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RESEARCH REPORT

Induction of antioxidant and detoxifying systems of *Chilo suppressalis* Walker after exposure to entomopathogenic fungi

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Abstract

The current study aimed to determine antioxidant and detoxifying responses of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) to *Beauveria bassiana* (Strains BBRR1, BBAL1, BBLN1, BBLN2), *Metarhizium anisopliae* and *Hirsutella subulata*. The interactions of insect humoral immune responses with the entered conidia of entomopathogenic fungi in addition to nodule formation and melanization caused the production of several reactive oxygenate species (ROS), such as hydrogen peroxidase (H_2O_2), hydroperoxides (ROOH), superoxide radicals (O^{2-}), and hydroxyl radical (OH^-). The highest activity of catalase was recorded by BBRR1 and BBAL1, treatment after 48 to 96 h while the larvae treated by BBRR1 showed the highest peroxidase activity. Both ascorbate peroxidase and glucose-6-phosphate dehydrogenase showed the highest activity in the larvae treated by BBRR1 after 48-96 h. The highest concentration of Malondialdehyde (MDA) reported in the larvae treated by BBRR1, BBAL1 and BBLN1, after 48 hours. The highest activity of general esterases was recorded in the larvae treated by BBRR1 after 48-96 hours. Similar results were recorded in the activity of glutathione-S-transferase but the enzyme had also the highest activity in the larvae treated by BBAL1 and BBLN2 after 48 hours. The larvae treated by BBRR1 and BBLN1 showed the highest activity of acid phosphatase (ACP) after 72 and 96 hours while the highest activity of alkaline phosphatase (ALP) was obtained in the larvae only treated by BBRR1 after 48-96 hours. The results clearly revealed that BBRR1 significantly and severely induced antioxidant and detoxifying systems of *C. suppressalis* larvae implying on virulence and immune induction of BBRR1 against the larvae.

Key Words: entomopathogen; antioxidant; *Chilo Suppressalis*; detoxification

Introduction

Entomopathogenic fungi are among the most successful microbial agents to combat insect pests that are exist in most aquatic and terrestrial habitats. They cause enzootic and epidemics in insect populations and serve as one of the alternatives to chemical insecticides in agroecosystems because of their adaptations and interactions with environmental factors such as pH, salinity, organic and inorganic materials as well as imposition of ecological and physiological costs on host insects (Lacey *et al*., 2015; Zibaee and Ramzi, 2018). Moreover, entomopathogenic fungi are the unique microbial agents that infect their host by penetrating thorough integument rather than bacteria or viruses that require to be ingested. Therefore, insect exposure to the fungal conidia in the contaminated environment will cause the onset of infection (Zibaee and Ramzi, 2018). Pathogenicity by entomopathogenic fungi initiates following attachment of conidia to the insect cuticle, passing through by germination, and proliferating after arrival into host hemocoel. Eventually, the entered fungal cells acquire yeast-like forms known as hyphal body that sequentially utilize food resources of host body and cause cellular death by producing secondary metabolites (Brownbridge *et al*., 2001).

Cell death imposed to different tissues of insects following penetration of fungal hyphae and the production of secondary metabolites causes lipid peroxidation and production of reactive oxygen

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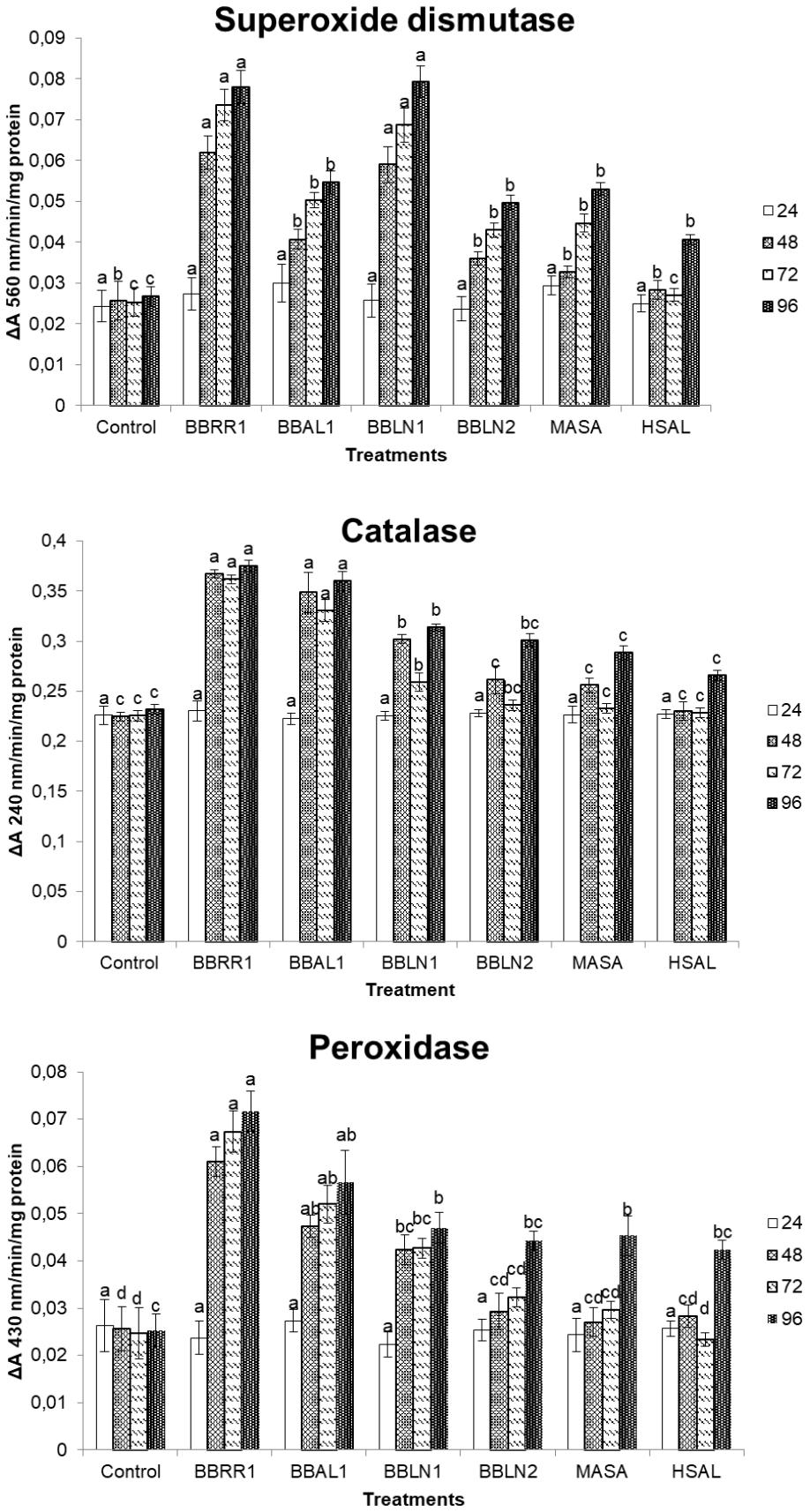

