ISOLATION, MOLECULER CHARACTERIZATION AND PATHOGENICITY OF *METARHIZIUM ANISOPLIAE* (METSCH.) SOROKIN (HYPOCREALES: CLAVICIPITACEAE) FROM SOIL IN ERZINCAN PROVINCE, TURKEY

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Abstract. Developing good microbial pesticides depends on isolating and knowing their pathogenicity. For this purpose, entomopathogenic *Metarhizium anisopliae* fungi were isolated from soils in Erzincan province and galleria bait method was used. Molecular identification of the isolates was performed by using ITS rDNA analysis. As a test organism, *Galleria mellonella* and *Tenebrio molitor* larvae were used and suspensions of $2x10^7$ conidia ml^{-1} were prepared adding Tween 80®. Bioassays were performed by dipping technique and mortality was recorded daily for 12 days. Each assay consisted of 3 replicates with 10 insects larvae. Ten fungal isolates were obtained from the bait method. Based on classical and molecular methods, all fungal isolates were identified as *M. anisopliae* and deposited into GenBank database with MH104853 - MH104862 accession numbers. All isolates of *M. anisopliae* were pathogenic to *G. mellonella* and *T. molitor* with a mortality rate of 63.3±3.3% - 83.3±3.3% and 30±5.8% - 66.7±3.3%, respectively 12 days after application. As a result, MaEMR1a and MaEO3 isolates can be applied as good biological control agents for pest insects.
Keywords: *biological control, Metarhizium anisopliae, G. mellonella, T. molitor*

Introduction

Entomopathogenic fungi (EPF) have an increasing prevalence in microbial control (Kılıç, E., 2009-Kılıç, E., 2014). EPF unlike other entomopathogenic microorganism (bacteria and viruses) which have to be ingested to cause diseases, typically infect insects by direct penetration of the cuticle followed by multiplication in the hemocoel (St. Leger et all., 2011). It is reported that EPF causes approximately, 60% of insect diseases (Faria and Wraight, 2007). EPF can be an alternative to chemical pesticides and are considered as non-harmful biological control agents in terms of human and environmental health (Faria and Wraight, 2007). *M. anisopliae* has a global distribution as a member of the soil flora (Zimmermann, 1992). This species has been reported in soils from widely differing climatic zones (Gillespie, 1988; McCoy et al., 1988; Kılıç, 2017). Some researcher pointed out that many isolates of *M. anisopliae* have entomopathogenic activity against a range of arthropod pests (Gillespie, 1988; McCoy et al., 1988). Many scientists reported that *M. anisopliae* has been effective in controlling more than 200 species of insect pests (Pu and Li, 1996; Sabbour, 2002;

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Brooks and Wall, 2005; Quesada-Moraga et al., 2008; Shanley et al.; 2009; Dimbi et al., 2009; Dickson et al., 2010; Marius et al., 2011; Niassy et al., 2011).

In this paper, we isolated and characterized pathogenic fungi *M. anisopliae* for possible use as microbial control agent of *G. mellonella* and *T. molitor* and its pathogenicity was determined. Both insect species caused economic losses at storage.

Material And Methods

Insect culture

Galleria mellonella Linnaeus (Lepidoptera: Pyralidae) was collected from different beekepping in 2014 in Erzincan province. At the same time *T. molitor* (Coleoptera: Tenebrionidae) was collected from flour factories in Erzurum and storages in Erzincan provience. *G. mellonella* and *T. molitor* were grown under laboratory conditions [(25ºC±2 and 70%±10 R.H.) (16: 8 h (L:D)] (Zimmermann, 1986).

Isolation and identification of entomopathogenic fungi

Insect-associated fungi were isolated from soil samples by using 'Galleria bait method' (Zimmermann, 1986). The wax moth larvae, *G. mellonella* were reared continuously in constant darkness at 26°C. The third or fourth instar larvae (approximately 30 days after hatching) were used as baits. Ten larvae were placed on the soil samples in each boxes and covered with a lid and incubated at 25±2°C for two weeks. The larvae were examined on days 7 and 14 after inoculation. Surface of dead larvae were sterilized by 3% sodium hypochlorite for 3 min and then rinsed twice with sterile distilled water. After removing free water of the larvae surface, they were placed onto PDA plates. The fungi were identified using morphological characteristics of reproductive structures with the aid of relevant taxonomic literature (de Hoog, 1972; Samson et al., 1988; Tzean et al., 1997; Domsch et al., 1980; Humber, R., 1997; Humber, R., et al., 2012) (*Table 1*).

