

Research Article

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

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**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-Hassanlou *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,

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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 gregaria* 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), *Ephesia 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 (Yazdani *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 °C, 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 Zibae, 2014).

### Enzymes assays

#### - Total protease activity

Total protease activity was assessed using the method described by Erlacher et al. (2006). The substrate was 250  $\mu$ l of 2% (w/v) azocasein solution in 20 mM Tris HCl buffer (pH 8.0). The reaction was initiated by loading 150  $\mu$ l 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  $\mu$ l of the clear supernatant were transferred to new microtube containing 700  $\mu$ l 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  $\mu$ l of 0.1 Mm) was mixed with 0.85 ml of 20 mM Tris-HCl buffer (pH 8.5) and 100  $\mu$ l of culture supernatant. The reaction mixture was incubated for 30 min at 28 °C, then terminated using 0.25  $\mu$ l 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  $\mu$ mol of *p*-nitroaniline per min under the above conditions. The experiment was conducted in three replicates.

#### - Chitinase activity

N-acetyl- $\beta$ -D-glucosaminidase (NAGase) activity was assessed according to the method of St. Leger et al. (1998). The reaction mixture contained 80  $\mu$ l of 0.1 M citrate buffer (pH 5.0), 10  $\mu$ l of 10 mM *p*-nitrophenol-N-acetyl- $\beta$ -D-glucosaminide, and 10  $\mu$ l of culture supernatant. Reaction was carried out at 37 °C for 30 min, then terminated by the addition of 100  $\mu$ l of 0.5 M NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> buffer. The release of *p*-nitrophenyl (*p*NP) was determined at 405 nm (Microplate reader, Bio-Tek, USA). Activities were expressed as 1  $\mu$ mol 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  $\mu$ l of the enzyme, 100  $\mu$ l of 20 mM Tris-HCl buffer (pH 7) and 50  $\mu$ l of 27 Mm *p*-nitrophenyl butyrate, were incorporated, thoroughly mixed and incubated at 37 °C. After 15 min of inoculation, 100  $\mu$ l of NaOH (1 N) was added to each tube and absorbance was read at 405 nm. One unit of enzyme releases 1  $\mu$ mol 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 ( $5 \times 10^7$  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  $25 \pm 1$  °C,  $70 \pm 5\%$  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  $\mu\text{m}$ . 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

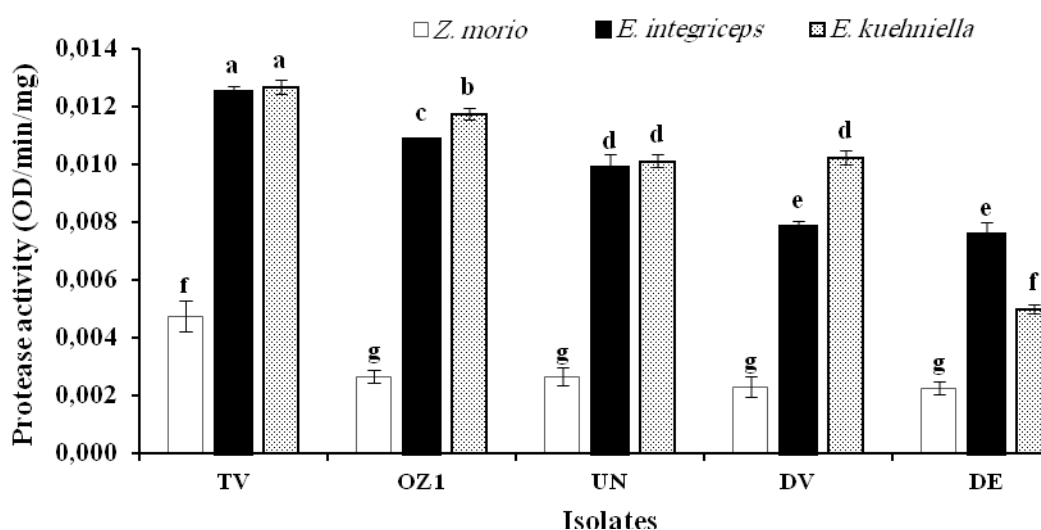
### Proteolytic activity

The proteolytic activity was significantly different among isolates ( $F_{4, 15} = 183.08$ ,  $p <$

