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RESEARCH REPORT

Host-pathogen interactions of the two native isolates of *Beauveria bassiana* to a predatory coccinellid, *Cryptolaemous montrouzieri* Mulsant (Coleoptera: Coccinellidae)

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Abstract

Fungi are among the most important microorganisms affecting population dynamics of insects. Although they are used as biocontrol agents for several decades but their interactions to insect pests, predators and parasitoids are still interesting in case of virulence, host physiology and environmental persistence. Understanding the possible synergistic or antagonistic interactions of entomopathogenic fungi with other biocontrol agents mainly predators is a critical factor to achieve a successful pest control program. In the current study, effects of the two native isolates of *Beauveria bassiana* (AM-118 and BB3) were studied on survival, cellular immunity and antioxidant system of *Cryptolaemous montrouzieri* Mulsant. Bioassay results showed that both AM-118 and BB3 caused significant mortality on the third instar larvae and the adults of *C. montrouzieri*. Moreover, they increased total and differential hemocyte counts and significantly induced phenoloxidase activity and nodule formation at 48, 72 and 96 h post-treatment. A considerable increase was also observed in the activities of antioxidant enzymes at 72 and 96 h post-treatment. Although the isolates caused mortality on both stages, induction of immune and antioxidant systems protect *C. montrouzieri* against infective conidia.

Key Word: *Cryptolaemous montrouzieri*; *Beauveria bassiana*; virulence; immune response; antioxidant system

Introduction

Mealybugs are the economically important pests of citrus and tea orchards that cause direct and indirect losses in production of well-quality crops. The chemical sprays to combat mealybugs may have no satisfactory efficiency because of their structural and behavioral capabilities to neutralize pesticide toxicity. These compounds may also endanger beneficial organisms like predators and parasites in cropping systems due to direct mortality or reproductive deficiencies (Rabindra and Ramanujam, 2007). Biological control procedures through pathogenic microorganisms and predators are much appreciable to control mealybugs in order to maintain sustainable production in orchards (Ramanujam *et al*., 2017). The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) and the predatory coccinellide, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) may be used simultaneously because of varying degrees of success in managing the mealybugs of citrus and tea orchards (Mani and Krishnamoorthy, 2008; Maqsoudi *et al*., 2018). *C. montrouzieri* is an important natural enemy in integrated pest management programs of orchards because it utilizes different life stages of mealybugs including *Pseudococcus viburni* (Signoret), *Maconellicoccus hirsutus* Green, *Planococcus citri* (Risso), *Ferrisia virgata* outbreaks and *Phenacoccus solenopsis* Tinsley (Thungrabeab and Tongma, 2007; Jiang *et al*., 2009; Ibrahim *et al*., 2011; Scorsetti *et al*., 2012). Moreover, *B. bassiana* is a successful entomopathogenic fungus naturally exists in soils and even lives as endophytes. It is extensively used as a mycoinsecticide to control different insect pests of lepidopterans, coleopterans, and dipterans (Toledo *et al*., 2014; Maistrou *et al*., 2018; Shahriari *et al*., 2021a). *B. bassiana* has several positive characteristcs containing high potential mortality on target pest population, high genetic variation of the

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Table 1 LC_{30} and LC_{50} concentrations of the two isolates of *B. bassiana* against *C. montrouzieri* after 14 days of exposure

Life stage	Isolates	LC_{30} (Cl 95 %) Conidia/mL	LC_{50} (Cl 95 %) Conidia/mL	X2 (df)	Slope±SE
3rd larvae	AM-118	4×10^4 (4.3×10^3 - 1.5×10^5)	6.4×10^5 (1.8×10^5 - 2×10^6)	0.619 (3)	0.438 ± 0.082
	BB3	2×10^4 (2.3×10^3 - 7×10^4)	2.2×10^5 (6.2×10^4 - 6.3×10^5)	0.527 (3)	0.497 ± 0.087
Adults	AM-118	1.3×10^5 (2.3×10^4 - 4.4×10^5)	2.6×10^6 (6.6×10^5 - 7.3×10^6)	0.262 (3)	0.450 ± 0.082
	BB3	8.1×10^4 (1.1×10^4 - 2.7×10^5)	1.2×10^6 (3.7×10^5 - 4×10^6)	0.438 (3)	0.446 ± 0.082

Note: calculations were carried out by POLO-Plus software

isolates, infection of different developmental stages of host insect, vertical and horizontal dispersal capacity depending on the host environment (Wraight *et al*., 2010; Jaronski, 2014).