Table 1. *Fungal material and their geographical origin (2014-2016)*

Fungus Species	**Isolate Code**	**Substrate (Soil)**	**Geographical Origin of Isolates**
Metharzium anisopliae	MaEMR1a	Vegetable Field	ERZİNCAN, Turkey
	MaEMR2b	Vegetable Field	ERZİNCAN-Üzümlü, Turkey
	MaEK1a	Vegetable Field	ERZİNCAN-Kemah, Turkey
	MaEİ3	Field (Barly-Weat)	ERZİNCAN-İliç, Turkey
	MaEKLY1a	Fruit Garden	ERZİNCAN-Kemaliye, Turkey
	MaER3	Field (Barly-Weat)	ERZİNCAN-Refahiye, Turkey
	MaEM1	Vegetable Field	ERZİNCAN-Mercan, Turkey
	MaET3	Field (Barly-Weat)	ERZİNCAN-Tercan, Turkey
	MaEÇ3	Field (Barly-Weat)	ERZİNCAN-Çayırlı, Turkey
	MaEO3	Field (Barly-Weat)	ERZİNCAN-Otlukbeli, Turkey

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The identification of the isolated entomopathogenic fungi were performed using ITS. For this purpose, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers were used for the PCR (White et al., 1990). Genomic DNA was isolated from fungal biomass following the protocols of the *EcoSpin.* Genomic DNA isolation kit (EcoTec Biotecnology, Türkey). The PCR cycling conditions were: 2 min at 95 ^{0}C, followed by 30 cycles at 95° C for 45 s, 55° C for 60 s, 72° C for 60 s with a final extension step of 72° C for 10 min. PCR products were analyzed in 1 % (w/v) agarose gels visualized by UV after staining with EtBr. The products were purified by following the protocols of the EcoSpin PCR Purification Kit (EcoTec Biotecnology, Türkey). After purification, ITS rDNA were sequenced in both directions with 4 same primers at OLIGOMER Biotecnology, Turkey. Sequences chromatograms were assembled into one complete sequence using Bioedit Program (Hall, 1999) and the sequences were compared to all known sequences by the use of BLASTN 2.2.26+ program (Zhang et al. 2000) and deposited into GenBank (*Table 2).*

Preparation of conidial suspension

M. anisopliae isolates conidia were harvested by scraping the surface of 3-week-old sporulating cultures grown on potato dextrose agar (PDA) in petri dishes in an incubation at 25ºC±2 for three weeks from sowing. Fungus spores were harvested after three weeks by pouring a suspension that was prepared by adding 0.01% Tween 80 in sterilised and distilled water in a sterilized glass Erlenmeyer flask, into the Petri dish developed by *M. anisopliae.* Liquid mixture with fungi was drained into the sterilised glass Erlenmeyer flasks from cheese cloth. Then it was rinsed on a rinsing device for five minutes. After that, the spores were counted in the suspensions using a haemocytometer to $2x10^7$ spores/ml (Klingen et al., 2002a; Klingen et al., 2002b; Klingen et al., 2002c; Vänninen et al., 1999).

Incubation of fungal spores and its treatment on G. mellonella and T. molitor

Dipping technique was used and the fungus isolate was dipped in the tested spore suspensions for 10 second, then left to dry at room temperature. Every petri dish included 10 larvae for the experiments. For control treatment, the same process was followed but 3ml of sterilized and distilled water with 0.01% of Tween 80 was used instead of fungus isolate for the dipping. After all of the application petri dishes were stored in the incubator (25ºC±2) (Butt et al., 1994; Butt, 2002; Safavi et al., 2010). Mortality was assessed daily for 12 days by direct inspection for any signs of mortality. The experiment was replicated three times.

Statistical analysis

The experimental design was a randomized complete block with three replicates, and each replicate consisted of 10 larvae. The collected data were analyzed by one-way ANOVA test using SPSS 15.0 and means were compered by Duncan test.

