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Evaluation of Some Entomopathogenic Fungi for Controlling the Green Shield Bug, *Palomena prasina* L. (Heteroptera: Pentatomidae)

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ABSTRACT

Green shield bug, *Palomena prasina* L. (Heteroptera: Pentatomidae) is a common pest species in Turkish hazelnut orchards. It feeds on hazelnut fruits and causes premature nut dropping in early season and later kernel damage during nut development. Controlling *P. prasina* is currently done by spraying insecticides that looks like the only option. Since hazelnut covers quite a large area in the country, possibility of using microbial control agents for controlling *P. prasina* is rather important. In this study, firstly hazelnut orchards were surveyed and 8 entomopathogenic fungi isolates were obtained from insects. Virulance of these isolates was evaluated against *P. prasina* nymphs in laboratory at 25°C and 90±5 RH. Isolates included 2 *Simplicillium lamellicola,* 4 *Lecanicillium muscarium* and one *Beauveria bassiana* and one *Isaria fumosorosea*. Isolates were bioassayed against nymphs. Dead individuals were counted daily following treatment for 12 days. Lethal time values (LT_{50} and LT_{90}) for entomopathogenic fungi were calculated. LT_{50} and LT_{90} values for the experimented isolates ranged from 3.20 to 8.48 days and from 9.32 to 40.30 days, respectively. At the end of 12 days post treatment, mortality rates were above (83%) at all treatments. Highest mortality rates (98.00 and 95.00%) were observed in at the isolate of *L. musacarium* and *B. bassiana*, respectively. Entomopathogenic fungi could be promising agents for controlling *P. prasina*.

Key words: Hazelnut, *Palomena prasina*, Entomopathogenic fungi, biocontrol.

INTRODUCTION

Hazelnut is one of the most important export products in Turkey and 70–75% of the world's hazelnut demand is supplied by the country. Turkey produces approximately 618 000 tons hazelnut and earns 2-3 billion dollars yearly. Moreover, 2 million people depend on income from hazelnut in Turkey (Anonymous, 2016).

Many insect pests damage hazelnut orchards and affect its production and quality in Turkey. Among these insect pests are the pentatomids that damage hazelnut kernel quality (Tavella *et al*., 2001 and Tuncer *et al.*, 2004) and among the pentatomids, the predominant species is the green shield bug *Palomena prasina* L. (Hemiptera: Pentatomidae) with (85%) prevalence in hazelnut orchards of Black Sea region of Turkey (Tuncer *et al.*, 2005). Spotted kernel damage by *P. prasina* was determined as (1.3-4.0%) in Italy (Tavella *et al.*, 2001).

Currently, the farmers have only the option of using insecticides to suppress insects in hazelnut orchards. Unfortunately, there are no alternative control methods against this pest. But, negative effects of the chemical control on human health and environment are well-known. Thus, alternative control methods should be developed. Biological control with entomopathogens might be an appropriate alternative control method. Entomopathogenic fungi (EF) are potential biological control agents of many arthropods worldwide (Roy *et al.*, 2006). Fungal entomopathogens such as *Beauveria bassiana* (Balsamo) Vuillemin, *Isaria farinosa*, *I. fumosorosea*, *Lecanicillium* spp. and *Simplicillium* spp. play an important role in the regulation of insect populations (Zimmermann, 2008). Several commercial products based on *B. bassiana* and *I. fumosorosea* alone and in combination with other entomopathogenic species have been developed in India, Colombia, Mexico and Venezuela as well as in the USA and in Europe (Zimmermann, 2008).

In Turkey, the entomopathogenic fungi, *B. bassiana*, *I. farinosa*, *L. muscarium* and *S. lamellicola* were isolated from different insect hosts (Anonymous, 2014) and their pathogenicity against different hosts was determinated (Saruhan *et al*., 2015), but there is no detailed study revealing the effect of such fungi on *P. Prasina,* despite its economic damage in hazelnut orchards (Tavella *et al*., 2001 and Tuncer *et al*., 2005).

The aim of this study was to determine the pathogenicity of 8 entomopathogenic fungi against 4th nymphal instar of *P. prasina* under laboratory conditions.

