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Fungal pathogens of *Amphimallon solstitiale* Linnaeus, 1758 (Coleoptera: Scarabaeidae)

Amphimallon solstitiale Linnaeus, 1758 (Coleoptera: Scarabaeidae)'nin fungal patojenleri

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Abstract

European June beetle, *Amphimallon solstitiale* Linnaeus, 1758 (Coleoptera: Scarabaeidae) is one of the most important soilborne pests in many parts of Turkey and the world. Entomopathogenic fungi are important microbial control agents that can be used to control soilborne pests, and it is desirable to obtain them from local insect populations. The study was conducted at Karadeniz Technical University, Faculty of Science, Microbiology Laboratory in 2017. In this study, fungal pathogens of *A. solstitiale* were investigated to find an effective microbial control agent. Fungi were isolated from infected larvae and morphological-molecular characterization of the isolates showed that all isolates were *Metarhizium flavoviride* Gams & Roszypal (Deuteromycotina: Hyphomycetes). Using phylogenetic analysis and pathogenicity tests, the isolates were found to be different genotypes of *M. flavoviride.* All isolates gave more than 80% mortality at a concentration of 10^6 conidia/ml, with one isolate (As2) causing 96% mortality. Therefore, dose-mortality experiments were conducted with As2, and the median lethal concentration was determined to be 3.87 x 10^3 conidia/ml. This study demonstrated that *M. flavoviride* As2 is an effective microbial control agent that can be used for biological control of *A. solstitiale.*

Keywords: *Amphimallon solstitiale,* biological control, entomopathogenic fungi, *Metarhizium flavoviride*

Öz

Avrupa Haziran böceği, *Amphimallon solstitiale* Linnaeus, 1758 (Coleoptera: Scarabaeidae) Türkiye'de ve dünyanın birçok bölgesinde önemli toprakaltı zararlılarından biridir. Entomopatojen funguslar bu zararlıların mücadelesinde kullanılabilecek önemli mikrobiyal mücadele ajanlarıdır ve bunların lokal böcek popülasyonlarından elde edilmesi tercih edilir. Çalışma 2017 yılında Karadeniz Teknik Üniversitesi, Fen Fakültesi, Mikrobiyoloji Laboratuvarında gerçekleştirilmiştir. Bu çalışmada, zararlıya karşı etkili bir mikrobiyal mücadele ajanı bulmak için zararlının fungal patojenleri araştırılmıştır. Bulaşık larvalardan fungus izolasyonu yapılmış ve morfolojik-moleküler karakterizasyonu sonucu izolatlar *Metarhizium flavoviride* Gams & Roszypal, (Deuteromycotina: Hyphomycetes) olarak tanımlanmıştır. Filogenetik analizler ve patojenite çalışmaları sonucunda izolatların *M. flavoviride*'nin farklı genotipleri olduğu belirlendi. En yüksek ölüm ise %96 ile As2 izolatında görülmüş olup, tüm izolatlar 10^6 konidya/ml konsantrasyonda %80'in üzerinde ölüme neden olmuştur. As2 izolatı ile doz denemeleri sonucunda, izolatın ortalama öldürücü konsantrasyonu 3.87 x 10^3 konidya/ml olarak belirlenmiştir. Bu çalışmalar, *M. flavoviride* As2 izolatının *A. solstitiale*'nin biyolojik mücadelesinde kullanılabilecek etkili bir mikrobiyal mücadele etmeni olduğunu göstermektedir.

Anahtar sözcükler: *Amphimallon solstitiale,* biyolojik mücadele, entomopatojen fungus, *Metarhizium flavoviride*

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Introduction

The larvae of scarab beetles (Coleoptera: Scarabaeidae), also known as white grubs, cause significant economic loses by feeding on the roots of several agricultural plants in many parts of the world. One of these insects, the European June beetle, *Amphimallon solstitiale* Linnaeus, 1758 (Coleoptera: Scarabaeidae), is a major soilborne pest of a wide range of crops throughout Europe and Turkey. It is also a serious pest of hazelnuts cultivated in the Black Sea Region of Turkey (Allen, 1995; de Goffau, 1996). The adults of the pest mostly feed on leaves whereas the subterranean larvae cause damage directly through feeding on plant roots and indirectly by creating wounds which allow entry of opportunistic plant pathogens. Larvae feeding on roots can cause drying, wilting and death of the plants.