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Results

All fungal isolates were pathogenic to *G. mellonella* and *T. molitor* with a mortality rate between 63.3±3.3% - 83.3±3.3% and 30±5.8% - 66.7±3.3%, respectively after 12 days from application. Microscopic investigations confirmed mycosis and fungi were reisolated from all dead individuals.

In this study, a total of 10 different isolates belonging to the genera *Metarhizium* were obtained. Based on clasicall and molecular methods, all fungal isolates were identified as *M. anisopliae* and deposited in the GenBank database under the accession numbers MH104853 - MH104862 as seen in *Table 2*.

Table 2. *Fungal material and their geographical origin (2014-2016)*

Species	Isolate Code	GenBank Accession Numbers
Metharzium anisopliae	MaEMR1a	MH104853
	MaEMR2b	MH104854
	MaEK1a	MH104855
	MaEİ3	MH104856
	MaEKLY1a	MH104857
	MaER3	MH104858
	MaEM1	MH104859
	MaET3	MH104860
	MaEÇ3	MH104861
	MaEO3	MH104862

Isolates of MaEMR1a, MaEM1 and MaEO3 caused mortality in more than 80% of *G. mellonella* larvae. There was no significant difference between these isolates ($p<0.05$). However, isolate MaEM1 led to earlier mortality than the others. Mortality was found to increase with time. The mortality percentages of isolates against *G. mellonella* are depicted in *Table 3*.

The most virulent isolates against T. molitor were MaEO3, MaET3, MaEKLY1a and MaEMR1a, which caused 60, 63.3, 66.7 and 63.3% mortality, respectively after 12 days from application. As seen in *Table 4*, M. anisopliae isolate MaEMR1a was consistently more virulent than other isolates because mortality caused by this isolate was found to be significantly different ($p<0.05$) on the 10^{th} day.

It is observed that the death rates have increased as time progressed. As the entomopathogenic fungi feed in the host and complete their development, secondary metabolites secreted to kill the host are exposed and the host is dying more rapidly. At the same time, the fungus develops inside the host and breaks down the integument of the intestine and hyphae appear on the host body surface.

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Table 3. *Mortality of M. anisopliae isolates against G. mellonella larvae (%)*