Insects have adapted themselves to colonize different habitats through developing a range of defense mechanisms. Immune system of insects inflicts invading pathogens by cellular and humoral responses. Upon distinguishing the pathogen entrance, cellular immune functions are triggered by hemocyte proliferation, micro-aggregation, nodulation and encapsulation followed by melanin deposition (Lavine and Strand, 2002). Additionally, a series of humoral responses including antimicrobial peptides, phenoloxidase cascades and generation of reactive oxygen species are simultaneously activated to fully disable pathogen dispersal and proliferation in body (Tsakas and Marmaras, 2010; Liu *et al*., 2013). Antioxidant enzymes are the other defense responses that act non-specifically against invading pathogens. These enzymes control concentration of reactive oxygen species (ROS) in insects (Karthi *et al*., 2018). In details, ROS including, hydroperoxides (ROOH), superoxide radicals (O_2^-), hydrogen peroxidase (H_2O_2) and hydroxyl radical (OH^-) are produced under exposure to pathogens damaging cell structure (Dubovskiy *et al*., 2008). Oxidative stress finally induces lipid peroxidation that disturbs cell membrane liquidity, DNA damage and apoptosis (Monaghan *et al*., 2009). Hence, regulating the content of ROSs is necessary to decrease their harmful effects and to modulate proper cell signaling pathways toward their concentrations (Shamakhi *et al*., 2020). Insect antioxidant system includes enzymatic and non-enzymatic components such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), glucose-6-phosphate dehydrogenase (GPDH), glutathione peroxidase, thiols, ascorbic acid and α-tocopherol (Dubovskiy *et al*., 2008; Shamakhi *et al*., 2020).

Table 2 LT_{50} concentrations (days) of the two isolates of *B. bassiana* against *C. montrouzieri* after 14 days of exposure

Life stage	Isolates	LT_{50} (Cl 95 %) days	X2 (df)	Slope±SE
3rd larvae	AM-118	8.12 (7.18 – 9.03)	3.237 (4)	5.433 ± 0.693
	BB3	8.23 (6.44 – 9.89)	6.414 (4)	5.664 ± 0.720
Adults	AM-118	9.90 (7.83 – 12.12)	6.280 (4)	4.845 ± 0.646
	BB3	9.72 (6.78 – 12.83)	11.569 (4)	5.264 ± 0.686