Fig. 1 Changes in superoxide dismutase, catalase and peroxidase activities of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

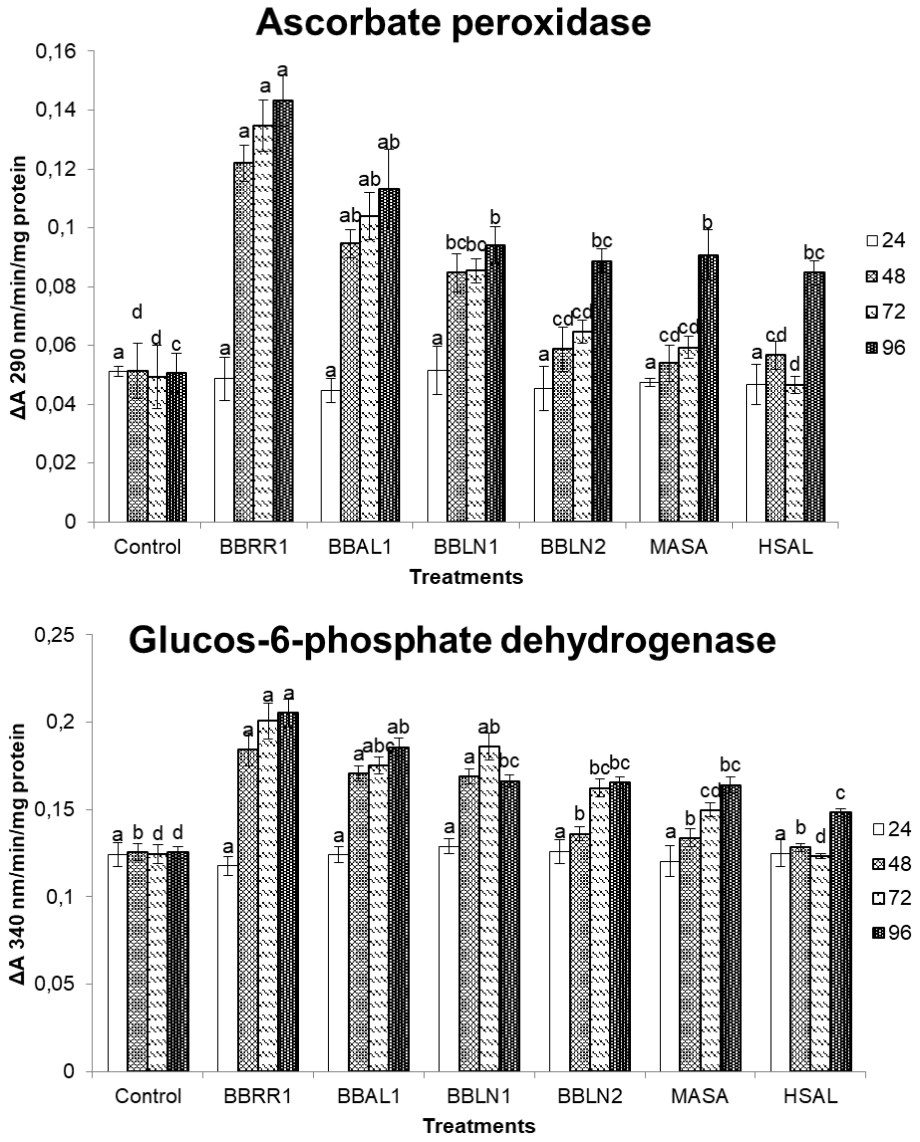

Fig. 2 Changes in ascorbate peroxidase and glucose-6-phosphate dehydrogenase activities of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

