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Research Article

Molecular identification of indigenous insect pathogenic fungi *Metarhizium pingshaense* (Ascomycota: Hypocreales) virulent to *Cnaphalocrocis medinalis* (Guenèe)

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ABSTRACT

Molecular sequencing of the EFα-1 region were performed for virulent *Metarhizium* isolates RMT16 (accession number: KC870069) and compared with EFα-1 sequences deposited at the NCBI and it was confirmed that the isolate belongs to the species *M. pingshaense*. *M. pingshaense* isolate virulent characteristic on different larval stages have been studied. The tested all larval stages were susceptible to the fungal isolate and do not differ significantly in mortality but differ in the lethal time with shorter lethal time were recorded in first instar larvae of *C. medinalis* and longest lethal time recorded in fourth instars larvae of *C. medinalis*. In estimation of the LC_{50} on third instar larvae, RMT16 show lower LC_{50}. In conclusion, the *M. pingshaense* RMT16 have found to high potent against the *C. medinalis in vitro* condition and found to be suitable *Metarhizium* isolate for the development as effective control agents against the *C. medinalis*

Keywords

Entomopathogenic Fungi, Elongation factor α-1, Metarhizium pingshaense, Cnaphalocrocis medinalis, Galleria bait method

1. INTRODUCTION

Entomopathogenic fungi (EPF) are natural enemies of the insect herbivorous and play significant role in natural regulation of insect populations in agro and forest ecosystem (Inglis et al., 2001; Zimmermann, 2007). EPF are widely distributed in a wide range of ecosystem such as aquatic, forest, agricultural and pasture and are recovered worldwide from infected/diseased insect and soil (Lacey et al., 1995; Chandler, 1997; Meyling and Eilenberg, 2006).

The insect pathogenic fungi species belong to Ascomycota phylum, Hpyocreales order, *Metarhizium* genera are natural pathogen of insects and used as effective biological control agents to manage the pest in agroecosystem (Inglis et al., 2001; Robert and Hajek, 2004; Zimmermann, 2007; Bischoff et al., 2009) and known to cause more numerous natural epizootics among the pest population in agricultural habitats of temperate regions than other habitats (Meyling and Eilenberg, 2006; 2007; Zimmermann, 2007).

Metarhizium have a cosmopolitan distribution as members of the natural soil flora. *Metarhizium* have been isolated from infected insects and soil of all continents except Antarctica (Roberts and St. Leger, 2004; Nishi et al., 2011) and known to isolate near the Antarctic Circle (Roddam and Rath, 1997). The species of *Metarhizium* genera composed of anamorphic fungi species that generally produce greenish, asexual, halopid conidiospore formed in chains on philalides on the cadavers of their hosts or culture media (Glare et al., 1996; Bischoff et al., 2009) The common name for *Metarhizium*-induced disease is known as ‘‘green muscardine’’ which is named based on the coating of insect cadavers with green conidia (Roberts and St. Leger, 2004; Bischoff et al., 2006).

The species of *Metarhizium* genera are known for its potential to kill a wide scale of insects from over 14 different insect orders including some non target orders such as Malacostrata (Amphipods), Acari, Ephemeroptera and Dermaptera and known to infect the species of superfamily Acridoidea, including locusts and grasshoppers (Zimmermann, 2007) and the *Metarhizium spp* is known parasite different life stage of the lepidopteran pests in *invitro* and field condition were reported from various studies (Ekesi et al., 2002; Tefera and Pringle, 2003a,b; Er et al., 2007; Anand et al., 2009; Godonou et al., 2009).

The conidia form an infective unit of the fungi, which germinate on the cuticle of a susceptible insect to produce a germ tube which penetrates into the body cavity with combined action of hyphae pressure and cuticle degrading enzymes (Charnley, 2003) and further kill the host by production of secondary metabolites, toxic protein and by proliferating hyphae in body of the host.

The mitosporic species of *Metarhizium* genera identification and discrimination is based on their morphological character of the *Metarhizium* in earlier days (Tulloch, 1976). The identification and classification of the fungi based on the morphology and colour do not provide satisfactory discrimination (Driver et al., 2000; Bischoff et al., 2006; 2009). Molecular analysis of regions of introns within EF-1α provides the greatest concentration of informative nucleotide variation and degree of phylogenetic resolution for terminal clades in *Metarhizium* (Bischoff et al., 2006; 2009).