Chemical pesticides are usually the first choice to control noxious insects. However, excess use of these has led to insecticide resistance, environmental pollution, and adverse effects on human, non-target fauna and flora (Ffrench-Constant et al., 2004). Therefore, scientists have focused on developing alternative methods for pest management (Wilson & Tisdell, 2001; Keller & Zimmermann, 2005; Danismazoglu et al., 2012; Gokce et al., 2013; Sevim et al., 2013). The use of microbial control agents such as viruses, bacteria, nematodes and fungi are the best alternative to chemical pesticides and the development of new commercial products an important area of research (Sevim et al., 2012; Kocacevik et al., 2016; Eski et al., 2017). Of these beneficial microorganisms, the mode of infection of entomopathogenic fungi (EPF) differs from other entomopathogens. There is no need to ingest EPF because they can infect their host directly through the cuticle (Shah & Pell, 2003; Ortiz-Urquiza & Keyhani, 2013). Therefore, EPF can infect non-feeding stages such as eggs and pupae (Anand et al., 2009). Due to their mode of action and high mortality rate of the pest, interest in EPF has increased significantly (Humber, 2008). Therefore, considerable efforts have been focused on the development and utilization of EPF, as they have the potential to be a key tool in sustainable pest management programs.

Although, some bacteria have been isolated from *A. solstitiale* and tested for biological control of this pest (Sezen et al., 2005), no study has investigated fungal pathogens as potential microbial control agents. In this study, isolation and characterization of EPF from *A. solstitiale* were performed, and their insecticidal effect were tested on *A. solstitiale* larvae.

Materials and Methods

Isolation of fungi

Amphimallon solstitiale larvae were collected from soil samples at different localities in Trabzon, Turkey between May and June 2016 and 2017, incubated in ventilated plastic boxes (30 ml) at 28ºC and 16:8 h L:D photoperiod and fed on hazelnut roots. The plastic boxes were checked daily. The infected larvae were removed, surface sterilized according to Mohammadyani et al. (2016) and incubated in the moist chamber for sporulation. After 4 d, fungi development of cadavers inoculated onto potato dextrose agar plus 1% yeast extract medium (PDAY) and 50 µg/ml ampicillin, the latter to avoid bacterial contamination (AppliChem, Darmstadt, Germany) and incubated 2 weeks at 28ºC, 65% RH and 16:8 h L:D photoperiod. A single colony was subculture from each isolate to obtain pure cultures. One hundred µl of conidial suspension of 1 x 10^6 conidia/ml pure culture was transferred to PDAY and incubated at 28ºC for 7 d under 16:8 h L:D photoperiod. After incubation, the growing colonies were transferred to the new medium and allowed to sporulated under appropriate conditions (28ºC for 2-3 weeks), and then stock cultures were prepared in 20% glycerol.

Morphological characterization

The macroscopic characterizations of isolates were determined by examining growth of the fungal colony and color of conidia. Based on these features, the initial identification of fungal isolates was

performed according to the fungal identification key (Humber, 2012) and confirmed by Dr. Humber (ARSEF Collection of Entomopathogenic Fungal Cultures, US Department of Agriculture, Agricultural Research Service, Washington DC, USA).

Molecular characterization

Hundred µl of conidial suspension of each isolate was spread on PDAY medium and incubated at 25ºC for 14 d to select colonies originated from single conidia, and each colony was subculture onto fresh medium. DNA was extracted from the mycelium of each isolate using the Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The isolated DNA was stored at -20ºC until used. In order to determine the molecular characteristics of fungal isolates, the partial sequences of ITS1-5.8S-ITS2*,* RPB1 (RNA polymerase II largest subunit), *β-tubulin* and *pr1* genes were amplified by polymerase chain reaction (PCR) using primer pairs listed in Table 1.

