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Determination of fungal pathogens of *Hypera postica* (Gyllenhall) (Coleoptera: Curculionidae): isolation, characterization, and susceptibility

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Abstract

Background: Fungal pathogens of *Hypera postica* (Gyllenhall) (Coleoptera: Curculionidae) were collected from the vicinities of Adana and Igdir in Turkey. The pathogenicity of the fungal isolates against the pest were investigated. According to morphologic (colony morphology, spore shape) and molecular (sequences of ITS1-5.8S ITS2 region and *EF*1-α, *Bloc*, and *RPB*1 genes) characterizations, the isolates were identified as *Beauveria bassiana* (Hpl-2, Hpl-6, Hpl-7, Hpl-10, HpA-3, HpA-4, HpA-5) and *Beauveria pseudobassiana* (Hpl-4). All these strains were isolated from *H. postica* for the first time. In order to determine pathogenesis of all isolates on the target pest, bioassays were conducted against larvae and adults, as screening of (1×10^7 conidia/ml) and dose-response (1×10^5, 1×10^6, 1×10^7, 1×10^8, conidia/ml), under laboratory conditions. The fungal isolates, closely related to each other, yielded significantly varied mortalities on larvae and adults. *H. postica* larvae were found more susceptible than adults to the fungal isolates in all tests. The highest mortality rates (100 and 98%) for larvae and adults, respectively, were obtained by *B. bassiana* strain HpA-5 within 14 days at 1×10^8 conidia/ml concentration. The median lethal concentration (LD_{50}) of HpA-5 required to kill the larvae and adults of *H. postica* at concentrations of 2.37×10^4 and 1.4×10^5 conidia/ml, respectively. These results are promising; therefore, the *B. bassiana* strain HpA-5 can potentially be used against *H. postica*.

Keywords: *Hypera postica*, Microbial control, *Beauveria bassiana*, Isolation, Susceptibility

Background

Alfalfa, *Medicago sativa* L., also called Lucerne, Purple Medic, and Trefoil, is a perennial flowering plant of the pea family (Fabaceae) and cultivated as significant forage crop lasting longer period than any other crop. Alfalfa has a very great product potential, and it is also one of the most palatable and nutritious forage crops. Due to the high protein and vitamin content, alfalfa is the main component of the diet of dairy cattle. Alfalfa is among the most valuable and cultivated plants worldwide (Mustafa et al. 2014). Also, it is one of the more productive nitrogen fixer in legumes, making it useful in long-term rotations as a soil builder to provide nitrogen.

Alfalfa weevil, *Hypera postica* (Gyllenhall) (Coleoptera: Curculionidae), is the most devastating insect pest of alfalfa, and a few closely related legumes which feeding terminals and new crown shoots thereby lowering crop yield and quality (Reddy et al. 2016). Anay and Kornosor (2000) recorded *H. postica* on alfalfa at Adana in Turkey. This pest occurs at several times of the growing season and reduces forage production in many ways. Heavily infested areas may appear silver or white; most of the leaves are turned into skeletons or completely consumed (Radcliffe and Flanders 1998). If large numbers of larvae or adults survive until harvest, they damage crown buds and stems, retarding regrowth (Fick 1976). Residual effects from severe damage decrease plant vigor, resulting

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in poor yields in subsequent harvests and lower stand density (Fick and Liu 1976). Several types of control have been used to decrease the economic damage incurred by this pest such as cultural, chemical, and biological control methods. Increasing concerns for insecticide resistance and environmental safety arising from a frequent use of synthetic insecticides affect the long-term control feasibility of alfalfa weevil management (Regev et al. 1983). Consequently, many alfalfa producers are looking for more environmentally friendly control methods all over the world to take over this destructive pest.

