J. Crop Prot. 2020, 9 (4): 563-576

Research Article

Variable induction of cuticle-degrading enzymes of *Beauveria bassiana* isolates in the presence of different insect cuticles

Farzaneh Sadat Seyed-talebi[1], Seyed Ali Safavi[1*], Reza Talaei-Hassanloui[2] and Alireza Bandani[2]

1. Department of Plant Protection, Faculty of Agriculture, Urmia University, Urmia, Iran.
2. Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.

Abstract: In the present study, the cuticle-degrading enzymes production potential of five native *Beauveria bassiana* (TV, OZ, UN, DV and DE) isolates was investigated in the presence of cuticles from *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio*. Furthermore, histopathology of infected insects by *B. bassiana* was studied. The level of cuticle degrading enzymes was the highest and lowest for TV (as the most virulent isolate) and DE (as the weakest isolate), respectively. *E. integriceps* nymphs as the most sensitive host produced the highest level of cuticle degrading enzymes (Pr2, exochitinase, and lipase) while *Z. morio* as the most resistant host, produced the lowest level of hydrolytic enzymes. According to histopathological study, the fungal isolate could not penetrate into *Z. morio* cuticle, as no mycelia or hyphae were observed in its tissues after inoculation, while fungal bodies were detected in microscopic slides of the other two insects. Overall, the chemical and topographical structure of insect cuticle had a substantial effect on the virulence of entomopathogenic fungus. Production of enzymes including proteases (especially Pr2), chitinase (*N*-acetyl-glucosaminidase), and lipases was positively related to virulence of fungus isolates. It can be concluded that not only the hydrolytic activity of *B. bassiana* isolates, but also host cuticle composition determine the pathogenesis and virulence cascade in fungus-insect interactions.

Keywords: *Beauveria bassiana*, cuticle-degrading enzymes, virulence, histopathology

Introduction

Fungal entomopathogens are important biological control agents all over the world and have been studied intensely for more than 100 years (Vega *et al.*, 2012). Among them, *Beauveria bassiana* can affect a wide range of arthropod pests such as coleopterans, hemipterans and mites, but its distinct isolates differ in their host range and specificity (Lacey *et al.*, 1999; Mayoral *et al.,* 2006; Talaei-Hassanloui *et al.*, 2007).

Entomopathogenic fungi can infect their hosts by direct penetration through the insect cuticle, which is composed of chitin embedded in a matrix with cuticular proteins, lipids, and other compounds (Richard *et al.*, 2010). Fungal penetration to the insect cuticle can be mediated by both mechanical processes and enzymatic attack (Vega *et al.*, 2012). Therefore, the physical and chemical properties of cuticle could affect the virulence of entomopathogenic fungi. Enzyme production by entomopathogenic fungi may be involved in many biological processes,

Handling Editor: Mohammad Mehrabadi

* **Corresponding author:** a.safavi@urmia.ac.ir
Received: 26 January 2020, Accepted: 07 July 2020
Published online: 04 August 2020

including the degradation of cuticular polymers during infection, assisting penetration through the insect exoskeleton, and additionally, providing nutrients for fungal growth (Goettel *et al.*, 1989). Proteases, chitinases, and lipases break down the cuticle, those are considered as the most important enzymes in the infection process (Mustafa and Kaur, 2009). Understanding the mode of relationship between enzyme activities and virulence could be useful in developing screening methods to identify new efficient fungal isolates (Gupta *et al.*, 1994).

Correlation between the virulence and the enzyme activity of fungal isolates have been previously reported in different articles (Pinto *et al.*, 2002; Dhar and Kaur, 2010; Zare *et al.*, 2014). A comprehensive study regarding the effects of three enzymes (Proteases, chitinases, and lipases) has been conducted by Pelizza *et al.* (2012). They indicated that the highest protease, chitinase, and lipase activities were recorded in the most virulent *B. bassiana* isolate on *Schistocerca cancellata* Serv (Orthoptera: Acrididae). Similarly, a positive correlation of the virulence with the protease and chitinase activities of *B. bassiana* isolates was demonstrated against *Spodoptera litura* Fabricius and *Helicoverpa armigera* Hübner (Padmini-Palem and Padmaja, 2013). Moreover, studies by Khan *et al.* (2012) on hydrolytic activities of some isolates of *B. bassiana* and *Verticillium lecanii* Zimm revealed that lipase participated more in total virulence to *Myzus persicae* Sulzer in comparison with protease and chitinase.

In this research, we studied the protease (Pr1 and Pr2), chitinase (*N*-acetyl glucosaminidase), and lipase activities of different *B. bassiana* isolates, in response to cuticles from *Eurygaster integriceps* (Hem: Scutelleridae), *Ephestia kuehniella* (Lep: Pyralidae) and *Zophobas morio* (Col: Tenebrionidae) to illuminate the effect of cuticles from disparate insect orders as substrate and their interaction with production of *B. bassiana* hydrolytic enzymes. Furthermore, the possible correlation between the virulence and the enzyme activities of fungal isolates and histopathology of infected insect cuticles by *B. bassiana* has been studied.

Materials and Methods

Insects rearing

Sunn pest, *E. integriceps*, adults were collected from wheat fields of Varamin, Tehran province, Iran and reared in plastic boxes (30×30×50 cm) on wet wheat seeds (*Triticum aestivum* var. Pishtaz), a piece of cotton soaked with water was used as a water source. Folded strips of paper were hung in containers as oviposition substrates. Newly emerged nymphs were transferred to plastic shelves with pots of wheat and wet wheat seeds. Fifth instar nymphs (identified by rudiments of hind wings and developmental periods) were used in experiments, as they are physiologically more stable than previous instars and their developmental period is long enough for successful penetration of the fungus. The flour moth colony was prepared from the Biological Control Laboratory, College of Agriculture and Natural Resources, University of Tehran and bred in plastic containers containing flour and yeast (10 g yeast per kg of flour), then fourth instar larvae identified by mean head capsule wide and moltings (Yazdanian *et al.*, 2005) were used in experiments. Initial colony of *Z. morio* beetle was obtained from a pet food store and reared in plastic containers (20 × 15 × 10 cm) containing wheat bran and pieces of potato. The last instar larvae of the new generation were used in experiments. Rearing condition was 25 ± 1 ºC, 70 ± 5% R. H. and 16:8 (L: D) h photoperiod for all insects.