The elongation factor-1 α (EF-1 α) gene is usually present in a single copy and encodes the translation elongation factor that controls the rate and fidelity of protein synthesis and from an essential component of the protein synthesis process in eukaryotes and archeabacteria (Baldauf, 1999).

In the present study elongation factor alpha-1 (EFα-1) gene sequencing was performed to identify the isolated *Metarhizium* isolate. Further, the effect of isolate on third-instar larvae of *Cnaphalocrocis medinalis* (Guenèe) was also studied.

2. MATERIALS AND METHODS

2.1. *Cnaphalocrocis medinalis* culture

C. medinalis culture methodology was adopted from the procedure as described by Senthil-Nathan et al. (2004).The *C. medinalis* culture was initiated with larvae collected from paddy field grown in and around the Tirunelveli district, Tamil-Nadu, India. *C. medinalis* larvae were reared in green house at 27±1°C under a 14:10 L:D photoperiod at 85% RH on potted rice plants placed in the cage (60×40×70cm^3). The pupae formed in the cage was transferred to oviposition cage for adults emergence (45×30×60cm^3), to maintain further culture, 13 female and 12 male moths were placed in the oviposition cage containing one potted rice plant. The moths were fed with 10% sucrose solution prepared with a few drops of vitamin mixture to increase their oviposition. After 4-7 days the potted plants were removed from the ovipositon cage. The leaf portions containing the eggs were clipped and placed on moist filter paper in a petridishes (100×15mm) (Hi-media, India). The eggs obtained were used to maintain the culture and newly hatched larvae from the eggs were placed on 50-day-old rice plants. Rice plants of Karnataka ponni strain used for the culture of *C. medinalis* were grown in earthenware pots, 18 cm tall with a 20cm diameter top and each pot held 12-15 plants. The pots containing rice plants were placed in about 10cm of water in a metal tray in the greenhouse. The rice plants were grown in the control condition without exposure to any chemical insecticides. The insects from the second generation culture were used for the biological assay studies.

2.2. *Galleria mellonella* culture

Galleria culture was maintained in laboratory at 27±1^{0}C, 90%RH. The *Galleria* larvae obtained were grown in the plastic container culture box (10 cm diam.×15 cm height) provided with artificial diet and incubated in dark condition. The pupae formed in the culture boxes were transfer to oviposition cage (40×20×45cm^3), the adults emerged from pupae was provided with sucrose solution with a few drops of vitamin mixture to enhance the oviposition of the adults. The oviposition cage was placed in laboratory provide with photoperiod of 8:16 L:D photoperiod at

27±1^{0}C, 90%RH. The wax-coated folded paper strips were placed in the oviposition cage, in which the adults female laid eggs in crevices of the folded paper. The eggs were carefully detached from paper strips without damaging eggs and placed in the culture boxes provided with artificial diet. The third and fourth instar *G. mellonella* larvae were used as bait for isolation of fungi from the soil.

2.3. Isolation of *Metarhizium* spp.

The fungal isolates used in the study were isolated from the soil sample collected from rice field habitat of Kottaimalai, Kadayanallur, Tirunelveli district, Tamil-Nadu state, India. *Galleria* bait method (Zimmermann, 1986) was employed to isolate the *Metarhizium* spp. from the soil samples. The soil samples were spread on a tray and air dried in aseptic condition to remove excess moisture from the soil to avoid entomopathogenic nematode infestation in *Galleria* larvae during baiting. From each soil samples, the 100g subsoil samples were placed in plastic boxes (6cm diam×9cm height) and third to fourth instar heat treated *Galleria* larvae were added to each plastic box and incubated at 29±1^{0}C in darkness. The lids of the plastic box were perforated to allow aeration to the soil in the box. Five replications were carried out for each sample and 15 to 20 larvae were used per replication. The larvae were inspected for mycosis from seven to fifteen days after bait. Dead larvae encountered in the boxes were removed and surface-sterilized in 3% sodium hypochlorite (Hi-media, India) for 3min and then three times washed in sterile distilled water and placed in moist chamber (petriplate with wetted filter paper sealed with parafilm) which provide humid condition for the external mycelia growth and aerial conidia production of fungi on cadaver. The humid chamber was incubated at 29±1^{0}C in complete darkness. The fungal propgules grown on the external surface of the *Galleria* cadavers were transferred using sterile inoculation loop to quarter strength SDA media amended with 500µg/ml chloramphenicol and 500µg/ml cyclohexamide (Hi-media, India).

