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RESEARCH REPORT

Effects of *Beauveria bassiana* and *Metarhizium anisopliae* on cellular immunity and intermediary metabolism of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae)

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Abstract

In the current study, fifth larval instars of *Spodoptera littoralis* were injected by spores of *Beauveria bassiana* and *Metarhizium anisopliae* to find their effects on cellular immunity and enzymes involved in intermediary metabolism. The highest numbers of plasmatocytes were observed 6 hours post-injection for both entomopathogenic fungi although there were the slight significant differences between time intervals of 3-12 hours. The highest numbers of granulocytes were observed 6 hours post-injection for both fungi although slight statistical differences were found by injecting spores of *B. bassiana* after 3-6 hours. Injection of larvae by *B. bassiana* spores caused the highest number of nodules 12 hours post-injection but spores of *M. anisopliae* caused the highest number of nodules after 1-6 hours of post-injection. The highest activity of phenoloxidase was obtained 6-12 hours post-injection by *B. bassiana* spores while the highest enzymatic activity was found 12 hours after injection by *M. anisopliae* spores. In case of assayed enzymes including, alanine aminotransferase, aspartate aminotransferase, δ-glutamyl transferase, acid phosphatase, alkaline phosphatase and lactate dehydrogenase, the highest activities were observed 6-12 hours post-injection by fungal spores. The results demonstrated that the highest physiological phenomena like immune responses and intermediary metabolisms were occurred 12 hours post-injection by entomopathogenic fungi. Determination of these processes could be helpful to improve quality of entomopathogenic fungi and their efficiency to decrease population outbreaks of pests.

Key Words: Entomopathogenic fungus, *Spodoptera littoralis*, Immunity, Intermediary metabolism

Introduction

The entomopathogenic fungi are the promising agents that are used against insect pests for several decades. These organisms include taxa of several fungal groups like Hypocreales of Ascomycota that *Beauveria bassiana* and *Metarhizium anisopliae* are the two most recognized species (Vincent *et al*., 2007). *B. bassiana* and *M. anisopliae* grow naturally throughout the world and acts as parasites of many arthropod species causing white and green muscardine diseases due to the color of their spores (Vincent *et al*., 2007). Besides entomopathogenic fungi caused natural mortality on insects, these agents are environmentally safe so there is a worldwide interest of their using and improvement for biological control of insects. When a spore adheres to cuticle of insects, a germ tube is generated and pass through the integument by mechanical and enzymatic (e.g. chitinases, proteases, lipases and etc) processe. When it reaches to the hemocoel, it produces blastospores which are the final pathogenic parts for host infection (Vincent *et al*., 2007).

Hemolymph of insects is a medium for several physiological processes like immune responses and intermediary metabolism. When an invader enters hemocoel of insects, hemocytes are engaged to remove non-self-target by phagocytosis, nodule formation, encapsulation, synthesis of antimicrobial peptides and reactive metabolites (Beckage, 2008). Intermediary metabolism consists of various pathways in which ingested and stored nutrients such as carbohydrates, lipids and proteins are processed to produce energy via their degradation or synthesis. In details, locked up energy in the nutrients is released by several biochemical reactions like glycolysis, β-oxidation of lipids, citric

