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Mortality and immune challenge of a native isolate of *Beauveria bassina* against the larvae of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae)

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Abstract

Background: Entomopathogenic fungi (EPF) attack a wide range of insects. They are considered environmental friendly alternatives to synthetic insecticides for pest control. In the present study, virulence of a native isolate of the EPF, *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) was evaluated against the least mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Crambidae), through bioassay, pathogenic pathways, and immune responses.

Results: The values of 2.6×10^4 conidia/ml and 3.54 days were determined as the median lethal concentration (LC_{50}) and median lethal concentration (LT_{50}) of AM-118 against the 4th instar larvae of *G. pyloalis*, respectively. The activities of proteases and chitinases in the culture medium containing the larval cuticle were higher than the control medium. Moreover, the total and the differential hemocyte counts of the larvae were significantly changed after injection with AM-118 spores. The highest numbers of total hemocytes and granulocytes were obtained 3 and 6 h post-injection, while the highest numbers of plasmatocytes and nodules were observed 6 h post-injection. The highest activity of phenoloxidase was determined 12 h post-injection by AM-118 spores.

Conclusions: The findings imply on virulence of the AM-118 isolate against the larvae of *G. pyoalis* although immune responses were triggered by the spores.

Keywords: *Glyphodes pyloalis*, *Beauveria bassiana*, Immunity, Mycoinsecticides, Virulence

Background

The lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae), is an important pest with annually sever damages on mulberry. The larvae intensively feed on leaves, fold them, and retain the black feces to lose quality of the leaves for silkworm rearing (Khosravi and Jalali Sendi 2010). Another important case is the capability of transmitting pathogenic diseases to silkworm (Watanabe et al. 1988). The main control measure is to spray chemical insecticides although the excessive use of these pesticides has resulted in adversely severe effects on non-target organisms mainly silkworm (Yazdani et al. 2013). Therefore, considering eco-friendly measures like natural enemies, predators, parasitoids, or entomopathogens seems to be necessary.

Several researches have studied the effects of entomopathogenic fungi (EPF) especially, *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) isolates against many lepidopteran insect pests. EPF not only have significant virulence against insects but they also play important roles in physiological functions by inactivation of enzymes or triggering some processes (Ramzi and Zibaee 2014; Baja et al. 2020). In infection pathway, conidia should be connected to the cuticle of host,

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germinated via different layers and reproduced after reaching the hemocoel. Then, produced blastospores led to kill the insect hosts by production of secondary metabolites and consumption of nourishing resources in host bodies (Brownbridge et al. 2001).

Before any suggestion for field application, it is necessary to understand the infection mechanism of EPF on the specific insect; therefore, the aims of the present research were to (a) bioassay the virulence of a native isolate of *B. bassiana* against the larvae of *G. pyloalis*, (b) evaluate the production of extracellular enzymes in presence of the host cuticle to find correlation between secretion of these compounds and virulence, and (c) evaluate the immune responses in the hemolymph of the larvae encountering the spores of *B. bassiana*.

Methods

Insects

Larvae of *G. pyloalis* were collected from infested mulberry plantation of Rasht, northern Iran (37° 19′ N, 49° 37′ E; -9 m). Rearing of insect was carried out in a growth chamber at 24 ± 2 °C, 70 ± 5% R.H., and 16:8 (L: D) h of photoperiod on fresh mulberry leaves (Yazdani et al. 2013). Adult moths were placed in transparent plastic containers (20 × 12 × 12 cm^3) and provided with fresh mulberry leaves for egg-laying and a cotton wool soaked in 10% honey for adult feeding. The containers were cleaned daily and fresh leaves were provided for the larvae.

Beauveria bassiana fungal culture

AM-118 isolate of *B. bassiana* was cultured on Potato Dextrose Agar (PDA) and kept at 25 ± 1 °C for 21 days. Then, the conidia were washed off by a 0.01% aqueous solution of Tween 80 to prepare a stock sample of fungal conidia. The isolate was collected from a rice field at Amol (26 25′ N, 52 21′ E; 17 m) and registered in herbarium of mycology.