Note: calculations were carried out by POLO-Plus software

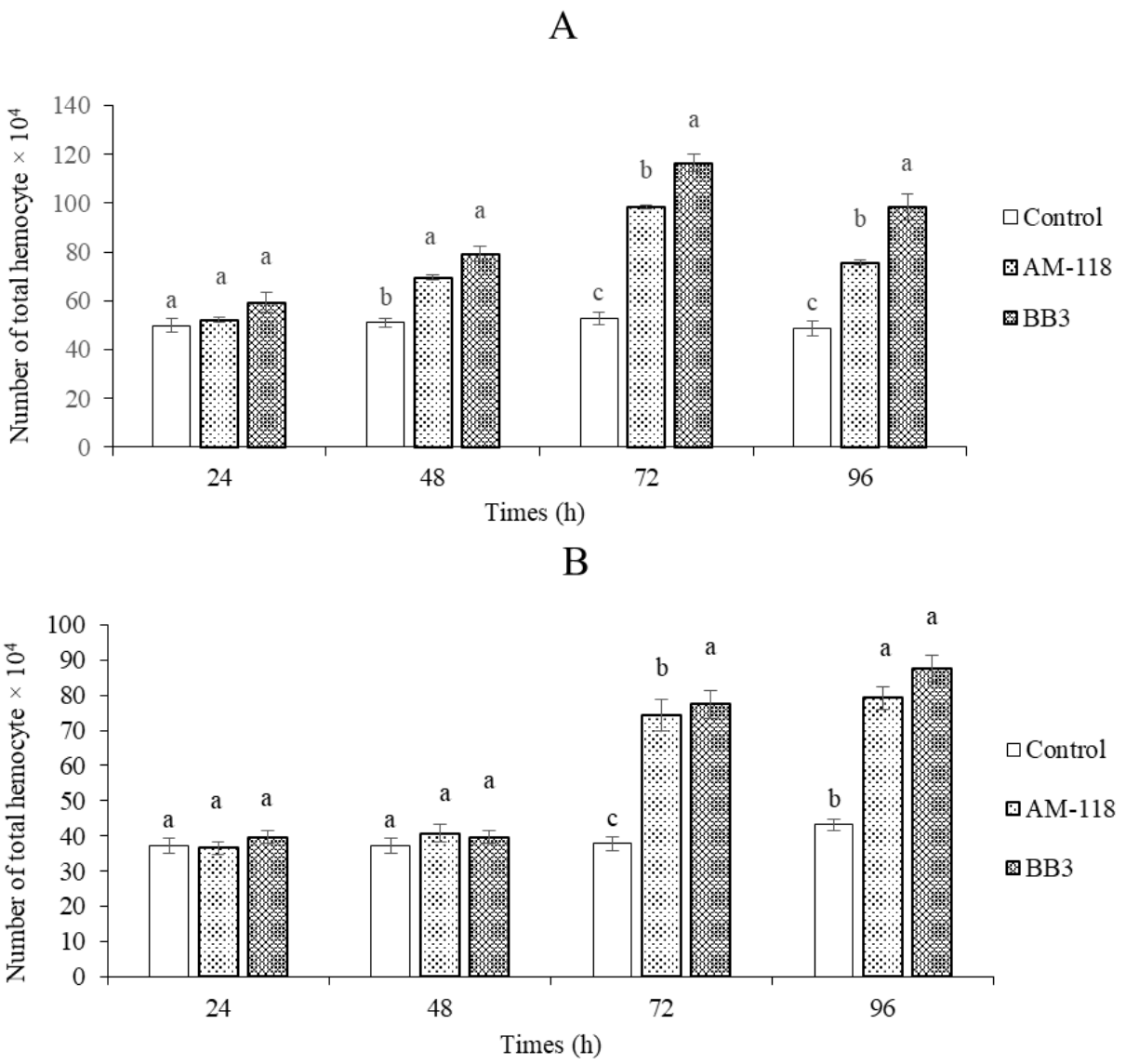

Fig. 1 Changes of the total hemocytes counts in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

Integrative use of entomopathogenic fungi and predators is essential to increment the role of biological agents to alleviate population outbreaks of pests with the least influence to the ecosystem (Wu *et al*., 2018). On the other hand, the successful use of entomopathogenic fungi in integrated pest management requires not only high virulence against insect pests, but also possible low or selective virulence against natural enemies (Portilla *et al*., 2017). Synergetic interactions between entomopathogenic fungi and insect predators may increase control efficacy, while antagonistic interactions lead to significant deficiency in pest control and impose financial and environmental costs (Roy *et al*., 1998). Our previous study revealed appropriate virulence of the two native isolates of *B. bassiana* against tea mealybug, *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae) (Maqsoudi *et al*., 2017). In case, it is important to investigate the effects of entomopathogenic fungi on *C. montrouzieri* to increase efficiency of mealybug control program. This would lead to implement a safe and sustainable pest control scheme. Therefore, the present study was done to increase our knowledge on side-effects of *B. bassiana* against *C. montrouzieri* from virulence and physiological aspects. Briefly, the third instar larvae and the adults of *C. montrouzieri* were treated with AM-118 and BB3 isolates of *B. bassiana*, then the sub-lethal concentration of the isolates was treated to determine immune and antioxidant responses.

Materials and Methods

Rearing of prey and predator

Cryptolaemous montrouzieri were provided from the Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension, Amol, Iran and reared at the laboratory conditions of 25 ± 2 °C, 70 ± 5 % RH, and 16L:8D photoperiod, in the glass jars (15 × 20 cm), on potato sprouts infested by *Pseudococcus viburn*i. The mealybugs population built-up on newly infested potato sprouts for two weeks and then transferred to beetle-rearing glass jars twice a week. Both larvae and adults were fed on the mealybugs but they kept at separate containers to avoid cannibalism (Aghdam and Malekshah, 2019).

