pupae, while *B. odoriphaga* adults were not susceptible. A temperature range of 18–28°C was optimum for the growth and sporulation of *M. hiemalis* BO-1 and virulence to *B. odoriphaga* larvae. At 3 and 5 d after inoculation with 10⁵ spores/ml at 23°C, the survival rates were 24.8% and 4.8% (2nd instar larvae), respectively, and 49.6% and 12.8% (4th instar larvae), respectively. The potted plant trials confirmed that *M. hiemalis* BO-1 exerted excellent control efficiency against *B. odoriphaga* larvae, and the control exceeded 80% within 5 d when the spore concentration applied exceeded 10⁷ spores/ml. In conclusion, these findings supported the hypotheses that this fungus could serve as an effective control agent against *B. odoriphaga* larvae and is worth being further tested to determine its full potential as a biocontrol agent.

Graphical Abstract

Key words: entomopathogenic fungi, *Bradysia odoriphaga*, biocontrol agent, pathogenicity bioassay, control efficiency
Biological control is an important component of Integrated Pest Management (IPM), owing to its reduced impact on the environment and its substantial efficacy (Shah et al. 2003, Hajek et al. 2007). The biological control agents of pests include predators, parasitic insects, entomopathogenic fungi, entomopathogenic bacteria, insect viruses, and entomopathogenic nematodes (Lacey et al. 2001). Entomopathogenic fungi (EPF) comprise a diverse group that includes potential biocontrol agents of many destructive pest species (Bale et al. 2008, Gabarry et al. 2012). Several entomopathogenic fungi have been used for pest control, including Beauveria bassiana (Balsamo), Metarhizium anisopliae (Metschnikoff), Nomuraea rileyi (Farlow), Verticillium lecanii (Zimmerman), and Paecilomyces fumosoroseus (Wite) (Coombes et al. 2016). However, despite the great variety of entomopathogenic fungi, only a few species have been successfully developed as biopesticides. Beauveria bassiana and Metarhizium anisopliae are potential candidates for the biological control of many agricultural pests, such as Lepidoptera, Coleoptera, and Diptera (Kassa et al. 2004, Khoury et al. 2019).

Bradysia odoriphaga Yang and Zhang (Diptera: Sciaridae), the Chinese chive root maggot, is a serious pest of Allium vegetables and oramental flowers in northern China (Yang and Zhang 1985, Xue et al. 2005). The larvae tend to aggregate in fields and directly damage plants by feeding on root and corm tissues, resulting in wilt or rot (Zhang et al. 2016). Since the damage caused by B. odoriphaga larvae is cryptic and primarily occurs on the underground portions of plants, it is difficult to effectively control (Zhu et al. 2017, Shi et al. 2018). To date, the most common management practice for B. odoriphaga larvae is applications of organophosphates, carbamates, and neonicotinoid insecticides (Ma et al. 2013). However, after many years of treatments, larvae have developed resistance to pesticides, and this has resulted in unsatisfactory control (Bai et al. 2016a). In addition, conventional chemical pesticide use has been increasingly restricted due to environmental pollution and human health concerns (Shan et al. 2020). Therefore, exploring biological control resources is important for reducing pesticide applications, decreasing environmental pollution, and improving pest population management.

Previous studies reported that compared to other agricultural pests, there are few applicable biological control agents of B. odoriphaga. These include Beauveria bassiana (entomopathogenic fungus), Bacillus thuringiensis (entomopathogenic bacteria), Stratiolaeaps scimitus (predatory mite), and Steinernema feltiae (entomopathogenic nematodes) (Chen et al. 2016, Zhou et al. 2014). Song et al. (2016) isolated a Beauveria thuringiensis strain JQ23 from soil, possessing high insecticidal activity against B. odoriphaga. This strain had a 72 hr LC50 of 8.38 x 10^6 spore/ml against 2nd instar larvae. Only Beauveria bassiana, Bacillus thuringiensis, and entomopathogenic nematodes have been applied to control B. odoriphaga in the field (Chen et al. 2016). Zhou et al. (2014) reported that when the dosage of Beauveria bassiana granules (15 billion spore/kg) was 4.5 kg/ha, the field control efficiency was 81.15% (7 d after application) and 85.19% (21 d after application). Although many entomopathogenic fungi possess good pathogenicity in the laboratory, their field efficacy depends on favorable environmental conditions, such as temperature, humidity, and characteristics of the microorganism (Ekesi et al. 1999, Fargues et al. 2003). For example, most entomopathogenic fungi exert good pathogenicity at optimum temperatures (Mwamburi et al. 2015), and the optimum temperatures of Beauveria bassiana that provided the best control efficiency ranged from 25 to 30°C (Fargues et al. 1997, Shimazu 2004). Bradysia odoriphaga is an underground insect in its immature stages and adults leave the soil. However, during the emergence peak of B. odoriphaga, the mean soil temperature in fields is typically below 20°C, which is not optimal for many biological insecticides (Shi et al. 2020). To sum up, finding an entomopathogenic fungus possessing high pathogenicity and broad environmental adaptability for use against B. odoriphaga appears to be key to improving bio-control efficiency.