Bioassay

The serial concentrations of 10^3, 10^4, 10^5, 10^6, and 10^7 conidia/ml of AM-118 were prepared in sterile distilled water containing Tween80 (0.02%); then, the early 4th instar larvae of *G. pyloalis* were randomly selected and separately dipped in each concentration for 5 s. The control larvae were dipped in aqueous solution of Tween 80 (0.02%) alone. After treatment, the larvae were placed on filter paper (Whatman No. 1), provided with fresh leaves, and kept at the rearing conditions. The bioassay was done in 3 replicates, containing 10 larvae/ replication. Mortality was recorded after 7 days and LC_{50} was determined using POLO-plus software. For estimation of LT_{50}, the mortality was recorded until death of all larvae at the highest conidia concentration.

Liquid culture for enzyme production

A liquid medium, including KH_2PO_4, 0.02%; $CaCl_2$, 0.01%; $MgSO_4$, 0.01%; Na_2HPO_4, 0.02%; $ZnCl_2$, 0.01%; and yeast extract, 0.01%, was used for enzymatic production of AM-118. Culture flasks were inoculated with a concentration of 10^8 conidia/ml and 5% (weight) of *G. pyloalis* larval cuticle and incubated for 8 days at 25 ± 1 °C on a rotatory shaker (70 rev/min) (Zibaee and Bandani 2009). In control flasks, starch and potato extract were added instead of larval cuticle.

Sample preparations for enzymatic assays

Eight days post-incubation, the mixture was harvested by centrifugation at 10,000×*g* and 4 °C for 30 min and washed in ice-cold Tris-HCl (25 mM, pH 8). Weighed mycelia were ground to a fine powder and suspended in distilled water. Then, the samples were homogenized and centrifuged at 20,000×*g* and 4 °C for 30 min to gain the supernatant for the enzymatic assays (Ramzi and Zibaee 2014).

Assay of proteases

Activities of subtilisin-like (Pr1) and trypsin-like (Pr2) as the 2 main fungal proteases were evaluated by 30 μl of succinyl-(alanine) 2-prolinephenylalanine-*p*-nitroanilide for Pr1 and benzoylphenylalanine-valine-arginine-*p*-nitroanilide for Pr2 in 100 μl of Tris–HCl buffer (20 mM, pH 8). Afterward, 20 μl of the enzyme solution was added to the mixture and incubated at 25 °C for 10 min. Then, 100 μl of trichloroacetic acid (TCA, 30%) was added and the absorbance was recorded at 405 nm (Zibaee and Bandani 2009).

Endo-chitinase assay

A reaction mixture, containing 50 μl of 0.5% colloidal chitin as substrate, 20 μl of enzyme sample, and 100 μl of Tris–HCl buffer (20 mM, pH 7), was prepared to assay endo-chitinase activity. The tubes containing reaction mixture were incubated in a water bath (30 °C) for 60 min. Then, 100 μl of DNS (dinitrosalicylic acid) was added and the incubation was prolonged for 10 min at boiling water before reading the absorbance at 545 nm (Miller 1959).

Exo-chitinase assay

Assay of exo-chitinase activity was carried out, using 200 μl of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNPg) solution (1 mg pNPg per ml of distilled water) as substrate, 500 μl of Tris-HCl (25 mM, pH 7), and 25 μl of culture filtrate. The mixture was incubated for 20 h at 30 °C, centrifuged at 20,000×*g* at 4 °C, and then, supernatant was added to 200 μl of sodium tetraborate-NaOH buffer (125 mM, pH 10) prior to reading the absorbance

at 400 nm. The extinction coefficient of 18.5 mM^{-1} cm^{-1} was calculated.

Protein assay

The method of Lowry et al. (1951) was used to measure the amount of protein in the enzymatic preparations. Twenty microliters of the enzyme sample was added into 100 μl of reagent (Ziest Chem. Co., Tehran-Iran) and incubated for 30 min before reading the absorbance at 545 nm.