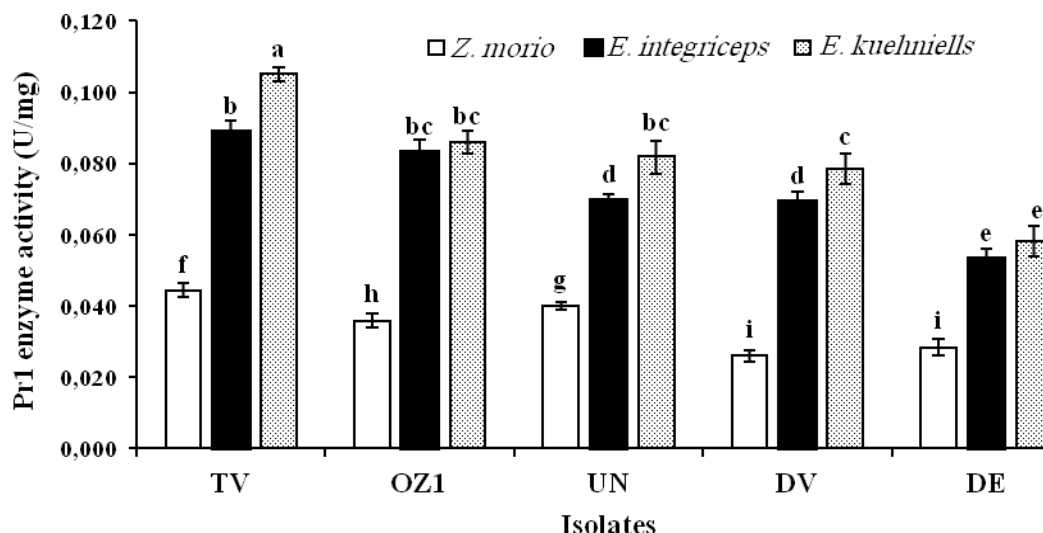
0.001) and insects ( $F_{2, 15} = 1377.70$ ,  $p <$  0.001). The lowest enzyme activities were recorded on *Z. morio* larvae cuticle (Fig. 1). Among the evaluated isolates, maximum proteolytic activity was observed for TV isolate. There was a positive correlation between the protease activity and the 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

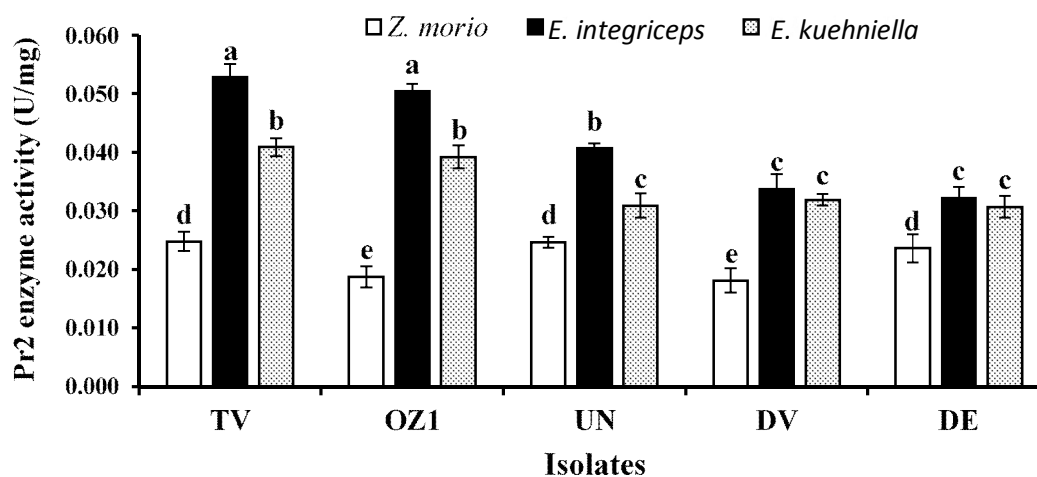
The Pr1 activity was statistically different among fungus isolates ( $F_{4, 15} = 30.09$ ,  $p <$  0.001) and insect cuticles ( $F_{2, 15} = 211$ ,  $p <$  0.001). In the same way, the Pr2 was variably produced by different *B. bassiana* isolates ( $F_{4, 15} = 10.12$ ,  $p <$  0.005) and on three distinct 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 ( $\pm$  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 ( $\pm$  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 ( $\pm$  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

