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ORIGINAL ARTICLE

Pathogenicity of endogenous isolate of *Paramyrothecium* (=*Myrothecium) roridum* (Tode) L. Lombard & Crous against the squash beetle *Epilachna chrysomelina* (F.)

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Abstract

The squash beetle *Epilachna chrysomelina* (F.) is an important insect pest which causes severe damage to cucurbit plants in Iraq. The aims of this study were to isolate and characterize an endogenous isolate of *Myrothecium*-like species from cucurbit plants and from soil in order to evaluate its pathogenicity to squash beetle. *Paramyrothecium roridum* (Tode) L. Lombard & Crous was isolated, its phenotypic characteristics were identified and ITS rDNA sequence analysis was done. The pathogenicity of *P. roridum* strain (MT019839) was evaluated at a concentration of 10^7 conidia · ml^{-1}) water against larvae and adults of *E. chrysomelina* under laboratory conditions. The results revealed the pathogenicity of the isolate to larvae with variations between larvae instar responses. The highest mortality percentage was reported when the adults were placed in treated litter and it differed significantly from adults treated directly with the pathogen. Our results documented for the first time that *P. roridum* has potential as an insect pathogen.

Keywords: *Epilachna chrysomelina*, *Paramyrothecium roridum*, pathogenicity, soil

Introduction

Paramyrothecium roridum (Tode) L. Lombard & Crous was introduced by Lombard *et al.* (2016) based on *Myrothecium roridum* Tode as the type species. The species is known to occupy different ecological niches. It has been reported as a pathogen on different host plants (Han *et al.* 2014; Haudenshield *et al.* 2018; Matic *et al.* 2019; Pappachan *et al.* 2019; Soliman 2020), as a seed-borne pathogen (Bharath *et al.* 2006; Abdullah and Al-Mosawi 2010), as an endophyte (Amitha *et al.* 2014; Liu *et al.* 2016; Shen *et al.* 2019), or as a soil and dead plant substrate inhabitant (Domsch *et al.* 2007; Lee *et al.* 2008; Abdullah *et al.* 2010; Kwon *et al.* 2014; Lombarad *et al.* 2016).

Paramyrothecium roridum produces several bioactive secondary metabolites or toxins such as trichothecens macrolides, including epiroridin E, mytoxin B (Liu *et al.* 2016), myrothecin A, 8-alpha-hydroxyroridin H (Soliman 2020), roridin E, verrucarin A (Bosio *et al.* 2017), myrothecines, hydroxymytoxin B and several other cyclotrichothecan derivatives (Shen *et al.* 2019). These compounds show cytotoxic activities on human cells (Shen *et al.* 2019) and tumor cell lines (Liu *et al.* 2016). Some metabolites also have clear antibiotic and antifungal effects against a wide range of bacteria and fungi (Liu *et al.* 2006). Moreover, due to its phytotoxic effects. *P. roridum* is considered to

be a promising agent for biological weed control (Lee *et al*. 2008).

Several studies showed that *P. roridum* isolates are capable of producing various extracellular hydrolytic enzymes such as cellulases (Cx) and polygalacturonase (PG) as revealed by (Okunowo *et al*. 2010; Talukdar and Dantre 2014; Soliman 2020). Production of toxins and extracellular hydrolytic enzymes contributed significantly to its pathogenicity (Okunowo *et al*. 2010; Soliman 2020).

The insecticidal activity of *M. roridum* has never been reported on insects except by Mou (1975) who found that some insects, such as *Macrotermes barneyi* and *Dendrolimus punctatus*, are parasitized by taxa which are morphologically similar to *M. roridum*.

In Iraq, in previous studies, *Paramyrothecium* (=*Myrothecium*) *roridum* (Tode) L. Lombard & Crous has been isolated from different sources including soil around Basrah (Ismail and Abdullah 1977), soil from date palm plantations (Abdullah and Zora 1993), surface sediments of the Shatt Al-Arab river (Abdullah and Abbas 2008), sunflower seeds (Abdullah and Al-Mosawi 2010) and soil from grapevine nurseries at Duhok (Abdullah and Saadullah 2013). However, the isolates were only identified according to their morphological characteristics. The squash beetle, *Epilachna chrysomelina* (F.) is a common pest of cucurbit plants in Iraq (Abdul-Rassoul 1976). Both larvae and adults cause severe damage to the leaves which reduces the vegetative production of the host plant and leads to yield reduction, growth discontinuation, fruit degradation and even complete dryness of the plants (Awadalla *et al*. 2011). In Iraq, the control of this pest has focused on using insecticides and no biological agents have been reported on *E. chrysomelina* except for the study of Hassan *et al*. (2019). They evaluated the pathogenicity of two isolates of *B. bassiana* against various stages of *E. chrysomelina* under laboratory and field conditions to develop application strategies suitable for future use in biological control.

