Egyptian Journal of Biological Pest Control, 25(1), 2015, 121-127

Identification of Turkish Isolate of the Entomopathogenic Fungi, *Purpureocillium lilacinum* (syn: *Paecilomyces lilacinus*) and its Effect on Potato Pests, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)

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(*Received: February 17, 2015 and Accepted: April 5, 2015*)

ABSTRACT

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops in Turkey as well in the world. Potato plants are attacked by a large number of insect pests such as aphids, beetles, leafhoppers and lepidopterous pests under both field and storage conditions. The Colorado potato beetle (CPB) [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)] and the potato tuber moth (PTM) [*Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae)] are the most destructive pests in potatoes grown areas. Many pesticides that used to control the potato pests are very toxic to environment and human. An alternative approach to the commercial pesticides is using biological agents like fungi. *Purpureocillium lilacinum* was described more than a century ago and is a commonly occurring fungus in soil. *P. lilacinum* (syn: *Paecilomyces lilacinus*) is the most widely tested fungus for the control of nematodes, aphids and insects. The fungus was determined in Turkey but its molecular identification has not been done yet. In this study, a Turkish isolate of *P. lilacinum* was identified by classical (morphologic and morphometric properties) and molecular methods. Also, the study concerned with the effect of different conidial concentrations of the fungal isolate on mortality rates of CPB and PTM at different temperatures. *P. lilacinum*was found to be most effective on the last larval instar of CPB and PTM (33.2 and 43.3% mortality, respectively) on 10th day of treatment with the fungal concentration of 10^8cfu ml^{-1}at 25°C. This fungus developed on all dead larvae and was highly susceptible at the tested temperatures, but its activity decreased at 15°C. Findings of this study showed a potential of the fungus as a bioagent against the CPB and the PTM.

Key words: Entomopathogen fungi, *Purpureocillium lilacinum*, identification, biological control, *Phthorimaea operculella, Leptinotarsa decemlineata.*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops in Turkey as well in the world. Under field conditions, potato plants are attacked by a large number of insect pests such as; aphids, beetles, leafhoppers, and lepidopteran pests. These insects cause an annual loss around 75% (Oerke, 2006). One of the most important polyphagous pests reducing potato production is the Colorado potato beetle [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)] (CPB). The pest pupates into the soil and overwinters as adults. Adults and larvae of CPB feed on green portions of the plants. The last instar larvae, in particular cause the most damages. CPB is also a vector of the bacterial ring rot disease. This disease causes significant losses when climatic conditions are suitable (Christie *et al.*, 1991).

Potato tuber moth (PTM), *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) is another destructive pest. It attacks not only potato but also other solanaceous plants such as tomato, tobacco, eggplant and pepper in tropical and subtropical countries. PTM larvae mine into the foliage, stems, and tubers (Islam *et al.*, 1990). The larvae also form blotches in leaves and fold leaves over for sheltering during feeding and for pupation (Mandour, 1997). New mines are always present in the upper part of the potato canopy. There is usually a 10% rate of tuber infestation with PTM, but when control methods are not used, the infestation rate may reach 100% (Sileshi and Teriessa, 2001). In potato storage facilities, infestation of PTM also causes partial and/or complete rotting by the subsequent infestation with fungi and/or bacteria, which render the infested tubers unmarketable (Sarhan, 2004).

Up to the last two decades, control of PTM and CPB has relied upon use of commercial insecticides (Sarhan, 2004). Chemical control has led to high levels of resistance where their control has become harder from year to year (Whalon *et al.*, 2011). Therefore, usage of alternative methods to chemical control against pests has been required; focusing on plant extracts (Scott *et al.*, 2003 and Gökçe*et al.*, 2007& 2012) and on biological control agents (Grissel, 1981; Toba *et al.*, 1983; Cantwell *et al.*, 1985 and Zehnder and Gelernter, 1989). In recent years, depending on biological control has gained further importance, with an increase of reactions to

use of pesticides, based on rise of the environmental conscience, particularly in the countries with advanced technology. Although more than 400 entomopathogenic fungi (EPF) have been defined, studies are concentrated only on 20 of them (Zimmermann, 1986). Among those, the genera; *Lagenidium, Entomophaga, Neozygites, Entomopytora, Erynia, Aschersonia, Lecanicillium, Nomuraea, Hirsutella, Matarhizium, Beauveria* and *Paecilomyces* are stated to be the most important (Robert and Wraight, 1986).