MATERIALS AND METHODS

Fungal cultures

A total of 6 isolates of entomopathogenic fungi were isolated from infected *P. prasina* males and females collected from hazelnut orchards in Giresun, Ordu, Samsun and Düzce provinces in Black Sea region of Turkey (Table 1). In addition, 2 isolates were isolated from infected pupae of *H. cunea.* Single-spore isolates were obtained by serial dilution

Table (1): Hosts and locations of the tested entomopathogenic fungi isolates

Species	Isolates denomination	ARSEF accession numbers	Hosts	Locations of collection
Simplicillium lamellicola	TR-01	ARSEF 11727	*Palomena prasina*	Giresun province, Turkey
S. lamellicola	TR-02	ARSEF 11728	*P. prasina*	Ordu province, Turkey
Lecanicillium muscarium	TR-04	ARSEF 11730	*P. prasina*	Ordu province, Turkey
L. muscarium	TR-05	ARSEF 11731	*P. prasina*	Samsun province, Turkey
L. muscarium	TR-07	ARSEF 11733	*P. prasina*	Ordu province, Turkey
L. muscarium	TR-08	ARSEF 11734	*P. prasina*	Düzce province, Turkey
B. bassiana	TR-55-3	ARSEF 12165	*Hyphantria cunea*	Samsun province, Turkey
Isaria fumosorosea	TR-78-7	ARSEF 12177	*H. cunea*	Samsun province, Turkey

(Dhingra and Sinclair, 1995), and identified as: *S. lamellicola* (TR-01 and TR-02 isolates), *L. muscarium* (TR-04, TR-05, TR-07 and TR-08 isolates), *B. bassiana* (TR-55-3 isolate) and *Isaria fumosorosea* (TR-78-7 isolate) (Table 1). The single-spore cultures were prepared and stored at 4°C on Sabouraud dextrose agar slants (SDA) (Merck Ltd., Darmstadt, Germany) and also in cryogenic tubes containing 15% glycerol at –80°C, and deposited in the fungal culture collection of the Mycology Laboratory at the Ondokuz Mayis University, Faculty of Agriculture's Department of Plant Protection in Samsun, Turkey and in the USDA-ARS Entomopathogenic Fungal Culture Collection in Ithaca, NY.

Insect culture

Fourth instar nymphs of *P. prasina* were used in bioassays. The nymphs were collected from different hazelnuts orchards by beating-sheet method during July in Samsun province. The insects were maintained in climate chamber with 25±1ºC, 70±5 RH and 16 h light and 8 h dark photoperiods. After the bugs were adapted to the laboratory conditions, the insect culture was fed with fresh bean pods (*Phaseolus vulgaris*) and the food was changed daily basis.

Conidial germination assessment

Aviability of conidia of the collected isolates was determined. A conidial suspension was adjusted to $1x10^4$ conidia ml^{-1}, and 0.1 ml was sprayed onto 6-cm-dia. Petri dishes containing potato dextrose agar (PDA: Oxoid Ltd, Basingstoke, UK). Petri dishes were incubated at 25±1°C. After 24 h of incubation, percentages of germinated conidia were counted using an Olympus CX-31 compound microscope (Olympus America Inc., Lake Success, NY) at 400X magnification. Conidia were regarded as germinated when they produced a germ tube at least half of the conidial length. Germination ratios for each fungus were calculated after examining a minimum of 200 conidia from each in 3 replicate plates (Saruhan *et al.*, 2015).

Inoculum of entomopathogen isolates

Collected isolates were grown on SDA at 25±1ºC for 15 days. Conidia were harvested with sterile distilled water containing 0.02% Tween 20. Mycelia were removed by filtering conidia suspensions through 4 layers of sterile cheesecloth. Conidia were counted under Olympus CX31 compound microscope using a Neubauer hemocytometer to calibrate a suspension of $1x10^8$ conidia mL^{-1} for each isolate.

Experimental design

Fourth nymphal instars of *P. prasina* were placed in 1L plastic ice-cream cups (sterilized by ethanol) containing two fresh bean pods which were 5-6 cm long. Ten 4th nymphs were placed into each cup. Bottoms of cups were covered by filter paper moistured by sterile-distilled water. Conidial suspensions ($1x10^8$ conidia ml^{-1}) of the collected fungi were applied to the nymphs of *P. prasina* (2 ml per cup) using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). The spray tower was cleaned with 70% ethanol and sterile distilled water after each application of the fungus suspensions for disinfecting the apparatus. Only sterile-distilled-water containing 0.02% Tween 20 was sprayed to control cups. They were incubated at 25±1°C and 75±5% RH, 16:8 h light: dark photoperiod for 12 days in a Binder incubator (Model KBWF 240; Germany). Ploye thylene sheets were used together with rubber bands to cover the cups. Relative humidity inside the cups was measured by hygrometer and was (90±5%). All cups were inspected daily for 12 days. Dead individuals on which the fungal sporulation observed, were counted under a Leica EZ4 educational stereomicroscope at 40-70X magnification. Mortality rate was recorded on daily basis and dead individuals were removed from the cups. Evidence of *Beauveria*, *Isaria*, *Lecanicillium* and *Simplicillium* on nymph cadavers was verified by microscopic inspection. The bioassays were experimented twice each with three replications.