In this study we aimed to identify *P. roridum* isolated from cucurbit plants and the soil by using morphological and molecular analysis with ITS-rDNA region amplification and sequencing. Then we evaluated its pathogenicity to squash beetle *E. chrysomelina*.

Materials and Methods

Isolation

Cucurbit plants and soil were collected from Amadia district (N 37.0917°, E43.4877°, 1122 m above sea level), Duhok province, Kurdistan region, Iraq. The soil samples were collected from agricultural soils cultivated with different cucurbit plants such as cucumber, squash, snake cucumber, melon, watermelon and pumpkin from seven villages in the Amadia district. The soil samples, about 500 g each, were taken randomly from a depth of 0–10 cm (standard depth of sampling for fungi) with a trowel after removing litter or weed plants. They were then placed in plastic bags, labeled and transferred to the laboratory (25 ± 2°C). Nine soil samples/cucurbit plant/village were taken. Before using, the samples were thoroughly mixed and passed through a fine mesh sieve to break up soil lumps and separate litter remnant. *Paramyrothecium* species were isolated with direct soil plating (Warcup 1960). The plates were incubated at 25°C for 7 days, then checked for the occurrence of *Paramyrothecium* spp. and other opportunistic fungi from the soil using oat amended with cetyl trimethyl ammonium bromide CTAB (0.6) as selective media. Pure cultures from the obtained growing colonies were transferred to fresh appropriate potato dextrose agar (PDA) media for identification depending on their morphological characteristics and reproductive structures with the aid of several taxonomic references (Tulloch 1972; Lombard *et al*. 2016). To isolate entophytic *Paramyrothecium*, cucurbit plants were collected and transferred to a laboratory. For each plant, healthy tissues (root, stem and leaves) were surface-sterilized according to Arnold (2007) and Macia-Vicente *et al*. (2008), plated on selective medium (30 g oat, 0.6 CTAB, 15 g agar, 0.25 g chloramphenicol, 1 l distilled water) and incubated at 25°C for 2 weeks to record the occurrence of *Paramyrothecium* spp. and other opportunistic fungi. Pure cultures from the obtained growing colonies were transferred to fresh appropriate PDA medium for identification. Colony morphology, growth rate and morphological characteristics of isolates were recorded.

Morphological observations

To produce monosporic cultures, a conidial suspension of 1×10 conidia $\cdot$ ml^{-1} was prepared from fungal cultures grown on PDA plates for 2 weeks. A single colony reproduced from single conidia was transferred to a new PDA dish and incubated at 25°C. Microscopic measurements of conidia were taken from slide-cultures produced by inoculation of a small amount of mycelium on a drop of methylene blue stain and covered with a cover slip. Measurements were performed with graticule lens.

Genomic DNA extraction, PCR and sequencing

To identify the *Paramyrothecium* isolates at a molecular level, they were grown on potato dextrose broth

for 7 days at 25°C. The mycelia samples were pelleted from liquid culture by filtering and then frozen and stored at –20°C. For polymerase chain reaction (PCR) amplification, genomic DNA was extracted from the prepared mycelium mat according to the commercial animal and fungi DNA preparation kit protocol (Jena Bioscience, Germany) (Hassan 2019). A set of Primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') was used to amplify an internal transcribed spacer (ITS) rDNA region (White *et al.* 1990). The sequences of 557–603 bp fragments of the ITS rDNA PCR product were determined by Macrogen (Seoul, Korea). The sequence results were then checked and aligned using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The sequences were submitted to GenBank. The similarity of the sequence with homologous sequences deposited in GenBank was calculated using the BLAST tool on the National Center for Biotechnology Information (NCBI) website. Alignment of selected sequences was done with clustalW. The phylogenetic tree was constructed using the Neighbor-Joining method with the Jukes-Cantor model in MEGA7. Branch support was estimated by bootstrap analysis with 1,000 replicates.