The genus Mucor includes many species of filamentous fungi widely distributed in soil that are usually associated with abundant humus. Most species are saprotrophic and widely applied in fermentation processes. Individual Mucor species are well known as pathogens of animals and humans, although there are a few Mucor species reported as insect pathogens. Reiss et al. (1993) reported that some Mucor hiemalis species were pathogenic to Artemia salina, an important arthropod. Konstantopoulos and Mazomenos (2005) isolated six species of entomopathogenic fungi from diseased Bactrocera oleae and Sesamia nonagrioides. Bioassays revealed that two strains of M. hiemalis were pathogenic to Ceratitis capitata larvae causing more than 80% mortality. This is the only report concerning the pathogenicity of M. hiemalis to Diptera.

In 2019, we isolated one strain of entomopathogenic fungi from infested B. odoriphaga larvae collected from a Chinese chive field. Morphological observation indicated that this strain was a Mucor species. In the process of culture, this strain was strongly pathogenic and infectious, causing laboratory population extinction within a few days once spore transmission spread occurred. We suspected that this entomopathogenic fungus had the potential for development as a biocontrol agent.

In this study, we isolated the strain from infected B. odoriphaga larvae using morphological and molecular analyses. The susceptibility of the life stages of B. odoriphaga to this entomopathogenic strain and the effects of temperatures on fungus growth and pathogenicity were evaluated. Additionally, the control efficiency was also conducted by a potted plant trial. Our results provide a new biological control agent for application in B. odoriphaga control and increase available options for exploring the green control techniques for crop root maggots.

**Materials and Methods**

**Insect Materials**

Samples of B. odoriphaga larvae were originally collected from a Chinese chive field in Tai’an, Shandong, in April 2015. The samples were maintained at the College of Agronomy, Liaocheng University, and reared on Chinese chives for more than 10 generations using the method described by Xue et al. (2005). Eggs, larvae, and pupae were reared in culture dishes (Φ = 9 cm), and pairs of newly emerged adults were placed in plastic oviposition containers (3 cm diameter × 1.5 cm high). Colonies were maintained in growth cabinets at 23 ± 1°C with 75 ± 5% relative humidity.

**Entomopathogenic Fungi Isolated From Infested Larvae**

Infested B. odoriphaga larvae were collected in winter from Chinese chives field in Liaocheng, Shandong (N 36.784, E 115.447, 10 November 2019). According to the method described by Barra et al. (2013), all symptomatic B. odoriphaga larvae with signs of fungal infection were surface sterilized with a 1% sodium hydrochloride solution dip for 1 min. Subsequently larvae were washed three times with distilled water and placed on Potato Dextrose Agar (PDA) plates (Φ = 9 cm). Plates were incubated at 23°C for 3 d to obtain growth of mycelia occurring internally, and the hypha at the edge of
the colony was picked and transferred to new PDA plates to obtain a pure strain.

Identification of Entomopathogenic Fungi Isolates
Morphological Description
The pure entomopathogenic fungus strain was incubated at 23°C, and the microscopic features (morphology of hyphae, fruiting bodies, and spores) and macroscopic features (colony morphology and color) were examined by microscopic observation methods described by Nyongesa et al. (2015) and Gerald (1995).

Molecular Phylogenetic Analysis
DNA from a fungal isolate was extracted using an EasyPure Fungi Genomic DNA Kit (ComWin Biotech, Beijing, China). Sequences containing the region encoding ITS was PCR amplified with primers described by Hinrikson et al. (2005) (ITS4: 5′-TCCTCCGCTTATTGATATGC-3′, ITS5:5′-GGAAGTAAAAAGTCTGAAACAAGG-3′). The fungal DNA was sequenced by BioSune Limited Company (Shanghai, China). Resulting ITS sequences were aligned using ClustalW in Mega v. 6.0, and their homologies were determined by BLAST searches within the NCBI (National Center for Biotechnology Information) database. A phylogenetic tree of the isolate was constructed using model strains of genus Mucor hiemalis (Taxonomy ID: 91627, Accession: NR_152948.1), Mucor fusiformis (Taxonomy ID: 1197867, Accession: NR_111660.1), Mucor sonoe (Taxonomy ID: 2054155, Accession: NR_165210.1), Mucor bacilliformis (Taxonomy ID: 1196329, Accession: NR_145283), Mucor zonatus (Taxonomy ID: 1095376, Accession: NR_105368), Mucor rudolphii (Taxonomy ID: 1776151, Accession: NR_152977), Mucor lilaneae (Taxonomy ID: 1776150, Accession: NR_152978), Mucor cortenius (Taxonomy ID: 101144, Accession: NR_168144), Mucor pseudocircinelloides (Taxonomy ID: 2021226, Accession: NR_169896), Mucor lusitanicus (Taxonomy ID: 29924, Accession: NR_126127), and Mucor ramosissimus (Taxonomy ID: 90264, Accession: NR_103627). This phylogenetic tree was constructed with MEGA v7.0, by the neighbor-joining method.