Beauveria bassiana isolates

Five *B. bassiana* isolates encoded TV, OZ, UN, DV and DE (soil origin, Seyedtalebi *et al.*, 2017) were grown on Sabouraud Dextrose Agar (SDA) plates and maintained at 25 ± 1 °C, 70 ± 5 % RH, and a photoperiod of 16:8 (L: D) h for 14 days. Cultures were scrapped after sporulation and conidia were obtained (Goettel and Inglis 1997). Virulence of the isolates has been previously studied on these insects, as TV and DE were the most and the least virulent

isolates, while the others had moderate virulence on *E. kuehniella* larvae and *E. integriceps* nymphs. None of the isolates significantly affected the survival of *Z. morio* larvae (Seyed talebi et al., 2018).

Cuticle preparation

For cuticle preparation, larvae of *E. kuehniella* and *Z. morio* and nymphs of *E. integriceps* were dissected and their internal organs were removed. Cuticles were rinsed several times with saline solution (6 mol/L NaCl), dried and ground to be used in liquid culture media (Ramzi and Zibaee, 2014).

Enzymes assays

- Total protease activity

Total protease activity was assessed using the method described by Erlacher et al. (2006). The substrate was 250 µl of 2% (w/v) azocasein solution in 20 mM Tris HCl buffer (pH 8.0). The reaction was initiated by loading 150 µl of sample to the substrate. The mixture was incubated at 37 °C for 30 min, then the reaction was stopped by addition of 1.2 ml of 10% (w/v) trichloroacetic acid (TCA). Thereafter, the samples were centrifuged (Universal 32R) at 8000 rpm for 4 min and 600 µl of the clear supernatant were transferred to new microtube containing 700 µl of 1.0 M NaOH and vortexed. The absorbance was read at 440 nm with a Microplate reader (Bio TeK, USA). The experiment was conducted in three replicates.

- Subtilisin (Pr1) and Trypsin (Pr2) activity

Pr1 and Pr2 activities were assayed using the described method by St. Leger et al. (1987) and specific synthetic substrates, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and Benzoyl-phenylalanine -valine-arginine-*p*-nitroanilide (BAPNA), respectively. Each substrate (50 µl of 0.1 Mm) was mixed with 0.85 ml of 20 mM Tris–HCl buffer (pH 8.5) and 100 µl of culture supernatant. The reaction mixture was incubated for 30 min at 28 °C, then terminated using 0.25 µl of 30% acetic acid. Absorbance was observed at 405 nm using Microplate reader (Bio TeK, USA) and the concentration of para-nitro aniline was determined. One unit of protease activity was defined as the amount of enzyme that produced 1 µmol of *p*-nitroaniline per min under the above conditions. The experiment was conducted in three replicates.

- Chitinase activity

N-acetyl-β-D-glucosaminidase (NAGase) activity was assessed according to the method of St. Leger *et al.* (1998). The reaction mixture contained 80 µl of 0.1 M citrate buffer (pH 5.0), 10 µl of 10 mM *p*-nitrophenol-N-acetyl-β-D-glucosaminide, and 10 µl of culture supernatant. Reaction was carried out at 37 °C for 30 min, then terminated by the addition of 100 µl of 0.5 M NaHCO3–Na2CO3 buffer. The release of *p*-nitrophenyl (*p*NP) was determined at 405 nm (Microplate reader, Bio-Tek, USA). Activities were expressed as 1 µmol of p-nitrophenol released per min. The experiment was conducted in three replicates.

- Lipase activity

The enzyme assay was carried out as described by Tsujita *et al.* (1989). Twenty µl of the enzyme, 100 µl of 20 mM Tris–HCl buffer (pH 7) and 50 µl of 27 Mm p-nitrophenyl butyrate, were incorporated, thoroughly mixed and incubated at 37 °C. After 15 min of inoculation, 100 µl of NaOH (1 N) was added to each tube and absorbance was read at 405 nm. One unit of enzyme releases 1 µmol of *p*-nitrophenol per min. The experiment was conducted in three replicates.

The protein concentration was determined by the method described by Bradford (1967), using BSA as standard for all experiments.

-Paraffin embedded histopathological sections

TV (as the most virulent) isolate was selected for this experiment. The individuals of sunn pest nymphs, the flour moth and king meal worm larvae were dipped into 20 ml of the fungal suspension (5×10^7 conidia/mL) for 10 s, distilled water containing 0.05% Tween-80 solution was used as control, based on our previous experiments (Seyed talebi *et al.*, 2018). There were 20 insects in each treatment. After dryness, the insects were transferred to a plastic dish containing relevant food and incubated under 25 ± 1 ºC, 70 ± 5% R.H. and 16:8 (L: D) h photoperiod conditions. At 24 h intervals, 3 - 4 larvae were picked out randomly for paraffin sectioning.

The larvae were fixed with Formaldehyde 10% for 24 h, and dehydrated in increasing graded (30, 50 and 75%) ethanol for 1 h. The fixed samples were embedded in paraffin wax and cut into sections of 5 - 6 µm. The sections were stained with hematoxylin and eosin then slide preparations were assessed under a Zeiss microscope and pictures were taken with Dino-Lite digital lens Dinocap®.

Statistical analysis

All experimental data were subjected to analysis of variance (ANOVA). Pooled data of two-time repeats of the whole assays were analyzed to determine possible significant differences among the treatments via F-LSD test post-significant ANOVA. A possible correlation between enzyme activity and virulence of isolates was analyzed through the Pearson correlation coefficient (SAS Institute, 2002).

Results

Proteolytic activity

The proteolytic activity was significantly different among isolates ($F_{4, 15}$ = 183.08, p < 0.001) and insects ($F_{2, 15}$ = 1377.70, p < 0.001). The lowest enzyme activities were recorded on *Z. morio* larvae cuticle (Fig. 1). Among the evaluated isolates, maximum proteolytic activity was observed for TV isolate. There was a positive correlation between the protease activity and the virulence of isolates for two of the insects, *E. kuehniella* larvae (r = 0.92, p < 0.001) and *E. integriceps* (r = 0.97, p < 0.001).

Pr1 and Pr2 activity

The Pr1 activity was statistically different among fungus isolates ($F_{4, 15}$ = 30.09, p < 0.001) and insect cuticles ($F_{2, 15}$ = 211, p < 0.001). In the same way, the Pr2 was variably produced by different *B. bassiana* isolates ($F_{4, 15}$ = 10.12, p < 0.005) and on three distinct insect cuticles ($F_{2, 15}$ = 72, p < 0.001). The highest activity of Pr1 and Pr2 was recorded for isolate TV in presence of *E. kuehniella* and *E. integriceps* cuticle, respectively (Figs. 2 and 3). There was a positive correlation between the Pr1 and Pr2 activity and the virulence of isolates on *E. kuehniella* larvae (r = 0.92, p < 0.001. r = 0.78, p < 0.007) and *E. integriceps* (r = 0.90, p < 0.0003. r = 0.77, p < 0.009).