Table 1. Primer pairs used for molecular characterization of fungal isolates

Primers	Primer sequence	Product size (bp)	Reference
ITS5	5'-GGAAGTAAAATCGTAACAAGG-3'	600	White et al., 1990
ITS4	5'-TCCTCCGCTTATTGATATGC-3'		
RPB1Af	5'-GARTGYCCDGGDCAYTTYGG-3'	800	Stiller & Hall, 1997
RPB1C	5'-CCNGCDATNTCRTTRTCCATRTA-3'		
T1	5'-AACATGCGTGAGATTGTAAGT-3'	1350	O'Donnell & Cigelnik,1997
T2	5'-TCTGGATGTTGTTGGGAATCC-3'		
METPR1	5 'CACTCTTCTCCCAGCCGTTC 3'	1200	Leal et al., 1997
METPR4	5'GTAGCTCAACTTCTGCACTC 3'		
METPR2	5'AGGTAGGCAGCCAGACCGGC 3'		
METPR5	5'TGCCACTATTGGCCGGCGCG 3'		

All targets except *pr1* were amplified with standard polymerase chain reactions using T100 thermal cycler (Bio-Rad, Watford, Hertfordshire, UK). Reaction mixture (50 µl total volume) contained 50 ng of DNA template, 10 µl 5X Phusion HF reaction buffer, 200 µM of each dNTPs, 1 µl (50 pmol) each primer, and 1-unit Phusion-DNA polymerase. PCR was performed under the following conditions: 98ºC for 30 s, followed by 98ºC for 10 s, 55ºC for 15 s, 72ºC for 1 min for 35 cycles with a final extension at 72ºC for 10 min.

Two successive nested-PCR amplifications of the *pr1A* gene were performed by using two primer pairs. In the first PCR, DNA from the samples as template and the outer pair of primers (METPR1 and METPR4) were used. In the second PCR, an aliquot of the first PCR as template and the inner pair of primers (METPR2 and METPR5) were used. PCR were applied as described above.

PCR products were analyzed by electrophoresis in 0.7% agarose gels and then visualized under UV light. The amplified products were purified and cleaned up using the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions, quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and addressed to sequencing (Macrogen, Amsterdam, the Netherlands).

Phylogenetic analysis

The sequences of the fungal isolates were aligned with the sequences in the NCBI GenBank. (www.ncbi.nlm.nih.gov/BLAST). The phylogenetic evolutionary analyses were performed using the neighbor-joining (NJ) method with MEGA X software (Kumar et al., 2018). Bootstrap tests were conducted with 1000 replicates in the NJ analysis.

Screening experiments

The conidial suspension used in the bioassay was prepared from 1-month-old fungus cultures, and fungal spores were harvested from agar surface using sterile 0.01% Tween 80 (AppliChem). The spore suspension was counted using a Neubauer hemocytometer, and the concentration was adjusted to 1 x 10^6 conidia/ml. The larvae of *A. solstitiale* were collected from agricultural fields in the vicinity of Trabzon, Turkey for bioassay, kept in plastic boxes (30 m^3), fed on hazelnut roots and allowed one week to adapt to the laboratory conditions. Ten healthy larvae were used in each bioassay, and the tests were replicated three times for each application. A dipping method was used for bioassay experiment. Larvae were dipped in 10 ml of 1 x 10^6 conidia/ml suspension for 5 s then put into plastic boxes (10 x 5 cm) containing sterile soil and hazelnut root pieces as food. Control larvae were exposed to 0.01% aqueous Tween 80. Experiments were performed at 20ºC under 16:8 h L:D photoperiod at 15 d. After the experiment, the dead larvae were removed from the boxes and surface sterilized and placed in incubator for mycosis.