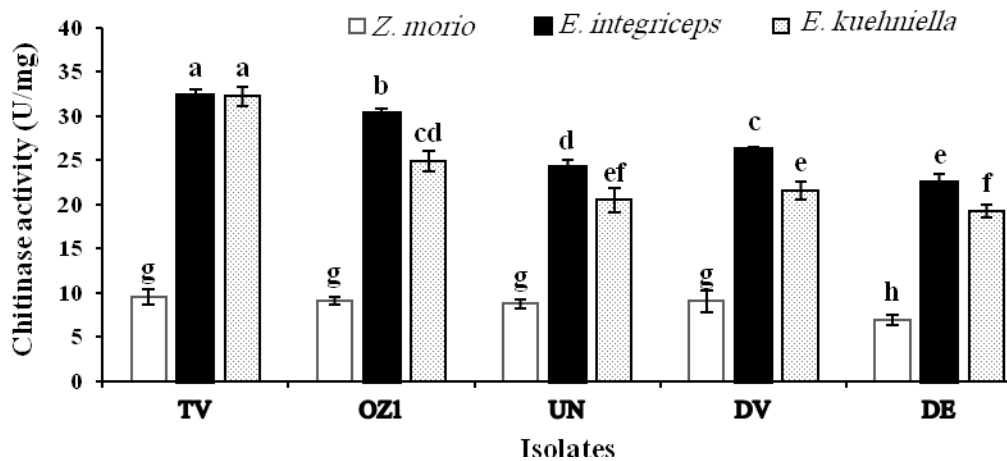
There were significant differences between isolates ( $F_{4, 15} = 19.12$ ,  $p < 0.001$ ) and insect cuticles ( $F_{2, 15} = 276.72$ ,  $p < 0.001$ ) in chitinolytic activity. TV isolate had the highest

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* ( $r = 0.83$ ,  $p < 0.003$ ).

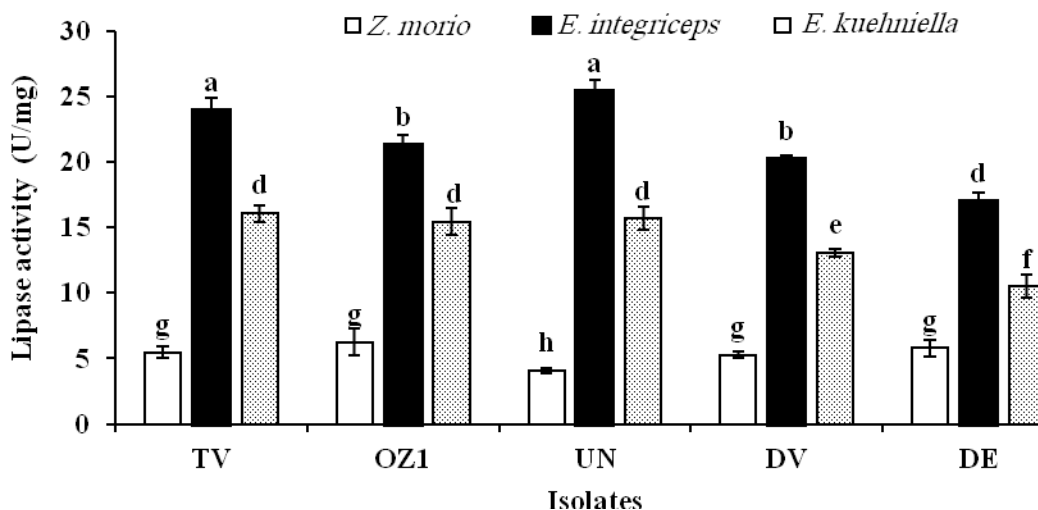
**Lipase assay**

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 ( $\pm$  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,  $P < 0.05$ ).