Culture of the squash beetle *Epilachna chrysomelina* for laboratory use

Adults of *E. chrysomelina* (females and males) were collected from infested fields near the village of Tilakru (37°03′45″ latitude, 42°51′35″ longitude and 637 m above sea level), located northwest of Duhok in early July 2019. Adults were placed in wooden cages (75 × 75 × 75 cm) which had one glass side, while the other sides were covered with sieves under growth chamber laboratory conditions (26 ± 2°C and 14 : 10 L : D) in the Plant Protection Department, College of Agricultural Engineering Sciences, Duhok University. The cages were supplied daily with pumpkin leaves, fixed inside a jar, and filled with water daily to keep the leaves fresh (Hassan 2003). The cages were also supplied with pieces of pumpkin fruit to enhance mating and egg laying to obtain different stages of *E. chrysomelina* for laboratory experiments.

Pathogenicity of *Paramyrothecium roridum* under laboratory conditions

A pathogenicity test of *P. roridum* strain isolated from snake cucumber soil (MT019839) was conducted against squash beetle *E. chrysomelina* larvae and adults under laboratory conditions. A suspension of 10^7 conidia · ml^{-1} water was prepared from 2 week old culture and used. Tween 80 at a concentration of 0.02% was added to the suspension. Conidia concentration was measured using a hemocytometer slide.

Replicates were determined (10 adults/replicate) in a small plastic container (20 × 10 × 10 cm) lined with moistened filter paper, supplied with fresh and clean pieces of pumpkin leaves and fruit when required. The replicates were sprayed using a new sprayer (50 ml capacity). The application was repeated twice.

For larvae instars, 10 larvae/instar/replicates were sprayed with 2 ml of fungal suspension. The mortality percent after 1, 2 and 3 days of treatment was recorded. For adults, two application methods were used: 1. Plant trials: 10 adults with pumpkin leaves (in a small plastic container 20 × 10 × 10 cm lined with moistened filter paper and supplied with fresh, clean pieces of pumpkin leaves and fruit when required) were sprayed directly with 5 ml of spore suspension/replicate; 2. Litter trials: on day of treatment, fallen, decomposing leaves under the pumpkin plants were collected, then the litter was cut and mixed (Parker *et al.* 2003). To each plastic jar, 25 g of litter was added, leaving about 2 cm of space in which the adult beetles could move around freely (Hassan 2019). To each jar, 5 ml of the fungus conidia suspension was sprayed onto the surface of the litter with a sprayer, and then after the litter dried, 10 adults/jar were added. The jars were held under laboratory conditions. On the 2nd day of treatment, to encourage the insects to crawl through the litter and come in contact with the fungus conidia, the jars were inverted. Four replicates (10 individuals/replicate/application method) were determined. For control treatment the replicates were sprayed with 5 ml of distilled water. The mortality percentage was recorded after 4, 8 and 12 days. Cumulative mortality counts obtained from experiments were corrected for natural mortality using Abbott's formula (Abbott 1925). Data were statistically analyzed by SAS program using a Complete Randomized Design (CRD) with four replicates and the means were compared, using Duncan's multiple range tests at $p \leq 0.05$.

Results

Morphological observations

A total of 40 isolates of different endophytic and soilborne fungi were isolated from cucurbit plants and soil collected from agricultural fields in Amadia district, Duhok province, Kurdistan region, Iraq. Based on microscopic observations, samples isolated from plants and soil displayed the typical morphological characteristics found in species of *Myrothecium* described by Tulloch (1972).

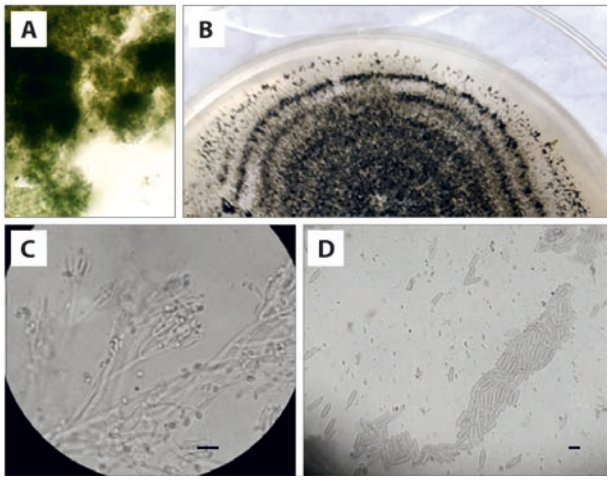

Fig. 1. Morphological features of *Paramyrothecium roridum*. A – sporodochia; B – colony morphology on potato dextrose agar (PDA); C – mycelia and conidiophores; D – conidia; scale bar = 5 µm