Effects of Environmental Temperatures on Fungus Growth
Pure fungal isolate agar disks (Φ = 8 mm) containing mycelium were made by a fungal puncher from the edge of the pure colony. Every agar disk was placed upside down at the center of a PDA plate (Φ = 9 cm). The plates were incubated in growth chambers at different temperatures (13, 18, 23, 28, and 33°C) according to a study on the effects of temperature gradients on B. odoriphaga (Li et al. 2015). At 23°C temperature was used as the control. Every temperature treatment contained five plates (replicates). The colony diameter was recorded daily using the cross-bonded method. Colony growth was recorded as mean perpendicular radius minus the diameter of the inoculum plug. After 10 d, the spore suspension of every plate was prepared using 50 ml 0.1% Tween 80 distilled water according to the description by Mwamburi et al. (2015), and the spore suspension concentration was calculated with blood counting chamber analysis.

Pathogenicity Bioassays
Pathogenicity to Biological Stages of B. odoriphaga
Serial spore suspension dilutions (1 × 103, 1 × 105, and 1 × 107 spores/ml) were made with 0.1% Tween 80 distilled water. Eggs, 2nd and 4th instar larvae, pupae, and adults were used as the test subjects. Bioassays on 2nd and 4th instar larvae (within 1 d after molt) were conducted using standard contact and stomach bioassay methods with slight modifications (Zhang et al. 2016). One piece of filter paper (Φ = 9 cm) was moistened by dropping 1 ml of spore suspension and placed in a culture dish, and fresh diet (fresh Chinese chives) was placed on the filter paper. Larvae were placed around the fresh diets. Eggs, pupae, and adults were reared in culture dishes (Φ = 9 cm) covered with filter paper moisturized by 1 ml spore suspension. This bioassay was conducted at 23°C, and pure water treatment was used as the control. Each treatment (concentration) contained five replicates and each replicate contained 20 individuals. B. odoriphaga mortality was recorded daily.

Pathogenicity Under Different Temperatures
According to the results of pathogenicity to biological stages, spore suspension (1 × 105 spores/ml) possessing moderate pathogenicity to B. odoriphaga larvae was chosen as the test dosage. The spore suspensions (1 × 107 spores/ml) were made with 0.1% Tween 80 distilled water. Bioassays on 2nd and 4th instar larvae were conducted using a previously described method. The culture dishes after adding spore suspension and test larvae were transferred to different temperature conditions (13, 18, 23, 28, and 33°C). Each treatment contained five replicates and each replicate contained 20 individuals. Larval survival was recorded daily for 7 d.

Trials with Potted Plants
Serial spore suspension dilutions (1 × 103, 1 × 105, and 1 × 107 spores/ml) were made with 0.1% Tween 80 distilled water. The 2nd and 4th instar larvae of B. odoriphaga were used as the test subjects. Potted Chinese chives (Xuejiu Variety) planted for two years were prepared. Pots with no larval damage were chosen as the experimental material. Before the trial, the aboveground part of Chinese chives was cut off and 2nd or 4th instar larvae were transferred into the rhizosphere soil. On the day of the experiment, 25 ml of spore suspension was added to the soil of every pot. Pure water (without spores) treatment was used as the control. Each treatment contained five replicates and each replicate contained 40 individuals. During the experiment, adequate water was added daily to maintain soil moisture. All the pots were maintained in growth chambers at 25 ± 1°C with 75 ± 5% relative humidity, and a 12:12 hr light: dark cycle. On the 3rd and 5th day after treatment, the larval survival of B. odoriphaga was recorded. The control efficiency was calculated according to Equations (1) and (2):

\[
\text{Survival rate} = \frac{\text{The number of survival insects}}{\text{The total number of tested insects}} \times 100\% 
\]

\[
\text{Control efficiency} = \frac{\text{Survival rate of control group} - \text{Survival rate of treatment group}}{\text{Survival rate of control group}} \times 100\% 
\]

Statistical Analysis
Statistical analysis was performed using SPSS statistics software (Version 19.0, SPSS, Chicago, IL). Values in the heat hardening treatments were compared by one-way ANOVA followed by Tukey’s HSD multiple comparison test (P < 0.05).

Results
Morphological and Molecular Characteristics
Macroscopic Morphological Analysis
This entomopathogenic fungi colonies cultured on PDA were white and slightly transparent, covered with thick long pile like cotton on the side and a rough surface (acellular hyphae) (Fig. 1A).
According to the description by Gerald (1995), small black dots (sporangium) were borne on the tip of the mycelium (Fig. 1A). Microscopic characterization of Mucor hiemalis BO-1 using an optical microscope showed that the hyphae were smooth, aseptate, and polyenular (Fig. 1C). The sporangiophores were globose to subglobose with smooth outer walls and wrapped in the sporangia (Fig. 1D), which were subglobose and at the top of individual aseptate hyphae (Fig. 1B).