Hemolymph collection and hemocyte counts

Fourth instar larvae were injected in the latest segment of thorax by 1 μl of a soluble Tween 80 containing LC_{50} concentration of *B. bassiana*. Then, larval hemolymph was collected at intervals of 1, 3, 6, 12, and 24 h after injection. Control larvae remained intact, while other larvae were injected by Tween 80 solution. Samples of hemolymph were bled into ice cold anticoagulant buffer in a ratio of 1–3 (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, pH 4.6) (Azambuja et al. 1991). Number of total hemocyte, granulocyte, plasmatocyte, and nodules were counted, using a Neubauer hemocytometer (Chemkind Co. China). For each treatment, 10 larvae were used and the experiment had 3 replicates.

Assay of phenoloxidase activity

The activity of phenoloxidase (PO) was assayed according to the procedure described by Wilson et al. (2002). Briefly, 10 μl of hemolymph was added into an Eppendorf tube (1.5 ml), centrifuged at 2000×*g* and 4 °C for 15 min. Supernatant was removed and 100 μl of ice-cold phosphate-buffered saline (20 mM, pH 7) was added to the pellets. To determine PO activity, samples were poured into each well of a plate containing 20 μl of 10-mM 3,4-dihydroxyphenylalanine (L-dopa). After 5 min of incubation at room temperature, the absorbance was recorded at 492 nm.

Statistical analysis

Probit analysis was performed to determine LC_{50} and LT_{50} values at the corresponding 95% confidence interval (CI) values by using POLO-Plus software. Biochemical data were compared by one-way analysis of variance (ANOVA), followed by *t* test and Tukey's test, where applicable. Differences between control and treatments were statistically analyzed at a probability less than 5% and marked by different letters.

Also, the extinction coefficient of 18.5 mM^{-1} cm^{-1} was considered for activity calculation based on the following formula:

$$\text{Volume activity (U/ml)} = [\Delta\text{OD (OD test} - \text{OD blank)} \times V_t \times \text{df}] / (18.5 \times t \times 1.0 \times V_s)$$

where V_t = total volume; V_s = sample volume; 18.5 = millimolar extinction coefficient of *p*-nitrophenol under the assay condition; 1.0 = light path length (cm); *t* = reaction time; and df = dilution factor (Miller 1959).

Results

Results of the Am-118 bioassay against the 4th instar larvae of *G. pyloalis* are presented in Tables 1 and 2. AM-118 showed a virulence against the larvae at different concentrations, and significant positive correlations among increasing concentrations of AM-118 and the larval mortality (Table 1).

The LC_{50} value of AM-118 was obtained as 2.6×10^4 (7.8×10^3–6.9×10^4) spore/ml (Table 1) and the LT_{50} value was calculated as 3.54 (2.99–4.08) days (Table 2). The activities of Pr1 and Pr2 significantly increased in the samples containing larval cuticle than in the control (Fig. 1). Similarly, high activities of endo- and exo-chitinase were observed in the cuticle treatment compared to control (Fig. 2).

Total number of hemocytes, number of granulocytes, and of plasmatocytes in *G. pyloalis* larvae demonstrated significant changes after AM-118 spore injection (Fig. 3). The total number of hemocytes increased in the larvae exposed to *B. bassiana* conidia in comparison to control and Tween 80 at all-time intervals. The highest number of total hemocyte was observed 3 and 6 h post-injection similar to granulocytes (Fig. 3). Also, injection of the spores led to the highest numbers of plasmatocytes at all-time intervals (Fig.3).

Table 1 LC_{50} value (conidia/ml) of *Beauveria bassiana* against the 4th instar larvae of *Glyphodes pyloalis*

Treatment	Concentrations (spore/ml)	Mortality (%) ± SE	LC_{50} (spore/ml) (95% CL)	χ^2 (df)	Slope ± SE
Beauveria bassiana	10^3	23.33 ± 3.3	2.6×10^4 (7.8×10^3–6.9×10^4)	0.433 (3)	0.52 ± 0.0088
	10^4	40 ± 5.7			
	10^5	60 ± 5.7			
	10^6	76.66 ± 3.3			
	10^7	93.33 ± 3.3			

Note: Calculation was carried out by POLO-Plus software

Table 2 LT_{50} value (days) of *Beauveria bassiana* against the 4th instar larvae of *Glyphodes pyloalis*