Isolates	3.day	4.day	5.day	6.day	7.day	8.day	9.day	10.day	11.day	12.day
MaEMR1a	0^{a}	$16.7^{\pm 6.7ab}$	$23.3^{\pm 6.7a}$	$40^{\pm 0ab}$	$43.3^{\pm 3.3c}$	$46.7^{\pm 3.3cd}$	$60^{\pm 0bcd}$	$63.3^{\pm 3.3bc}$	$70^{\pm 0ab}$	$83.3^{\pm 3.3a}$
MaEMR2b	0^{a}	$16.7^{\pm 3.3ab}$	$30^{\pm 0a}$	$40^{\pm 0ab}$	$46.7^{\pm 3.3bc}$	$56.7^{\pm 3.3abc}$	$63.3^{\pm 3.3abc}$	$66.7^{\pm 3.3bc}$	$73.3^{\pm 3.3ab}$	$76.7^{\pm 3.3ab}$
MaEK1a	0^{a}	$13.3^{\pm 3.3abc}$	$23.3^{\pm 3.3a}$	$33.3^{\pm 8.9b}$	$40^{\pm 5.8c}$	$40^{\pm 5.8d}$	$50^{\pm 0d}$	$63.3^{\pm 3.3bc}$	$63.3^{\pm 3.3bc}$	$76.7^{\pm 3.3ab}$
MaEİ3	0^{a}	$10^{\pm 0bc}$	$23.3^{\pm 3.3a}$	$33.3^{\pm 3.3b}$	$40^{\pm 5.8c}$	$46.7^{\pm 3.3cd}$	$56.7^{\pm 3.3cd}$	$63.3^{\pm 3.3bc}$	$73.3^{\pm 3.3ab}$	$76.7^{\pm 3.3ab}$
MaEKLY1a	0^{a}	$13.3^{\pm 3.3abc}$	$30^{\pm 5.8a}$	$36.7^{\pm 6.7b}$	$40^{\pm 5.8c}$	$53.3^{\pm 3.3bc}$	$60^{\pm 0bcd}$	$60^{\pm 5.8c}$	$60^{\pm 5.8c}$	$63.3^{\pm 3.3c}$
MaER3	$3.3^{\pm 3.3a}$	$20^{\pm 5.8ab}$	$36.7^{\pm 3.3a}$	$46.7^{\pm 6.8ab}$	$50^{\pm 5.8bc}$	$56.7^{\pm 3.3abc}$	$63.3^{\pm 3.3abc}$	$73.3^{\pm 3.3ab}$	$73.3^{\pm 3.3ab}$	$73.3^{\pm 3.3ab}$
MaEM1	0^{a}	$20^{\pm 5.8ab}$	$26.7^{\pm 3.3a}$	$43.3^{\pm 3.3ab}$	$53.3^{\pm 3.3bc}$	$63.3^{\pm 3.3ab}$	$70^{\pm 0ab}$	$80^{\pm 0a}$	$80^{\pm 0a}$	$83.3^{\pm 3.3a}$
MaET3	$3.3^{\pm 3.3a}$	$16.7^{\pm 3.3ab}$	$30^{\pm 5.8a}$	$46.7^{\pm 3.3ab}$	$53.3^{\pm 3.3bc}$	$56.7^{\pm 3.3abc}$	$63.3^{\pm 3.3abc}$	$63.3^{\pm 3.3bc}$	$70^{\pm 0ab}$	$70^{\pm 0bc}$
MaEÇ3	0^{a}	$10^{\pm 5.8bc}$	$26.7^{\pm 3.3a}$	$43.3^{\pm 3.3ab}$	$66.7^{\pm 3.3a}$	$66.7^{\pm 3.3a}$	$66.7^{\pm 3.3abc}$	$70^{\pm 0abc}$	$73.3^{\pm 3.3ab}$	$76.7^{\pm 3.3ab}$
MaEO3	$3.3^{\pm 3.3a}$	$26.7^{\pm 3.3a}$	$36.7^{\pm 3.3a}$	$53.3^{\pm 3.3a}$	$60^{\pm 0ab}$	$66.7^{\pm 3.3a}$	$73.3^{\pm 3.3a}$	$73.3^{\pm 3.3ab}$	$76.7^{\pm 3.3a}$	$83.3^{\pm 3.3a}$
Control	0^{a}	0^{c}	0^{b}	0^{c}	0^{d}	0^{e}	0^{e}	0^{d}	0^{d}	$3.3^{\pm 3.3d}$

*All values are mean ± standard error of three determinations (n=3). Same alphabet letters in the same column are not significantly different at $p<0.05$.

Table 4. *Mortality of M. anisopliae isolates against Tenebrio molitor larvae (%)*