In culture, the fungal colony reached 46 mm diameter on PDA after 7 days at 25°C in the dark. Initial colonies of isolates were white, floccose mycelium and developed olivaceous to dark green sporodochia (Fig. 1A) bearing slimy spore masses surrounded by a white setose fringe (Fig. 1B). Conidiophores were branched repeatedly (Fig. 1C). Conidia were hyaline and cylindrical with both ends being rounded, with an occasional blunt end, and 5.3 to 7.8 × 1.1 to 2.8 µm (Fig. 1D). All characteristics were consistent with the description of *P. roridum* (Lombard *et al*. 2016).

Sequencing of ITS and phylogenetic analysis

The results of rDNA-ITS sequencing showed 557–603 bp of special DNA fragment sequenced. The obtained sequences confirmed that the taxon of five isolates belonged to *P. roridum* (Table 1).

Using BLAST search tool, to compare the sequence results of this study with the sequences of rDNA in the Genbank (NCBI; http://www.ncbi.nlm.nih.gov), phylogenetic analysis showed that the obtained sequences shared 100% homology to *P. roridum* strains: USA isolate (MH473919), Iranian isolate (KX826477), Australian isolate (KU059976), Chinese isolate (MT077143), Tunisian isolate (MF401576) and Italian isolate (MF401576). In the phylogenetic analysis, the isolates were grouped with other members of *Paramyrothecium* in amonophyletic clade with 100% bootstrap value (Fig. 2). Based on morphological characteristics and molecular data these fungi were identified as *P. roridum.*

Pathogenicity of *Paramyrothecium roridum* under laboratory conditions

Pathogenicity results (Fig. 3) indicated that P. roridum was pathogenic to *E. chrysomelina* larvae, however there were differences between larvae instar respones. For the 1st instar larvae, the mortality percentage ranged between 86.7 and 100% after 3 days of treatment and was significantly higher than the natural mortality in the control treatment (16.7–26.7). The mortality percentage after 3 days of 2nd instar larvae treatment was 86.67% compared to 60.67% and 46.67% for 3rd and 4th instar larvae mortality after the same period of treatment. With age the mortality percent decreased with larvae development. In 4th instar larvae, mortality percent ranged 0–44.83% after 3 days of treatment. Effected larvae were characterized by color changes from shiny yellow to brown.

The data in Figure 4 show the mortality percent of *E. chrysomelina* adults according to two application methods. The highest mortality percent was 33.33% after 8 days of treatment when the adults placed in treated litter differed significantly from the mortality percent of adults treated directly with fungus suspension. After 8 days of direct spray application on adults, 16.67% mortality was recorded and it was significantly higher than in the control treatment (0.00%).

Under high humidity, dark green sporodochia was produced on larvae and adult abdominal sternums after 3 and 9 days of treatment (Fig. 5).

Table 1. *Paramyrothecium roridum* isolates code, locality, hosts, Genbank accession numbers and conidia measurements

Isolate	Locality	Host/Substrate	Genbank No.	Conidia [µm]
cq-32	Qidishi	cucumber soil based	MT019838	1.1–1.2 × 6.8–7.3
sq-33	Qidishi	snake cucumber soil based	MT019839	1.8–2.8 × 5.3–7.1
mb-39	Bawan	melon stem	MT019840	1.2– 1.3 × 7.0–7.4
mb-40	Bawan	melon stem	MT019841	2.5–2.8 × 7–7.9
mm-41	Mergi	melon stem	MT019842	1.2–1.3 × 6.6–6.9