Biological control by entomopathogenic fungi (EPF) is an attractive alternative to the use of traditional pesticides, mainly because these fungi are safer to animals, plants, and the environment. EPF play an important role in the regulation of insect populations in Turkey as well as all over the world (Sevim et al. 2010). Besides, reports related to fungi are quite limited, so there is a need to evaluate the potential of fungi as a group of biological control agents against this pest. Limited attempts have been carried out to study the effects of EPF on the control of *H. postica*, including the studies of Hedlund and Pass 1968 and Sakurai et al. 1998 who recorded the infection of *H. postica* with *Beauveria bassiana* and *Metarrhizium brunneum*. Most of the studies, conducted for the control of *H. postica*, showed that a negative influence of the pest on alfalfa is still continuing.

In the present study, the pathogenicity of eight EPFs, isolated from *H. postica*, was tested against both the larvae and adults of the pest under laboratory conditions.

Methods

Collection of the insect

H. postica larvae and adults were collected from alfalfa fields in Adana and Igdir, Turkey, between 2014 and 2015 and transferred to the laboratory in plastic boxes. The insects were checked once a week, and those showed fungal diseases or death symptoms were transferred to a moist chamber for 7 days to stimulate fungal sporulation. The naturally infected collected larvae were also transferred from the field to the laboratory in eppendorf tubes. Fungus isolation was made from mycosed larvae and adults. Healthy individuals were selected and used for bioassays.

Isolation of fungi

Fungi were isolated from infected larvae and adults by cutting out portions of mycosed cadavers and cultured on potato dextrose agar medium with 1% yeast extract (PDAY medium, Merck, Darmstadt, Germany) including 50 μg/ml ampicillin and 50 μg/ml tetracycline (AppliChem) to prevent bacterial growth. The cultures were incubated for 1–2 weeks at 25–28 °C to facilitate growth and sporulation. Then, all isolates were subcultured from a single colony to acquire a pure colony. Therefore, conidial suspension of 1×10^6 conidia/ml was prepared, plated on PDAY, and incubated at 28 °C for 1 week under 12-h L and 12-h D photoperiod. After 6–7 days, a single colony was cut out, transferred to a fresh PDAY medium, and incubated at 28 °C for 2–3 weeks until the plates were fully overgrown. Single colony spore suspensions were stored in entomopathogen culture collection at Microbiology Laboratory, Karadeniz Technical University, Trabzon, Turkey.

Morphological identification

The appearances of fungal infection on larvae and adults, colony morphology, spore size, and spore shape of fungal isolates were used in the first identification process. Spore sizes were measured, using a phase-contrast microscope. Initial identification was made according to the key of Humber (1997). The fungal cultures were deposited in the Microbiology Laboratory at Karadeniz Technical University (Trabzon, Turkey).

Molecular characterization of isolates

Sequencing of the *ITS1-5.8S-ITS2* region between 18 S and 28 S rRNA subunits and the partial sequencing of *EF*1-*α*, *Bloc*, and *RPB1* genes was conducted to confirm the identity of the isolates. Hyphae and spores, obtained from pure cultures, were inoculated into flasks containing 100 ml of potato dextrose broth (PDB medium, Difco, NJ, USA), and the cultures were incubated at 25 °C in a rotary shaker (GFL 3031) at 230 rpm for 1 week under 12/12 photoperiod. After the incubation, cultured media were filtered, mycelial mass was harvested on sterile filter paper, and each sample was frozen in liquid nitrogen and then crushed. Isolation of genomic DNA was extracted, using the ZR Fungal/Bacterial DNA MiniPrep (50, ZYMO RESEARCH) from 50 mg of crushed mycelium. The *ITS1-5.8S-ITS2* region of each fungal isolate was amplified, using the primer pair of ITS5, as a forward primer and ITS4 as a reverse primer (White et al. 1990) in a 50-μl reaction volume containing 10 μl *Phusion* HF DNA polymerase reaction buffer, 200 μM of each dNTPs, 0.05 nmol of each opposing amplification primer, 1 unit *Phusion* DNA polymerase (Thermo Scientific), and 50 ng genomic DNA. PCR conditions were as follows: initial denaturation at 98 °C for 30 s; 35 cycles of 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min.