Statistical analysis

Since insect material were limited for experiment, multiple observations were made for each of dose group at a series of times after treatment. Because standart probit analysis techniques are not applicable to serial time-mortality data (Throne *et al*.,1995 and

Robertson *et al*., 2007), serial-time mortality data from bioassays were analyzed by probit analysis program (PROBIT2-PP) to calculate LT_{50} and LT_{90}, despite of that this valid method produced high Chi-square and heterogeneity value. Slopes of regression lines were compared by each others by their standard errors. Mortality rates were compared by one-way analysis of variance (ANOVA), followed by Duncan's studentisized test when significant differences were found at P<0.05 (SPSS, Version 21).

RESULTS AND DISCUSSION

The results showed that all 8 isolates of the entomopathogenic fungi were pathogenic to *P. prasina* (Table 2). When considering LT_{50} values of the tested isolates, the TR-07 isolate ranked first among the isolates showing the fastest effect within 3.20 days. TR-55-3 isolate came second with 5.01 days, and the slopes of these two isolates took a part in the same group. These isolates followed by the TR-01 with 5.36 days, TR-08 with 6.41 days, TR-05 with 6.90 days, and the other isolates (Table 2). In addition, when considering LT_{90} values, the fastest effect was also occured in the TR-07 isolate within 9.32 days, followed by TR-55-3 isolate with 17.08 days, and the others. The Slope±SE and Chi-square values of bioassays are given in table (2).

At the first day post treatment, there was almost no mortality at all isolates as expected. After 3rd day of treatment, mortality rates started to increase slightly reaching (48%) in TR-07 isolate of *L. muscarium*. After 5 days, mortality percentage increased to above (50%) for most isolates, with (83.33%) in TR-07 isolate of *L.muscarium*. From 5 to 12 days after treatment, mortality rates in some isolates in which most of the mortality occurred during the first 5 days (TR-01, TR-05, TR-07, TR-08 and TR-55-3) increased gradually but slowly whereas in some isolates (TR-02, TR-04 and TR-78-7) they increased rapidly. Mortality after 12 days post treatment varied from (83 to 98%) among the tested isolates. Two tested isolates of *S. lamellicola*, TR-01 and TR-02 produced similar mortality rates (P>0.05). Four tested isolates of *L. musacarium* TR-04, TR-05, TR-07 and TR-08 produced (83.33 to 98.33%) mortality. The isolate TR-07 differed from other isolate TR-08 of *L. muscarium* (P<0.05). Except TR-08 isolate of *L. muscarium*, other 3 isolates did not show any significant difference (P>0.05). *B. bassiana* isolate TR-55-3 gave (95%) mortality after 12 days post treatment, which was very close to the most virulent isolate TR-07 of *L. muscarium*, but with no significant difference (P>0.05). *I. fumosorosea* isolate TR-78-7 showed the lowest mortality (83.33%) as with TR-08 isolate of *L. muscarium*. The highest rate of mortality (98.33%) of *P. prasina* nymphs by the end of 12th day was recorded at the TR-07 isolate of *L. muscarium.* Among the tested fungi, the TR-07 isolate of *L. muscarium* emerged as the most promising against *P. prasina* reaching (98.33%) after 12 days. Furhermore, this isolate showed the most rapid effect and caused (83.33%) mortality after 5th day. Also, *B. bassiana* isolate showed high efficiacy, reaching (60.00%) mortality on 5th day and (95.00%) after 12 days. It was clear that all isolates belonging to the 4 different fungi species were satisfactorily effective against *P. prasina*, after 12 days treatments (Table 3). The present results are consistent with earlier reports that effectiveness of some strains of *B. bassiana*, *I. fumosorosea*, *L. muscarium* and *S. lamellicola* that were approved as entomopathogenic fungi on different insect pests (Hussein *et al*., 2013 and Saruhan *et al*., 2015).