Dose-response experiments

Metarhizium flavoviride Gams & Roszypal (Deuteromycotina: Hyphomycetes) isolate As2 (obtained during this study) was used in dose-response experiments as it had the highest virulence based on the screening experiments. Healthy larvae were collected from the farmland and used for dose-response experiments. They were treated with five conidial concentrations (10^8, 10^7, 10^6, 10^5 and 10^4 conidia/ml). The control group was treated only with 0.01% Tween 80. Bioassays were conducted as described for the screening test.

Temperature sensitivity and UV resistance

Temperature and UV tolerance of *M. flavoviride* isolate As2, shown the highest mortality on *A. solstitiale* were determined. Fungus was grown on PDA medium (Difco, Laboratories, Detroit, MI, USA) at 28ºC and under a 16:8 h L:D photoperiod. The spores were harvested from Petri dishes by adding 10 ml of sterile 0.01% Tween 80 onto the 4-weeks-old cultures. The prepared conidial suspension was filtered into 50 ml plastic universal bottle by a sterile cheesecloth to remove mycelium and vortexed for 5 min to obtain homogeneous suspension. Subsequently the concentration of conidial suspension was adjusted to 10^5 conidia/ml using a Neubauer hemocytometer. The experiment was designed according to the method of Bidochka et al. (2002). Ninety-six-well cell culture plates were filled with 100 µl of PDA and inoculated with 10^5 conidial suspension. Evaluation of the fungal growth was performed at 8, 15, 25 and 37ºC. Criteria from Bidochka et al. (2002) were adapted for assessment of positive growth; at 8ºC and OD_{630}>0.15 after 14 d; at 16ºC and OD_{630}>0.50 after 5 d; at 25ºC and OD_{630}>0.50 after 2 d; and at 37ºC and OD_{630}>0.25 after 3 d. UV tolerance of the fungal isolates was tested by exposing fungal conidia to UV radiation (306 nm) for 30 and 60 min. The cell culture plates were then incubated at 25ºC and OD_{630}>0.25 after 2 d was chosen as indicative of UV tolerance.

Statistical analysis

The control mortalities were corrected using Abbott's formula (Abbott, 1925). Statistical differences between the isolates were evaluated by one-way ANOVA, followed by Tukey's post-hoc test ($p < 0.05$). Each fungal isolate and control group were compared for mortality and mycosis. The LC_{50} value of *M. flavoviride* isolate As2 was estimated by probit analysis (Finney, 1971). Chi-square test was used to analyze growth of isolate As2 isolate in response to different temperatures and UV exposure using SPSS v 22.0 (IBM Corp., Armonk, NY, USA).

Results

Isolation and characterization of fungi

The specimens were classified as infected if mycelia growth was evident on the outside of cadavers. Four fungal isolates were obtained from *A. solstitiale* larvae. The isolates were identified based on the shape and size of conidia when grown on PDAY according to Humber (2012). In this way, the four isolates were determined to be *Metarhizium* sp. Sorokin, 1879. One isolate (As2) was differed from the others in colony morphology producing a darker green color 10 d after inoculation.

After amplification for ITS1-5.8S-ITS2, RPB1, *β-tubulin* and *pr1,* 600, 800, 1350 and 1200 bp amplicons were visualized in agarose gels, respectively. The partial sequences of ITS1-5.8S-ITS2, RPB1, and *β-tubulin* and *pr1* were used to construct phylogenetic trees. All isolates were determined to be identical to *M. flavoviride* as described by Bischoff et al. (2009). The four isolates As1 and As19, and As2 and As18 formed distinct two group on the phylogenetic trees (Figures 1 & 2). The sequences were deposited in the GenBank database under the accession numbers KY327805, KY348739, KY348740 and KY348741.