Approximately 1200 bp segments of *EF*1-α, 1500 bp segment of *Bloc* (the nuclear intergenic region), and 700 bp segments of *RBP1* (RNA polymerase II largest subunit) were amplified and sequenced for further characterization of *Beauveria* isolates, according to the study of Rehner et al. (2011). PCR conditions were adapted, essentially as described earlier (Rehner and Buckley 2005 and Rehner et al. 2011). The primers used in this study are given

in Table 1. PCR products were loaded on 1.0% agarose gels stained with ethidium bromide and scanned under UV light for visualization. After checking PCR products, they were purified, using Nucleospin Gel and PCR Clean-up (Macherey-Nagel) and then sent to MACROGEN (Netherlands) for sequencing. Obtained sequences were subjected to BLAST searches, using the NCBI GenBank database and phylogenetic analysis to compare with known species (Benson et al. 2012). *ITS1-5.8S-ITS2*, *EF*1-α, *bloc*, and *RPB1* gene sequences of our isolates and the sequences from the study of Rehner et al. (2011) were included for comparison of *Beauveria* isolates.

Screening tests

A total of eight isolates were used for the pathogenicity test against larvae and adults of *H. postica*. Therefore, each fungal isolate was plated on PDAY and incubated at 25 °C for 4 weeks. Conidial suspensions were prepared by adding 10 ml of 0.1% Tween 80 (AppliChem) into the 4-week-old Petri dishes, and the conidia were taken from the agar surface, using a sterile inoculation loop to dislodge. The concentrations of conidial suspensions were determined, using a Neubauer hemocytometer and adjusted to 1×10^7 conidia/ml. For the screening test, *H. postica* larvae and adults were collected from infested alfalfa fields in the Adana and Igdir, Turkey. Healthy larvae and adults were randomly selected and used for bioassays. For bioassays, 30 third instar larvae and adults were used for each test and each fungal isolate. All experiments were repeated three times on different days, using fresh collected larvae. Larvae and adults were inoculated by dipping into 1 ml of 1×10^7 conidia/ml suspension, prepared from different cultures at different times for 4–5 s. Thereafter, they were placed in plastic boxes (15 cm wide × 8 cm deep) including alfalfa leaves. The control groups of larvae and adults were treated by a sterile water supplemented with 0.1% Tween 80. All boxes were incubated at 20 °C for 1 week, at 12/12 photoperiod. Fresh alfalfa leaves were provided daily for 7 days. The mortality rates of larvae and adults were estimated daily after inoculation for 14 days. Dead insects were surface sterilized by dipping into 1% sodium hypochlorite for 3 min followed by 70% ethanol for 3 min and washed three times in sterile distilled water. Then, they were placed on a wet filter paper in sterile plastic Petri dishes, covered with parafilm, and incubated at 25 °C to stimulate fungal sporulation on the cadavers. Finally, the pathogenicity of the isolates was measured by determination of mortality rates and calculation of percentage of mycoses by using Schneider-Orelli's formula (Püntener 1981). The isolates that caused more than 90% of mortality (total or confirmed) were selected to the next phase of the study.

Dose-mortality response test

Dose-mortality response tests were conducted, using the *B. bassiana* strain HpA-5, based on the screening test. Conidial suspensions of fungal isolates were prepared as described above, and a series of dilutions was prepared as 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml. The spore suspension (1 ml) was applied by using a sterile sprayer. Thirty healthy larvae and adults were used for each replicate, and all experiments were repeated three times in different days. The experimental design was similar with that mentioned for the screening test. Mortality rates of larvae and adults were estimated daily over 14 days of bioassays. Finally, the mortality data were corrected using Schneider-Orelli's formula (Püntener 1981), and lethal concentration (LC_{50}) value was calculated by probit analysis using MS Excell (Finney 1952).