2.4. Morphological confirmation of *Metarhizium spp.*

The fungal propugules from pure colony of the fungal isolate were placed in the glass-slide using inoculation needle and mounted with lactophenol-cotton blue (Hi-Media, India) and observed through microscopic at 40×(Optika-Fluo series B-600TiFL, Italy) and the fungal genera were identified and confirmed based on morphological character using taxonomic keys (Humber, 1997). The taxonomic keys such as conidial color, size and reproductive structure of fungi were used for identification of the fungi.

2.5. DNA Extraction and EFα-1 Sequencing

The *Metarhizium* isolate RMT16 was cultured in the 100ml conical flask containing 50ml quarter strength SD broth amended with 1% yeast. The culture was incubated at 28^{0}C for 4-7 days on

an incubator shaker (Lark, India) at 200rpm. The mycelium was obtained from broth by filtration and weighed 50mg approximately, lyophilized and crushed into ground using microcentrifuge tubes and pestles (Sigma-Aldrich, USA) placed in liquid nitrogen. The DNA was extracted from crushed mycelium using the GeneiPure™ Plant Genomic DNA Purification Kit (Genei, India) according to instruction mentioned by the manufactures.

The region of the nuclear gene EF-1α was amplified in the thermal cycler (Eppendorf Mastercycler®personal, Germany) under the PCR conditions followed by Rehner and Buckley (2005) by using following pair of primers: Forward primer EF1T 5'ATGGGTAAGGARGACAAGAC-3' and reverse primer PGEF2R 5'GAACTTGCADGCRATGTGVG-3'. The 50µl of the PCR reaction mixture contains 5.0µl of 10×reaction buffer (100 mM Tris– HCl, pH 8.8, 50 mM KCl, 0.1 Triton X-100 and 1.5 mM $MgCl_2$), 5.0µl 2mM dNTP Mix; 10 pmol each of the opposing amplification primers; 2.0µl DNA template (50ng) and 0.5µl Taq DNA polymerase (5U/µl).

The obtained PCR product was analysed by electrophoresis in 1.0% agarose gels, the gel containing DNA bands were stained with ethidium bromide and photographed on a UV transilluminator using the gel documentation system (Photostation-Silver plus gel documentation system, Lark, India). PCR products were purified using GeneiPure™ Quick PCR Purification Kit (Genei, India) and purified products were sequenced.

The sequence obtained was aligned and edited with BioEdit version 7.1.9 .The EF-1α sequence of *Metarhizium* was analysed and compared with the other *Metarhizium* isolates in Genbank, NCBI (National Center for Biotechnology Information) using Basic Local Alignment Search Tool (BLASTn) program. The phylogenetic dendogram was constructed with the computer software MEGA5 (Tamura et al., 2011).

2.6. Conidial suspension preparation

The conidial suspension of *M. pingshaense* for the bioassay was harvest from 15-20 days- old culture. The conidia were scraped from the medium surface using sterile inoculation loop and transfer to 30ml universal glass bottle (Essco Glass Ampoules and Vials, India) containing 20ml of sterile 0.05% Triton-X-100 (Hi-media) solution with glass beads (3mm) (Hi-media, India). The conidial suspension was vortex for 15 min to break the conidial clumps to obtain the homogenous suspension. The concentration of the initial stock conidial suspension was determined by counting under microscope (Optika-Fluo series B-600TiFL, Italy) at 40× using improved Neubauer haemocytometer chamber. Then it was diluted to obtain different concentration ranging from 1×10^4 - 1×10^8 conidia/ml.

2.5. Effect of *M. pingshaense* isolate RMT16 on larval stages of *C. medinalis*

Bioassay was performed with the *M. pingshaense* isolate RMT16 with different concentration (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml) on larval stages (2^{nd}, 3^{rd} and 4^{th} instar) of *C. medinalis* to estimate LC_{50}. The leaves of rice were sprayed with the 5ml conidial suspension at different concentrations (1×10^4-1×10^8 conidia/ml), the control leaves were treated with 0.05% Triton-X-100 solution using regulator-controlled spray applicator. The newly moulted third instars larvae were placed in treated leaves and allowed to feed on the leaves. Five replications were carried out for each treated concentration and twenty larvae were used per replication. The mortality of the larvae was recorded from the second to ninth day after the treatment. The mortality of third-instar larvae was recorded and percentage of the mortality was calculated and corrected with Abbott formula (Abbott, 1925). Lethal concentration (LC_{50}) and Lethal time (LT_{50})required by *M. pingshaense* isolate to kill third-instar larvae of *C. medinalis* was calculated by probit analysis (Finney, 1971).