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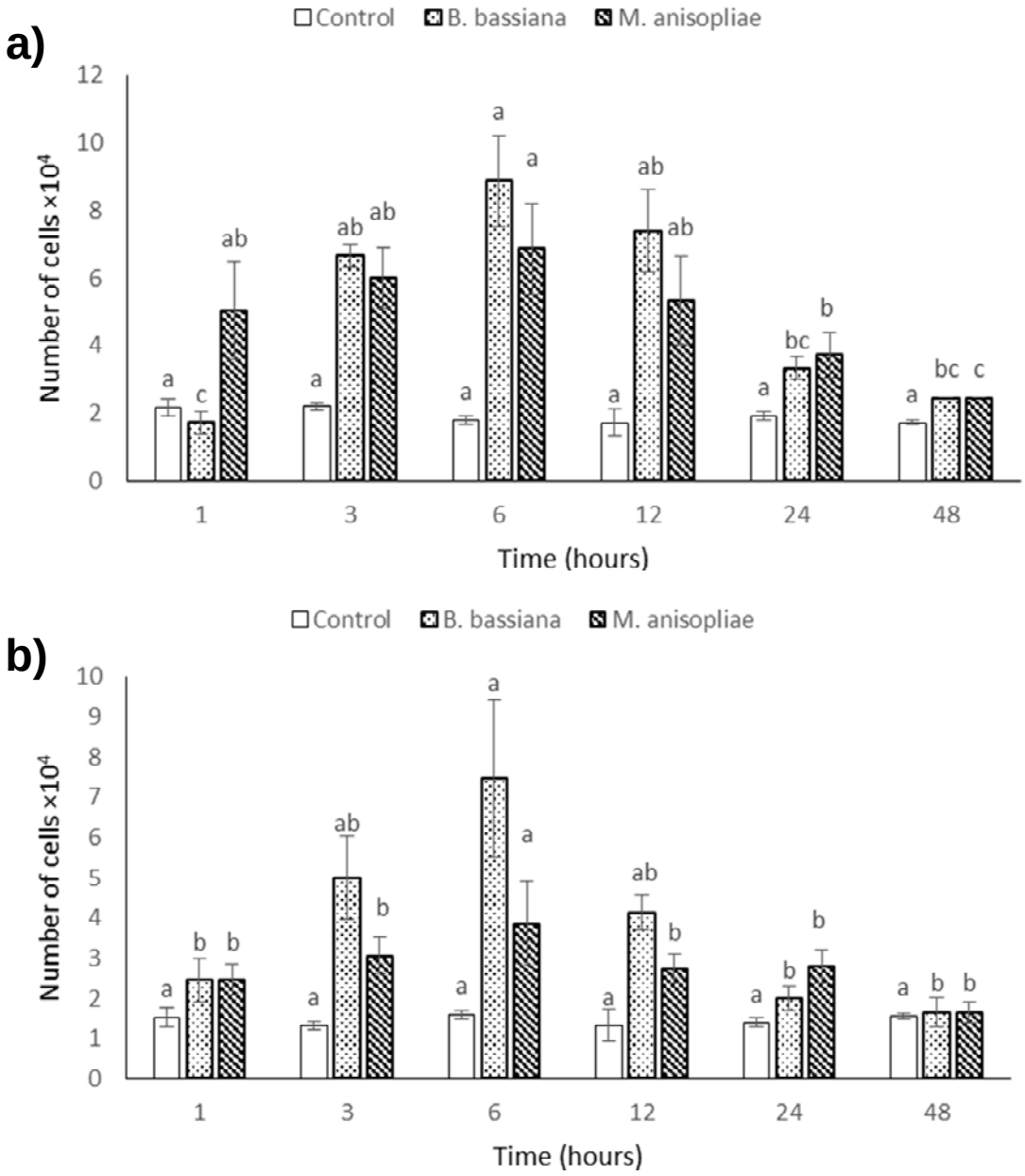

Fig. 1 Effects of *B. bassiana* and *M. anisopliae* injections on number of plasmatocytes (a) and granulocytes (b) in *S. littoralis* larvae. Statistical differences have been shown by various letters ($p \leq 0.05$).

acid cycle, electron transport system, transaminations and etc (Nation, 2008).

Spodoptera littoralis Boisduval (Lepidoptera: Noctuidae) known as African Cotton Leaf-worm or Mediterranean Brocadeis is one of the most destructive agricultural pests in subtropical and tropical regions that causes severe damages of plants belonging to 44 different families including grasses, legumes, crucifers and deciduous fruit trees by highly economic importance (Abdel-Megeed, 1975). Larvae intensively fed on leaves and sometimes fruits of crops. The pest could be active nine months of a year and complete a generation within 30 days (Gharib, 1979). Although cultural procedure, sanitation and chemical control are used to decrease population outbreaks of the pest but it annually causes several damages worldwide.

In addition to activation of immune responses, microbial infections could alter intermediary metabolism of insects by affecting activity of involved enzymes and detoxifying ones although majority of studies have been concentrated on evaluation of general esterases and phosphatases