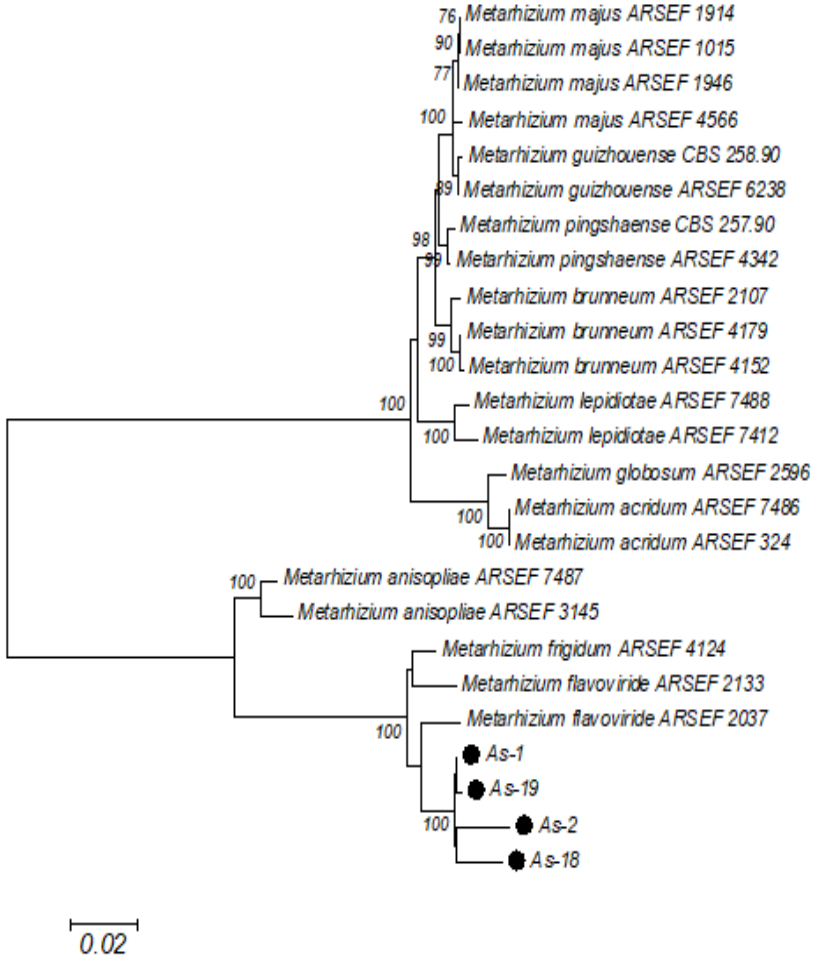

Figure 1. Taxonomic position of *Metarhizium flavoviride* As1, As2, As18 and As19 within the genus *Metarhizium* based on the combined data from ITS1-5.8S-ITS2, RPB1*, β-Tubulin* sequences. The reference isolates were taken from the study of Bischoff et al. (2009). The dendrogram was constructed by using the neighbor-joining analysis with *p*-distance model in MEGA X. Bootstrap values C ≥ 70% are labeled.