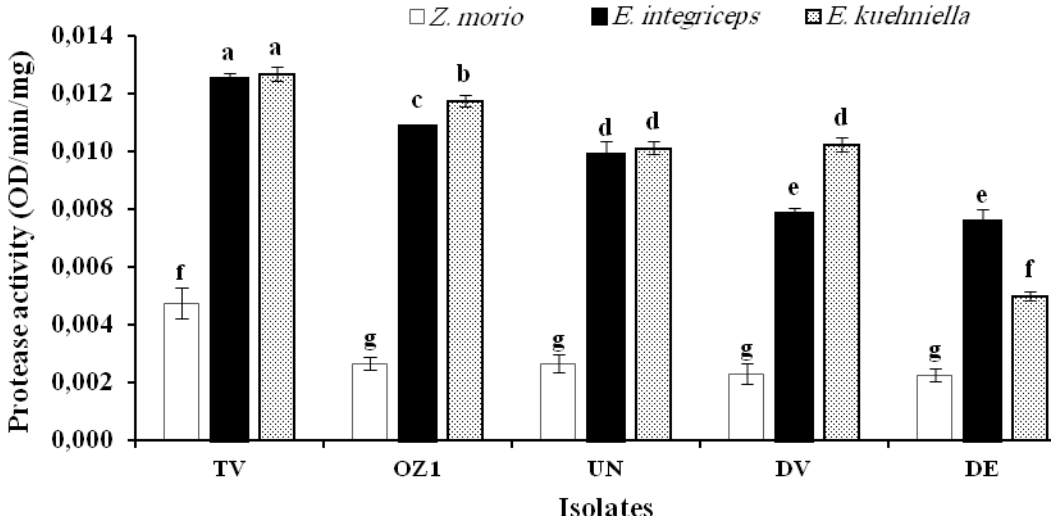

Figure 1 Mean activities of proteases (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, P < 0.05).

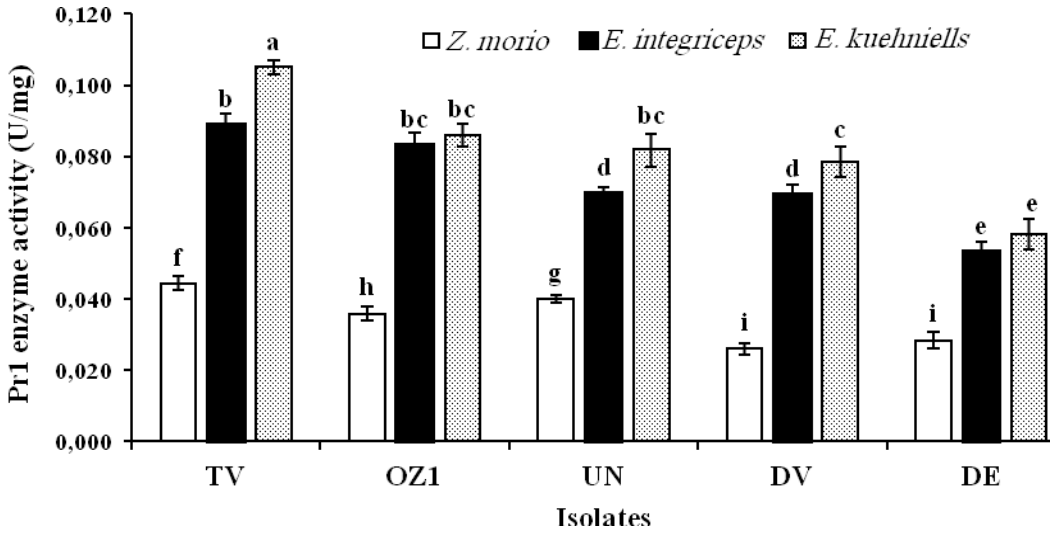

Figure 2 Mean activities of Pr1 enzyme (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, P < 0.05).

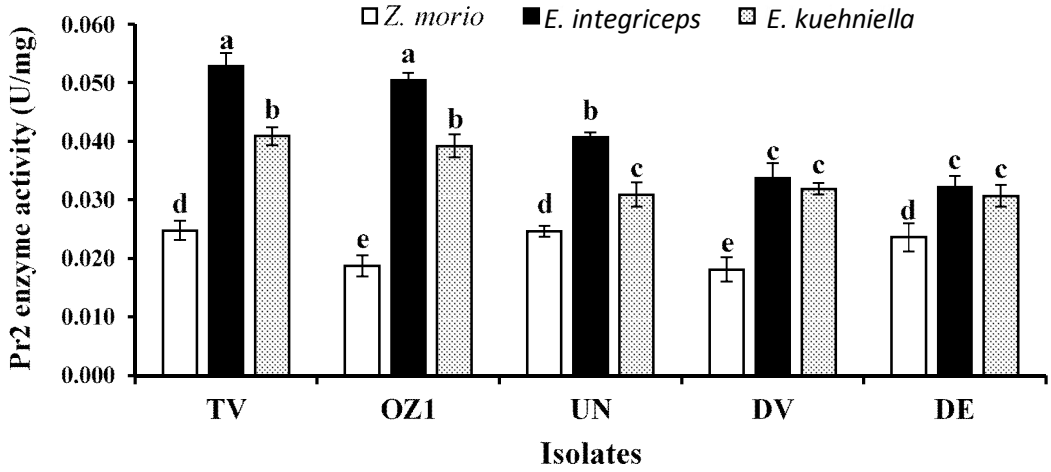

Figure 3 Mean activities of Pr2 enzyme (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, P < 0.05).

Chitinase activity

There were significant differences between isolates ($F_{4, 15}$ = 19.12, p < 0.001) and insect cuticles ($F_{2, 15}$ = 276.72, p < 0.001) in chitinolytic activity. TV isolate had the highest enzymes activity (Fig. 4). There was a positive correlation between the chitinase activity and the virulence of isolates on *E. kuehniella* (r = 0.94, p < 0.001) and *E. integriceps* (r = 0.83, p < 0.003).

Lipase assay

Lipase activity was significantly different among isolates ($F_{4, 15} = 0.19$, $p < 0.003$) and insects ($F_{2, 15} = 6.71$, $p < 0.001$). The highest and lowest enzyme activities were observed for *E. integriceps* nymphs and the *Z. morio* larvae, respectively (Fig. 5). There was a positive correlation between the lipase activity and the virulence of isolates for *E. integriceps* ($r = 0.81$, $p < 0.004$) unlike *E. kuehniella* ($r = 0.61$, $p > 0.6$).