In Turkey, few literature records have been found on the effect of EPF, originating on the pest groups of economical significance, particularly the target pests *L. decemlineata* and *P. operculella*. Therefore, this study aimed to indentify the entomopathogenic fungi using classical and molecular techniques and to determine the effect of different conidial concentrations of the identified fungus *Purpureocillium lilacinum* on mortality of PTM and CPB.

MATERIALS AND METHODS

Fungus Sources

A culture of the Turkish isolate of the entomopathogenic fungus, *Purpureocillium lilacinum* TR1 (syn: *Paecilomyces lilacinus*) was provided by the Plant Protection Central Research Institute, Ankara, Turkey. *P. lilacinum* was isolated from root-knot nematodes in the tomato plant roots in Sarıcakaya (Eskisehir) within the scope of the project "Determination of fungal and bacterial pathogens of root-knot nematodes, a problem for greenhouse vegetable growing in the cities of Burdur, Isparta and Eskisehir" that carried out between 2002 and 2007. As a result of the study conducted to determine the fungal pathogens of root-knot nematodes in the country, the Turkish isolate of *P. lilacinum* was attained (Kepenekci *et al.*, 2009). It was stored at +4°C on Potato Dextrose Agar (PDA) and at–80°C. For mass-culturing of *P. Lilacinum*, the fungus was subcultured on PDA (DifcoTM, Becton Dickinson and Company, USA) at 25±1°C for 7-14 days.

Mass-culturing of *P. lilacinum*

The fungus was subcultured on PDA (DifcoTM, Becton Dickinson and Company, USA) with the help of a sterilized bacteriological loop. The dishes were closed by Parafilm at 25±1°C for 7-14 days. The conidia were harvested using a sterilized rubber loop attached to 1 ml borosilicate pipette at the angle of 45°. Scraped material was shifted into sterilized Petri dishes and stored at 4°C in refrigerator. The harvested fungal conidia were incorporated to sterile 0.05% Tween-80 solution and the material was stirred for complete homogeneity (Wakil *et al.*, 2012). The serial dilutions were prepared and the number of conidia was measured under haemocytometer to achieve the 10^6, 10^7 and 10^8cfu ml^{-1}concentrations.

P. lilacinus morphological identification

Colony characteristics of the fungal isolate were observed after the cultures were grown on PDA for 7-14 days. Morphological features (*e.g.*, conidiophores, phialides) of the isolate were examined by a light microscope (Leica DM4000 B LED) and measured using the software LAS (Leica Application Suite) program. Morphological identification based on Samson (1974).

DNA extraction

DNA from fungal cultures was extracted according to DNAeasy Tissue and Blood Kit (Qiagen, Hilden, Germany), following manufacturer's instructions, using species specific PCR assays. The primer sequence *PaeF*and *PaeR* (PaeF: 5' CTC AGT TGC CTC GGC GGG AA 3'; PaeR: 5' GTG CAA CTC AGA GAAGAA ATT CCG 3') (Atkins *et al.*, 2005) were used for DNA analysis. PCR amplification was performed in a total volume of 25 ml containing 10XPCR Buffer, 0.2 mMdNTP, 0,4 mM of each primer, 2 mM $MgCl_2$, 20 ng of template DNA and 1 Unit Taq DNA Polymerase (Fermentase). PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in Tris-EDTA (TAE) buffer with 2% agarose gel stained with Pronosafe (Condalab, Spain) at 80 V for 1 h and then visualized under UV light.