Molecular Phylogenetic Analysis of ITS Region
The ITS rDNA region of Mucor hiemalis BO-1 was amplified and sequenced. The length of the ITS sequence was 936 bp and it was deposited in the GenBank database (accession number MN686205). The sequence similarity search using ITS sequences of Mucor hiemalis BO-1 in BLASTN program revealed that this strain and Mucor hiemalis (CBS201.65) were present in the same cluster with 96% sequence similarity, which were well supported by a bootstrap value of 100% (Fig. 2).

Pathogenicity of Mucor hiemalis BO-1 Against B. odoriphaga Larvae
A total of 100 B. odoriphaga larvae treated with Mucor hiemalis BO-1 (1.0 × 10^6 spores/ml) were raised in moist petri dishes and observed with a stereoscopic microscope. In the early stages of infection (24 hr after inoculation), the larval movement became slower. Few food particles were present in larval guts (Fig. 3A). In the mid-course of pathogenesis (48 hr after inoculation), larval behavior was more sluggish, and the larvae barely moved even after being touched by brush. The larval body became transparent gradually. At 48 hr, the larval feeding capacity was maintained, but the food consumption declined dramatically and only a small amount of diet material was seen in the digestive tract (Fig. 3B). At the end of pathogenesis (72 hr after inoculation), the larvae were moribund (only the head and mouthparts moved slightly) and the body was yellow, turbid, and supple (Fig. 3C). In the growth period of the hyphae (120 hr after inoculation), numerous white aerial hyphae grew from the dead larval body, and black sporangia were present at the top of the mycelium (Fig. 3D).

Pathogenicity of M. hiemalis BO-1 to Biological Stages of B. odoriphaga
We observed the infection of M. hiemalis BO-1 on different stages of B. odoriphaga, and the bioassay results indicated that M. hiemalis BO-1 was more pathogenic effect to B. odoriphaga larvae than to eggs, pupae, and adults (Fig. 4). For 2nd instar larvae, the survival rates after inoculation decreased to 75.2% (10^3 spores/ml), 44% (10^3 spores/ml), and 32% (10^3 spores/ml), while that of the control was 96.8%. With the extension of time, the survival rates are declined rapidly. At 4 d after inoculation, the survival rates were 36.8% (10^3 spores/ml), 6.4% (10^3 spores/ml), and 3.2% (10^3 spores/ml). The 4th instar larvae were less susceptible to M. hiemalis BO-1 than the 2nd instar larvae. At 4 d and 5 d after treatment, the survival rates were 53.6% and 42.4% (10^3 spores/ml), respectively, 12% and 5.6% (10^5 spores/ml), respectively, and 5.6% and 4% (10^7 spores/ml), respectively. These rates were higher than those of the 2nd instar larvae.

M. hiemalis BO-1 showed only slight pathogenicity to B. odoriphaga eggs and pupae. At 5 d after treatment, the egg hatchability was 84.12% (10^3 spores/ml), 71.29% (10^5 spores/ml), and 59.51% (10^7 spores/ml), and most newly hatched larvae died within 1 d. The adult emergence rates were 78.4% (10^3 spores/ml), 73.6% (10^5 spores/ml), and 68.8% (10^7 spores/ml). B. odoriphaga adults were scarcely affected based on no significant differences in longevity among the different treatments.

Effects of Temperature on the Growth and Pathogenicity of Mucor hiemalis BO-1

Fungus Growth
The culture results showed that the growth and sporulation quantity of M. hiemalis BO-1 were affected by temperature (Fig. 5). Temperatures ranging from 18 to 28°C were beneficial to mycelial growth, while the mycelium grew poorly at 33°C (Fig. 5A). At 5 d culture, the colony diameter was 5.83 cm (13°C), 8.52 cm (18°C), 8.66 cm (23°C), 8.23 cm (28°C), and 2.84 cm (33°C) (F_{4,9} = 398.397, P < 0.001). After 10 d culture, the sporulation quantities were 5.5 × 10^9 spores/ml (13°C), 1.69 × 10^9 spores/ml (18°C), 3.07 × 10^9 spores/ml (23°C), 1.17 × 10^9 spores/ml (28°C), and 0.86 × 10^9 spores/ml (33°C), and there were significant differences between different treatments (F_{4,10} = 19.969, P < 0.001) (Fig. 5B). We found that 23°C was the optimum temperature for growth and sporulation of M. hiemalis.