Detection of the *pr*1 genes

*pr*1 genes were screened in the isolates to detect the presence of Pr1 proteases. Therefore, nested-PCR amplifications of the *pr1* B gene were performed by using two primer pairs. The outer primer pair was TGCCAA CATCGGACAAGACA (Pr1B1) and CATGGACGACCC CGAAAGAG (Pr1B2), and the inner pair was AGCG TTCCCGGCAGTTACCATT (Pr1B3) and CCCGGCG CAAAAATATCAAC (Pr1B4) (Wang et al. 2002). The 50 μl reaction buffer contained 200 μM of each dNTP,

Table 1 Primers and their sequences used in this study

Gene	Primer name	Sequences	References
ITS1-5.8S-ITS2	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	(White et al. 1990)
	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	
EF1-α	EF1T	5′-TGGGTAAGGARGACAAGAC-3′	(Rehner and Buckley 2005)
	1567R	5′-CHGTRCCRATACCACCSATCTT-3′	
RPB1	RPB1Af	5′-GARTGYCCDGGDCAYTTYGG-3′	(Stiller and Hall 1997)
	RPB1C	5′CCNGCDATNTCRTTRTCCATRTA-3′	
Bloc	B5.1F	5′-CGACCCGGCCAACTACTTTGA-3′	(Rehner et al. 2006)
	B3.1R	5′-GTCTTCCAGTACCACTACGCC-3′	

1 unit of GoTaq Flexi-DNA-Polymerase (Promega, Madison, WI, USA), 10 μl 5× GoTaq Flexi Buffer, 4 μl $MgCI_2$, and 50 ng of DNA template. PCR conditions were as follows: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products were loaded on 1.0% agarose gels stained with ethidium bromide and scanned under UV light for visualization. After checking PCR products, they were purified using Nucleospin Gel

Fig. 1 Neighbor-joining tree of eight *Beauveria* isolates isolated from alfalfa fields in Adana and Igdir, Turkey, and from the study of Rehner and Buckley (2005) based on *ITS1-5.8S-ITS2*, *EF*1-α, *bloc*, and *RPB*1 gene regions. Bootstrap values shown next to nodes are based on 1000 replicates. The tree was rooted using isolate *Cordyceps* cf. *militaris* 5050 as outgroup. Bootstrap values ≥ 60% are labeled

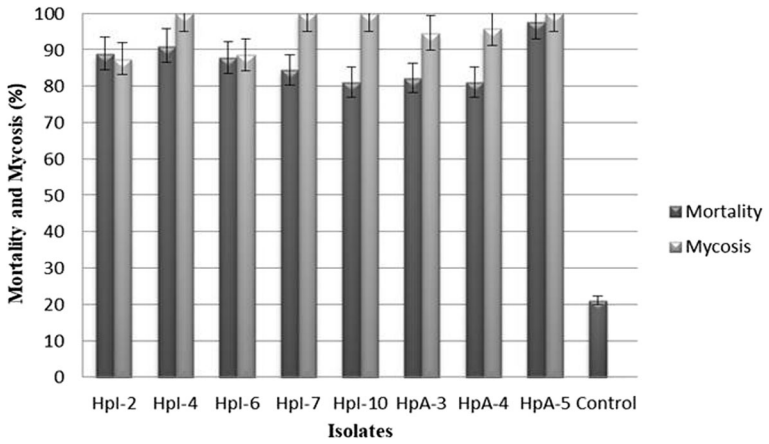

Fig. 2 Mortality of *Hypera postica* larvae after application of eight entomopathogenic fungal isolates within 14 days after application of 1×10^7 conidia/ml

and PCR Clean-up (Macherey-Nagel) and then sent to MACROGEN (Netherlands) for sequencing.

Statistical analysis

DNA sequences of the isolates were assembled and edited with Clustal W packed in BioEdit (version 7.09) and aligned (Hall 1999). Sequences obtained from *ITS*, *EF*1-α, *bloc*, and *RPB*1 gene regions were compared with the NCBI GenBank accessions, using Blast to confirm identifications of all isolate strains. Genomic analysis using the neighbor joining was performed using with MEGA 6.0 software (Saitou and Nei 1987). The reliability of the dendrogram was tested by bootstrap analysis with 1.000 replicates using MEGA 6.0 (Felsenstein 1985 and Tamura et al. 2013).