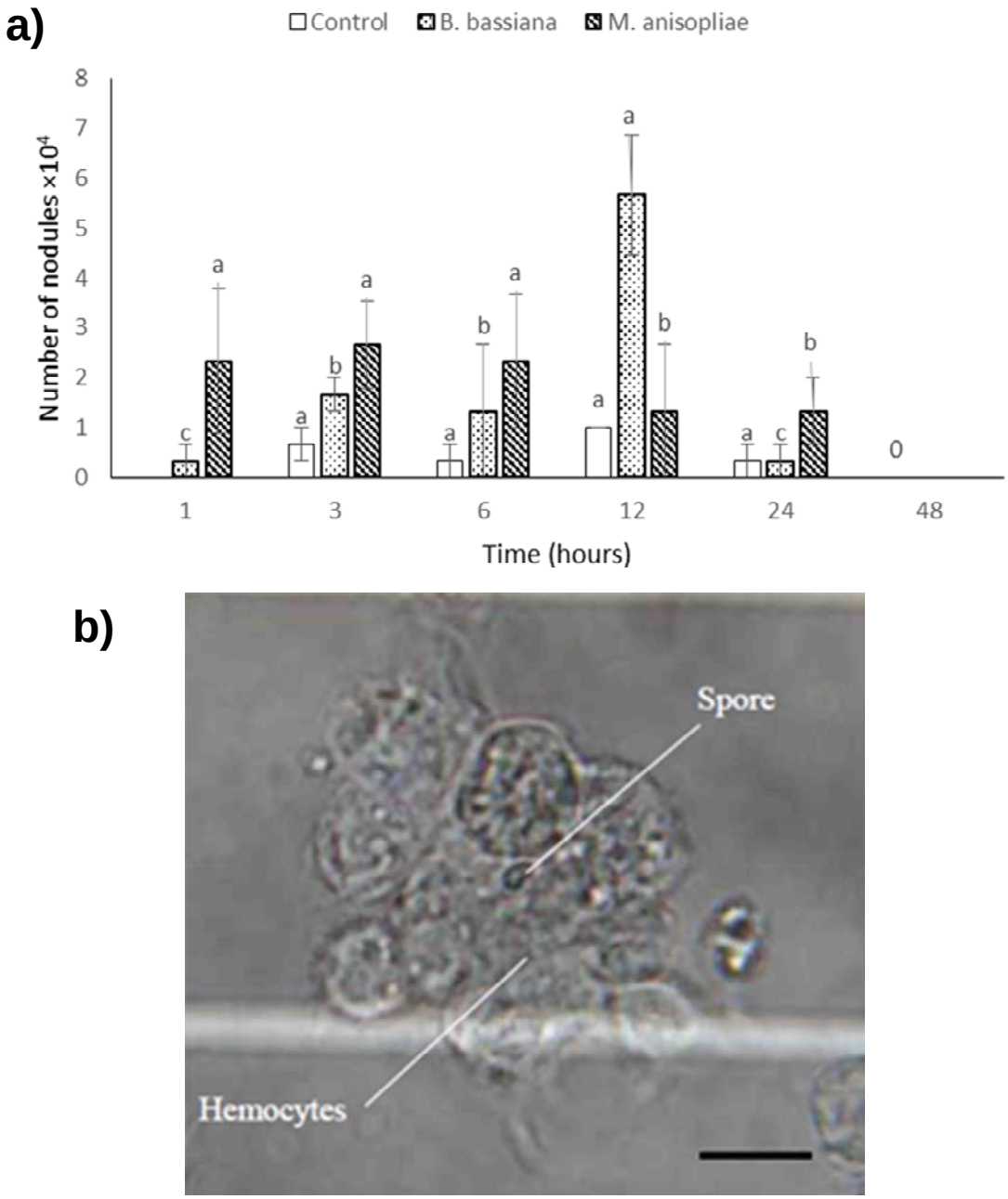

Fig. 2 Effects of *B. bassiana* and *M. anisopliae* injections on nodule formation in *S. littoralis* larvae (a). An image of observed nodule (b). Statistical differences have been shown by various letters ($p \leq 0.05$).

(Sokolova and Sundukov, 1999; Xia *et al*., 2000; Xia *et al*., 2001). Since *B. bassiana* and *M. anisopliae* are the two main entomopathogenic fungi, their interaction with immune system and intermediary metabolism of *S. littoralis* could be useful to improve efficiency of these microbial agents. Hence, objectives of the current studies are (i) determination of larval cellular responses to *B. bassiana* and *M. anisopliae*, and (ii) determination of changes in intermediary metabolism by measurements of involved enzymes.

Materials and Methods

Insect Rearing

Larvae were collected from Strawberry field in Pirbazar, Rasht (north of Iran) and reared on the same leaves at 25±1 °C, 70% of relative humidity