species (ROS) (Gulpov *et al*., 2003; Karthi *et al*., 2018; Shamakhi *et al*., 2020). These molecules are involved in cell signaling and stimulate the genes engaged in host defense. The increased concentrations of ROS inflict damages to cell structure under a series of reactions under oxidative stress (Dalton *et al*., 1999; Kamata and Hirata, 1999; Shamakhi *et al*., 2019, 2020). Moreover, the interactions of insect humoral immune responses with the entered conidia of entomopathogenic fungi mainly nodule formation and melanization caused the production of several (ROS), such as hydrogen peroxidase (H_2O_2), hydroperoxides (ROOH), superoxide radicals (O_2^-), and hydroxyl radical (OH^-). These molecules act bilaterally to disable the pathogenic infliction of invading organism and to impose some harmful effects on insect tissues including dissociation of cell membrane, apoptosis and DNA damage that led to suppress developmental and reproductive disorders (Robinson and Badwey, 1994; Whitten and Ratcliffe, 1999; Monaghan *et al*., 2009). Therefore, insects have recruited several components of antioxidant systems to regulate ROS level within body. These enzymatic and non-enzymatic molecules include catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), asocorbate peroxidase (APOX), glucose-6-phosphate dehydrogenase (GPDH), ascorbic acid, thiols, and α-tocopherol (Wang *et al*., 2001; Dubovskiy *et al*., 2008; Shamakhi *et al*., 2020).

There are several reports to imply induction of detoxifying system of insects after microbial

infections that may trigger insect resistance to chemical insecticides (Sokolova and Sundukov, 1999; Xia *et al*., 2000, 2001; Zibaee *et al*., 2009). Glutathione-S-transferase (GST) and general esterases (ESTs) are the multifunctional and the important enzymes in detoxification of xenobiotics in insects. GST conjugates the reduced glutathione to the electrophilic center of xenobiotics and it may engage in both detoxification and cellular antioxidant responses (Substrate, 2002; Li *et al*., 2007). ESTs catalyze the esters of higher fatty acids within metabolic process of insects and xenobiotic hydrolyzation (Li *et al*., 2007). Nevertheless, these enzymes have been reported to engage in degradation of toxic molecules synthesized during fungal penetration (Serebrov *et al*., 2006; Dubovskiy *et al*., 2008; Zibaee *et al*., 2009).

Understanding the physiological interaction of entomopathogenic fungi with their hosts is critical to success of agricultural pest control. These interactions can vary depending on the fungal virulence and the host's immune as well as biochemical responses. In the previous study, the cellular and humoral immunity of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) to the different entomopathogenic fungi was investigated by evaluating the changes in hemocyte count and the expression of antimicrobial peptide genes at different time intervals (Shahriari *et al*., 2021a). These studies are of interest because *C. suppressalis* is an economically important rice pest in north of Iran. The larvae severely fed on stems causing “dead heart” and “white head” of rice plants. Such deficiencies significantly decrease rice yield of imposed field requiring insecticide treatment. Hence the current study was done to determine the effects of four isolates of *Beauveria bassiana*, one isolate of *Metarhizium anisopliae* and *Hirsutella subulata* on the antioxidant and detoxification system of *C. suppressalis* to elucidate other aspects of fungi-insect interactions.

Materials and Methods

Insect rearing

The field collected adults were transferred to the laboratory and allowed to lay eggs on rice leaves of Hashemi variety. The hatched larvae were gently transferred on rice stems and kept in sterile containers at 28 ± 2 °C, 80% relative humidity (RH) and 16h light:8h dark (LD 16:8). Fresh stems were provided daily until the fourth instars. Two generations were reared on laboratory to have a cohort with the least environmental stresses (Zibaee and Malagoli, 2014).

Entomopathogenic fungi culture

Four isolates of *B. bassiana* including BBRR1, BBAL1, BBLN1 and BBLN2 in addition to one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA) were selected based on the results of our previous studies, and cultured on Potato dextrose agar at 25 ± 2 °C. After 14 days, conidia were gently gathered and added onto a 0.01% solution of Tween 80 (Sigma Aldrich, USA) to prepared desirable concentration.

Larval treatment and sample preparations

The fourth instar larvae of *C. suppressalis* were randomly immersed into 10^5 conidia/mL concentration of each fungal isolate (each containing 30 specimens in the three replicates of 10) while the control larvae were dipped in Tween-80 (0.02%) alone. After 24, 48, 72 and 96 h of post-treatment, the total bodies of the larvae were separately homogenized in distilled buffer by a glass pestle in a 1.5 ml of microtubes then centrifuged at 20000 ×*g*, 4 °C for 20 min. The supernatants were stored at -20 °C and used in the biochemical experiments.

Antioxidant enzymes

Catalase (EC 1.11.1.6)

Briefly, 50 µL of enzyme sample was added into 500 µL of hydrogen peroxide (1%) and incubated at 28 °C for 10 min. Then, the absorbance was read at 240 nm (Wang *et al*., 2001).