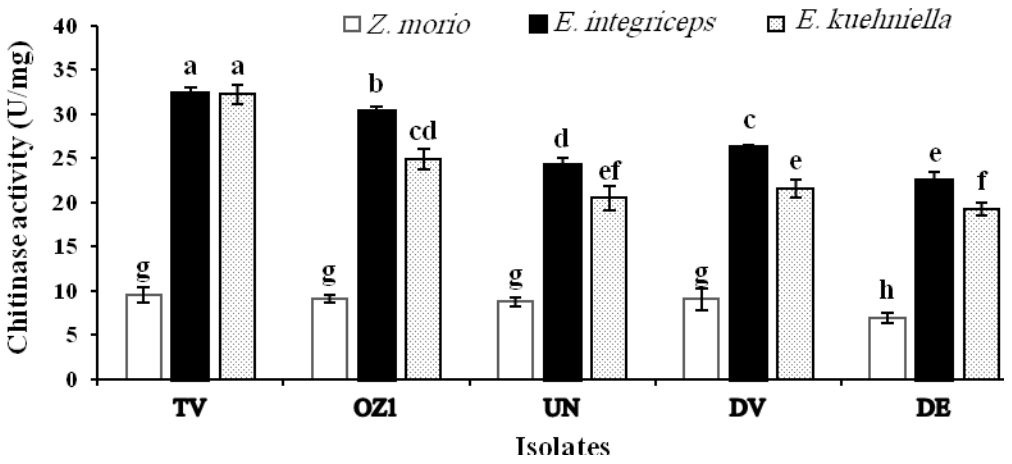

Figure 4 Mean chitinase activity (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, $P < 0.05$).

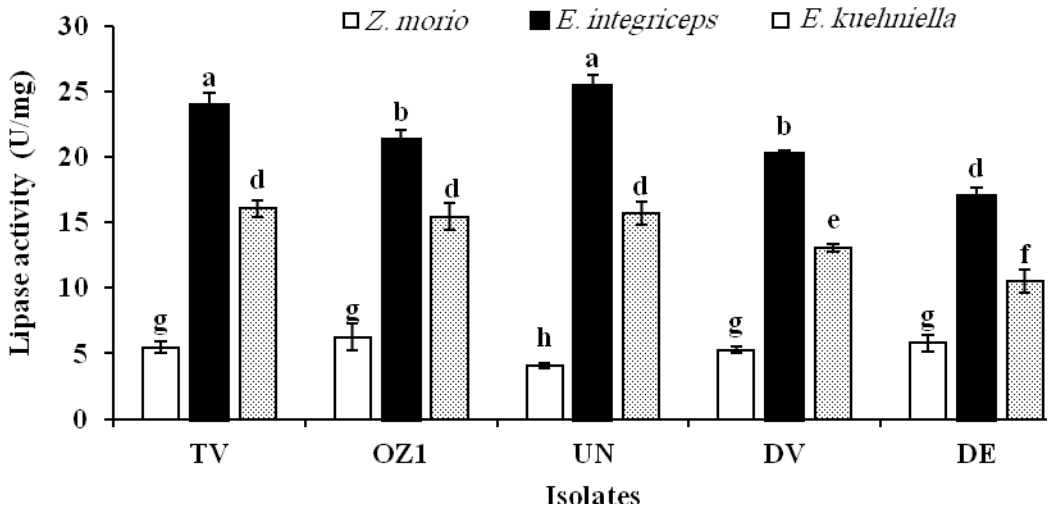

Figure 5 Mean lipase activity (± SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test, $P < 0.05$).

Histopathological study

Microscopic slides indicated different morphological characteristics in the cuticles of the studied insects. The cuticle of the flour moth larvae had numerous folding on the surface (Fig. 6A), Sunn pest cuticle was characterized with many microscopic thistles in different parts (Fig. 6B) and in the case of king meal worm larvae it had a flat surface, it was also thicker (Fig. 6C). Histological examinations, after 96 h exposure to *B. bassiana*, revealed the growth of fungal hyphae in the tissues of the infected *E. kuehniella* (Fig. 7A) and *E. integriceps* (Fig. 7B). In the exposed *Z. morio* larvae, fungal growth was not detected (Fig. 7C).

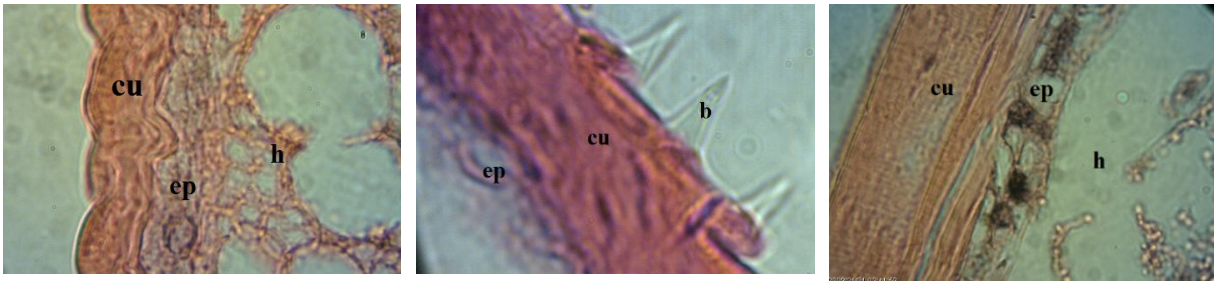

Figure 6 The cuticle (× 1000) of flour moth larvae, *Ephestia kuehniella* (left), Sunn pest, *Eurygaster integriceps* (middle) and king meal worm, *Zophobas morio* (right). (cu: cuticle, ep: periderm, h: tissue and b: thistle).

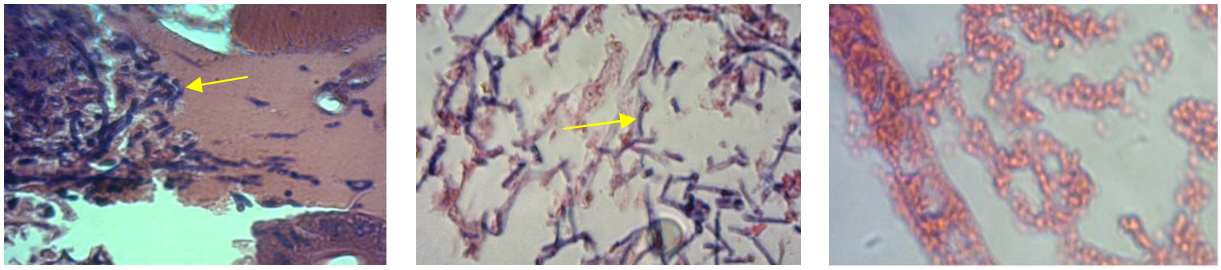

Figure 7 Fungal hyphae of *Beauveria bassiana* in the body tissues of the infected *Ephestia kuehniella* (left) and *Eurygaster integriceps* (middle), with no fungal growth in the body tissues of *Zophobas morio* (right).