Sequence Analysis

Primers ITS4 (5'TCCTCCGCTTATTGATATG C-3') and ITS5 (5'GGAAGTAAAAGTCG TAACAAGG-3') (White *et al.*, 1990) were used to amplify the ITS region of rDNA. The 50 mL of reaction mixture contained 10 ng templates DNA, 1 mM of each primer, 100 mM of each dNTP, 5 mL 10x PCR buffer, 1.5 mM $MgCl_2$, and 2.5 Units of Taq Polymerase. PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 55°C for 1 min, 72°C for 1 min, 95°C for 2 min, with a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in Tris-EDTA (TAE) buffer with 2% agarose gel stained with Pronosafe (Condalab, Spain) at 80 V for 1 h and then visualized under UV light. Sequencing data wereanalyzedusing Chromas Pro 1.7.6 (Technelysium Pty Ltd., Qld, Australia). Sequences were 100 and 98% of the similarity when compared to NCBI data.The sequences of *P. lilacinum* TR1were generated for the first time in this study and filling a void in the GenBank data base.

Insect sources

Last instar larvae (L4) of *L. decemlineata* (CPB) and *P. operculella* (PTM) were obtained from the laboratory colonies, maintained at the Entomology Division, Plant Protection Central Research Institute in Ankara, Turkey. The larvae were used within two hours in the experiments. Larvae of CPB and PTM were collected from untreated potato plants grown in Central Anatolia and were fed on fresh potato leaves daily until the larvae completed feeding and developed to prepupae (inactive, nonfeeding stage prior to pupation). For PTM larvae, they were reared on potato tubers in a rearing room at 28±2°C, 14:10 L:D. Newly formed 4th instar was used within two hours in the experiments.

Bioassay

Effect of temperature and fungus concentration on mortality of PTM and CPB

In this study, plastic plates with 12 cells (each of 24 mm diameter and 20 mm depth) were used. Application in each cell was done by filling it with sterile sand of 0.2 g. Three different concentrations of *P. lilacinum* spores' suspension (10^6, 10^7 and 10^8cfu ml^{-1}) were applied to each cell. Plates were closed by adding into each cell one PTM or CPB larva (totaly 10 from each were used in each treatment for bioassay test) and they were kept into incubators at three different temperatures (15, 25 and 30°C and >70% Relative Humidity at 16L: 8D photoperiod). In the control, sterile water was added to the surface of the cells. Each treatment and bioassay test was repeated independently three times (totally 30 larvae from each were used). Data were recorded after 2nd, 4th, 6th, 8th and 10^{th}days' interval. Bioassay tests were performed in June-August, 2013 at Plant Protection Central Research Institute, Entomology Department, Nematology Laboratory.

Data Analysis

One-way ANOVA was used to compare the mortality rates of *L. decemlineata* and *P. operculella*. Means were compared at the P=0.05 level, and Tukey's test was used to separate means (SPSS, 1999). Arcsine transformation was carried out on mortality (%) before analyses.

RESULTS AND DISCUSSION

Identification of the entomopathogenic fungus, *Purpureocillium lilacinum* TR1

P. lilacinum colonies developed to a diameter of 5-7 cm within 7-14 days at 25°C on PDA. Colony color started white and changed vinaceous to violet (Fig.1-A). Conidiophores erected arising mainly from sub-merged hyphae, with occasional formation of synnemata. Conidiophores erected 400-600 µm in length. Conidiophore stipeses were 3-4 µm wide, yellow to purple and rough-walled. Vegetative hyphae were hyaline, straight and 5.0-4.0 µm wide. Verticillate branches appeared with whorls of 2 to 4 phialides. Phialides were cylindrical or ellipsoidal in the swollen basal part, narrowing abruptly into a short neck approximately 1 µm wide and measured 8.4-13.5×1.9-3.1 µm. Conidia were formed in divergent chains, which sometimes became slightly roughened to ellipsoidal fusiform, smooth-walled to slightly roughened, 2.1-3.4×2.0-2.2 µm. Chlamydospores were absent. Morphological diagnosis was confirmed by molecular tools. When the species-specific primers *PaeF* and *PaeR* were used for amplification of the ITS region, approximately 130 bp patterns were obtained (Fig.1-C). Also, cDNA sequences of PCR products were attained using general ITS-4 and ITS-5 primers. cDNA sequences of the isolate that was diagnosed molecularly were maintained in the GenBank and their access numbers were received (GenBank accession no. KF706346).