The 2 isolates of *S. lamellicola* and the 4 isolates of *L. muscarium* showed considerably high pathogenicity and gave (88.33%) (TR-01) and (91.67%) (TR-02) mortality rates after 12 days post treatment (Table 3). Mortality rate reached up to (83.33%) after 5 days in one isolate (TR-07). Saruhan *et al.* (2015) determined the efficacy of isolates *L. muscarium* (TR-08), *S. lamellicola* (TR-09) and the commercial bioinsecticide *Verticillium lecanii* against *Aphis fabae*, at 20 and 25°C and found that these three fungi caused about (100%) mortality

Table (2): Lethal times (LT_{50} and LT_{90}) values of 4th instar nymphs of *Palomena prasina* treated with entomopathogenic fungi under laboratory conditions

Species	Isolates	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)	Slope±SE	χ^2
Simplicillium lamellicola	TR-01	5.36 (2.90-10.94)	22.78 (11.12-116.36)	2.04±0.25 b*	24.07
S. lamellicola	TR-02	7.12 (4.79-11.27)	23.66 (14.21-61.51)	2.46±0.30 ab	20.67
Lecanicillium muscarium	TR-04	7.15 (4.52-12.36)	22.13 (12.71-72.77)	2.61±0.30 a	30.28
L. muscarium	TR-05	6.90 (1.00-1082.60)	45.52 (10.37-8.76x10^{10})	1.56±0.22 c	69.72
L. muscarium	TR-07	3.20 (2.18-4.66)	9.32 (6.15-18.47)	2.76±0.29 a	14.89
L. muscarium	TR-08	6.41 (4.87-8.62)	23.87 (16.30-41.86)	2.24±0.27 ab	10.60
Beauveria bassiana	TR-55-3	5.01 (2.87-9.15)	17.08 (9.31-62.10)	2.41±0.27 ab	35.84
Isaria fumosorosea	TR-78-7	8.48 (4.40-21.57)	40.30 (17.19-387.49)	1.90±0.26 bc	31.67

*Slopes followed by same letters do not differ significantly.

Table (3): Mortality of 4th instar nymphs of *Palomena prasina* treated with entomopathogenic fungi under laboratory conditions

Isolates	Mean % mortality (±SE) on days					
	1	3	5	7	9	12
TR - 01	00.00±0.^{0a*}	28.33±6.54^{bc}	61.67±6.01^{b}	71.67±4.77^{b}	81.67±3.07^{ab}	88.33±3.07^{abc}
TR - 02	1.67±1.67^{a}	16.67±3.33b^{cd}	35.00±6.71^{c}	66.67±4.94^{bc}	73.33±5.58^{bcd}	91.67±4.77^{abc}
TR - 04	1.67±1.67^{a}	6.67±2.11^{d}	33.33±7.15^{c}	53.33±5.58^{cd}	66.67±5.58^{cd}	91.67±4.77^{abc}
TR - 05	0.00±0.0^{a}	15.00±2.24^{cd}	50.00±7.75^{bc}	61.67±4.01^{bc}	66.67±2.11^{cd}	86.67±4.22^{abc}
TR - 07	0.00±0.0^{a}	48.33±3.07^{a}	83.33±2.11^{a}	86.67±2.11^{a}	88.33±1.67^{a}	98.33±1.67^{a}
TR - 08	0.00±0.0^{a}	21.67±5.43^{bc}	51.67±9.10^{bc}	65.00±5.63^{bc}	75.00±5.00^{bc}	83.33±4.47^{c}
TR-55-3	0.00±0.0^{a}	30.00±7.30^{b}	60.00±8.94^{b}	70.00±4.47^{b}	80.00±3.65^{ab}	95.00±3.42^{ab}
TR-78-7	1.67±1.67^{a}	16.67±4.94^{bcd}	33.33±7.15^{c}	46.67±6.15^{d}	61.67±3.07^{d}	83.33±6.67^{bc}
Control	0.00±0.0^{a}	3.33±2.11^{d}	6.67±2.11^{d}	10.00±3.65^{e}	11.67±30.7^{e}	13.33±2.11^{d}

*****Within columns followed by same letters do not differ significantly (Duncan's multiple range test P<0.05).

after 7 days; and LT_{50} values for the isolates of *L. muscarium, S. lamellicola* and the commercial product Bio-Catch WP (*V. lecanii*) were (1.77, 2.12 and 2.33 days), respectively.