2.6. Statistical analysis

The mortality data were expressed as the mean of five replications and normalized by arcsine-square root transformation of percentages. The transformed percentages were subjected to analysis of variance (ANOVA). Differences between the treatments were determined by Tukeys multiple range test ($p < 0.05$) (SAS Institute, 2001). Lethal concentration (LC_{50}) required by the fungal isolate were estimated by probit analysis (Finney, 1971). The statistical analysis was performed using Minitab®16 statistical software package.

3. RESULT

3.1. Molecular identification of RMT16 *Metarhizium* isolate

EFα-1 gene region of nuclear genome was amplified and sequenced in order to identify the species of isolated *Metarhizium* isolates. Agarose gel analysis of the amplified PCR products of two isolates, have shown that EFα-1 gene is nearly 1500 bp in length. The BLAST analysis of the RMT16 isolate EFα-1 gene sequence shows that these isolate belong to the species of *M. pingshaense*. The isolates RMT16 have shown 100% similarity respectively to others *M. pingshaense* isolates. Subsequently the sequence of RMT16 was submitted to NCBI GenBank database and accession numbers were obtained (accession number: KC870069).

The phlyogenetic analysis of *M. pingshaense* isolates RMT16 was carried out by Neighbour-Joining (NJ) method based on the Kimura 2-parameter model with the computer programme

MEGA5 (Figure. 1). The phlyogenetics dendogram has shown that the isolate RMT16 is closely related to the *M. pingshaense* species.