Treatment	Days	Mortality (%) ± SE	LT_{50} (day) (95% CL)	χ^2 (df)	Slope ± SE
Beauveria bassiana	1	3.33 ± 0.33	3.54 (2.99–4.08) days	6.655 (6)	4.094 ± 0.475
	2	16.66 ± 0.33			
	3	40 ± 0.57			
	4	50 ± 0.57			
	5	63.33 ± 0.33			
	6	80 ± 0.57			
	7	93.3 ± 0.66			
	8	100 ± 1.15			

Note: Calculation was carried out by POLO-Plus software

Injection of larvae with *B. bassiana* spores caused the highest number of nodules 6 h post-injection but control and tween 80 larvae showed no nodules (Fig. 4). The activity of PO statistically increased in the larvae injected by AM-118 spores at all-time intervals. The highest PO activity was found 12 and 6 h post-injection by AM-118 spores, respectively (Fig. 4).

Discussion

Obtained results showed the virulence of *B. bassiana* on the 4th instar larvae of *G. pyloalis* and the induction of

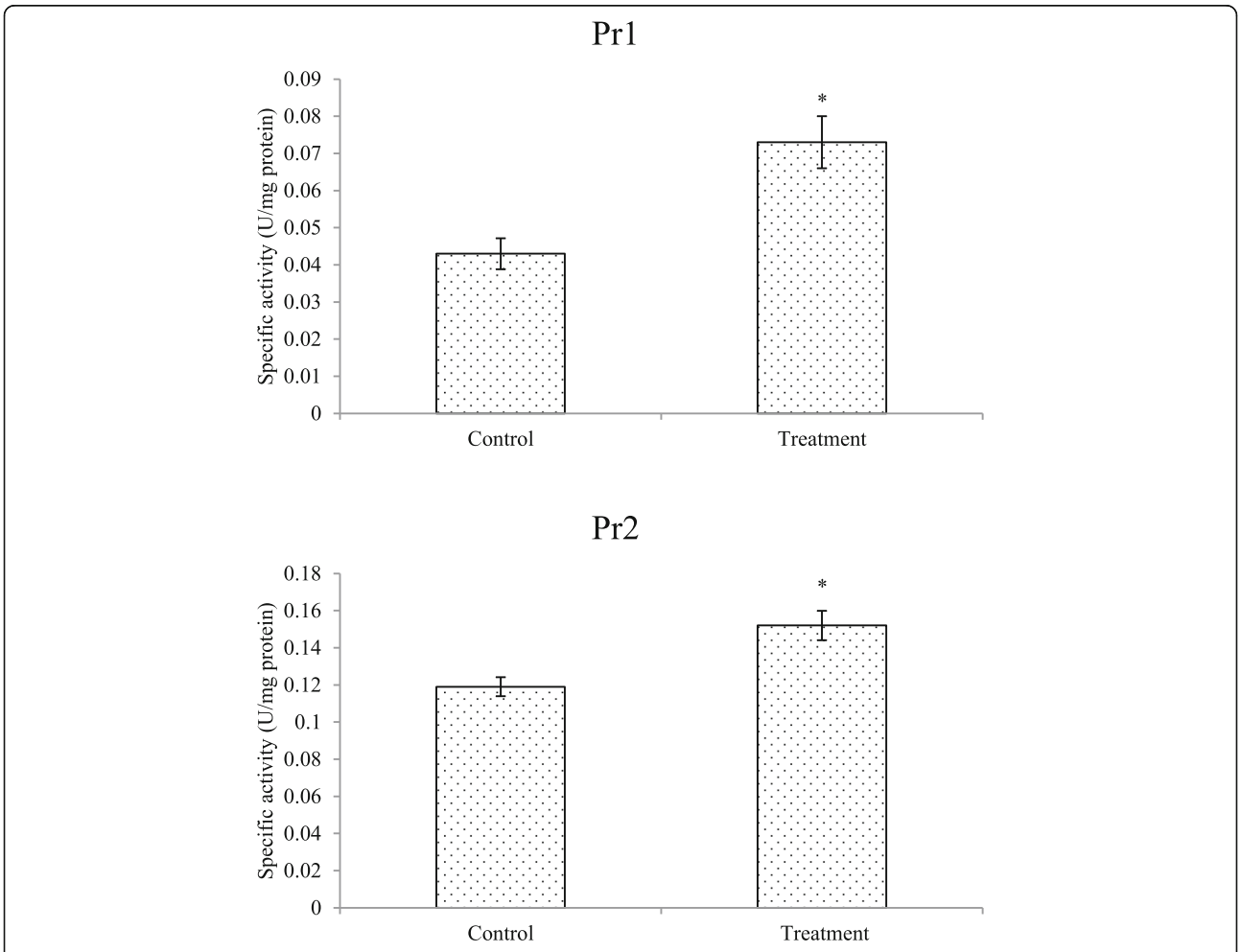

Fig. 1 Activities of proteases (U/mg protein) in the cultured *Beauveria bassiana* on liquid medium containing *Glyphodes pyloalis* cuticle. Statistical differences are shown by asterisks (*t* test, $p \leq 0.05$)

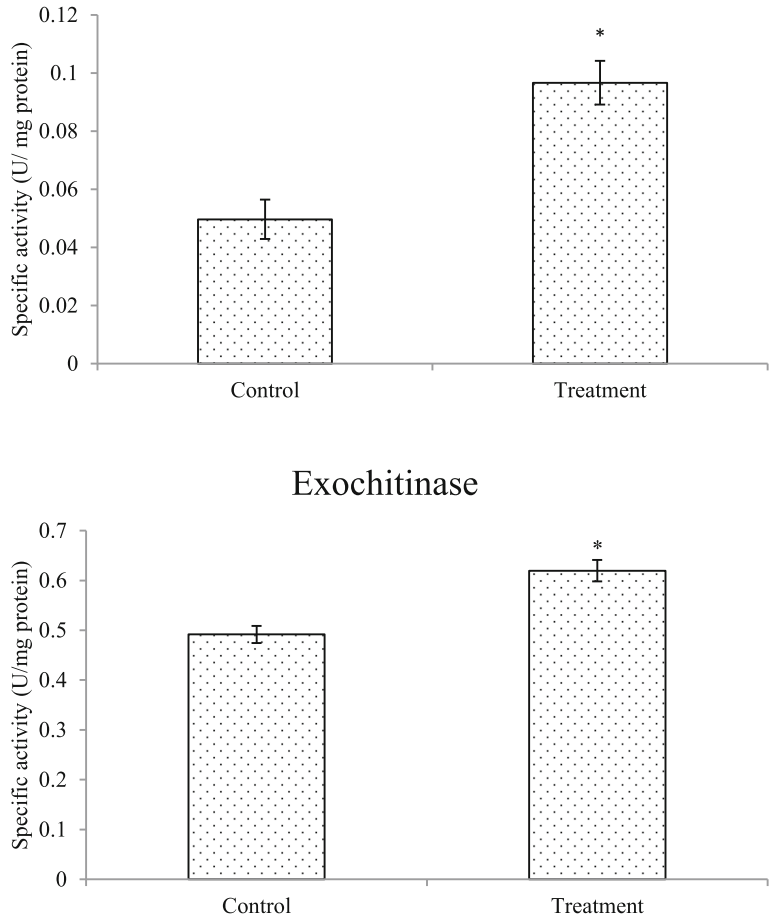

Fig. 2 Activities of chitinases (U/mg protein) in the cultured *Beauveria bassiana* on liquid medium containing *Glyphodes pyloalis* cuticle. Statistical differences are shown by asterisks (*t* test, $p \leq 0.05$)