Superoxide dismutase (EC 1.15.1.1)

The method of McCord and Fridovich (1969) was used to measure SOD activity. Briefly, 50 µL of enzyme sample was added into 500 µL of a solution containing 70 µM of NBT, 125 µM of xanthine, dissolved in PBS (20 mM, pH 7.1). Then, 100 µL of xanthine oxidase solution added by 10 mg of bovine serum albumin was mixed with the earlier medium. Finally, 100 µL of xanthine oxidase (5.87 units/ml) was added and incubated at darkness, 28 °C for 20 min. At the end of incubation time, the absorbance was read at 560 nm.

Peroxidase (EC 1.11.1.x)

The reaction mixture contained 50 µL of enzyme sample, 250 µL of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer [pH 7.0]) and 250 µL of H_2O_2 (1%). The absorbance was continuously recorded every 30 s for 2 min at 430 nm (Addy and Goodman, 1972).

Ascorbate peroxidase (EC 1.11.1.11)

The enzyme assay was done by preparing a reaction mixture containing 50 µL of sample, 150 µL potassium phosphate buffer (67 mM, pH 7.0), 70 µL ascorbic acid (2.5 mM) and 200 µL H_2O_2 (30 mM). The absorbance was read every 40 s for 5 min at 290 nm (Asada, 1992).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

The enzyme assay was done by mixing 100 µL Tris-HCl (100 mM, pH 8.2), 50 µL NADP (0.2 mM) and 30 µL $MgCl_2$ (0.1 M). After 1 min, 50 µL water, 50 µL of the sample and 100 µL GPDH (6 mM) was added to the earlier mixture. Finally, the absorbance was read at 340 nm after 5 min (Balinsky and Bernstein, 1963).

Malondialdehyde (MDA)

The MDA concentration was measured by mixing 100 µL of 20% trichloroacetic acid and 50 µL of supernatant. The mixture was centrifuged at 15,000 *g* for 10 min at 4 °C. Then, 100 µL of 0.8% TBA reagent was added into the gained supernatant

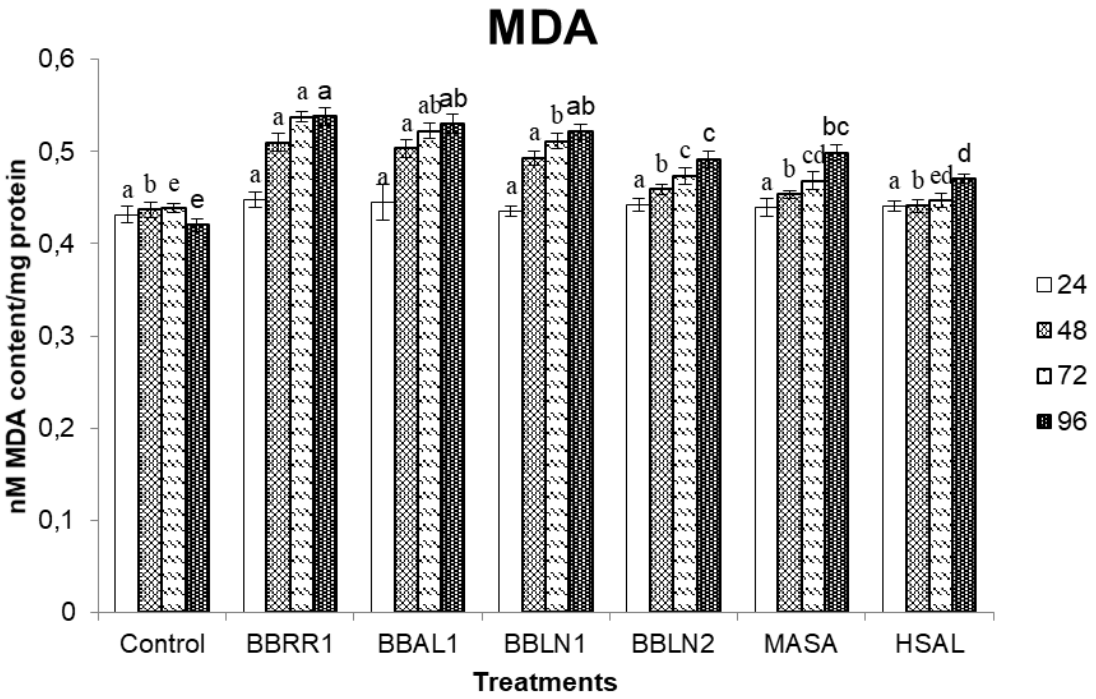

Fig. 3 Changes in malondialdehyde content of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

and incubated at 100 °C for 60 min before to read absorbance at 535 nm. The MDA concentration was calculated using a molar extinction (Bar-Or *et al*., 2001).

Detoxifying enzymes

General esterase (EC 3.1.1.1.)

Seventy-five microliters of the two substrates, α-naphthyl acetate and β-naphthyl acetate (10 mm) was separately added into 75 µL fast blue RR salt (1 mm). Then, 50 µL of the enzyme solution was added to the reaction mixture and the absorbance was read at 450 nm w at intervals of 10 s for 1 min (Han *et al*., 1995).

Glutathione-S-transferase (EC 2.5.1.18)

Twenty microliters of CDNB (20 mm) or DCNB (40 mm) were separately was added into 100 µL of enzyme solution. Then the absorbance was read at 340 nm at intervals of 9 s in 1 min (Oppenoorth *et al*., 1979).