Pathogenicity
The bioassay results indicated that the temperature affected the pathogenicity of M. hiemalis BO-1 to B. odoriphaga larvae and temperatures ranging from 18 to 28°C were the most effective for M. hiemalis BO-1 (Fig. 6). For 2nd instar larvae, at 3 d after inoculation with 10^5 spores/ml, the survival rates were 38.4% (18°C), 24.8% (23°C), and 50.4% (28°C). These rates were significantly lower than survival at 13°C (76%) and 33°C (71.2%). For 4th instar larvae, at 3 d after inoculation with 10^3 spores/ml, the survival rates were 58.4% (18°C), 49.6% (23°C), and 65.6% (28°C). These were significantly lower than survival rates at 13°C (85.6%) and 33°C (84%). At 5 d after inoculation, a similar data trend was observed. The relatively high 33°C temperature might suppress the pathogenicity of M. hiemalis BO-1. The survival rates were still 55.2% (4th instar) and 51.2% (2nd instar) until 7 d, and the mortality might have been affected by high temperature. The optimum temperature

Fig. 1. Macroscopic morphological characteristics of Mucor hiemalis BO-1. A (a) white and slightly transparent aerial hyphae on potato dextrose agar (PDA) medium; B (b) small black sporangium; (c) vertical and uniramous sporangiophore; C (d) smooth, aseptate and polynuclear hyphae; (e) branched hyphae; D (f) broken sporangium releasing sporangiospores.
for the pathogenicity of *M. hiemalis* BO-1 was 23°C followed by 18°C and 28°C.

**Results of Potted Plant Trials**

*M. hiemalis* BO-1 provided superior control of *B. odoriphaga* 2nd and 4th instar larvae when the spore suspension concentration exceeded $10^7$ spores/ml. At 3 d after the 2nd instar larvae treatment, the control efficiencies were 33.16% (1 x $10^5$ spores/ml), 50.51% (1 x $10^7$ spores/ml), and 63.77% (1 x $10^9$ spores/ml) (Fig. 7). For 4th instar larvae, the control efficiencies were 16.25% (1 x $10^5$ spores/ml), 36.04% (1 x $10^7$ spores/ml), and 48.22% (1 x $10^9$ spores/ml), respectively. At 5 d after the 2nd instar larvae treatment, the control efficiencies were 45.99% (1 x $10^3$ spores/ml), 62.03% (1 x $10^5$ spores/ml), 81.28% (1 x $10^7$ spores/ml), and 93.78% (1 x $10^9$ spores/ml), respectively (Fig. 7). For 4th instar larvae, the control efficiencies were 29.32% (1 x $10^3$ spores/ml), 49.74% (1 x $10^5$ spores/ml), 70.16% (1 x $10^7$ spores/ml), and 84.29% (1 x $10^9$ spores/ml), respectively.

**Discussion**

In this study, we isolated a new entomopathogenic fungus strain from infested *B. odoriphaga* larvae. Based on morphological and molecular characters, this strain was identified as *Mucor hiemalis*. The unique morphological features of this strain include white colonies covered with fluffy mycelia and globose sporangia on the tips of the mycelium. These features revealed that this isolate was *Mucor hiemalis* (Zhang et al. 2018). Molecular identification (ITS sequence analysis) with supplementary elements was performed to confirm the identification (Ardeshir et al. 2016). Reiss et al. (1993) reported the pathogenicity of 15 species of Mucorales (including *M. hiemalis*) to brine shrimp (*Artemia salina*) larvae, and Bibbs et al. (2013) reported that a new strain of *Mucor fragilis* Bainier had strong pathogenicity to brown widow spiders (*Latrodectus geometricus* Koch). Therefore, this entomopathogenic fungi strain was named *Mucor hiemalis* BO-1.

Biological assays revealed that *Mucor hiemalis* BO-1 had a substantial pathogenic effect on *B. odoriphaga* larvae. After infection, *B. odoriphaga* larval movement slowed, and the insect body became transparent. During infection, food residue in the alimentary canal decreased. Therefore, it appears that *Mucor hiemalis* BO-1 infection disrupted the physiological and digestive functions of *B. odoriphaga* because of nutritional plunder, degrading enzymes, and toxic proteins. Previous studies have reported that *Beauveria bassiana* and *Metarhizium anisopliae* produce insecticidal mycotoxins against pests (Sowjanya et al. 2008, Khoury et al. 2019, Yin et al. 2021). Wei et al. (2017) reported that *Beauveria bassiana* infection resulted in dysbiosis of mosquito gut microbiota and decreasing bacterial diversity, which promoted mortality of the mosquito larvae. Most entomopathogenic fungi gain access to the body cavity through the external cuticle, where they consume nutrients, produce toxins, destroy host cells and eventually kill the hosts (Wang and Wang 2017). A few entomopathogenic fungi invade the body through the digestive tract by direct feeding of insects, where they disrupt larval feeding capacity primarily during the initial stage of pathogenesis (Kubicek...
Fig. 4. Pathogenic effects of *Mucor hiemalis* BO-1 on different stages of *Bradysia odoriphaga*. Data in the figure are the mean ± SE. Different letters over the same column indicate significant differences between different spore concentration treatments at the $P < 0.05$ level as indicated by one-way ANOVA.