cellular immune responses of the larvae. Ramzi and Zibaee (2014) reported that two isolates of *B. bassiana* (BB1 and BB2) had a high virulence against *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae compared to *Metarhizium anisopliae* Metchnikoff (Hypocreales: Clavicipitaceae), *Isaria fumosoroseus* Wize (Hypocreales: Clavicipitaceae), and *Lecanicilium lecanii* Zare & Gams (Hypocreales: Cordycipitaceae). Wraight et al. (2010) demonstrated a high virulence of *B. bassiana* isolates against *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), *Spodoptera exigua* Fabricius (Lepidoptera: Noctuidae), *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae), *Pieris rapae* L. (Lepidoptera: Pieridae), and *Trichoplusia ni* Hubner (Lepidoptera: Noctuidae). Laznik et al. (2012) investigated the effects of *Beauveria brongniartii* Vuillemin and *B. bassiana* against June beetle, margined vine chafer, and garden chafer. The authors reported significant effects of the fungi on the total number of the white grubs in April and May once the population was higher than the economic threshold. Also, the highest virulence of *B. bassiana* was observed on the *Duponchelia fovealis* Zeller (Lepidoptera: Crambidae) larvae (Baja et al. 2020). *B. bassiana* (different isolates) are among the most virulent EPF against insects and it has shown a significant capability to be used as an efficient biocontrol agent in agro-ecosystems. Moreover, AM-118, the native isolate of *B. bassiana* in north of Iran, has shown a virulence against *C. suppressalis* and *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae). Lesser mulberry is the third case of pests which is susceptible to the isolate, which implied on the pathogenic ability of AM-118 in a wide range of crop and orchard pests. This may be attributed to the isolate adaptability to the climate of northern Iran and host-associations which emboss virulence of AM-118 microbe.