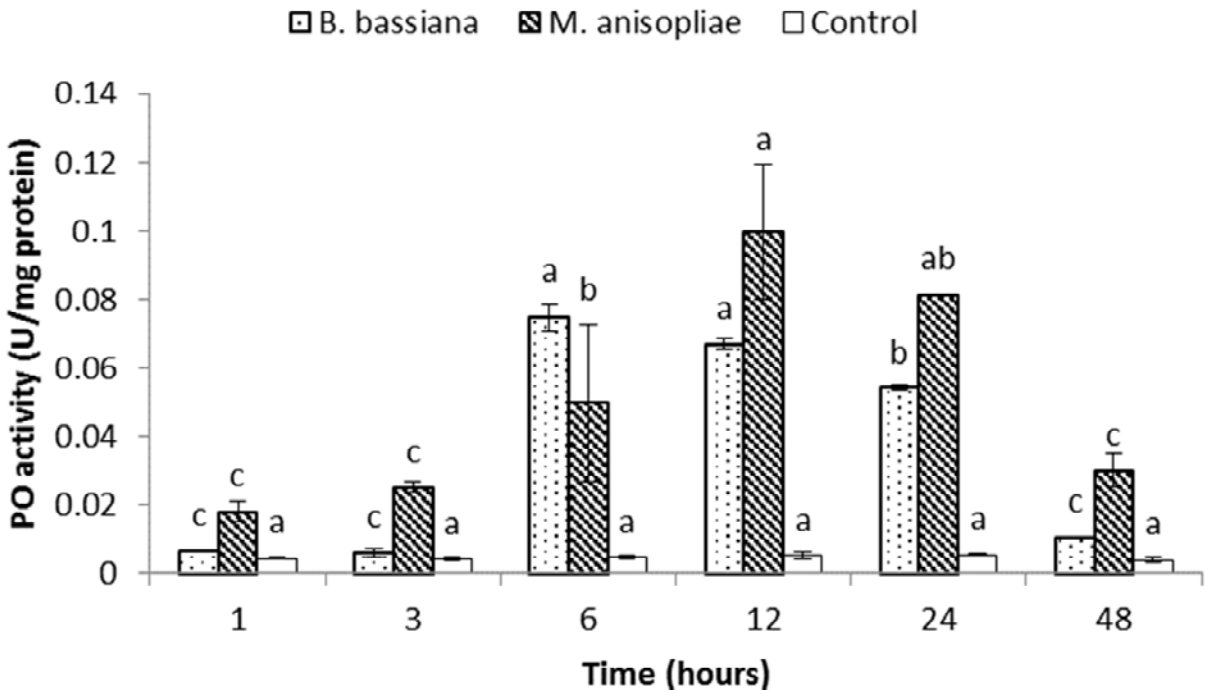

Fig. 3 Effects of *B. bassiana* and *M. anisopliae* injections on phenoloxidase activity in *S. littoralis* larvae. Statistical differences have been shown by various letters ($p \leq 0.05$).

and 16L:8D of photoperiod (The larvae was identified by Dr. Jalil Hajizade, Professor of Insect taxonomy in our department). Fifth larval instars were used to carry out the experiments.

Entomopathogenic fungi culture

B. bassiana (Isolate B3 Isolated from Fashand soil-Iran) and *M. anisopliae* (isolated from rice fields) were cultured at 25 ± 1 °C on Sabouraud Dextrose Agar (Merck Co., Germany) (pH = 5.6) amended with 1% yeast extract. After 14 days, conidia were washed off with a 0.01% aqueous solution of Tween 80 (Sigma Aldrich Co., Austria) and different concentrations of spores were prepared.

Effect of fungal spore on hemocyte numbers

To determine possible changes of hemocyte numbers followed by treatment of entomopathogenic fungi, fifth larval instars were injected laterally into the latest segment of thorax with 1 µL of a 10^5 spores/mL concentration of mentioned fungal isolates. Hemolymph was collected at intervals of 1, 3, 6, 12, 24 and 48 hours after injection. Samples of hemolymph were bled into 1 mL of ice–cold anticoagulant buffer in 1.5 mL plastic tubes. The tubes were gently inverted 5 to 7 times to facilitate mixing, and both total and different hemocyte numbers were counted using an improved Neubauer hemocytometer (Chemkind Co. China). For each treatment, 6 larvae were used and the experiment had five replicates (N=30, n=5).

Effect of fungal spores on nodulation

Injections were carried out as described in the previous section to find number of formed nodules in response to spores of different entomopathogenic fungi. Number of nodules was calculated at intervals of 1, 3, 6, 12, 24 and 48 hours post-injection. Injected larvae were chilled on ice, hemolymph was gathered in a capillary tube, and then 200 µL of samples in three replicates were poured in a hemocytometer and nodules were counted.

Effects of fungal spores on phenoloxidase activity (PO)

After injecting of larvae with fungal spores, hemolymph was collected at mentioned intervals. A hemocyte lysate supernatant was prepared after injections based on Leonard *et al*. (1985). Collected hemolymph from larvae was mixed with anticoagulant buffer and centrifuged at 13,000 rpm for 5 min; the supernatant was discarded and the pellet washed gently twice with a phosphate buffer (0.02 M, pH = 7.1). Cells were homogenized in 200 µL of phosphate buffer centrifuged at 13,000 rpm for 15 min, and the hemocyte lysate supernatant was used in PO assays. Samples (10 µl) were pre-incubated with phosphate buffer at 30 °C for 3 min before the addition of 20 µL of 10 mM aqueous solution of L-dihydroxyphenylalanin (Sighma-Aldrich Co., USA) as substrate. The mixture was incubated for an additional five min at 30 °C and PO activity was measured at 495 nm. One unit of PO activity