Alkaline- (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatase

The enzyme assay was done based on the method of Bessey (1954). The buffered substrate, *p*-nitrophenol phosphate in Tris-HCl (20 mM) pH 8 and (pH 5) was separately used to assay alkaline- and acid phosphatase, respectively. Twenty microliter of the enzyme assay was added into the buffered substrate separately and incubated for 5 min before reading the absorbance at 450 nm.

Protein Assay

The protein content of the samples in both control and fungal treated was assayed by the method of Lowry *et al*. (1951) (recommended by Ziest Chem. Co., Tehran-Iran).

Statistical Analyses

The date of antioxidant and detoxification experiments were compared by one-way analysis of variance (ANOVA) using Tukey's test (SAS 9.3 2010). The statistical differences were marked by different letters at a probability less than 5%.

Results and Discussion

Exposure of *C. suppressalis* larvae to the six native isolates of *B. bassiana*, *M. anisopliae* and *H. subulata* significantly enhanced antioxidant and detoxifying activities in the fourth instar larvae *C. suppressalis*. The activities of SOD, CAT and POD in the larvae treated by BBRR1, BBAL1 and BBLN1 significantly increased at 48 and 72 h of post-treatment while no significant difference was observed between fungal treatments and control larvae after 24 h (Pr>F: 52.88; df= 6; P<0.0001, Fig. 1). Moreover, the activities of SOD, CAT and POX in the larvae significantly elevated at 96 h post-treatment for all isolates (Pr>F: 71.23; df= 6; P<0.0001, Fig. 1). Entomopathogens and their toxins have been recognized as one of the important exogenous resources that generated free radicals (Jia *et al*., 2016; Karthi *et al*., 2018;

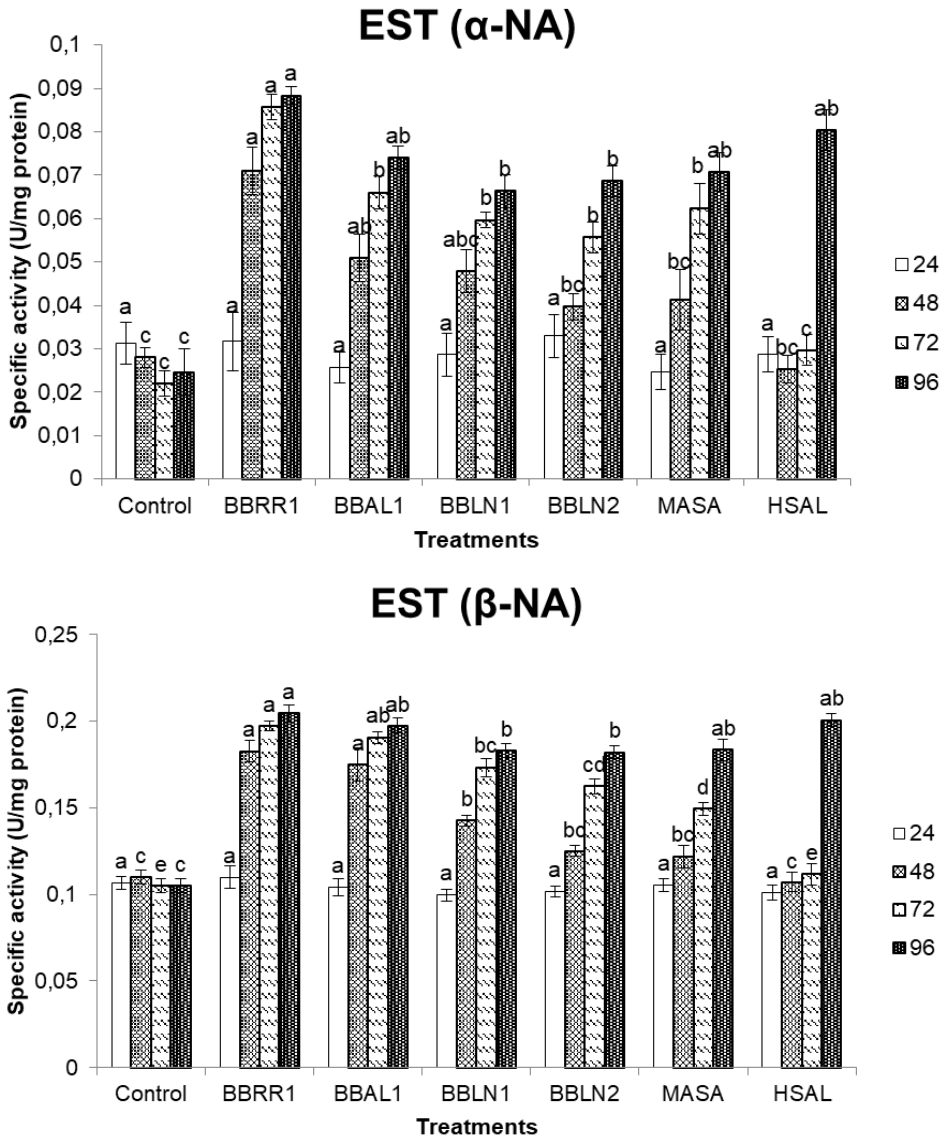

Fig. 4 Changes in general esterase activity of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