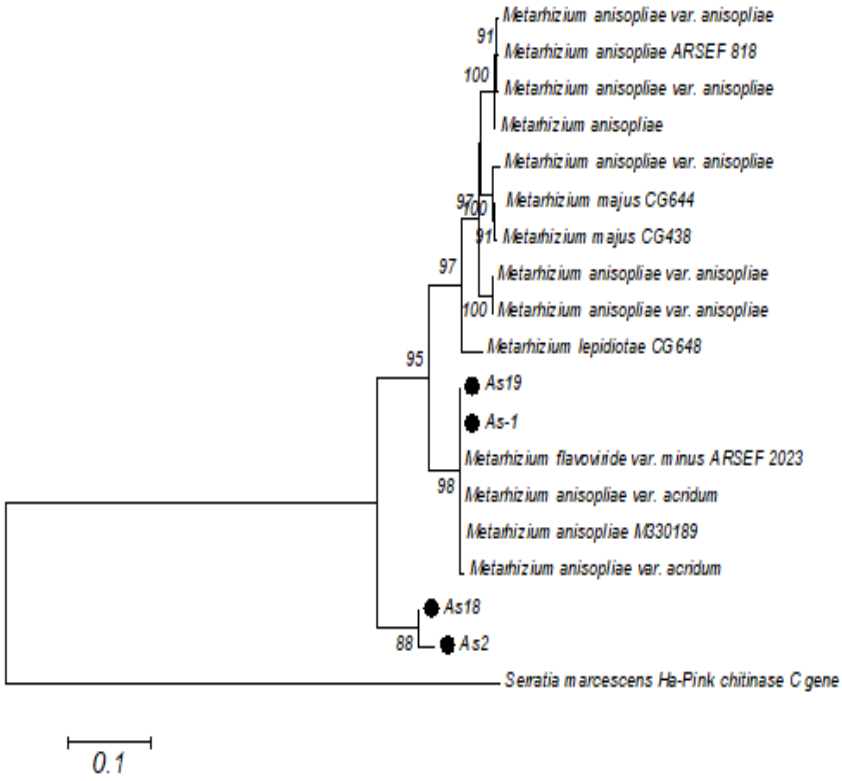

Figure 2. Taxonomic position of *Metarhizium flavoviride* As1, As2, As18 and As19 within the genus *Metarhizium* based on the combined sequence of *pr1 (*protease-type subtilisin) gene. The dendrogram was constructed by using the neighbor-joining analysis with *p*-distance model in MEGA X. Bootstrap values C ≥ 70% are labeled. The reference isolates were taken from the study of Bischoff et al. (2009). *Serratia marcescens* Ha-Pink chitinase C gene was used as an outgroup.

Screening test and dose-response experiments

The four isolates of *M. flavoviride* isolates (As1, As2, As18 and As19) gave high pathogenicity to *A. solstitiale* larvae 15 d post inoculation. Mortalities ranged from 86 to 97%, with isolates having different mycosis rates. Isolates As2 and As18 gave the highest mortality (93 to 96%, $p < 0.05$; Table 2). Although the mortality caused by these two isolates was equivalent, with isolate As2 all cadavers exhibited mycosis.

Table 2. Screening the pathogenicity test of locally isolated fungi against *Amphimallon solstitiale*

Fungal treatment at 10^6 conidia/ml	Mortality (%)±SE			
	5 DAT	10 DAT	15 DAT	Mycosis
As1	26±3.2 d	43±2.0 c	83±2.1 c	97±2.0 a
As2	53±3.5 b	83±3.1 a	96±3.8 a	100±0.0 a
As18	41±4.2 c	51±2.5 b	93±2.0 ab	98±2.1 a
As19	64±3.0 a	80±1.5 a	88±2.5 bc	97±2.1 a

Each value is mean of three replicates; Data analyzed by one-way ANOVA and Tukey's test. Values in a column followed by the same letter are not significantly different (p < 0.05); SE, standard error; and DAT, days after treatment.

In the dose-response experiments, *M. flavoviride* isolate As2 gave complete mortality of *A. solstitiale* larvae within 15 d after treatment with a conidia suspension of 1 x 10^8 conidia/ml (Figure 3). The LC_{50} of the isolate was estimated as 3.87 x 10^3 conidia/ml (Table 3). All concentrations caused a significantly higher mortality than control ($p < 0.05$).