Fig. 5. Effects of temperature on mycelial growth (A) and conidiation (B) of *Mucor hiemalis* BO-1. Data in the figure is the mean ± SE. Different letters over the same column from the top indicate significant differences between different temperature treatments at the $P < 0.05$ level as indicated by one-way ANOVA.
Entomopathogenic fungi have been considered as biopesticides because they are an environmentally friendly alternative to chemical insecticides (Shah and Pell 2003, Barra et al. 2013). Clarifying the susceptibility of biological stages of pests to entomopathogenic fungi is crucial for field application and this information be used to effectively target the most damaging insect stages (Shah and Pell 2003, Angel et al. 2005). The susceptibility bioassay results showed that B. odoriphaga larvae are more susceptible to M. hiemalis BO-1 than other life stages, and early-instar larvae are more susceptible than older larvae. In addition, M. hiemalis BO-1 also showed mild pathogenicity to eggs and pupae. At 120 hr after treatment with 10⁷ spores/ml, the survival rate was 59.51% (eggs) and 68.8% (pupae). Although some larvae from treated eggs successfully eclosed, they died within 24 hr. This may have resulted from the strong pathogenicity of M. hiemalis BO-1 to early-instar larvae. However, B. odoriphaga adults were not susceptible to M. hiemalis BO-1. In contrast, Daniel and Wyss (2009) observed higher mortality of Rhagoletis cerasi adults and larvae in contact bioassay for all the fungi (Beauveria bassiana, Metarhizium anisopliae, and Isaria fumosorosea) tested as compared to the other life stages, and the adult life span of R. indifferentes treated with M. anisopliae was shortened and fertility was reduced. Yee and Lacey (2005) also reported that Rhagoletis indifferentes adults were more susceptible to Metarhizium anisopliae than larvae and eggs. These dissimilar results could be caused by differences among the entomopathogenic fungi species, which possess different infection modes, or different types of insect species. In this study, M. hiemalis BO-1 exhibited strong pathogenicity to B. odoriphaga larvae, which is the stage causing the greatest plant damage and economic losses (Zhang et al. 2016).

If an entomopathogenic fungus is used for pest control in the field, it must exert satisfactory insecticidal activity under a variety of environmental conditions (Kubicek and Druzhinina 2007). Temperature is a key constraint restricting the ability and speed of which entomopathogenic fungi to infect and colonize host insects (Fargues et al. 2003, Yeo et al. 2003, Kryukov et al. 2018). Our results confirmed that 18–28°C was the optimum temperature for growth and sporulation of M. hiemalis BO-1. At 13°C and 33°C, growth and sporulation of M. hiemalis BO-1 were reduced in comparison with 23°C. A pathogenicity bioassay confirmed that M. hiemalis BO-1 also possessed stronger pathogenicity against B. odoriphaga larvae at 18–28°C. This agrees with other studies showing little germination and growth of Metarhizium spp at low temperatures (Ekesi et al. 1999, Bai et al. 2016b). The pathogenicity bioassay results were consistent with the biorational assay results. At 13°C M. hiemalis BO-1 exerted a slightly more pathogenic effect than that at 33°C. It is possible that the low temperature merely slowed the pathogenic process of the fungus while the high temperature blocked the pathogenic process. Similarly, Fargues and Luz (2000) and Soyel et al. (2020) reported that a temperature between 20 and 25°C was optimum for the pathogenicity of B. bassiana against Rhodnius prolixus nymphs and Musca domestica larvae and adults, and the fungal virulence declined rapidly at temperatures exceeding 30°C. This indicated that extreme temperatures not only restricted the growth and infection of the fungus, but also accelerated nutrient metabolism resulting in less extracellular enzymes and toxic proteins (Bai et al. 2016b). Previous studies reported that during the emergence peak of B. odoriphaga larvae, the mean soil temperature fluctuates between 15 and 25°C, which would be suitable for the use of M. hiemalis BO-1 to control B. odoriphaga based on our results (Shi et al. 2018, 2020). To sum up, M. hiemalis BO-1 application could provide satisfactory control efficiency during the spring and autumn in open fields, or during winter in the greenhouse. During these seasons, the soil temperature ranges between 10 and 25°C (Shi et al. 2018). After summer and winter, M. hiemalis BO-1 reapplication could increase the soil abundance of this fungus and increase its efficacy. However, the pathogenicity of entomopathogenic fungi is also dependent on humidity and other abiotic factors. In general, dry conditions and poor nutrition are generally unsuitable for the propagation of fungi (Garrido-Jurado et al. 2011). Further studies on the interactive relationships among temperature, humidity, and the pathogenicity of fungi should be conducted.

Many entomopathogenic fungi exhibit good insecticidal activity in the laboratory, but the field control was unsatisfactory. This performance failure has been attributed to complex natural environment conditions (Hussein et al. 2010, Garrido et al. 2011). We simulated field experiments using potted Chinese chives plants to evaluate the potential of M. hiemalis BO-1 for the control of root maggots. The results confirmed that when the concentration of spore suspension exceeded 1 × 10⁷ spores/ml, the control efficiency was satisfactory. At 5 d after treatment, the control efficiencies were 81.28% (1 × 10⁷ spores/ml) and 93.58% (1 × 10⁷ spores/ml).