Despite of that obtained from *H. cunea* pupae, *B. bassiana* induced one of the highest mortality rates (95.00%) among the tested isolates after 12 days post treatment. This isolate showed also more rapid action and mortality rate reaching up to 60.00%, after 5 days of treatment. Some of the above mentioned entomopathogenic fungi caused various mortality rates depending on experimental conditions. Three *B. bassiana* and 2 *Metarhizium anispoliae* isolates were evaluated against the pentatomid adult *Halyomorpha halys* and *M. anispoliae* isolates produced lower mortalities than the *B. bassiana* isolates. The results showed the potential of managing *H. halys* with entomopathogenic fungi (Gouli *et al.*, 2012). Mustu *et al.* (2011) determined that *B. bassiana* isolate displayed consistently high virulence than *I. farinosa* isolate on the pentatomid wheat stink bug, *Aelia rostrata*, and both isolates were more effective at 95% RH and $1x10^8$ conidial concentrations (ml^{-1}) than 70% RH and the same conidial concentrations. Also, in the study, *B. bassiana* caused (100%) mortality in the 9th day of incubation. Mustu *et al.* (2014) tested the same fungi on the scutellerid bugs *Eurygaster integriceps* and *E. austriaca* and had similar effectiveness, with (85%) mortality after 12 days post treatment. Similarly, Parker *et al.* (2003) found that several isolates of *B. bassiana* and *M. anisoplia* displayed consistently high virulence against *E. integriceps.*

Isaria fumosorosea isolate which was obtained from *H. cunea* pupae, was one of the weak isolates in terms of effectiveness in the test with (83.33%) mortality after 12 days. Hussein *et al.* (2013) determined the high efficacy of the new strain CCM 8367 of *I. fumosorosea* on different stages of the Egyptian cotton leafworm, *Spodoptera littoralis* under laboratory conditions, where this strain was detected to be mycoinsecticidal effect on the insect pest.

Entomopathogenic fungi are more likely to have ecological compatibility with pest species, due to their geographical locations and habitat types (Sevim *et al.*, 2013). They screened 13 entomopathogenic fungal strains, including *B. pseudobassiana, B. bassiana, M. anisopliae* and *I. fumosorosea* obtained from different sources in Middle and East Black Sea region of Turkey, with respect to pathogenicity against *Corythucha ciliata* (Say) (Hemiptera: Tingidae), and they demonstrated that *B. bassiana* KTU – 24 was the most virulent with (86%) mortality. The present study showed that almost all local isolates caused significant mortality rates against *P. prasina* under laboratory conditions. In this respect, all isolates appeared to be significant candidates for controlling *P. prasina,* especially in Turkey, considering the origins of these isolates. Moreover, other local isolates obtained from different host insects and tested in present study against *P. prasina* were also highly effective. Tanada and Kaya (1993) revealed that strains of fungi within a species isolated from a specific host, are more virulent for that host than those isolated from another host.

The most important abiotic environmental constraints for fungi are temperature, humidity or moisture and solar radiation. These factors are also responsible for effective, commercial use of entomopathogenic fungi (Zimmerman, 2007). In addition, rain plays an important role in transmission of entomopathogenic fungi (Goettel *et al*., 2005). For instance, *B. bassiana* optimal RH for germination of conidia is (92-100%) (Hallsworth and Magan, 1999) and optimum growth temperature is between 20 and 30 ºC (Tefera and Pringle, 2003). In agreement with these results, high mortality rates on *P. prasina* for all isolates belong to 4 entomopathogenic fungi were found under laboratory conditions, with 25±1°C and 90±5% RH. The Middle and Eastern Black Sea region, which has largest hazelnuts growing area of

Turkey, has favorable environmental conditions to use entomopathogenic fungi in biocontrol of *P. prasina* because it is rainy, humid and has low annual temperatures. It is possible to use the tested entomopathogenic fungi (especially TR-07 and TR-55-3) as potential biocontrol agents against *P. prasina* in quite large hazelnut plantations of Turkey, rather than using detrimental chemical pesticides. However, it is also necessary to evaluate efficacy of the isolates on *P. prasina* under field conditions to set a succesfull biocontrol program. Forecasting field performance according to *in vitro* efficacy is difficult and sometimes same results may not be obtained in the field (Legaspi *et al*., 2000).

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