Isolates	3.day	4.day	5.day	6.day	7.day	8.day	9.day	10.day	11.day	12.day
MaEMR1a	0	$10^{\pm 5.8ab}$	$16.7^{\pm 3.3bcd}$	$20^{\pm 5.8cde}$	$20^{\pm 5.8cd}$	$36.7^{\pm 3.3bcd}$	$40^{\pm 5.8bcd}$	$53.3^{\pm 3.3abc}$	$56.7^{\pm 3.3ab}$	$63.3^{\pm 3.3a}$
MaEMR2b	0	$6.7^{\pm 3.3ab}$	$13.3^{\pm 8.9cde}$	$20^{\pm 5.8cde}$	$26.7^{\pm 12bcd}$	$30^{\pm 11.5cde}$	$43.3^{\pm 8.9abc}$	$43.3^{\pm 8.9cd}$	$43.3^{\pm 8.9bc}$	$53.3^{\pm 8.9abc}$
MaEK1a	0	$10^{\pm 5.8ab}$	$20^{\pm 5.8abcd}$	$26.7^{\pm 3.3abc}$	$30^{\pm 5.8abc}$	$43.3^{\pm 3.3abc}$	$53.3^{\pm 3.3ab}$	$56.7^{\pm 3.3abc}$	$56.7^{\pm 3.3ab}$	$56.7^{\pm 3.3ab}$
MaEİ3	0	$6.7^{\pm 3.3ab}$	$13.3^{\pm 3.3cde}$	$13.3^{\pm 3.3}$	$23.3^{\pm 3.3cd}$	$26.7^{\pm 3.3de}$	$26.7^{\pm 3.3de}$	$33.3^{\pm 3.3de}$	$33.3^{\pm 3.3c}$	$40^{\pm 5.8cd}$
MaEKLY1a	0	$20^{\pm 5.8a}$	$30^{\pm 5.8ab}$	$36.7^{\pm 3.3a}$	$36.7^{\pm 3.3abc}$	$53.3^{\pm 3.3a}$	$53.3^{\pm 3.3ab}$	$63.3^{\pm 3.3a}$	$63.3^{\pm 3.3a}$	$66.7^{\pm 3.3a}$
MaER3	0	$3.3^{\pm 3.3b}$	$10^{\pm 5.8de}$	$10^{\pm 5.8ef}$	$10^{\pm 5.8de}$	$20^{\pm 5.8e}$	$20^{\pm 5.8e}$	$23.3^{\pm 3.3e}$	$30^{\pm 5.8c}$	$30^{\pm 5.8d}$
MaEM1	0	$20^{\pm 5.8a}$	$26.7^{\pm 3.3abc}$	$30^{\pm 0abc}$	$33.3^{\pm 3.3abc}$	$43.3^{\pm 3.3abc}$	$46.7^{\pm 3.3abc}$	$46.7^{\pm 3.3bcd}$	$50^{\pm 5.8ab}$	$53.3^{\pm 3.3abc}$
MaET3	0	$10^{\pm 5.8ab}$	$23.3^{\pm 3.3abcd}$	$23.3^{\pm 3.3bcd}$	$43.3^{\pm 3.3ab}$	$43.3^{\pm 3.3abc}$	$46.7^{\pm 3.3abc}$	$60^{\pm 5.8ab}$	$63.3^{\pm 3.3a}$	$63.3^{\pm 3.3a}$
MaEÇ3	0	$10^{\pm 0ab}$	$13.3^{\pm 3.3cde}$	$13.3^{\pm 3.3}$	$20^{\pm 5.8cd}$	$30^{\pm 5.8cde}$	$36.7^{\pm 3.3cd}$	$43.3^{\pm 3.3cd}$	$43.3^{\pm 3.3bc}$	$43.3^{\pm 3.3bcd}$
MaEO3	0	$13.3^{\pm 3.3ab}$	$33.3^{\pm 3.3a}$	$33.3^{\pm 3.3ab}$	$46.7^{\pm 3.3a}$	$50^{\pm 0ab}$	$56.7^{\pm 6.7a}$	$60^{\pm 5.8ab}$	$60^{\pm 5.8a}$	$60^{\pm 5.8a}$
Control	0	0^{b}	0^{e}	0^{f}	0^{e}	0^{f}	0^{f}	0^{f}	0^{d}	0^{e}

*All values are mean ± standard error of three determinations (n=3). Same alphabet letters in the same column are not significantly different at $p<0.05$.

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Discussion

Entomopathogenic fungi are used in the control of harmful insects and especially in the Integred pest management system (IPM) and they are used successfully nowadays. It is important to know the virulence of the newly developed mycoinsecticides, the killing rates, the molecular characterization of these fungi and their genetic structure as soon as possible.

According to this study, MaEMR1a, MaEM1 and MaEO3 isolates of *M. anisopliae* have been determined to have potential to suppress *G. mellonella larvae*; MaEO3, MaET3, MaEKLY1a and MaEMR1a isolates have potential to suppress *T. molitor* larvae. Among them these isolates, MaEMR1a and MaEO3 isolates possessed a good potential against both *G. mellonella* and *T. molitor* larvae. Our results overlap with other pataogentite tests on *T. molitor* and *G. mellonella larvae* (Pajar et al., 2013; Mora, E.A.M., 2016). Our results indicated that these two isolates (MaEMR1a and MaEO3) have a broad host range and can be used as biocontrol agents for *G. mellonella* and *T. molitor* and also they can be used as storages of biological control agents.

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