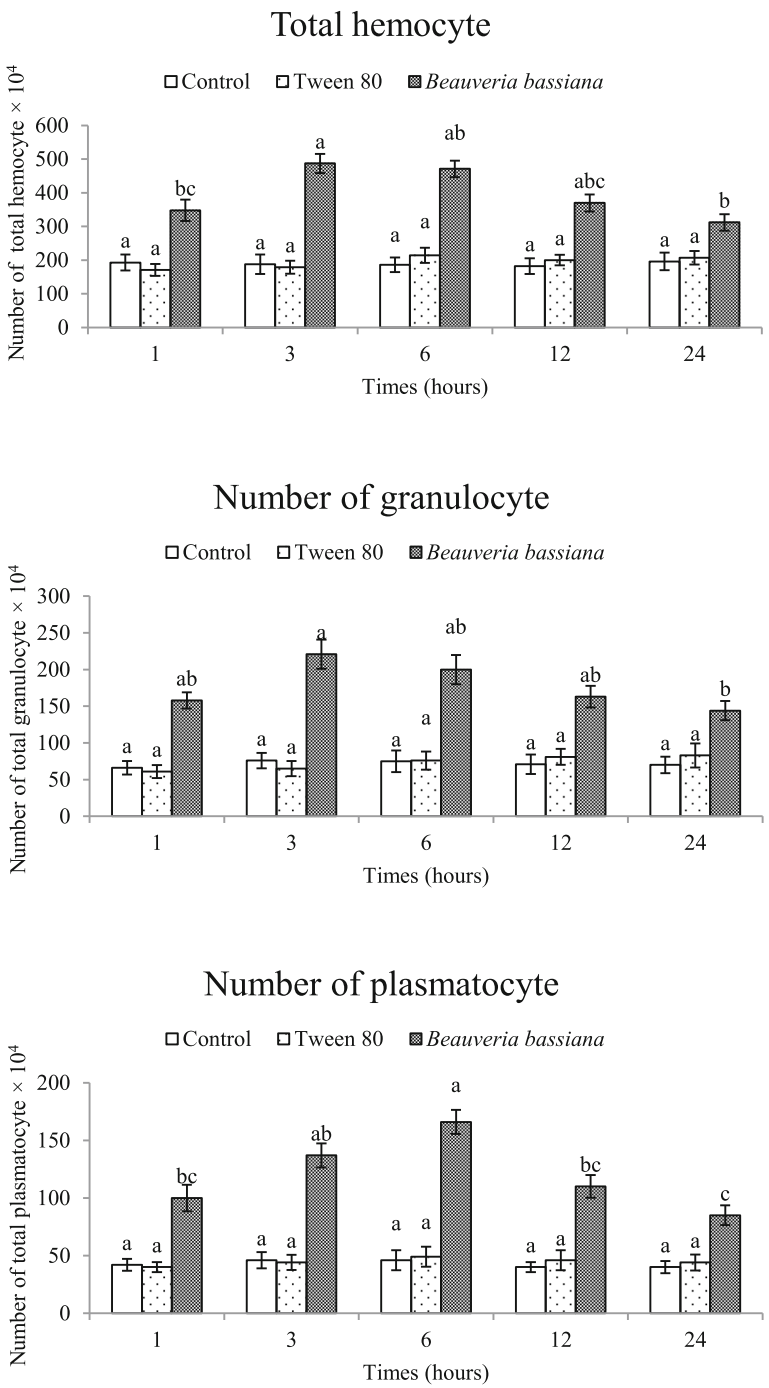

Fig. 3 Changes of hemocytes numbers in *Glyphodes pyloalis* larvae injected by 2.6 × 10^5 spore/ml of *Beauveria bassiana* (AM-118). Different letters indicate significant differences among treatments in time intervals ($p \leq 0.05$)

Once fungal conidium attached to the integument of insects, it generates a germ tube and penetrates by passing through cuticle, using extracellular enzymes and mechanical pressure. Proteases and chitinases are the necessary enzymes to facilitate fungal penetration through insect cuticle (Ramzi and Zibaee 2014). Subtilisin-like (Pr1) and trypsin-like (Pr2) are the 2 types of proteases important in pathogenic process of EPF. Pr1 catalyzes widely the cuticular protein of insects and is secreted in the initial steps of penetration, while Pr2 plays as a supplementary protease to accomplish protein degradation along with Pr1 (Dias et al. 2008). Chitinases speed up the hydrolysis of b-(1, 4)-linked polymer of *N*-acetyl-D-glucosamine in insect cuticle although they play

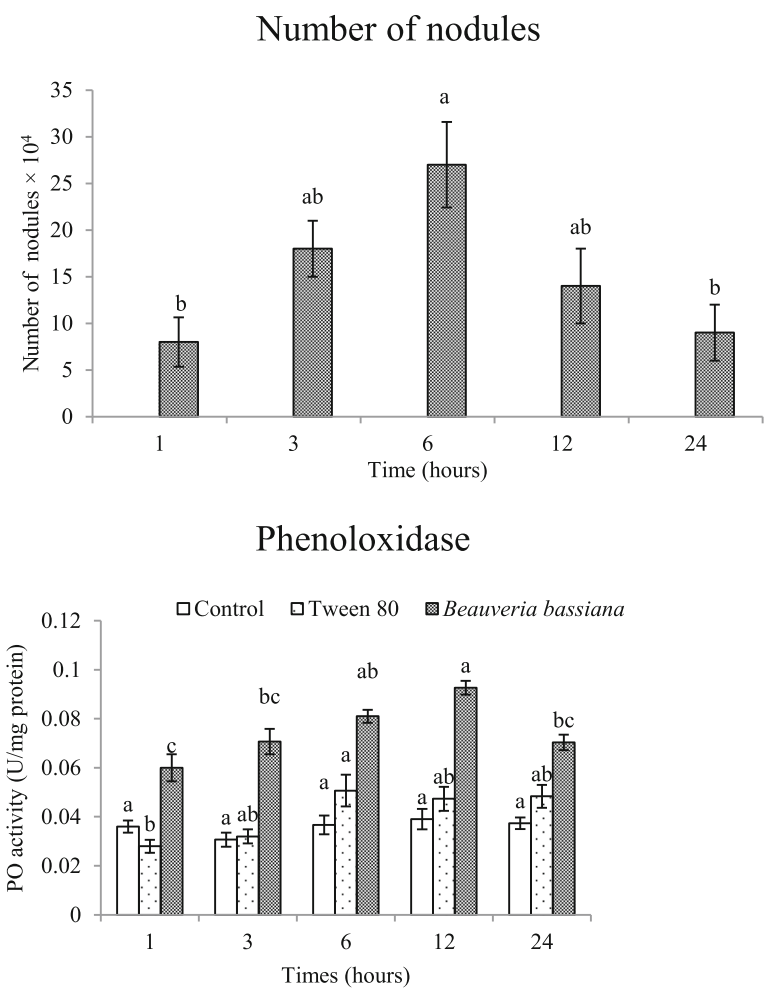

Fig. 4 Changes of nodules numbers and PO activity (U/mg protein) in *Glyphodes pyloalis* larval hemolymph after injection with 2.6×10^5 spore/ml of *Beauveria bassiana* (AM-118). Different letters indicate significant differences among treatments in time intervals ($p \leq 0.05$)