Discussion

Our results revealed that the enzymatic activity of *B. bassiana* was significantly different based on both fungus isolates and insect cuticles. Although some pre-penetration aspects related to conidial size, vigor, germination speed, and attachment affect the host-fungus interaction (Altre *et al.*, 1999; Safavi *et al.*, 2007; Safavi, 2011; Faria *et al.*, 2015), penetration through the insect cuticle is carried out via a combination of mechanical pressure and hydrolytic enzymes (Lu and St. Leger, 2016). Degradation of cuticular hydrocarbons starts with the reaction of enzyme complexes (Barra *et al.*, 2015). These cuticle-degrading enzymes are important in infection process, as they hydrolyze the major components of insect cuticle including polymer protein, chitin and lipid complexes (Petrisor and Stoian, 2017).

Different induction of hydrolytic enzymes on three insect cuticles in our experiments revealed the crucial role of the host cuticle in the infection process. The composition of the components of host cuticle may induce or inhibit fungal development. This depends on a variety of different factors involved in the specificity of the interaction between the insect cuticles and entomopathogenic fungi (Crespo *et al.*, 2002). The most sensitive and resistant hosts to *B. bassiana* were *E. integriceps* nymphs and *Z. morio* larvae, respectively

(Seyedtalebi *et al.*, 2018). Also, in most cases the maximum enzymatic activity was observed in the presence of *E. integriceps* cuticle, unlike *Z. morio* cuticle which induced minimum levels of enzymatic activity. Regulation of degradative enzymes is in response to differences in the composition of insect cuticle which varies from species to species (Gupta et al., 1994). Experiments by El-Sayed *et al.* (1993) revealed that expression of hydrolytic enzymes was different when cuticles of *Trichoplusia ni* Hübner and *Helicoverpa zea* Boddie were used as substrates.

Based on our findings, TV isolate, as the most virulent isolate (Seyed talebi et al. 2018), had the highest proteolytic activity. The entomopathogenic fungus can produce diverse enzymes as virulence factors in response to different insect cuticles. Comparisons among isolates for virulence and production of enzymes indicate the great variability within a fungal species for numerous factors, many of which may influence cuticle-degrading enzyme activity (Petrisor and Stoian, 2017). Extracellular enzymes such as proteases, chitinases and lipases are in some way related to virulence of entomo- pathogenic fungi (Fang *et al.*, 2005; Kaur and Padmaja, 2009; Safavi, 2012; Dhawan and Joshi, 2017). In the same way, our data indicated that there was a positive correlation between protease, chitinase, and lipase activities and fungal isolate virulence. Astudy by Gupta *et al.* (1994) supports the notion that cuticle degrading enzymes may determine not only specific virulence, but also host specificity of fungal isolates. However, other studies showed no correlation between the virulence of fungus and enzyme activities (Rosato *et al.*, 1981).

Insect cuticle is essentially composed of proteins and chitin associated with lipids and phenolic compounds, but protein content of this complex and composite structure is higher than the others. There are different types of proteins in insect cuticle, which vary among different insects. Thus, main focus has been directed on the role of proteases in penetration process (Andersen *et al.,* 1995; Dombrovsky *et al.*, 2003; Charnley, 2003). Although proteases initiate degradation, they act synergistically with chitinases in solubilization of the insect cuticle (Smith *et al.*, 1981; St. Leger *et al.*, 1986). Subtilisin-like serine protease (Pr1) and trypsin-like enzyme (Pr2) are synthesized in the early stages of cuticle colonization, suggesting their critical role in degrading proteins (St Leger *et al.*, 1988; Mohanty *et al.*, 2008). The significantly variable protease activities that was evident in our experiments are in line with other studies indicating degrees of variation in production of cuticle degrading proteases in different isolates of entomopathogenic fungi (Clarkson and Charnley, 1996; Pinto *et al.*, 2002; Boldo *et al.*, 2009; Dhar and Kaur, 2010; Revathi *et al.*, 2011). Also, different insect cuticles induced diverse levels of protease activities. The maximum levels of total protease and Pr1 activity was observed when *E. kuehniella* cuticle was used as substrate, while Pr2 activity was mostly stimulated with *E. integriceps* cuticle. Moreover, as there was a positive correlation between proteolytic activities and fungal isolate virulence, a direct relationship of *B. bassiana* and activity of cuticle degrading enzymes is proposed (Chui-Chai *et al.*, 2012). Besides, positive correlation of proteolytic enzymes and virulence has been proved previously (St. Leger *et al.,* 1996; Feng, 1998; Gillespie *et al.*, 1998; Zare *et al.*, 2014).

Our results showed a crucial role of chitinolytic enzyme in virulence, because more virulent fungal isolates secreted more chitinase. The highest enzyme level was produced by TV isolate in the presence of *E. integriceps* and *E. kuehniella* cuticles. The hydrolysis of chitin by chitinolytic enzymes occurs through the endochitinases, and the exochitinases. N-acetylglucosaminidase is an exochitinase that cleaves chitin from its non-reducing end, releasing dimers $(GlcNAc)_2$ (Duo-Chuan, 2006; van Aalten *et al.*, 2011). Studies have shown that chitinase is secreted later into the cuticle, after the protease, and plays a secondary role in infection process (St. Leger, 1993; St. Leger *et al.*, 1996). Production of the exochitinases in entomopathogenic fungi was studied previously

(St. Leger *et al.*, 1996; Qazi and Khachatourians, 2008; Rustiguel *et al.*, 2012). Moreover, Gupta *et al.* (1994) showed a positive correlation between the production of high levels of N-acetylglucosaminidase and proteases with the virulence of *B. bassiana* against *Gallera mellonela* L. and *Trichoplusia ni*. Also, Montesinos-Matias *et al.* (2011) reported production of proteases, and β-N-acetylglucosaminidase in three isolates of *B. bassiana* in the presence of the cuticle of *T. molitor*, with the highest Pr1 and chitinase activities in the most virulent isolate. In contrast, a positive correlation between virulence with protease but not with chitinase activity of different *B. bassiana* isolates was observed on *Diatraea saccharalis* Fabricius (Svedese *et al.*, 2013).