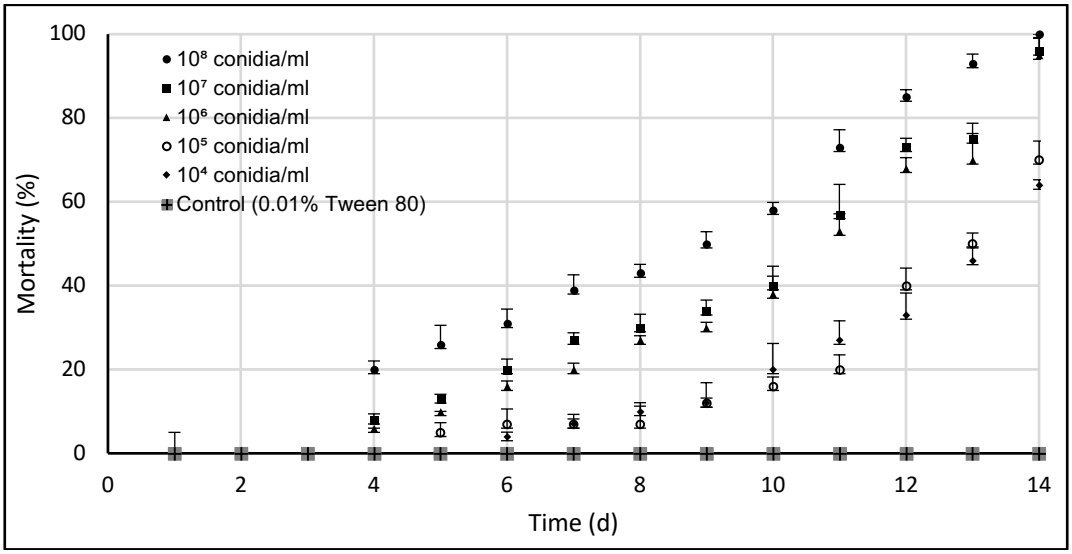

Figure 3. Cumulative mortality of *Amphimallon solstitiale* larvae after application of five different doses of the spore concentration of *Metarhizium flavoviride* strain As2.

Table 3. Median lethal concentration (LC_{50}) of *Metarhizium flavoviride* strain As2 against *Amphimallon solstitiale* larvae in the laboratory conditions

Isolate	LC_{50} (conidia/ml) (*FL*, %95)	Slope±*SE*	LC_{95} (conidia/ml) (*FL*, %95)	*df*	X^2
As2	3.87×10^3 ($1.7 - 2.77 \times 10^4$)	0.53± 0.071	3.38×10^6	3	8.32

FL: Fiducial limit, *SE*: Standard error, *df*: Degree of freedom, X^2: Chi square.

Effects of temperature and UV exposure on the growth of *Metarhizium flavoviride* isolate As2

The effect of temperature and UV exposure on *M. flavoviride* isolate As2 were compared using Chi-square test and there were not significant effects measured (p>0.05).

Discussion

Synthetic chemical insecticides are still the most commonly-used control strategy for white grubs. However, the use of chemicals to eliminate harmful insects have negative impacts on parasitoids of the grubs, evolution of insect resistance, contamination of environment and harmful effects on human. Entomopathogenic fungi represents a better alternative for eco-friendly management of white grubs.

In this study, isolation and identification of EPF from *A. solstitiale* was conducted and, four *M. flavoviride* isolates (As1, As2, As18 and As19) were obtained. The predominantly entomopathogenic fungal genus *Metarhizium* has a global distribution and includes several common species such as *Metarhizium anisopliae* Metschn.*, M. flavoviride* and *M. brunneum* Petch (Keller et al., 2003; Zimmermann, 2007; Kepler et al., 2014; Alkhaibari et al., 2016). In recent years, the taxonomy of the genus *Metarhizium* has been revised with the inclusion of other genera. In addition, some *M. flavoviride* variants were raised to species level (Kepler et al., 2014). Kepler & Rehner (2013) reported that additional genomic regions are needed to determine the identity to species level. In order to ensure the full recognition of the diversity of the genus belonging to *Metarhizium*, biological and distribution studies should be supported by molecular-based studies. Rarely all isolates of an EPF from a pest will belong to same genus. Sevim et al. (2010) characterized five *Beauveria* Vuillemin (Hyphomycetes: Moniliales) isolates from *Thaumetopea pityocampa* Denis & Schiffermüller, 1775 (Lepidoptera: Notodontidae) with all having distant taxonomic positions according to ITS and *EF1α* sequences. In the present study, the four *Metarhizium* isolates had different

taxonomic positions according to phylogenetic analysis. Also, the phylogenetic tree base on *pr1* gene encoding serine-like protease indicated that isolates were different genotypes of *M. flavoviride.* Studies on *Galleria mellonella* larvae indicated that the *pr1* gene is upregulated during pathogenesis and enhances virulence (Small & Bidochka, 2005). Presence of *pr1A* gene in our isolates may be indicative of enhanced virulence. Cito et al. (2014) tested the efficacy of various *Metarhizium* isolates against *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Dryophthoridae) and found that mortality caused by isolates with high Pr1 enzymatic activity was greater.