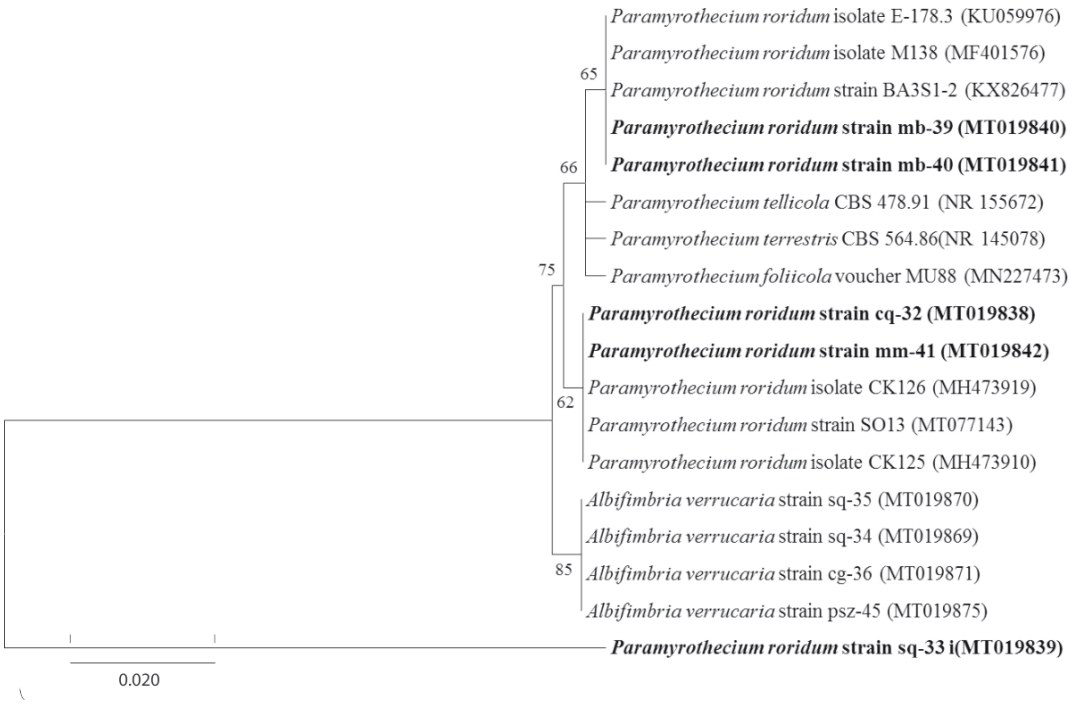

Fig. 2. Phylogenetic tree of *Paramyrothecium roridum* based on Neighbor-Joining analysis with 1,000 bootstrap replicates of ITS-rDNA sequences of the new strains from Iraq (in bold) and related *Paramyrothecium* species from NCBI. GenBank accession numbers provided next to species names

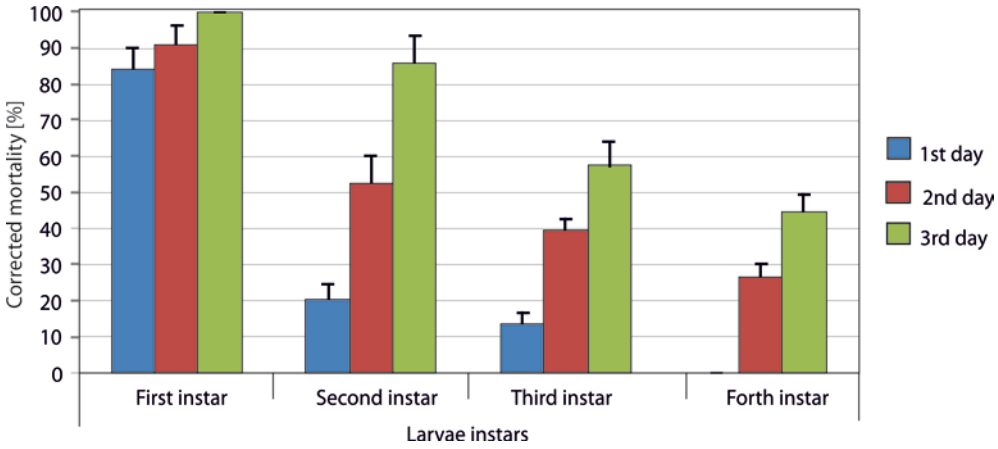

Fig. 3. Pathogenicity of *Paramyrothecium roridum* to squash beetle *Epilachna chrysomelina* larvae