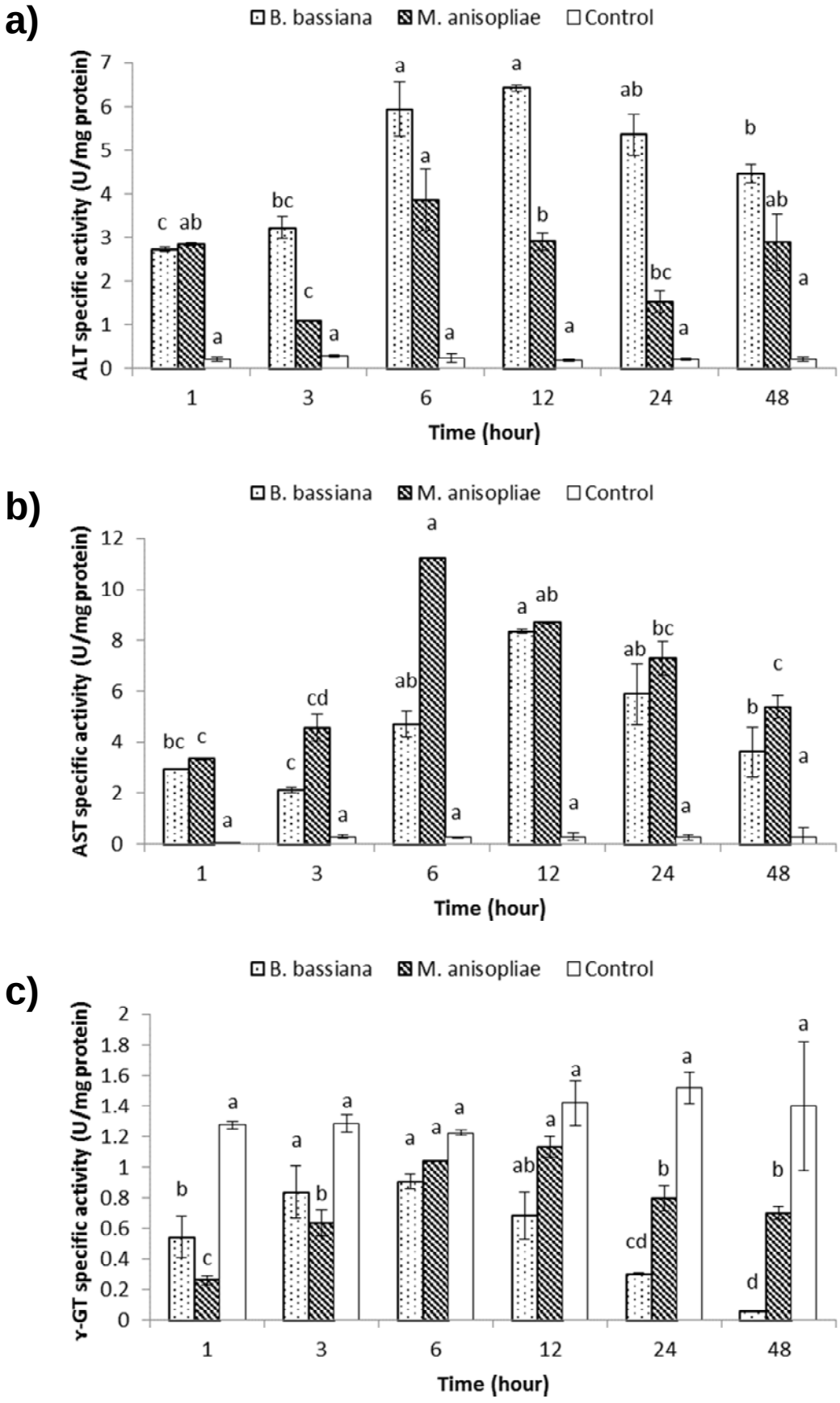

Fig. 4 Effects of *B. bassiana* and *M. anisopliae* injections on ALT (a), AST (b) and δ-GT (c) activities in *S. littoralis* larvae. Statistical differences have been shown by various letters ($p \leq 0.05$).

represents the amount of enzyme required to produce an increase in absorbance of 0.01 min^{-1} (Dularay & Lackie, 1985). Activity in treated assays was compared with that of controls (n=3).

Sample preparation

Hemolymph samples gathred from larvae in each time intervals were poured in 1.5 ml tubes containing 100 µl of anticoagulant solution, centrifuges and supernatant was used as enzymatic source of intermediary metabolism.

Estimation of aspartate (EC 2.6.1.1) and alanine aminotransferases (EC 2.6.1.1)

Alanine aminotrasferase (ALT) and aspartate aminotransferase (AST) were measured using Thomas' (1998) procedure. This assay was done by AST and ALT kit (Biochem Co, Iran). On this basis, solution 1 and 2 were mixed (4:1). Then, samples were added and absorption was read at 340 nm.

Estimation of δ-glutamyl Transferase

The method described by Tate and Meister (1985) was used to assay activity of □-glutamyl transferase. In a kit by Ziest-Chem. Co. (Tehran, Iran), reagent A was incubated with samples for 5 min, then reagent B was added and absorbance was read at 405 nm.

Assay of estimation of acid (EC 3.1.3.2) and alkaline phosphatases (EC 3.1.3.1)

The enzyme assays were carried out as described by Bessey *et al*. (1946). The buffered substrate (phosphate buffer, 0.02 m, pH 7.2) was incubated with samples for 30 min. Alkali were added to stop the reaction and adjust the pH for the determination of concentration of the product formed. The spectral absorbance of *p*-nitrophenolate was maximal at 310 nm. The molar absorbance of p-nitrophenolate at 400 nm is about double that of p-nitrophenyl phosphate at 310 nm. On converting the p-nitrophenolate into p-nitrophenol by acidification, the absorption maximum is shifted to about 320 nm with no detectable absorption at 400 nm.

Estimation of lactate dehydrogenase (EC 1.1.1.27)

For evaluating lactate dehydrogenase (LDH), the King (1965) method was used. To standardize volumes, 0.2 ml NAD+ solution was added to the test tubes and 0.2 ml of water was added to control test tubes, each containing 1 ml of the buffered substrate. The sample containing 0.01 ml was also added to the test tubes. Test tube samples were incubated for exactly 15 min at 37°C and then arrested by adding 1 ml of color reagent (2,4-dinitrophenyl hydrazine) to each tube and the incubation continued for an additional 15 min. After cooling at room temperature, 10 ml of 0.4N NAOH was added to each tube to make the solutions strongly alkaline. At exactly 60 s after the addition of alkali to each tube, the intensity of color was measured at 440 nm.