There have not been any reports of EPF isolated from *A. solstitiale*. However, Sezen et al. (2005) determined the bacteria associated with *A. solstitiale* and showed that one *Bacillus cereus* Frankland & Frankland, 1887 strain gave 90% mortality of *A. solstitiale.* In addition, they tested a mixture of another *B. cereus* strain and a *Bacillus thuringiensis* strain isolated from *Melolontha melolontha* Fabricius, 1775 (Coleoptera: Scarabaeidae) and obtained complete mortality of *A. solstitiale* under laboratory conditions. However, bacteria have a low efficacy against soilborne insects under field conditions because bacterial agents must be ingested to be effective and it is too difficult to deliver bacterial agents and products to soilborne pests such as *A. solstitiale*.

Use of entomopathogenic fungi against similar pests provided several additional advantages such as continuity in natural environments and horizontal transportation in pest populations. All isolated *Metarhizium* showed significant insecticidal activity against the pest. Also, the pathogenicity of *M. flavoviride* on *A. solstitiale* was determined in the present study. According to the screening tests, the highest mortality of *A. solstitiale* larvae was 97% within 15 d with isolate As2. All cadavers also exhibited mycosis in a humidity camber (Table 2). At the end of the dose-response tests conducted with this isolate, mortality reached 100% within 14 d (Figure 3). Several other studies on the insecticidal potential of *M. flavoviride* isolated from soil and insects have been conducted against harmful insects (Moore et al., 1992; Seyoum et al., 1994; Thomas & Jenkins, 1997). Magalhaes et al. (1997) tested *M. flavoviride* CG 423 (Brazilian isolate) and CG 291 (Australian isolate) against *Rhammatocerus schistocercoides* Rehn, 1906 (Orthoptera: Acrididae) and observed high mortality (>85%) at 10^7 conidia/ml 8 d post inoculation. Similarly, Li et al. (2012) noted that *M. flavoviride* Mf82 had the highest virulence against *Nilaparvata lugens* (Stål, 1854) (Hemiptera: Delphacidae) with 83.5% mortality within 10 d post inoculation. An *M. flavoviride* isolate obtained from soil sample using *Tenebrio*-bait method was tested against *Riptortus pedestris* Fabricius 1775 (Hemiptera: Alydidae), *Plutella xylostella* Linnaeus, 1758 (Lepidoptera: Plutellidae) and *Tenebrio molitor* Linnaeus, 1758 (Tenebrionidae: Coleoptera) and caused 15, 100 and 95% mortality, respectively (Kim et al., 2018). These studies show that geographic conditions where the EPF were isolated, the genus of the applied insect and, bioassay method affects the virulence. In addition, fungal pathogens show higher insecticidal effect against their hosts compared to other insects (Tanyeli et al., 2010; Sönmez et al., 2016). Sevim et al. (2010) showed that a *Beauveria bassiana* isolate from *Thaumetopoea pityocampa* Denis & Schiffermüller, 1775 (Lepidoptera: Thaumetopoeidae) could cause complete mortality its larvae. Similarly, *M. flavoviride* isolated from *A. solstitiale* gave complete mortality on its larvae.

In conclusion, EPF of the European June beetle were isolated for the first time and their insecticidal potential determined. The results indicate that the isolates obtained are strong candidates for microbial control of this pest. In particular, *M. flavoviride* isolate As2, which gave the highest mortality at low conidial concentration was determined to be the most effective isolate. In the further studies, this isolate should be formulated as a mycoinsecticide to protect it from adverse environmental conditions and tested under the field conditions against the *A. solstitiale* and the other root-feeding white grubs.

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