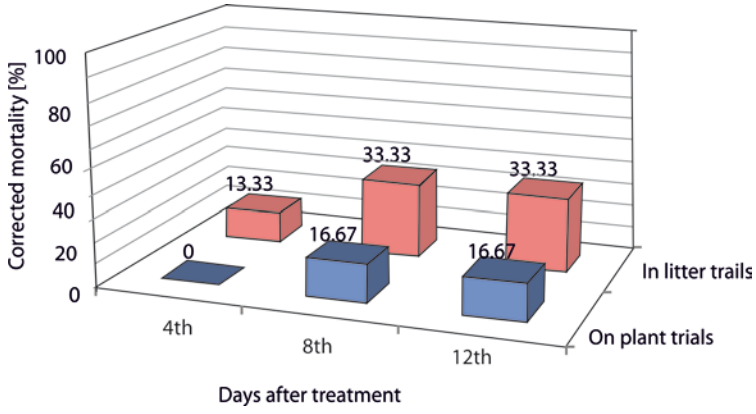

Fig. 4. Pathogenicity of *Paramyrothecium roridum* to squash beetle *Epilachna chrysomelina* adults

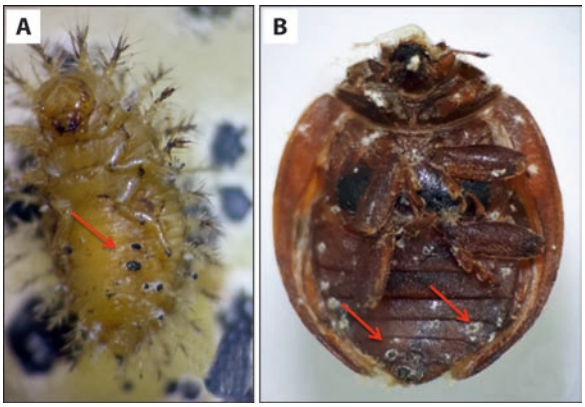

Fig. 5. Dark green sporodochia (indicated by red arrows) on abdominal sternums. A – larva; B – adult

Discussion

Myrothecium-like species (Stachybotryaceae) are polyphyletic and are characterized by producing sporodochia which are irregular in outline, are composed of a slimy mass of conidia and are surrounded with a white to gray fringe. A phylogenetic revision to the genus *Myrothecium* Tode led to the introduction of 13 new genera with *Myrothecium*-like morphology, including *Paramyrothecium* Lombard & Crous (Lombard *et al.* 2016).

The genus *Paramyrothecium* is currently comprised of 13 species (Lombard *et al.* 2016; Liang *et al.* 2019). The former genus *Myrothecium* is restricted to *M. inudatum* Tode as the type species of the genus and a newly described species *M. simplex* Lombard & Crous (Lombard *et al.* 2016).

Paramyrothecium roridum has rarely been documented as an insect pathogen, except for the report of Mou (1975), who found that some insects such as *Macrotermes barneyi* and *Dendrolinus punctatus* were infected with the fungus. In contrast, *Albifimbria* (=*Myrothecium*) *verrucaria* (Alb. & Schwein.) L. Lombard & Crous was reported with high levels of cuticle degrading enzymes such as chitinases that hydrolyze the insect cuticle (Vidhate *et al.* 2015; Chavan *et al.* 2017). However, a strain of *P. roridum* was recently reported among the fungi associated with insects and its ability to produce different compounds of antifungal microcyclic trichothecenes was confirmed (Li *et al.* 2019). The mortality reported in both larvae and adults of *E. chrysomelina* could be attributed mainly to several mycotoxic metabolites produced by *P. roridum* as revealed by several studies (Liu *et al.* 2016; Bosio *et al.* 2017; Shen *et al.* 2019).

Our study indicated that an indigenous isolate of *P. roridum* showed biocontrol activity against both larvae and adults of the squash beetle (*E. chrysomelina*) under laboratory conditions. To our knowledge, this is the first report of *P. roridum* as an insect pathogen. Further studies are required to test the potential virulence of *P. rordium* against other important pests under laboratory and field conditions. The results show promise of being effective for the control of other insects. Although it is a phytopathogen, this fungus as an entomopathogenic fungus requires a unique management strategy to avoid the risk of damage to plants. Field application of entomopathogenic fungi towards various insects often deponds on the behavior of insects in a natural habitat. Soil is the natural habitat of fungi and, since squash beetle adults hibernate under litter, herbs, cracks, trunks and inside the cleavages of soil, particularly around roots until the next season, it is theoretically possible to infect the soil with fungal spores (Gindin *et al.* 2006; Assaf *et al.* 2011; Hassan *et al.* 2019). Because the leaf litter (especially fallen decomposing leaves), used in the second method of adult insect inoculation was not sterilized previously, it may contain spores of the fungus from natural conditions. This might have contributed to the final pathogenicity; therefore, the results of this method may not be meaningful.

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