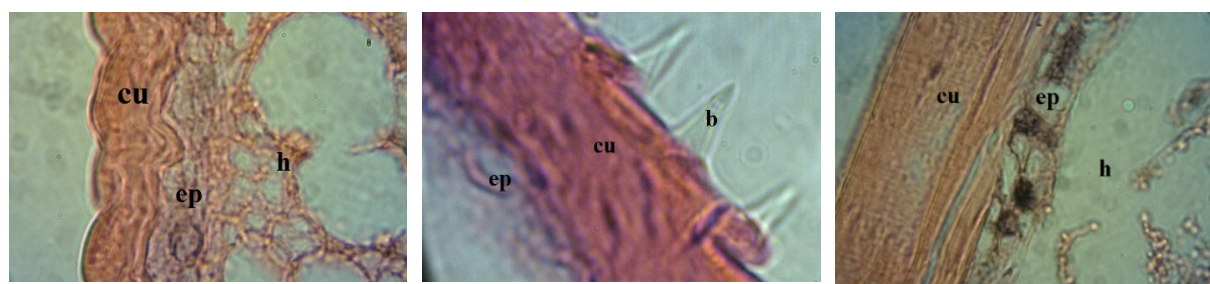


**Figure 5** Mean lipase activity ( $\pm$  SE) in the cultured *Beauveria bassiana* isolates on liquid medium containing *Zophobas morio*, *Ephestia kuehniella* and *Eurygaster integriceps* cuticles (Means with common or similar letters are not significantly different, F-LSD test,  $P < 0.05$ ).

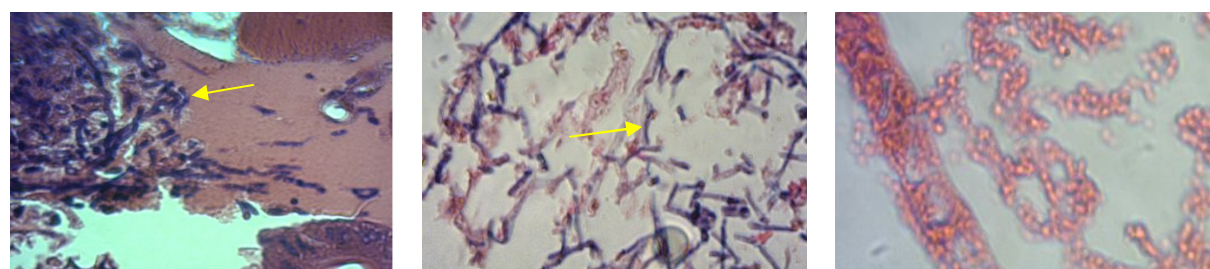
### Histopathological study

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 ( $\times 1000$ ) 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 Stoiian, 2017). Extracellular enzymes such as proteases, chitinases and lipases are in some way related to virulence of entomopathogenic 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. A study 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)<sub>2</sub> (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 *Galleria mellonella* L. and *Trichoplusia ni*. Also, Montesinos-Matias *et al.* (2011) reported production of proteases, and  $\beta$ -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.

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**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.

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

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**چکیده:** در تحقیق حاضر تولید آنزیم‌های تجزیه‌کننده کوتیکول در پنج جدایه از *Beauveria bassiana* (*DE* و *DV*، *UN*، *OZ*، *TV*) در حضور کوتیکول حشرات *Ephestia*، *Eurygaster integriceps* 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 عدم حضور 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 گلوکزآمینیداز) و لیپاز می‌تواند به‌عنوان شاخص زهرآگینی برای تشخیص قوی‌ترین جدایه‌ها به‌کار گرفته شود.

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