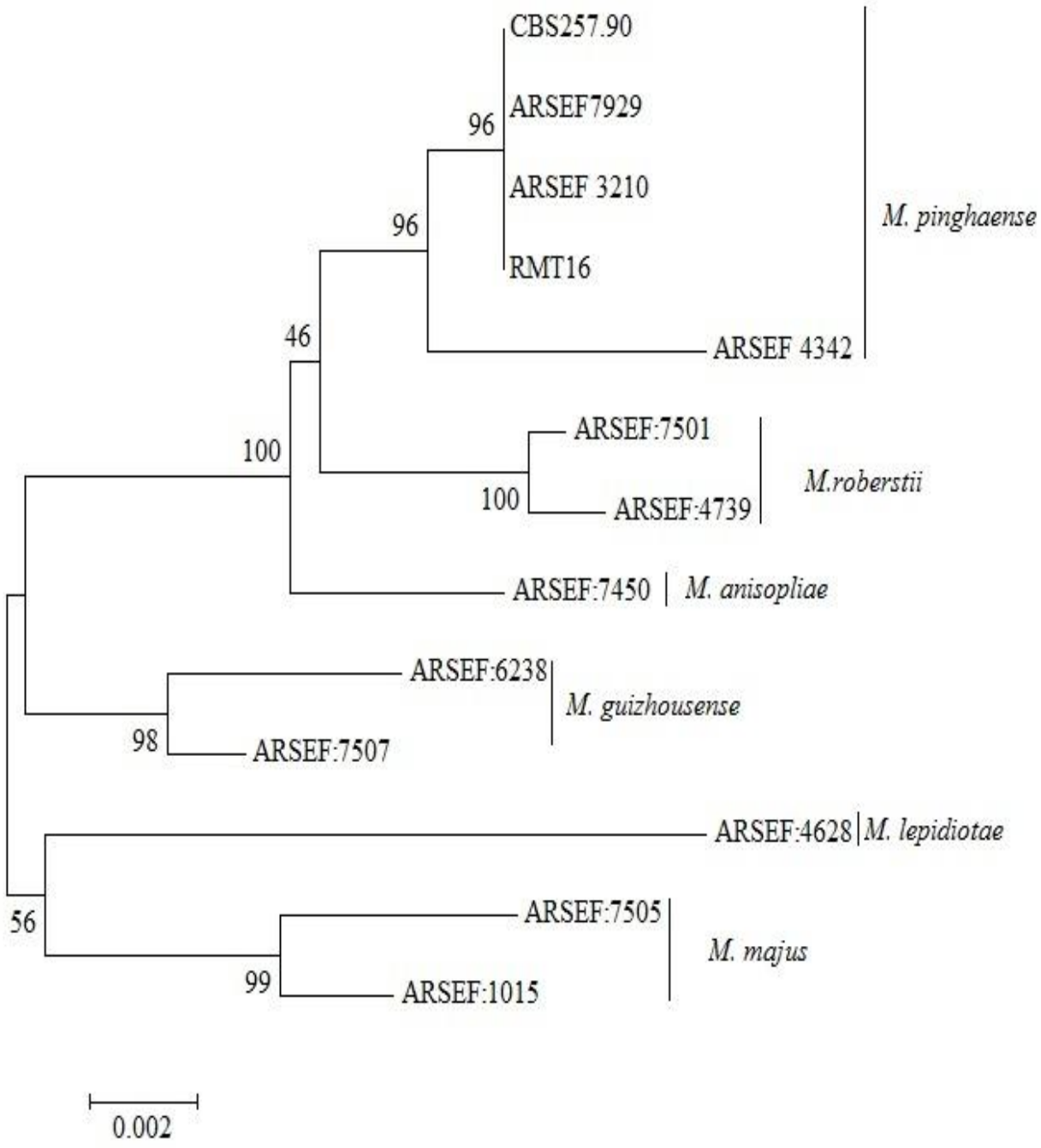

Figure 1. The phylogenetics tree of *Metarihizium* genus elongation factor 1-alpha (EF1-α) gene constructed using Neighbor-Joining method. The tree show genetic relationship between *M. pinghaense* isolate RMT16 and other *Metarhizium* species EF1-α gene obtained from the study of the Bischoff et al. (2009). Bootstrap values shown next to nodes are based on 100 replicates.

3.2. Larval age dependent mortality of *C. medinalis* treated with *M. pingshaense* RMT16

Second, third and fourth instar larvae of *C. medinalis* were treated with the *M. pingshaense* RMT16 at the concentration of 1×10^8 conidia/ml. All tested larval stages were susceptible to the both fungal isolates and there was no significant difference in mean percentage mortality among the larval stages in treatment with *M. pingshaense* ($F_{2,12}$=2.80, P<0.0001) (Figure 2). However there was significant difference in lethal time (LT_{50}) among different larval stages treated with *M. pingshaense* RMT16 ($F_{2,12}$=42.05, P<0.0001) (Table 1). The second instar larvae treated with fungal isolate were highly susceptible which show shorter lethal time than other tested larval instar of *C. medinalis* (Table 1).

Shorter LT_{50} of 4.84 days were recorded in second instar of *C. medinalis* followed by third and fourth instar larvae in treatment with *M. pingshaense* RMT16 (Table 1). The lethal time (LT_{50}) value of RMT4 (5.32 days) on the fourth instar of *C. medinalis* show that 4th instar larvae were less susceptible to the both fungal isolates than other tested stages (Table 1).

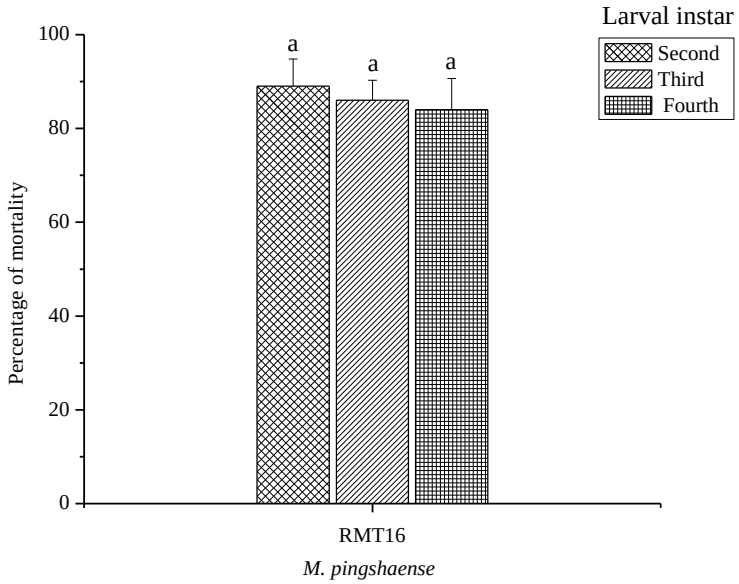

Figure 2. Percentage [d] to 4th instar larvae of *C. medinalis* treated with *M. pingshaense* RMT16 at the concentration of 1×10^8 conidia/ml.
Mean (SE±) followed by the same letters above bars indicate no significant difference ($P \leq 0.05$) according to a Tukey's test.