Shamakhi *et al*., 2020). Overproduction of ROSs by biological and chemical toxic compounds, apart from its nature of origin, resulted in oxidative stress that eventualy leads to lipid peroxidation and damage of DNA (Dubovskiy *et al*., 2008). Several reports have shown involvements of SOD, CAT, POD, GPDH, APOX and nonenzymatic components (e.g., MDA and α-tocopherol) in the antioxidant defenses of insects (Dubovskiy *et al*., 2008; Rahimi *et al*., 2018; Shahriari *et al*., 2019; Shamakhi *et al*., 2019, 2020). However, there are few reports regarding the effects of entomopathogenic fungi on antioxidant induction of insect pests. SOD has an important role to catalyze O_2^- radicals into H_2O_2. Then, H_2O_2 changed to H_2O and oxygen by the activities of CAT and POD (Dubovskiy *et al*., 2008). Because the activities of CAT and POD significantly increased in the larvae treated by the all six isolates, it seems that metabolites produced from fungal conidia may induce contents of superoxide radicals and hydrogen peroxide which subsequently elevated activation of the antioxidant enzymes. Moreover, the enhanced activities of CAT and POD may be attributed to the higher activity of SOD which generates hydrogen peroxides in the larvae of *C. suppressalis*. Similar results were reported regarding the effects of *B. bassiana* on *C. suppressalis* larvae (Shamakhi *et*

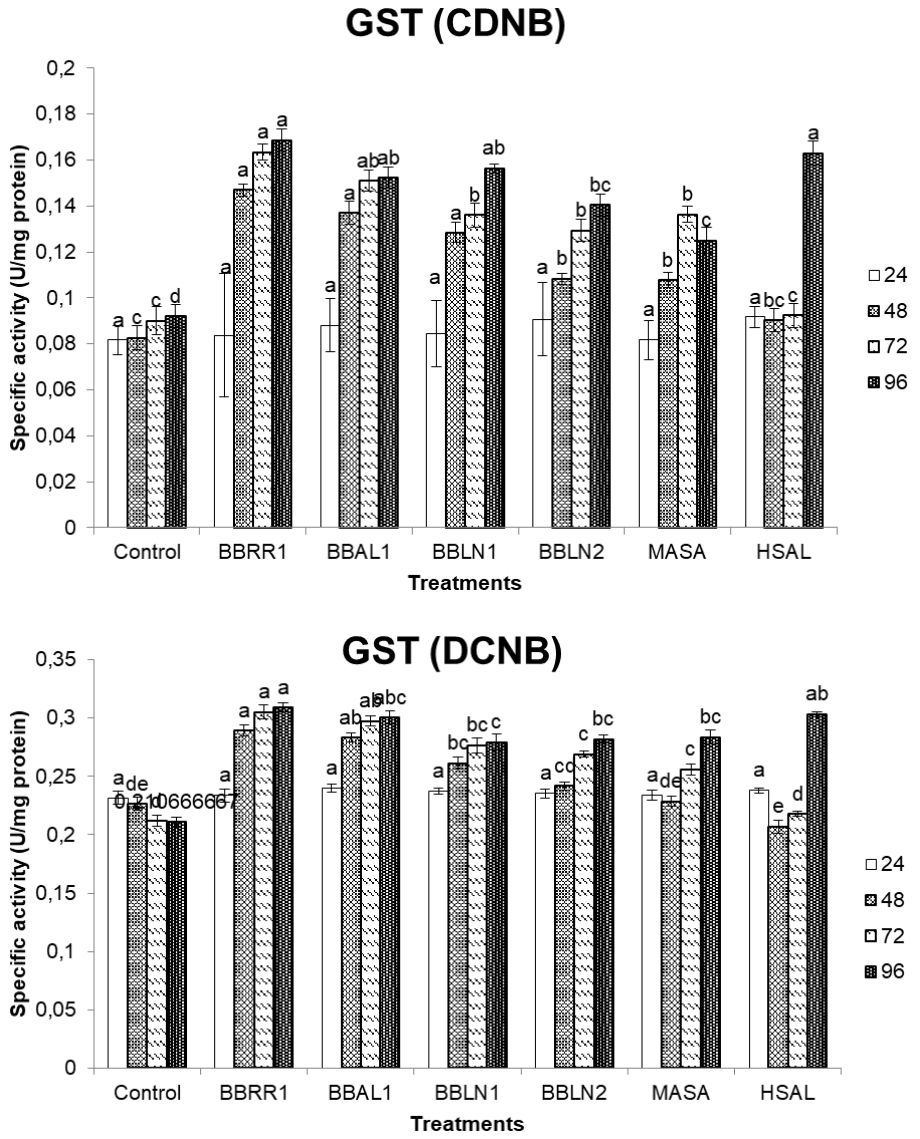

Fig. 5 Changes in glutathione *S*-transferase activity of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

al., 2020). Karthi *et al*. (2018) demonstrated that infection of *Spodoptera litura* (Fabricius) (Lepidoptera; Noctuidae) by *Aspergillus flavus* conidia caused the higher activities of SOD, CAT and POD. Jia *et al*. (2016) reported that *M. anisopliae* caused the higher activities of SOD, CAT, and POD in *Locusta migratoria* (Meyen) (Orthoptera: Acrididae). The highest activity of antioxidant enzymes in *C. suppressalis* larvae was observed in the larvae treated by BBRR1, BBAL1 and BBLN1. In our previous study, BBRR1, BBAL1 and BBLN1 showed the highest virulence in the shortest time against the larvae of *C. suppressalis* (Shahriari *et al*., 2021b). Moreover, the authors demonstrated the highest activities of the extracellular enzymes in BBAL1, BBRR1 and BBLN1 therefore the conidia of these isolates pass through the cuticle of larvae in a faster time than other isolates (Shahriari *et al*., 2021b). These differences may be correlated to the presence of different molecules in the cell membrane of conidia and production of toxins by the isolates which impose oxidative stress in the larvae.