![Fig. 6. Effects of environmental temperatures on the pathogenicity of Muco hiemalis BO-1 to Bradysia odoriphaga 2nd (A) and 4th (B) instar larvae. Data in the table are the mean ± SE. Different letters over the same column from the top indicate significant differences between different temperature treatments at the P < 0.05 level as indicated by one-way ANOVA.](image-url)
Fig. 7. Control efficacy of Mucor hiemalis BO-1 against Bradysia odoriphaga 2nd and 4th instar larvae. Data in the table are the mean ± SE. Different letters over the same column from the top indicate significant differences between different treatments at the *P* < 0.05 level as indicated by one-way ANOVA.

spores/ml for 2nd instar larvae, and 70.16% (1 × 10^7 spores/ml) and 84.29% (1 × 10^9 spores/ml) for 4th instar larvae. This is an exceptional result compared to other entomopathogenic fungi, which display excellent control efficiency in the field only when the concentration of the spore suspension exceeds 1 × 10^11 spores/ml (Zhou et al. 2014, Coombes et al. 2016, Murigu et al. 2016). M. hiemalis BO-1 possessed excellent control efficiency against B. odoriphaga larvae, and control was rapid, with only three 3–5 d being required to achieve a high level of control. Other biocontrol agents, such as Beauveria bassiana (Zhou et al. 2014), Bacillus thuringiensis (Song et al. 2016) and entomopathogenic nematodes (Wu et al. 2017) require more than 7 d to achieve the same effect. However, combinations with other entomopathogenic fungi or chemical insecticides could provide better control and these should be studied further.

In conclusion, we isolated a new entomopathogenic fungi strain from infested B. odoriphaga larvae. This was identified as Mucor hiemalis BO-1 based on the morphological and molecular characteristics. Bioassay results confirmed that Mucor hiemalis BO-1 exhibited the greatest pathogenicity to B. odoriphaga larvae at 18–28°C, a temperature range that was beneficial to fungal growth and sporulation. A pot experiment confirmed that M. hiemalis BO-1 possessed efficient control efficiency against B. odoriphaga larvae, and control exceeded 80% within 5 d. M. hiemalis BO-1 should be further evaluated for use as a biocontrol agent for B. odoriphaga.

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Author Contributions

The study was jointly conceived by GZ, WD and MX. Experiments were designed by GZ and MX. GZ prepared the manuscript; YZ, MX, WD, ML, and ZL edited the manuscript, and GZ and WD carried out the experiments.

References Cited

Angel-Sahagún, C., R. Lezama-Gutiérrez, J. Molina-Ochoa, E. Galindo-Velasco, M. López-Edwards, O. Rebollo-Dominguez, C. Cruz-Vázquez, W. Reyes-Velázquez, S. Skoda, and J. E. Foster 2005. Susceptibility of biological stages of the horn fly, Haematobia irritans, to entomopathogenic fungi (Hyphomycetes). J Insect Sci, 5: 1–8.

Ardeshir, Z., Z. Mohammadali, B. Mansour, and H. Jamal, 2016. Molecular identification of Mucor and Lichtheimia species in pure cultures of Zygomycetes. Jundishapur J. Microbiol, 9(4): e35237.

Bai, G. Y., H. Xu, Y. Q. Fu, X. Y. Wang, G. S. Shen, H. K. Ma, X. Feng, J. Pan, X. S. Gu, Y. Z. Guo, et al. 2016a. A comparison of novel entomopathogenic nematode application methods for control of the chive gnat, Bradysia odoriphaga (Diptera: Sciaridae). J Econ. Entomol. 109: 2006–2013.

Bai, Y., Y. Cui, N. Cao, Y. Liu, A. Ghulam, and B. Wang, 2016b. Effects of Luz humidity and temperature on the pathogenicity of Beauveria bassiana against Stephanitis nashi and Locusta migratoria manilensis. Chinese J Biol Control. 32: 735–742.

Bale, J. S., J. C. van Lenteren, and F. Bigler. 2008. Biological control and sustainable food production. Philos. Trans. R. Soc. Lond. B Biol Sci. 363: 761–776.

Barra, P., L. Rosso, A. Nesi, and M. Etcheverry, 2013. Isolation and identification of entomopathogenic fungi and their evaluation against Tribolium confusum, Sttiphus zeamais, and Rhyzopertha dominica in stored maize. J Pest Sci. 86: 217–226.

Benny, G. I. 1995. Classical morphological in zygomycete taxonomy. Can. J. Bot. 73: S725–S730.

Bibbs, C.S., A.M. Vitoreli, G. Benny, C.L. Harmon, and R.W. Baldwin 2013. Susceptibility of Latrodectus geometriseus (Araneae: Theridiidae) to a Mucor strain discovered in north central Florida, USA. Fla Entomol. 96: 1052–1061.