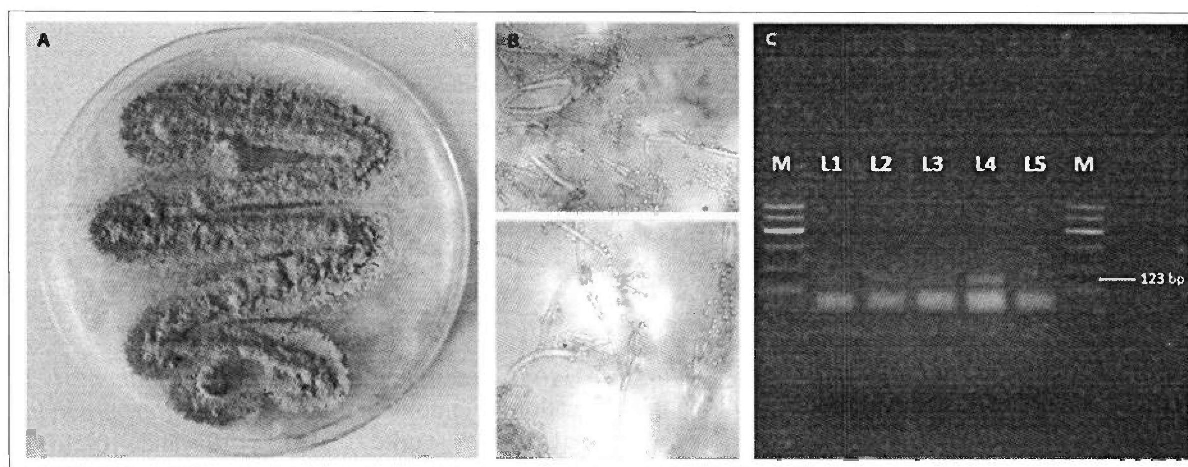

Fig. (1): A: *Purpureocillium lilacinum* TR1; B: *P. lilacinum* as shown under microscope (conidiophore, conidia and phialid); C: Restriction pattern of PCR product from *P. lilacinum*. M (Marker, 600+ bp); 1, 2 and 3 *P. lilacinum*TR-1 isolate; 4 Positive control; 5- Negative control (water).