The mortality data for screening and dose-mortality response tests were subjected to analysis of variance (ANOVA) and subsequently to Dunnett's one-tailed *t* test to compare test isolates against the controls with respect to mortality and mycoses. To determine the difference among isolates, the data were subjected to ANOVA and subsequently to LSD multiple comparison test. All analyses were performed by using SPSS 20.0 statistical software.

Results and discussion

Isolation and morphological identification of fungi

A total of eight fungal isolates was obtained from *H. postica* larvae and adults, infected naturally by fungi, collected from alfalfa fields. The infected individuals were recognized according to the mycelial growth outside cadaver (Greenfield et al. 2016). Also, isolates were identified morphologically based on conidial morphology, shape, and size according to the most current identification key (Humber 1997). The isolates were morphologically identified as *Beauveria bassiana* (× 7 isolates) and *Beauveria pseudobassiana* (× 1 isolate).

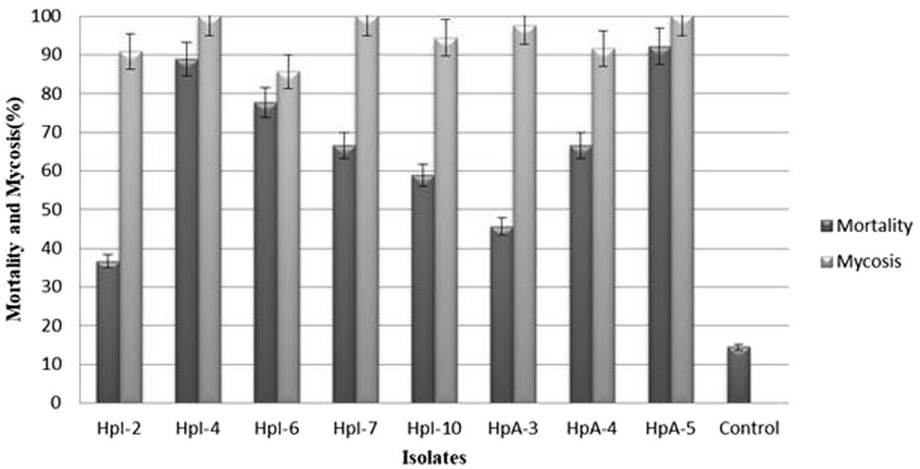

Fig. 3 Mortality of *Hypera postica* adult after application of eight entomopathogenic fungal isolates within 14 days after application of 1×10^7 conidia/ml

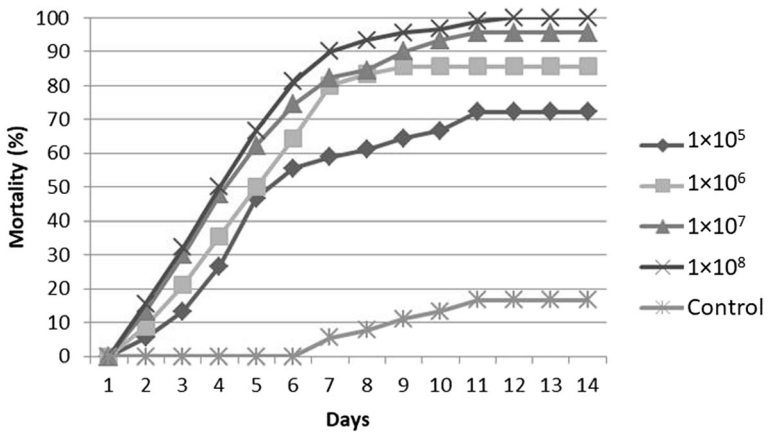

Fig. 4 Daily cumulative mortality of *Hypera postica* larvae after application of four different doses of the spore concentration of *Beauveria bassiana* strain HpA-5. Concentration unit is conidia/ml