Protein assay

Protein concentrations were assayed according to the method described by Lowry *et al*. (1951).

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentisized test when significant differences were found at $P \leq 0.05$ and marked in tables with letters.

Results and Discussion

It was found that injection of *S. littoralis* larvae by *B. bassiana* and *M. anisopliae* spores significantly affected cellular immunity and phenoloxidase activity at various time intervals. The highest numbers of plasmatocytes were observed 6 hours post-injection for both injected spores of the entomopathogenic fungi although there were the slight significant differences between time intervals of 6-24 hours (F= 7.65, Pr>F: 0.0019; F=14.87, Pr>F: 0.0001) (Figure 1a). The highest numbers of granulocytes were observed 6 hours post-injection for *B. bassiana* and 3-6 hours for *M. anisopliae* (F= 5.31, Pr>F: 0.0084; F=56.26, Pr>F: 0.0001) (Figure 1b). Meanwhile, injection of *B. bassiana* spores caused higher numbers of these hemocytes in the larvae and overall numbers of plasmatocytes were higher than that of granulocytes (Fig. 1a,b). Several studies have been reported fluctuation of hemocyte numbers in the immune challenged insects such as *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae), *Schistocerca gregaria* L. (Orthoptera: Acrididae), *Periplaneta americana* L. (Blattaria: Blattidae) *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae), *Galleria mellonella* L. (Lep., Pyralidae), *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae), *Oxya japonica* Thunberg (Orthoptera: Acrididae), *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), (Bidochka and Khachatourians, 1987; Gunnarsson, 1988; Hung and Boucias, 1992; Sewify and Hashem, 2001; Chouvenc *et al.,* 2009; Anggraeni *et al*., 2011; Zibaee *et al*., 2011). These fluctuations could be attributed to some factors like; taking part of hemocytes in nodule formation after injection, cytotoxic effect of fungal secondary metabolite on hemocytes and composition of spore surface mainly hydrophobin proteins. Meanwhile, differences in numbers of plasmatocytes and granulocytes followed injection by *B. bassiana* and *M. anisopliae* could be attributed to different properties of fungal spores in production of secondary metabolites and composition of spore surface.

Nodule formation is one of the major cellular responses of insects against pathogens since it is considered to be the last defensive line (Chouvenc *et al.,* 2009). Injection of larvae by *B. bassiana* spores caused the highest number of nodules 12 hours post-injection (F= 7.52, Pr>F: 0.0013) but spores of *M. anisopliae* caused the highest number of nodules 3-6 hours (F= 11.23, Pr>F: 0.0052) although spores of *B. bassiana* caused higher number of nodules versus *M. anisopliae* (Fig. 2). These findings are correspondence with the higher number of plasmatocytes and granulocytes after 3 hours of injection. Hence it could be concluded that higher production of plasmatocytes and granulocytes is due to involvements of these hemocytes in nodule formation.