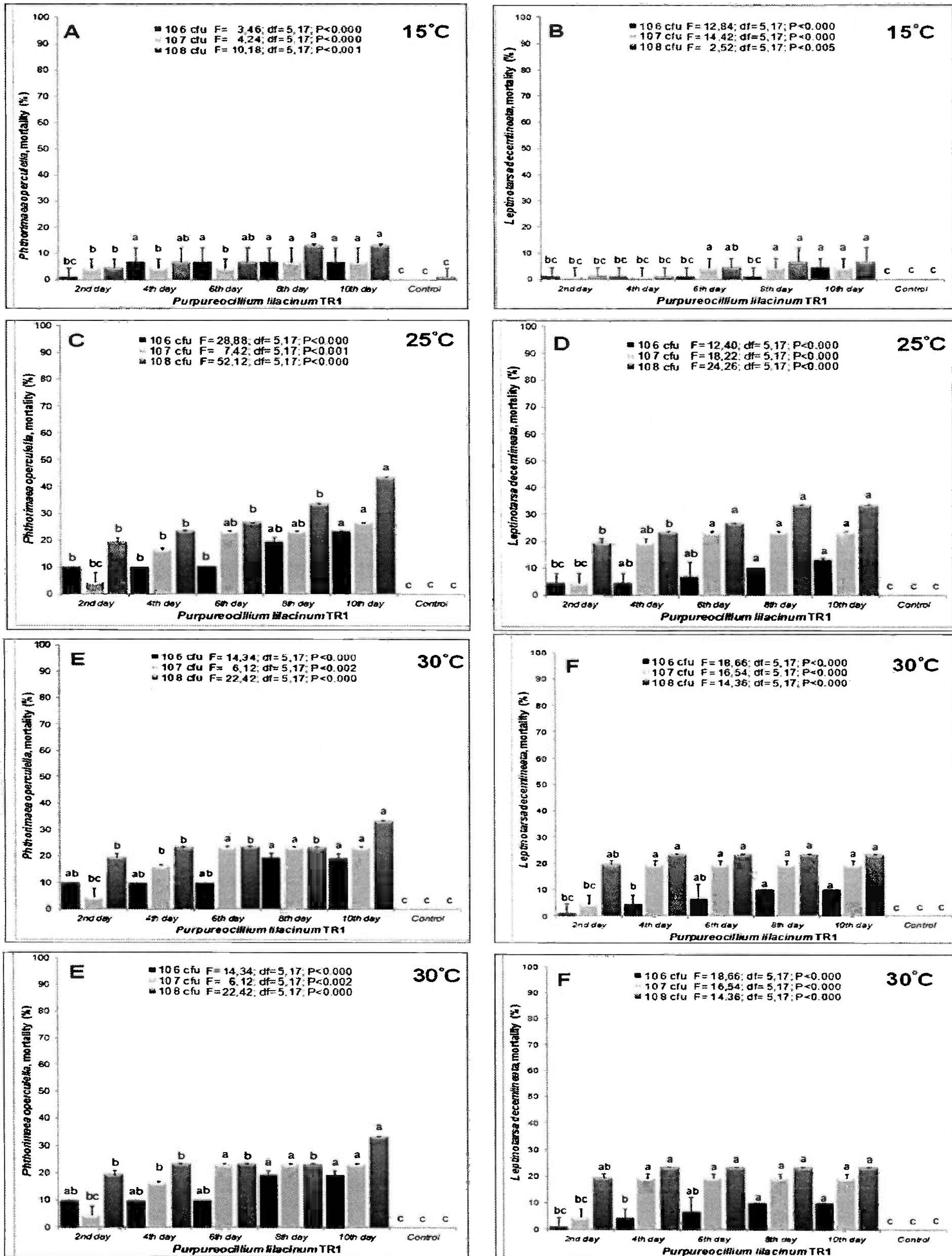

Fig. (2): Mortality (%) of *Phthorimaea operculella* (PTM) (A, C and E) and *Leptinotarsa decemlineata* (CPB) (B, D and F) of last larval instars, following application of entomopathogenic fungi *Purpureocillium lilacinum* TR1 (syn: *Paecilomyces lilacinus*) (isolated from Turkey) at 10^6, 10^7 and 10^8cfu ml^{-1} concentrations at different temperatures [15°C (A, B) 25°C (C, D) and 30 °C (E, F)]. Data are expressed as mean±SE. The same letter above the error bars indicates no significant difference (P>0.05; Tukey test).