other roles in development, nutrition, and morphogenesis of fungi (Tsigos and Bouriotis 1995). High activities of fungal enzymes in the presence of larval cuticle implies on better adaptations of these enzymes with integument composition of host rather than pure supplements in control media. In fact, there is a coevolution in host-microbe associations in which the EPF recruit the enzymatic isoforms to facilitate integument rupture and to enhance achieving of fungus to the haemocoel of host.

Microbial infections are generally lethal for insects, but several studies demonstrate that some physiological functions neutralize the damage of infectious injury (Gorman et al. 2007; Vengateswari et al. 2020). Immunity is important to protect insects against microbial agents. Immune responses of insects rely on circulating hemocytes such as prohemocyte, granulocyte, plasmatocyte, and oenocytoids (Russo et al. 2001; Schmid-Hempel 2005; Borges et al. 2008; Zibaee and Malagoli 2014). The hemocytes act through different functions including nodule formation, phagocytosis, and encapsulation to entrap and kill pathogens in the hemolymph (Borges et al. 2008). In the present study, the results demonstrated that injecting of Am-118 spores to *G. pyloalis* larvae led to increase of total and differentiated hemocyte counts than to tween 80 and control larvae. Similar findings were observed in case of number of nodules, which highlight the roles of circulating hemocytes in alleviating or removing deleterious effects of entomopathogenic infection. Similar studies demonstrated a direct correlation between EPF infection and number of differential hemocytes in insects. For example, Mirhaghparast et al. (2013) showed that injection of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) larvae by *B. bassiana* and *M. anisopliae* increased the number of hemocytes and nodules. In another research, Zibaee and Malagoli (2014) reported that the number of hemocytes and nodules in *C. suppressalis* increased after injection with different EPF.

Phenoloxidase (PO) is an important enzyme in immunity of insect, which has a key role in catalyzing hydroxylation of monophenols to diphenols and conversion of diphenols to quinones (Gorman et al. 2007). Then, quinones are converted to the melanin pigments. In insects, melanin is involved in wound healing, cuticle sclerotization, and defense reactions against pathogens, such as encapsulation and nodule formation (Zdybicka-Barabas et al. 2014). In the present study, there is a correlation between number of hemocytes and PO activity because the highest PO activity was obtained after 6 post-injections, which it coincided with the hemocytes high numbers in the given time of post injection. Since PO is secreted and stored within hemocytes, increased PO activity may be attributed to the highest number of hemocytes after injection of fungi conidia. Several studies have been demonstrated an increase of hemocytes' numbers causes increase PO activity of insects (Catalán et al. 2012; Mirhaghparast et al. 2013; Zibaee and Malagoli 2014; Shamakhi et al. 2019).

Conclusion

The present study revealed detailed results on the function of a native *B. bassiana* isolate from the pathogen infection–host interaction perspective. Injection of AM-118 led to mortality of *G. pyloalis* larvae by increased secretion of extracellular enzymes and affected insect host immunity. Increased knowledge on the physiological interplays among EPF and the insect host could provide new strategies for pest management.

Abbreviations

PDA: Potato Dextrose Agar; Pr1: Subtilisin-like; Pr2: Trypsin-like; TCA: Trichloroacetic acid; DNS: Dinitrosalicylic acid; pNPg: *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide; L-dopa: 3,4-Dihydroxyphenylalanine; CI: Confidence interval; PO: Phenoloxidase

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Authors' contributions

SAP: Investigation, methodology, formal analysis, writing; AZ: supervision, writing—review and editing; MGR: methodology; HH: writing—review and editing; MS: supervision, formal analysis, methodology. All authors have read and approved the manuscript.

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Competing interests

The authors declare no competing interests.

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