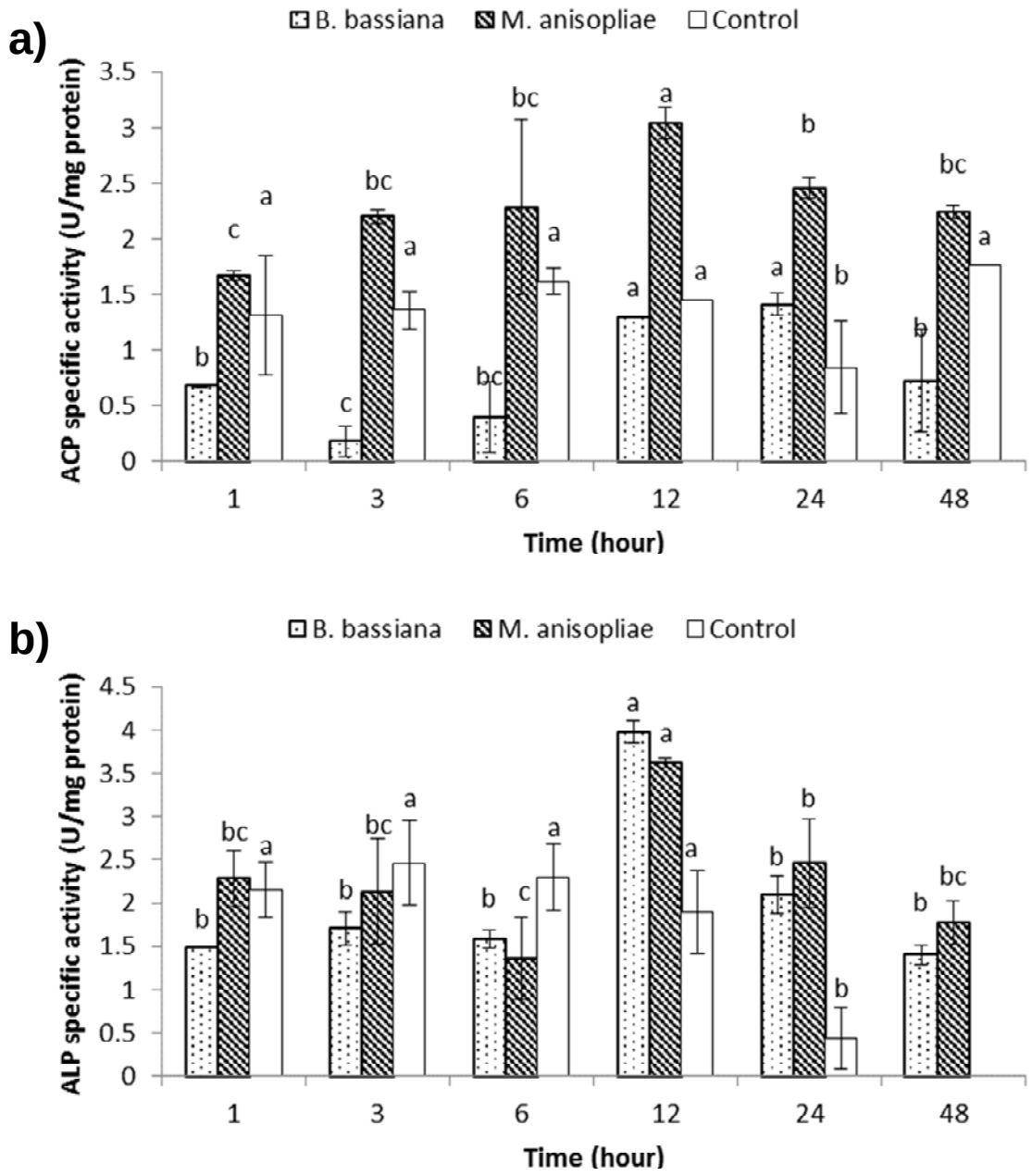

Fig. 5 Effects of *B. bassiana* and *M. anisopliae* injections on ACP and ALP activities in *S. littoralis* larvae. Statistical differences have been shown by various letters ($p \leq 0.05$).

The highest activity of phenoloxidase was obtained 6-12 hours post-injection by *B. bassiana* spores (F= 214.9, Pr>F: 0.0001) while the highest enzymatic activity was found 12 hours after injection by *M. anisopliae* spores (F= 12.2, Pr>F: 0.0043) (Fig. 3). Overall activity of the enzymes in the larvae injected by *M. anisopliae* spores was higher than that of *B. bassiana* spores (Fig. 3). Phenoloxidases are activated upon wounding or infection as part of the innate immune response (Kanost and Gorman 2008). The enzymes have two biochemical functions in hydroxylation of tyrosine to form dihydroxyphenylalanine and oxidizing *o*-diphenols to form quinones (Gorman *et al.,* 2007). After forthcoming reactions, quinones changes to form melanin, which is deposited on the surface of encapsulated parasites, hemocyte nodules, and wound sites (Kanost & Gorman 2008). Results of the current study are attributed to melanin deposition to complete of nodule formation process.

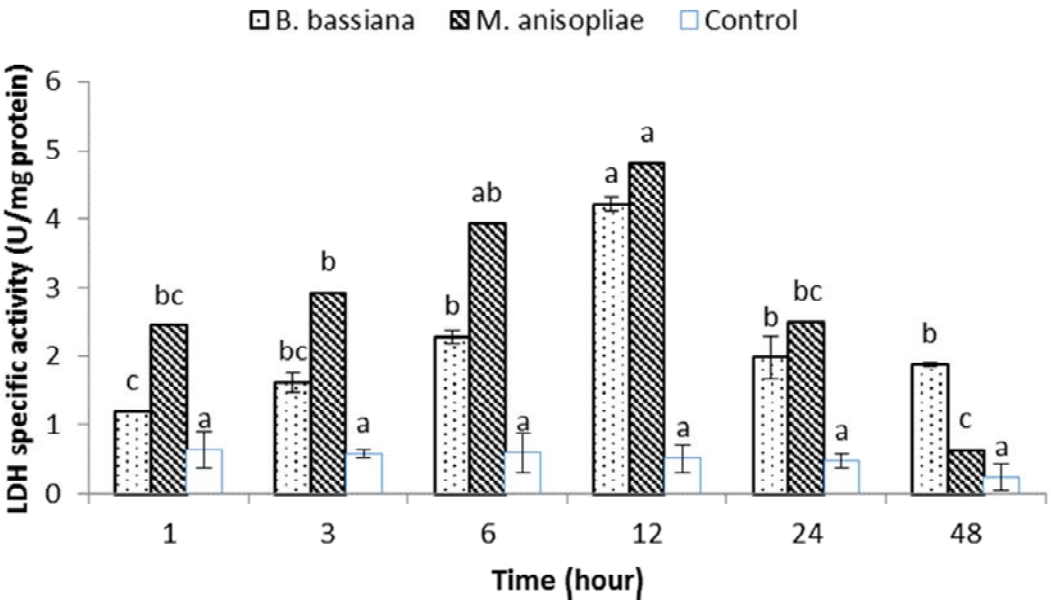

Fig. 6 Effects of *B. bassiana* and *M. anisopliae* injections on LDH activity in *S. littoralis* larvae. Statistical differences have been shown by various letters ($p \leq 0.05$).