Table 1. LT_{50} and 95% confidence interval of *M. pingshaense* RMT16 isolate on different larval stages of *C. medinalis.*

Fungal isolates	LT_{50} ±SE (95% Confidence Interval)		
	Larval instar		
	Second	Third	Fourth
M. pingshaense RMT16	4.84±0.9^{c} (4.67 - 4.99)	4.76±0.9^{b} (4.96 - 4.09)	5.32±1.1^{a} (5.21 - 5.43)

Means (±SE) within rows followed by the same letter are not significantly different (Tukey's test, P≤0.05).

In determination of the LC_{50} value of *M. pingshaense* RMT16 on the second, third and fourth instar larvae of *C. medinalis*, the larval stages were treated with different concentration of conidia suspension (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml). The LC_{50} value of *M. pingshaense* RMT16 shown great variation among the larval stages of *C. medinalis*. The RMT16 isolate has shown lower LC_{50} value of 8.55×10^4 conidia/ml on second instar with high mortality rate of 89%, followed by third (2.87×10^5 conidia/ml) and fourth instar larvae (9.49×10^5 conidia/ml) of *C. medinalis* (Table 2). The LC_{50} value of fungal isolate on the different larval stages has shown the second instar larvae were high susceptible with high mortality rate of 89% in treatment with RMT16. The higher LC_{50} was observed on the fourth instar in fungal treatment, which shows that fourth instar larvae were less susceptible among the tested larval instars.

Fungal isolate	Larval instar	LC_{50} conidia/ml	Confidence interval	χ^2	df	*P*
M. pingshaense RMT16	Second	8.55×10^4	$8.03 - 9.55\times10^4$	63.98	28	<0.0001
	Third	2.87×10^5	$2.08 - 3.90\times10^5$	46.51	28	<0.0001
	Fourth	9.49×10^5	$9.03 - 9.88\times10^5$	35.94	23	<0.0001

Table 2. LC_{50} and 95% confidence interval of *M. pingshaense* RMT16 isolate on different larval stages of *C. medinalis*

4. DISCUSSION

The isolated *Metarhizium* spp. (RMT16) suggested the necessity of confirming their taxonomic identity previously identified morphologically as *Metarhizium*, it is not possible to distinguish *Metarhizium* taxa based on morphology (Bischoff et al., 2009). Molecular confirmation and identification were needed to identified and differentiate the *Metarhizium* species (Driver et al., 2000; Bischoff et al., 2006; 2009; Nishi et al., 2011).

The EF-1α amplified fragment with approximately 1500 bp of the tested isolate were compared with the sequences obtained from NCBI database of the isolates used by Bischoff et al. (2009). Our analysis of EF-1α nucleotide sequences from isolate clearly showed that the isolates RMT4 belongs to species *M. pingshaense*. The EF-1α is to date the most informative region to use for routine species identification within the genus. This region requires only two primers and is easily amplified (Bischoff et al., 2009; Nishi et al., 2011).

Treatment with *M. pingshaense* RMT16 isolate on the different larval stages of *C. medinalis* shown that the early second instar larval stage was highly susceptible with shorter lethal time than third and fourth instar larval stages. The tested *M. pingshaense* RMT16 required comparatively lower LC_{50} to kill second larval stages of *C. medinalis* than other larval stages. The insects larval stage varies in their susceptibility to infection by entomopathogenic Hyphomycetes (Inglis et al., 2001). Hafez et al. (1997) reported that early larval instars of the potato tuber moth *Phthorimaea operculella* Z. were more susceptible to *B. bassiana* than older larval stages. Nguyen et al. (2007) shown that early larval instar of *H. armigera* was highly susceptible to entomopathogenic fungi than late larval stage. In contrast Vandenberg et al. (1998) reported that third and fourth instar of diamondback moth *P. xylostella* was more susceptible to *B. bassiana*. Kirubakaran et al. (2014) reported that second instar of *C. medinalis* show higher susceptibility over third and fourth instars when treated with *Metarhizium* (*M. anisopliae*)

In conclusion, our results show that *M. pinghaense* RMT16 are very effective against all *C. medinalis* larval stages. Exploring the full potentials of tested fungal strains as possible biological control agents requires further studies, especially those toward possible application methods, long term effects in agricultural environment should also be investigated.

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