B. bassiana was the most commonly detected fungus in alfalfa collected from fields.

Molecular characterization

To confirm identifications of the *Beauveria* isolates, the *ITS1-5.8S-ITS2* region and partial sequence of *EF1-α*, *RPB1*, and *Bloc* genes were determined and used for genomic analysis. The isolates showed different base sequences in the target regions according to each other. Based on the genomic analysis, seven isolates were found to be identical as *B. bassiana* and one isolate as *B. pseudobassiana*, according to the study of Rehner et al. (2011) (Fig. 1). Sevim et al. (2010) characterized five strains as *Beauveria* from *Thaumetopeae pityocampa*. Nevertheless, all of them took place so far taxonomic positions based on partial sequence of both *ITS* and *EF1-α*. In the present study, eight *Beauveria* strains showed also different taxonomic positions according to *ITS*, *Bloc*, and *RPB1* regions.

Screen tests

Fungal isolates caused different mortality rates in both larvae and adults in the screening tests. In the case of larvae, the rates ranged (81–97%) depending on the tested strain. The highest mortality rate (97%) on larvae was recorded by *B. bassiana* strain HpA-5, within 14 days (Fig. 2). All isolates produced high mycosis ratios. The mortality rates on adults ranged 36–92% based on the isolate. The highest one (92%) was also obtained by *B. bassiana* strain HpA-5 within 14 days as the larvae (Fig. 3). All isolates produced very high mycosis ratios on adults after sporulation period. With respect to susceptibility to fungal isolates, a significant difference between larvae and adults was found. Also, *H. postica* larvae were more susceptible to fungal isolates ($P < 0.05$) than adults. Generally, early instar larvae or nymphs are more susceptible than older ones (Butt 2002).

Dose-response tests

B. bassiana strain HpA-5 was selected for dose application based on its highest mortality rate and mycosis ratio on *H. postica* larvae and adults at the end of screening tests. In the dose-response tests of *B. bassiana* strain HpA-5 larvae, mortality reached 100% within 14 days after inoculation of the conidial concentration of 1×10^8 conidia/ml, and there was a significant difference among concentrations ($df = 2$, $P < 0.05$) (Fig. 4). The LC_{50} values of isolate *B. bassiana* HpA-5 were calculated as 2.37×10^4 and 1.4×10^5 conidia/ml against larvae and adults based on probit analysis, respectively (Table 2). Adults' mortality rate also reached 98% within 14 days after application of the 1×10^8 conidia ml^{-1} concentration, and there was a significant difference among concentrations ($df = 2$, $P < 0.05$) (Fig. 5).

Hedlund and Pass (1968) investigated infection of *B. bassiana* on the alfalfa and indicated that high rate of infection required relative humidity of 98 to 100%. In addition, they showed that all stages of the *H. postica* were found to be susceptible against the fungus, although spores were not produced from infected larvae as they were from infected adults. In another study, comparative pathogenicity of *B. bassiana*, *M. anisopliae*, *Clonostachys rosea*, and *Lecanicillium lecanii* to adults of *H. postica*, displayed that, especially *B. bassiana* was the most effective species against adults of the pest (Mustafa et al. 2014). Fungal strains showed higher pathogenic effect against insects from which they

Table 2 Probit analysis parameters from the multiple concentration bioassays performed with the *B. bassiana* HpA-5 isolate against larvae and adults of *H. postica*

Isolate	LC_{50}	Slope ± SE	df	X^2
HpA-5 (larvae)	2.37×10^4 (0.08–65.23)	0.3 ± 0.73	2	0.842
HpA-5 (adults)	1.4×10^5 (0.14–14.73)	0.42 ± 0.52	2	0.961