Melanin deposition prevents absorption of nutrients to kill parasites due to starvation (Chen and Chen, 1995). Also, formation of cytotoxic reactive oxygen and nitrogen intermediates during melanin synthesis causes to kill invading organisms (Nappi and Christensen, 2005).

Alanin amino transferase (ALT) and aspartate aminotransferase (AST) are the enzymes that are involved in transamination process of various tissues (Nation, 2008). These enzymes catalyze conversion of alanine, aspartate and α-ketoglutarate to oxaloacetate and glutamate. Any changes in activities of ALT and AST are due to existence of a physiological challenge in body such as microorganism infections, damage to some tissues or being a toxic material (Giboney *et al*., 2005). Meanwhile, □-glutamyl transferase is another aminotransferase. Also, it plays a key role in the gamma-glutamyl cycle, a pathway for the synthesis and degradation of glutathione and drug and xenobiotic detoxification (Tate and Meister, 1985). In the current study, the highest activities of two aminotransferase were observed 6-12 hours post injection (Figure 4a,b; F= 12.24, Pr>F: 0.0043; F=12.09, Pr>F: 0.0042). δ-Glutanyl transferase (δGT) showed the highest activity 3-12 hours post injection (Figure 4c; F= 12.24, Pr>F: 0.0042; F=26.32, Pr>F: 0.0003). Although activity of δGT had no significant differences in the larvae injected by *B. bassiana* and *M. anisopiae* spores but the larvae treated by *B. bassiana* had higher activity of ALT and larvae treated by *M. anisopliae* had higher activity of AST (Fig. 4). These results implies that immune challenge of larvae by entomopathogenic fungi cause a protein shortage due to differentiation of hemocytes and detoxifying of fungal secondary metabolites. So, enzymes involve in transamination get the higher activity to increase availability of amino acids for physiological processes. These observations are similar to insects treated by chemical insecticides (Ender *et al*., 2005; Etebari *et al*., 2005; Zibaee *et al*., 2008; Zibaee *et al*., 2011).

Acid (ACP) and alkaline phosphatases (ALP) are the hydrolytic enzymes responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids in alkaline and acidic conditions, respectively under name of dephosphorylation (Zibaee *et al.,* 2011). Also, these enzymes are involved in lipid hydrolysis in several tissues like midgut, hemolymph and fat bodies (Zibaee *et al*., 2011). Overall activities of ACP and ALP in the larvae injected by *M. anisopliae* were higher than those by *B. bassiana* (Fig. 5) But both enzymes showed the highest activity 12 hours post injection (Fig. 5a,b; F= 19.38, Pr>F: 0.0012; F=31.09, Pr>F: 0.0003). Activity elevations of these enzymes in immune challenged *S. littoralis* could be due to energy demands for compensatory mechanisms like treated insects by chemical insecticides.

Lactate dehydrogenase catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD+ in glycolysis cycle (Kaplan and Pesce, 1996). Shortage in oxygen or tissue breakdown elevates levels of LDH considering as a medicinal biomarker (Kaplan and Pesce, 1996). In the current study, the highest activity of LDH was observed 12 hours post-injection although there was a slight statistical difference between intervals 6-12 hours (Fig. 6; F= 26.36,

Pr>F: 0.0001; F=43.27, Pr>F: 0.0001). Now, it has been determined that entomopathogenic fungi produce toxic secondary metabolites to disable immune system of insects. These materials kill hemocytes and intervene in phagocytosis or nodule formation against parasites (Zibaee *et al*., 2011). So, this could be one of the reasons for elevation of LDH activity in immune challenged larvae of *S. littoralis*. Another reason could be energy demands for removing parasite from hemolymph by elevation of glycolysis rate leading to conversion of pyruvate to lactate.

Results of the current study clearly revealed different effects of *B. bassiana* and *M. anisopliae* spores on cellular immunity and phenoloxidase activity in the fifth larval instars of *S. littoralis*. These differences were due to surface properties of spores and their capability in production of secondary metabolites which are toxic on hemocytes and other tissues. Moreover, findings on changes of enzymatic activity involved in intermediary metabolism support results of previous studies on effect of fungal spores and secondary metabolite on chemical composition of insect hemolymph (Madziara-Borusiewiez and Kucera, 1978; Sujak *et al*., 1978; Kol'chevskaya and Kol'chevskii, 1988; Shiotsuki and Kato, 1996; Sokolova and Sundukov, 1999; Xia *et al*., 2000, 2001; Serebrov *et al*., 2006; Zibaee *et al*., 2009). Meanwhile, it was obtained that 6-12 hours post-injection the highest physiological phenomena were observed in both immune responses and intermediary metabolisms. Hence, it could be concluded that pathogenicity of an entomopathogenic fungi may occur via overcoming on immune responses and discrepancies of intermediary metabolisms. These finding show significant role of these agents against agricultural pests and might be used to improve their quality and efficiency in future.

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