Effect of temperature and fungus spore concentration on mortality of PTM and CPB

The data generally showed that all concentrations were effective against *P. operculella* (PTM) and *L. decemlineata* (CPB) in their last instar larvae (Fig. 2 A-F). Results of this study were recorded at 10^6, 10^7 and 10^8cfu ml^{-1} concentration at 15, 25 and 30°C, after 2nd, 4th, 6th, 8th and 10th days of application. Mortality rates of PTM and CPB were around 13 and 6.6%, respectively, at 15°C for all concentrations. At 25°C, PTM recorded the highest mortality rate (43.3%) after 10 days of treatment with 10^8cfu ml^{-1} concentration. Effects on mortality under the other concentrations did not exceed 26.5%. CPB showed significantly less susceptible than PTM. The highest mortality rate of CPB (33.2%) was recorded after 10 days of exposure to 10^8cfu ml^{-1} concentration at 25°C. Effect on mortality rates in other concentrations did not exceed 23.1%. The highest effect on PTM and CPB were found in the treatment with 10^8cfuml^{-1} concentrations at 25°C. No mortality was observed in the CPB used as control up to the end of the experiment. But in PTM, (1.1%) mortality was recorded only at the control under 15°C. The highest mortality rate on PTM (33.2%) was found at 10^8cfu ml^{-1}concentration under 30°C, 10 days after application. On 8th day of treatment at 10^6cfu ml^{-1} concentration, the highest mortality rate was recorded but with insignificant difference. At the concentration of 10^7cfu ml^{-1}, the highest mortality rate (23.1 %) was recorded on 6th day; on 10th day insignificant difference was recorded. The highest mortality rate among CPB larvae (23.1%) was recorded at 10^8cfu ml^{-1} and (19.1%) at 10^7cfu ml^{-1} concentrations under 30°C on the 4th day of application; insignificant difference was observed between both concentrations on 10th day. The concentration of 10^6 cfu ml^{-1} at 30°C on the 8th day of exposure recorded the highest mortality rate (9.9%), while in the other days, mortality rate was lower.

In this study firstly, diagnosis of EPF was done by morphological, morphometric and molecular identification. *P. lilacinum* TR1, DNA extraction was performed, using DNeasy Blood and Tissue Kit (Qiagen, Germany), and 123 bp bands were attained using primer set specific to species. Also, cDNA sequences of PCR products were derived using general ITS primers and these cDNA sequences were compared to NCBI data. cDNA sequences of the isolate that was diagnosed molecularly were maintained in the GenBank and their access numbers were received. Subsequently, the effect of *P. lilacinum* TR1, which has been recorded previously in Turkey on the last instar larvae of *L. decemlineata* and *P. operculella* and negatively impacted potato production, was bioassyed under laboratory conditions.

The data generally showed that all fungal concentrations had high affects at 25°C than 15 and 30°C against the last instar larvae of PTM and CPB. At 15 and 30°C, EPF caused less than 13 and 33% mortality rates, respectively. Ansari *et al.*(2004) found that mortality rate depends on the concentration of conidial suspension, exposure time and temperature. Similar to obtained results, effect of different conidial concentrations of *Verticillium lecanii*, *Paecilomyces fumosoroseus* and *Metarhizium anisopliae* against *Brevicoryne brassicae* showed that mortality of aphids increased with increase of the spores' concentration and exposure time (Asi *et al.*, 2009). Also, the virulence of *B. bassiana* against *Myzus persicae* was examined and the results showed that aphids had higher mortality rates at 28 and 21°C than those at 16 and 11°C (YinQuan *et al.*, 2000). The optimum growth temperature for the fungus was 26-30°C, while it was less effective at 15°C. It was observed that the efficacy of all treatments showed increasing trends up to 10 days post application. It may be appropriate to test obtained results against the two pests under field conditions. Encouraging results were obtained by the studies of *P. lilacinum* TR1 against the primary agricultural pests in Turkey; great spruce bark beetle [*Dendroctonus micans* (Kugelann) (Coleoptera: Scolytinae)], black cherry aphid [*Myzus cerasi* Fabricus (Hemiptera: Aphididae)] and stored grain insect pests (Kepenekci *et al.*, 2014a, b and Kepenekci *et al.*, 2015).

ACKNOWLEDGMENTS

A part of this study (preparation of *P. lilacinum*) was supported by the Scientific and Technical Research Council of Turkey (TÜBITAK 110O784). Thanks are due to Drs. F.D. Erdoğuş and M. Alkan (Dept. of Entomology, Plant Protection Central Research Institute, Ankara-Turkey) for their assisstance throughout this study.

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