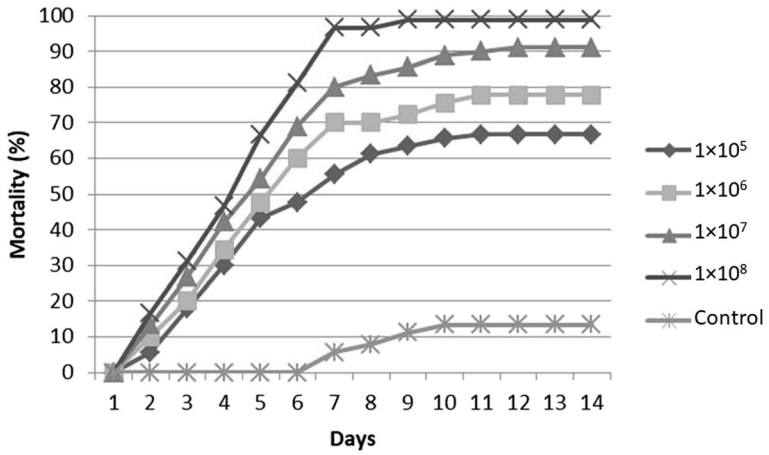

Fig. 5 Daily cumulative mortality of *Hypera postica* adults after application of four different doses of the spore concentration of *Beauveria bassiana* strain HpA-5. Concentration unit is conidia/ml

isolated as compared to others (Tanyeli et al. 2010 and Sonmez et al. 2016). Namely, a strain of *B. bassiana* isolated from *T. pityocampa* showed a 100% insecticidal effect toward its larvae (Sevim et al. 2010). All of these studies suggest that the current isolate of *B. bassiana* (HpA-5) has potential to be used against alfalfa weevil. On the other hand, local isolates have more advantages than exotic species due to ecological adaptability.

Detection of the *pr*1 genes

The *pr*1B gene was found in all of the isolates. The presence of proteases in isolates is a virulence enhancing factor. Fang et al. (2009) reported that *B. bassiana* Pr1A homolog (CDEP1) increases fungal virulence. In the direction of the results, HpA-5 isolate had Pr1 B proteases as well as high mortality and mycosis ratio (Fig. 6).

Conclusions

The pathogenicity and virulence of EPF isolates to *H. postica* under laboratory conditions was determined. *B. bassiana* strain HpA-5 was the most promising isolate according to its highest mortality rate and mycosis value. Further studies should include determination of the virulence of this isolate under field conditions and its productivity in the industrial culture media. Additionally, horizontal transmission studies between adults and larvae are also warranted. Moreover, the side effects of the *B. bassiana* strain HpA-5 against natural enemies of *H. postica* should be also investigated.

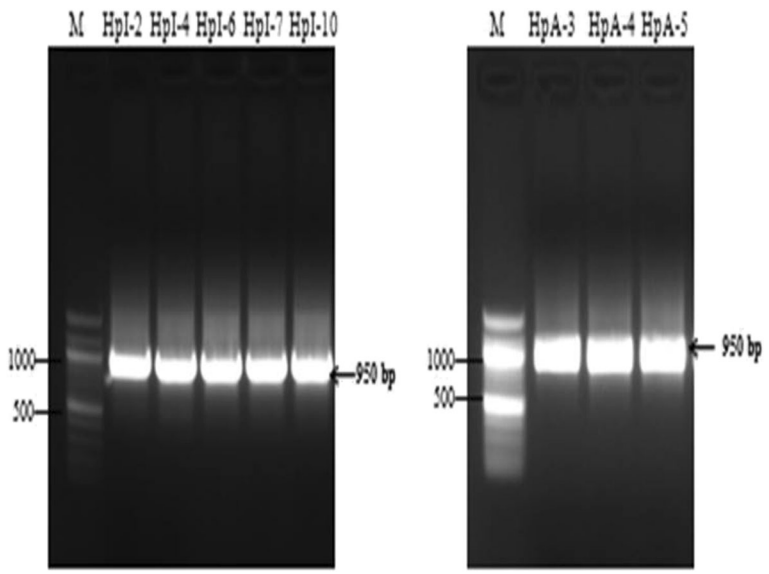

Fig. 6 PCR detection of protease-type subtilisin (*pr*1) gene in *Beauveria* sp. strains

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Authors' contributions
The whole team jointly planned the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate
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Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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