Exposure of BBRR1, BBAL1, BBLN1 and BBLN2 on *C. suppressalis* larvae increased the activities of APOX and GPDH after 48 and 72 h so that these enzymes showed the highest activities compared to control at 96 h post-treatment (Pr>F: 65.23; df= 6; P<0.0001, Fig. 2). GPDH and APOX are the two antioxidant enzymes which engaged in detoxification of pro-oxidant compounds within insects (Asada, 1992). APOX eliminates H_2O_2 in

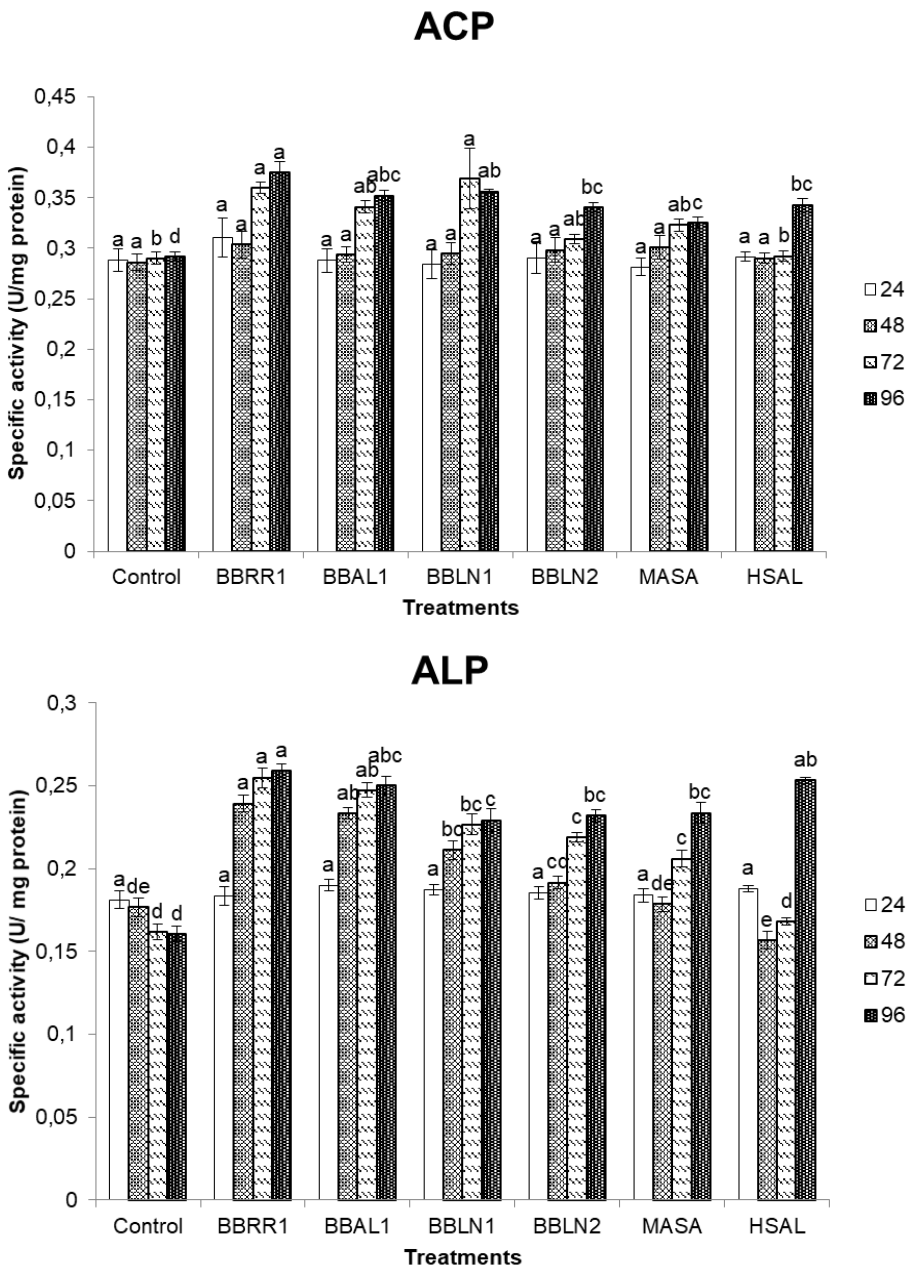

Fig. 6 Changes in acid- and alkaline activities of *C. suppressalis* larvae treatments by different isolates of entomopathogenic fungi. Different letters indicate statistical differences at $p < 0.05$ in each time interval. *B. bassiana* includes BBRR1, BBAL1, BBLN1 and BBLN2 strains as well as one isolate of *M. anisopliae* (MASA) and *H. subulata* (HSLA)

chloroplasts, cytoplasm, and mitochondria, while GPDH involved in deleting oxidant compounds in cytosol via transduction of NADPH to $NADP^+$ (Asada, 1992). Similar to our results, the larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) infected by *Bacillus thuringiensis* showed also the higher activities of APOX and GPDH compared to control (Dubovskiy *et al*., 2008). Shamakhi *et al*. (2020) demonstrated significant higher activities of APOX and GPDH in *C. suppressalis* injected with *B. bassiana* conidia. The higher GPDH activity led to elevation of NADPH synthesis to remove products of APX activity. Moreover, NADPH reduces the toxic effects via transporting electrons to free radicals (Barbehenn, 2002; Shamakhi *et al*., 2020).