As can be seen from Fig. 5, although the lipase activity of used *B. bassiana* isolates was higher in the presence of sunn pest cuticle, some lipolytic reactions were recorded on the cuticle of the other two insects. Lipases hydrolyze the ester bonds, fats and wax layers of the insect integument (Clarkson and Charnely, 1996; Kachatourians and Qazi, 2008). Lipolytic enzymes of many pathogenic microorganisms described so far, play an important role in the infection process (Schofield *et al.*, 2005). Lipase activity and virulence are usually positively correlated in *B. bassiana* (Feng, 1998; Kaur and Padmaja, 2009; Robledo-Monterrubio *et al.*, 2009). Our data on *E. integriceps* cuticle showed a positive correlation between virulence and lipase activity. Similarly, Pelizza *et al.* (2012) demonstrated the most virulent *B. bassiana* isolate had the highest proteases, chitinase and lipase activity to *S. cancellat.* However, lipolytic activity of fungal isolates on *E. kuehniella* and *Z. morio* cuticles was lower and correlated less with virulence. This indicates substrate specification and inherent differences among fungal isolates. Some studies have shown that virulence of *B. bassiana* isolates against *S. litura* and *H. armigera* was not correlated with lipase activity in enzyme assays (Padmaja and Palem, 2013).

Histopathological study showed that the fungal isolate could not penetrate to *Z. morio* cuticle because no mycosis was observed in its tissues after infection, while fungal hyphae were detected in microscopic slides of the other two insects. In a comparative study, Bogus *et al.* (2007) assessed the effect of the entomopathogenic fungus *Conidiobolus coronatus* Costantin against three insect species including *Dendrolimus pini* L., *Galleria mellonella* L. and *Calliphora vicina* Robineau-Desvoidy. *C. vicina* was resistant host and no signs of fungal penetration were found in the fly cuticle and also its internal organs remained unchanged.

There were many microscopic thistles on the cuticle of *E. integriceps* while the cuticle of *Z. morio* was very smooth; possibly, this ragged and thistly surface increases chance of fungal conidia attachment. Similarly, Boucias and Pendland (1991) and Toledo *et al.* (2010) reported that conidia of *B. bassiana* attach to all body regions, with a preference for surfaces containing hairs in which they are trapped and tightly bound.

In conclusion, not only the chemical composition and structure of insect cuticle play a substantial role in virulence of *B. bassiana*, but also the insecticidal effects of fungus isolates could be directly linked with the activity of hydrolytic enzymes. Further research is needed to identify probable synergistic interaction of cuticle-degrading enzymes. This interplay might enhance fungal penetration and pest control ability.

Acknowledgments

This publication is part of the first author's Ph.D. thesis funded by the Urmia University. We thank the Biological Control Laboratory, College of Agriculture and Natural Resources, University of Tehran, Karaj for technical support.

Conflict of Interest: The authors declare that they have no conflict of interest.

Declarations: The manuscript has not been published before and is not under consideration

for review and publication elsewhere. All authors are aware of and consent to this submission. All authors have equally contributed to this research.

Author contributions: This manuscript is based on the first author thesis and the first draft of the manuscript was written by her and improved substantially by Seyed Ali Safavi. All authors commented on primary draft of the manuscript. All authors read and approved the final manuscript.

References

Altre, J. A., Vandenberg, J. D. and Cantone, F. A. 1999. Pathogenicity of *Paecilomyces fumosoroseus* isolates to diamondback moth, *Plutella xylostella*: correllation with spore size, germination speed, and attachment to cuticle. Journal of Invertebrate Pathology, 73: 332-338.

Andersen, S. O., Hojrup, P. and Roepstorff, P. 1995. Insect cuticular proteins. Insect Biochemistry and Molecular Biology, 25: 153-176.

Barra, P., Etcheverry, M. and Nesci, A. 2015. Improvement of the insecticidal capacity of two *Purpureocillium lilacinum* strains against *Tribolium confusum*. Insects, 6: 206-223.

Boldo, J. T., Junges, A., Amaral, K. B., Staats, C., Vainstein, M. H. and Schrank, A. 2009. Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*. Current Genetics, 55: 551-560.

Boucias, D. G. and Pendland, J. C. 1991. Attachment of mycopathogens to cuticle. The initial event of mycoses in arthropod hosts. In: Cole, G. T. and Hoch, H.C. (Eds.) The fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, New York, pp. 101-127.

Bradford, M. M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Charnley, A. K. 2003. Fungal pathogens of insects: cuticle degrading enzymes and toxins. Advances in Botanical Research, 40: 241-321.

Chui-Chai, N., Krutmuang, P., Nalumpang, S., Mekchay, S., Khanongnuch, C. and Chanbang, Y. 2012. Insecticidal activity and cuticle degrading enzymes of entomopathogenic fungi against *Plutella xylostella* (Lepidoptera: Plutellidae). Journal of Nature and Science, 11(1): 147-155.

Clarkson, J. M. and Charnely, A. K. 1996. New insights into mechanisms of fungal pathogenesis in insects. Trends in Microbiology, 4: 197-204.

Crespo, R., Juárez, M. P., dal Bello, G. M., Padín, S., Calderón Fernández, G. and Pedrini, N. 2002. Increased mortality of *Acanthoscelides obtectus* by alkane-grown *Beauveria bassiana*. BioControl, 47: 685-696.

Dhar, P. and Kaur, G. 2010. Cuticle-degrading proteases produced by *Metarhizium anisopliae* and their induction in different media. Indian Journal of Microbiology, 50: 449-455.

Dhawan, M. and Joshi, N. 2017. Enzymatic comparison and mortality of *Beauveria bassiana* against cabbage caterpillar *Pieris brassicae* LINN. Brazilian Journal of Microbiology, 48: 522-529.

Duo-Chuan, L. 2006. Review of fungal chitinases. Mycopathologia, 161: 345-360.

Dombrovsky, A., Huet, H., Zhang, H., Chejanovsky, N. and Raccah, B. 2003. Comparison of newly isolated cuticular protein genes from six aphid species. Insect Biochemistry and Molecular Biology, 33: 709-715.

El-Sayed, G. N., Ignoffo, C. M., Leathers, T. D. and Gupta, S. C. 1993. Effects of cuticle source and concentration on expression of hydrolytic enzymes by an entomopathogenic fungus, *Nomuraea rileyi*. Mycopathologia, 122: 149-152.

Erlacher, A., Sousa, F., Schroeder, M., Jus, S., Kokol, V., Cavaco-Paulo, A. and Guebitz, G. M. 2006. A new cuticle scale hydrolysing protease from *Beauveria brongniartii*. Biotechnology Letters, 28: 703-710.

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., Feng, J., Yang, X., Zhang, Y. and Pei, Y. 2005. Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. Applied and Environmental Microbiology, 71: 363-370.

Faria, M., Lopes, R. B., Souza, D. A. and Wraight, S. P. 2015. Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi. Journal of Invertebrate Pathology, 125: 68-72.

Feng, M. 1998. Reliability of extracellular protease and lipase activities of *Beauveria bassiana* isolates used as their virulence indices. Acta Microbiologica Sinica, 38: 461-467.

Gillespie, J. P., Bateman, R. and Charnley, A. K. 1998. Role of cuticle-degrading protease in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. Journal of Invertebrate Pathology, 71: 128-137.