Chen, H., Y. Wang, X. Zhou, H. Gao, Y. Zhai, and Y. Yi, 2016. Status and prospect on biological control of Bradysia odoriphaga. Shandong Agric. Sci. 48: 158–161.

Coombes, C.A., M. P. Hill, S. D. Moore, and J. F. Dames, 2016. Entomopathogenic fungi as control agents of Thsammatotithia leuceotreta in citrus orchards: field efficacy and persistence. Biocontrol. 61: 1–11.

Daniel, C., and E. Wyss, 2009. Susceptibility of different life stages of the European cherry fruit fly, Rhagoletis cerasi, to entomopathogenic fungi. J. Appl. Entomol. 133:473–483.

Eksei, S., N. Maniania, and K. Ampong-Nyarko, 1999. Effect of temperature on germination, radial growth and virulence of Metarhizium anisopliae and Beauveria bassiana on Megalurothrips sjostedti. Biocontrol. Sci. Technol. 9: 177–185.

Farques, J., and C. Luz. 2000. Effects of fluctuating moisture and temperature regimes on the infection potential of Beauveria bassiana for Rhodinus prolincus. J. Invertebr. Pathol. 75: 202–211.

Farques, J., M. Goettel, N. Smits, A. Ouedraogo, and M. Rougier, 1997. Effect of temperature on vegetative growth of Beauveria bassiana isolates from different origins. Mycologia. 89: 383–392.

Farques, J., C. Vidal, N. Smits, M. Rougier, T. Boulard, M. Mermier, P. Nicot, P. Reich, B. Jeannequin, and G. Ridray, 2003. Climatic factors on
entomopathogenic hyphomycetes infection of Trialeurodes vaporariorum
(Host: Aleyrodinae) in Mediterranean glasshouse tomato. Biol.
Control. 28: 320–331.
Gaharty, A., H. Salem, M. Fouda, A. Abas, and A. Ibrahim, 2012. Pathogenicity
induced by the entomopathogenic fungi Beauveria bassiana and
Metarhizium anisopliae in Agrotisip sil (Hufn.). J. Radiat. Res. Appl.
Sci. 7: 95–100.
Garrido-Jurado, I., J. Torrent, V. Barron, and E. Quesada-Moraga, 2011.
Soil properties affect the availability, movement, and virulence of
entomopathogenic fung conidia against puparia of Ceratitis capitata
(Diptera: Tephritidae). Biol. Control. 58: 277–285.
Hajek, A.E., M. L. McManus, and I. D. Junior, 2007. A review of introduc-
tions of pathogens and nematodes for classical biological control of insects
and mites. Biol. Control. 41: 1–13.
Hinrikson, H. P., S. F. Hurst, L. De Aguirre, and C. J. Morrison, 2005.
Kubicek, C.P., and I. S. Druzhinina, 2007.
Kryukov, V. Y., O. N. Yaroslavtseva, M. M. A. Whitten, M. V. Tyurin,
K. J. Ficken, C. Greig, N. R. Melo, V. G. Glupov, I. M. Dubovskiy,
and T. M. Butt, 2018. Fungal infection dynamics in response to temperature in
the lepidopteran insect Galleria mellonella. Insect Sci. 25: 454–466.
Kwyckov, V. Y., N. O. Yaroslavtseva, M. A. M. Whitten, V. M. Tyurin,
K. J. Ficken, C. Greig, N. R. Melo, V. G. Glupov, I. M. Dubovskiy,
and T. M. Butt, 2018. Fungal infection dynamics in response to temperature in
the lepidopteran insect (Hypocreales), against Spodoptera litura (Lepidoptera:
Noctuidae) larval stages. Pest Manag. Sci. 64: 119–125.
Song, J., J. W. Cao, and S. Feng, 2016. Effectiveness of high virulence Bt
resources as a means of controlling Bradysia odoriphaga larvae. Chinese
J. Appl. Entomol. 53: 1217–1224.
Sowjanya Sree, K., V. Padmaja, and Y. L. Murthy, 2008. Insecticidal activity
of destruxin, a mycotoxin from Metarhizium anisopliae (Hypocreales),
against Spodoptera litura (Lepidoptera: Noctuidae) larval stages. Pest
Manag. Sci. 64: 119–125.
Song, J., W. Cao, and S. Feng, 2016. Effectiveness of high virulence Bt
resources as a means of controlling Bradysia odoriphaga larvae. Chinese
J. Appl. Entomol. 53: 1217–1224.
Sowjanya Sree, K., V. Padmaja, and Y. L. Murthy, 2008. Insecticidal activity
of destruxin, a mycotoxin from Metarhizium anisopliae (Hypocreales),
against Spodoptera litura (Lepidoptera: Noctuidae) larval stages. Pest
Manag. Sci. 64: 119–125.
Song, J., W. Cao, and S. Feng, 2016. Effectiveness of high virulence Bt
resources as a means of controlling Bradysia odoriphaga larvae. Chinese
J. Appl. Entomol. 53: 1217–1224.