Content of MDA in the larvae treated by BBRR1, BBAL1 and BBLN1 were significantly induced after 48 h while other isolates caused the MDA elevation in the larvae of *C. suppressalis* after 72 and 96 h (Pr>F: 67.23; df= 6; P<0.0001, Fig. 3). MDA is an oxidative stress indicator to show enhancement in radical oxidative stresses. The

increased content of MDA indicated occurrence of oxidative stress following lipid peroxidation (Rahimi *et al*. 2018). On the other hand, ROSs led to catalysis of polyunsaturated acids, generation of MDA and elevation of toxic compounds within cells (Wang *et al*., 2001). Results of our study demonstrated that lipid peroxidation was higher in the larvae treated by fungal isolates than control so it can be concluded that conidia proliferation and secretion of secondary metabolites increased lipid peroxidation in hemocoel that leads to cytotoxicity. Some research reported a direct correlation between fungal infection and lipid peroxidation in insects. For example, the induction of MDA under mycoses showed in *C. suppressalis* larvae injected by *B. bassiana* (Shamakhi *et al*., 2020), as well as *S. litura* larvae infected by *A. flavus* (Karthi *et al*., 2018).

Activity of general esterase significantly increased after 72 and 96 h for all isolates except for BBRR1, BBAL1 and BBLN1, while no significant difference was observed among isolates and control after 24 h (Pr>F: 49.73; df= 6; P<0.0001, Fig. 4). Similar results were obtained for GSTs activity in which the exposure of all isolates led to enhanced activity of GSTs at 72 and 96 h of larval post-treatment (Pr>F: 49.18; df= 6; P<0.0001, Fig. 5). In spite of efforts to develop chemical and biological pesticides, insects are also looking for ways to neutralize the effects of these pesticides. ESTs and GSTs are the two important groups of detoxifying enzymes that involve in the breakdown and neutralization of toxic compounds entering insect body (Zhu *et al*., 2011). Findings of our study support results of earlier investigations regarding the effect of fungal conidia on detoxifying enzymes of insects (Xia *et al*., 2000, 2001; Serebov *et al*., 2001, 2006; Dubovskiy *et al*., 2008; Zibaee *et al*., 2009; Fan *et al*., 2013; Petlamul *et al*., 2019). In our study, treatment of *C. suppressalis* larvae by conidia of the entomopathogenic fungi increased activities of ESTs and GSTs especially in the larvae treated by BBRR1, BBAL1 and BBLN1. This could be a nonspecific response to integument damage. In fact, tissue damage is the most common feature of infection by entomopathogenic fungi causing secretion of detoxifying enzymes which could be the usual response of larvae to infection irrespective of the agent nature (Xia *et al*., 2001; Serebov *et al*., 2006; Zibaee *et al*., 2009).

ACP activity in the larvae treated by all isolates significantly increased at 96 h of post-treatment while no significant difference was observed among isolates and control after 24 and 48 h (Pr>F: 34.12; df= 6; P<0.0001, Fig. 6). Activity of ALP significantly induced after 96 h for all isolates except for BBRR1, BBAL1 and BBLN1 isolates but no significant difference was observed among isolates and control after 24 h of treatment (Pr>F: 47.26 df= 6; P<0.0001, Fig. 6). ACP and ALP are the two hydrolytic enzymes responsible for removing phosphate groups from some molecules like proteins, nucleotides and alkaloids in acidic and alkaline conditions, respectively (Ramzi *et al*., 2014). These enzymes also are able to support, modulate and accelerate phagocytosis (Karthi *et al*., 2018). In the present study, the activities of ACP and ALP significantly increased in the larvae treated by all isolates. The higher phosphatase activity was found in the larvae exposed to conidia of BBRR1, BBAL1 and BBLN1. Our findings are parallel to the research of Karthi *et al*. (2018), who demonstrated ACP and ALP activities increased in *S. litura* after exposure to *A. flavus*. Similarly, *B. bassiana* and *M. anisopliae* significantly increased the activities of ACP and ALP in *S. littoralis* (Mirhaghparast *et al*., 2013). In contrast, Shaurub *et al*. (2020) reported that the LD50 of *B. bassiana* did not affect ALP activity in the 4th-instar larvae of *S. litura*. These findings are expected because enzyme activity is dependent on several factors, such as pathogen strain, dose, host insect and etc.

Conclusions

Results of our research reported the induction of antioxidant system in the larvae of *C. suppressalis* exposed to several isolates of entomopathogenic fungi that may be the result of ROSs production after larval immune responses and damages to tissues caused by entomopathogenic fungi infection. Also, larval infection by the isolates increased the activities of detoxifying enzymes. Therefore, it may be concluded that the antioxidant and detoxifying systems of *C. suppressalis* is a part of larval defense mechanisms against fungal infection in addition to humoral or cellular responses which have been previously reported.

Acknowledgments

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