Goettel, M.S. and Inglis, G. D. 1997. Fungi: hyphomycetes. In: Lacey, L. A. (Ed), Manual of Techniques in Insect Pathology, Academic Press, San Diego, pp. 213-249.

Goettel, M. S., St. Leger, R. J., Rizzo, N. W., Staples, R. C. and Roberts, D. W. 1989. Ultrastructural localization of a cuticle degrading protease produced by the entomopathogenic fungus, *Metarhizium anisopliae* during penetration of host cuticle. Journal of General Microbiology, 135: 2223-2239.

Gupta, S. C., Leathers, T. D., El-Sayed, G. N. and Ignoffo, C. M. 1994. Relationships among enzyme activities and virulence parameters in *Beauveria bassiana* infections of *Galleria mellonella* and *Trichoplusia ni*. Journal of Invertebrate Pathology, 64:13-17.

Kaur, G. and Padmaja, V. 2009. Relationships among activities of extracellular enzyme production and virulence against *Helicoverpa armigera* in *Beauveria bassiana*. Journal of Basic Microbiology, 49: 264-274.

Khachatorians, G. G. and Qazi, S. S. 2008. Entomopathogeneic fungi: biochemistry and molecular biology. In: Brakhage, A. A. and Zipfei, P. F. (Eds.), The Mycota, VI: Human and Animal Relationships, 2nd ed. Springer-Verlag, Berlin, pp. 33-61.

Khan, S., Guo, L., Shi, H., Mijit, M. and Qiu, D. 2012. Bioassay and enzymatic comparison of six entomopathogenic fungal isolates for virulence or toxicity against green peach aphids *Myzus persicae*. African Journal of Biotechnology, 11(77): 14193-14203.

Lacey, L. A., Horton, D. R., Chauvin, R. L. and Stocker, J. M. 1999. Comparative efficacy of *Beauveria bassiana*, *Bacillus thuringiensis*, and aldicarb for control of Colorado potato beetle in an irrigated desert agroecosystem and their effects on biodiversity. Entomologia Experimentalis et Applicata, 93: 189-200.

Lu, H.-L. and St. Leger, R. J. 2016. Insect immunity to entomopathogenic fungi. Advances in Genetics, 94: 251-285.

Mayoral, F., Benuzzi, M. and Ladurner, E. 2006. Efficacy of the *Beauveria bassiana* strain ATCC 74040 (Naturalis®) against whiteflies on protected crops. Integrated Control in Protected Crops, Mediterranean Climate, IOBC/WPRS Bulletin, 29: 83-88.

Mohanty, S. S., Raghavendra, K. and Dash, A. P. 2008. Induction of chymoelastase (Pr1) of *Metarhizium anisopliae* and its role in causing mortality to mosquito larvae. World Journal of Microbiology and Biotechnology, 24(10): 2283-2288.

Montesinos-Matias, R., Viniegra-González, G., Alatorre-Rosas, R. and Loera, O. 2011. Relationship between virulence and enzymatic profiles in the cuticle of *Tenebrio molitor* by 2-deoxy-D-glucose-resistant mutants of *Beauveria bassiana* (Bals.) Vuill. World Journal of Microbiology and Biotechnology, 27: 2095-2102.

Mustafa, U. and Kaur, G. 2009. Extracellular enzyme production in *Metarhizium anisopliae* isolates. Folia Microbiologica, 54: 499-504.

Padmini Palem, P. C. and Padmaja, V. 2013. Functional interplay of certain pathogenicity determinant factors governing virulence of the entomopathogenic fungal species *Beauveria*. International Journal of Research in Biosciences, 2 (2): 83-92.

Pelizza, S. A., Eliades, L. A., Scorsetti, A. C., Cabello, M. N. and Lange, C. E. 2012. Entomopathogenic fungi from Argentina for the control of *Schistocerca cancellata* (Orthoptera: Acrididae) nymphs: fungal pathogenicity and enzyme activity. Biocontrol Science and Technology, 22(10): 1119-1129.

Petrisor, C. and Stoian, G. 2017. The role of hydrolytic enzymes produced by entomopathogenic fungi in pathogenesis of insects. Romanian Journal for Plant Protection, 10: 66-72.

Pinto, F. G. S., Fungaro, M. H. P., Ferreira, J. M., Valadares-Inglis, M. C. and Furlaneto, M. C. 2002. Genetic variation in the cuticle degrading protease activity of the entomopathogen *Metarhizium flavoviride*. Genetics and Molecular Biology, 25: 231-234.

Qazi, S. and Khachatourians, G. 2008. Addition of exogenous carbon and nitrogen sources to aphid exuviae modulates synthesis of proteases and chitinase by germinating conidia of *Beauveria bassiana*. Archives of Microbiology, 189: 589-596.

Ramzi, S. and Zibaee, A. 2014. Biochemical properties of different entomopathogenic fungi and their virulence against *Chilo suppressalis* (Lepidoptera: Crambidae) larvae. Biocontrol Science and Technology, 24(5): 597-610.

Revathi, N., Ravikumar, G., Kalaiselvi, M., Gomathi, D. and Uma, C. 2011. Pathogenicity of three entomopathogenic fungi against *Helicoverpa armigera*. Plant Pathology and Microbiology, 2: 1-4.

Richard, J. S., Neal, T. D., Karl, J. K. and Michael, R. K. 2010. Model reactions for insect cuticle sclerotization: participation of amino groups in the cross-linking of *Manduca sexta* cuticle protein MsCP36. Insect Biochemistry and Molecular Biology, 40: 252-258.

Robledo-Monterrubio, M., Alatorre-Rosas, R., Viniegra-Gonzalez, G. and Loera, O. 2009. Selection of improved *Beauveria bassiana* (Bals.) Vuill. strains based on 2-deoxy-D-glucose resistance and physiological analysis. Journal of Invertebrate Pathology, 101: 222-227.

Rosato, Y. B., Messias, C. L. and Azevedo, J. L. 1981. Production of extracellular enzymes by isolates of *Metarhizium anisopliae*. Journal of Invertebrate Pathology, 38: 1-3.

Safavi, S. A. 2011. Successive subculturing alters spore-bound Pr1 activity, germination and virulence of the entomopathogenic fungus, *Beauveria bassiana*. Biocontrol Science and Technology, 21(8): 883-890.

Safavi, S. A. 2012. Attenuation of the entomopathogenic fungus *Beauveria bassiana* following serial in vitro transfers. Biologia, 67(6): 1062-1068.

Safavi, S. A., Shah, F. A., Pakdel, A. K., Rasoulian, G. R., Bandani, A. R. and Butt, T. M. 2007. Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. FEMS Microbiology Letters, 270: 116-123.

SAS Institute. 2002. SAS for Windows, Release 9.1, Cary, NC, USA.

Schofield, D. A., Westwater, C., Warner, T. and Balish, E. 2005. Differential *Candida albicans* lipase gene expression during alimentary tract colonization and infection. FEMS Microbiology Letters, 244(2): 359-65.

Seyedtalebi, F. S., Safavi, S. A., Talaei-Hassanloui, R. and Bandani, A. R. 2017. Quantitative comparison for some immune responses among *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio* against the entomopathogenic fungus *Beauveria bassiana*. Invertebrate Survival Journal, 14: 174-181.

Seyed talebi, F. S., Safavi, S. A., Talaei-Hassanloui, R. and Bandani, A. R. 2018. Study of the virulence and conidial germination types for some *Beauveria bassiana* isolates. Biological Control of Pests and Plant Diseases, 7(1): 65-73.

St. Leger, R. J. 1993. Biology and mechanisms of invasion of deuteromycete fungal pathogens. In: Beckage, N. C., Thompson, S. N. and Federici, B. A. (Eds.), Parasites

and Pathogens of Insects. Academic Press, San Diego, pp. 211-229.

St. Leger, R. J., Charnley, A. K. and Cooper R. M. 1987. Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Archives of Biochemistry and Biophysics, 253: 221-232.

St. Leger, R. J., Durrands, P. K., Charnley, A. K. and Cooper, R. M. 1988. Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*. Journal of Invertebrate Pathology, 52: 285-293.

St. Leger, R. J., Joshi, L., Bidochka, M. J., Rizzo, N., Roberts, D. W. 1996. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Applied and Environmental Microbiology, 62: 1257-1264.

St. Leger, R., Joshi, L. and Roberts, D. W. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Applied and Environmental Microbiology, 64(2): 709-713.

Svedese, V., Vieira, P., Pereira, J., Mesquita, L., Luna, E. and Figueiredo, A. 2013. Pathogenicity of *Beauveria bassiana* and production of cuticle-degrading enzymes in the presence of *Diatraea saccharalis* cuticle. African Journal of Biotechnology, 12(46): 6492-6497.

Talaei-Hassanloui, R., Kharazi-Pakdel, A., Goettel, M. S., Little, S. and Mozaffari, J. 2007. Germination polarity of *Beauveria bassiana* conidia and its possible correlation with virulence. Journal of Invertebrate Pathology, 94: 102-107.

Toledo, A. V., Remes Lenicov, A. M. M. and Lopez Lastra, C. C. 2010. Histopathology caused by the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, in the adult planthopper, *Peregrinus maidis*, a maize virus vector. Journal of Insect Science, 10:35.

Tsujita, T., Ninomiya, H. and Okuda, H. 1989. P-nitrophenyl butyrate hydrolyzing activity of hormone sensitive lipase from bovine adipose tissue. Journal of Lipid Research, 30: 997-1004.

van Aalten, D. M. F., Komander, D., Systand, B., Gaseidnes, S., Peter, M. G. and Eijsink, V. G. H. 2011. Structural Insights into the Catalytic Mechanism of a Family 18 Exochitinase. Proceedings of the National Academy of Sciences, 98(16): 8979-8984.

Vega, F. E., Meyling, N. V., Luangsa-Ard, J. J. and Blackwell, M. 2012. Fungal entomopathogens. In: Vega, F. E. and Kaya, H. K. (Eds.) Insect Pathology. Elsevier, London, pp. 171-220.

Yazdanian, M., Haddad Irani Nejad, K. and Mashhadi Jafarloo, M. 2005. Determining the number of larval instars of the Mediterranean flour moth, *Anagasta kuehniella* (Lepidoptera, Phycitidae) in laboratory conditions. Agricultural Science 15: 45-54 (In Persian).

القای متغیر آنزیم‌های تجزیه‌کننده کوتیکول در جدایه‌های *Beauveria bassiana* در حضور کوتیکول حشرات مختلف

فرزانه سادات سیدطالبی[1]، سیّدعلی صفوی[1*]، رضا طلایی حسنلویی[2] و علیرضا بندانی[2]

۱- گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه ارومیه، ارومیه، ایران.
۲- گروه گیاه‌پزشکی، دانشکده کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: a.safavi@urmia.ac.ir
دریافت: ۶ آذر بهمن؛ پذیرش: ۱۷ تیر ۱۳۹۹

چکیده: در تحقیق حاضر تولید آنزیم‌های تجزیه‌کننده کوتیکول در پنج جدایه از *Beauveria bassiana* (TV، OZ، UN، DV و DE) در حضور کوتیکول حشرات *Eurygaster integriceps*، *Ephestia kuehniella* و *Zophobas morio* مورد ارزیابی قرار گرفت. علاوه بر آن آسیب‌شناسی بافتی حشرات آلوده به *B. bassiana* بررسی شد. سطح آنزیم‌های تجزیه‌کننده کوتیکول برای جدایه TV (به‌عنوان قوی‌ترین جدایه در زهرآگینی) در بیش‌ترین سطح و برای DE (به‌عنوان ضعیف‌ترین جدایه در زهرآگینی) در کم‌ترین مقدار بود. کوتیکول پوره سن پنجم *E. integriceps* به‌عنوان حساس‌ترین میزبان بالاترین سطح از آنزیم‌های تجزیه‌کننده کوتیکول (Pr2، اگزوکیتیناز و لیپاز) را القا کرد درحالی‌که کم‌ترین مقادیر در حضور کوتیکول *Z. morio* که مقاوم‌ترین میزبان بود مشاهده شد. براساس بررسی‌های آسیب‌شناسی مشخصات کوتیکول در بین این حشرات متفاوت بود و عدم‌حضور و رشد میسلیوم قارچی در بافت‌های بدن *Z. morio* نشان‌دهنده این مطلب است که قارچ امکان عبور از کوتیکول این حشره را نداشته است درحالی‌که رشد میسلیوم در برش‌های بافتی دو حشره دیگر دیده شد. به‌طور کلی مشخصات فیزیکی و شیمیایی کوتیکول حشرات نقش اساسی در زهرآگینی قارچ‌های بیمارگر دارد. سطوح فعالیت آنزیم‌های پروتئاز (به‌خصوص Pr2) ، کیتیناز (ان استیل گلکوزآمینیداز) و لیپاز می‌تواند به‌عنوان شاخص زهرآگینی برای تشخیص قوی‌ترین جدایه‌ها به‌کار گرفته شود.

واژگان کلیدی: *Beauveria bassiana*، آنزیم‌های تجزیه‌کننده کوتیکول، شاخص زهرآگینی، آسیب‌شناسی بافتی