Fungal isolates and culture

The isolates of *Beauveria bassiana* (AM-118 and BB3) were cultured at 25 ± 2 °C on Potato Dextrose Agar (PDA) supplied with yeast extract (1 %). The cultures were kept for three weeks, and a hemocytometer was used to determine conidia concentration for stock and intended suspensions (Maqsoudi *et al.* 2018).

Bioassay

Dipping method was used to assay the effects of fungal isolates against the larvae and the adults *C. montrouzieri*. At first, a series of primary tests were done to estimate the concentration range causing 15 to 85 % mortality. Then, five concentrations were prepared at logarithmic intervals of 10^4, 10^5, 10^6, 10^7, and 10^8 conidia/mL within 0.02 % solution of Tween-80. The third instar larvae and adults were randomly dipped into the prepared concentrations of each isolate although the control insects were dipped in an aqueous solution of 0.02% Tween-80 alone. Three replicates including 10 individuals were considered for the experiment (N = 180). After two weeks, mortality was recorded and values of LC_{30} and LC_{50} were calculated by the probit analysis using POLO-Plus software. Also, LT_{50} value was determined by exposing different groups of the larvae and the adults to 10^8 conidia/mL concentration of each isolate. Mortality recorded for eight days to death of the last individual.

Cellular immunity

Insect treatment, hemolymph collection, and hemocyte counts

The third instar larvae and the adults of *C. montrouzieri* were randomly dipped into 10^4 conidia/mL concentration of AM-118 and BB3 isolates while the control insects were immersed in Tween-80 (0.02 %) alone. The hemolymph of treated and control individuals was separately collected at intervals of 24, 48, 72 and 96 h by cutting the last thoracic leg with a microscissor. Hemolymph was transferred into the ice-cold anticoagulant buffer (0.01M ethylenediaminetetraacetic acid, 0.1M glucose, 0.062 M NaCl and 0.026 M citric acid, pH 4.6) in the ratio of 3:1. The total and differential hemocyte (granulocyte and plasmatocyte) counts as well as the number of nodules were specified using a Neubauer hemocytometer (Chemkind Co. China). In each time intervals, sixty insects were used in six replicates.

Assay of phenoloxidase activity

PO activity was measured at all-time intervals for both control and fungi-treated specimens as described by Leonard *et al*. (1985). The obtained hemolymph was added into the anticoagulant buffer and centrifuged at 10,000 × g for 8 min. The supernatant was discarded, and the pellet was washed with phosphate buffer for three times (0.02 M, pH 7.1). The current samples were homogenized in 100 µL of phosphate buffer after a night incubation in -20 °C and centrifuged at 12,000×g for 15 min. The final mixture for PO assay contained 30 µL of dihydroxyphenylalanine (10 mM), 50 µL of phosphate buffer (pH 7.1), and 15 µL of the supernatant. The absorbance was recorded at 492 nm following 5 min of incubation at 30 °C.

Assay of antioxidant components

Sample preparation

The third instar larvae and the adults of *C. montrouzieri* were exposed to 10^4 conidia/mL concentration of each isolate to evaluate fungal effects on enzymatic and non-enzymatic antioxidant components. After 24, 48, 72 and 96 h of post-treatment, total bodies of the insects were homogenized in distilled water, and centrifuged at 12000 *g* for 15 min at 4 °C. Then, the supernatant was used in the biochemical experiments.

Superoxide dismutase (SOD)

Activity of superoxide dismutase (SOD) was measured by suppression of declined level of NBT (nitro blue tetrazolium) by the superoxide anion to be produced after xanthine oxidation (McCord and Fridovich, 1969). Enzyme solution (60 µL) was added into 500 µL of the reaction solution (125 µM of xanthine; 70 µM of NBT; both dissolved in PBS) and 20 µL of xanthine oxidase solution [10 mg of bovine albumin; 100 µL of xanthine oxidase (5.87 units/mL); dissolved in 2 mL of PBS]. The reaction mixture was maintained in darkness at 25 °C for 20 min. Finally, the absorbance was recorded at 560 nm and reported as ΔA 560 nm/min/mg protein.

Catalase (CAT)

Activity of CAT was determined by the decomposition amount of hydrogen peroxide (H_2O_2) (Wang *et al*., 2001). Fifty microlitre of enzyme solution was added into 500 µL H_2O_2 in PBS (1 %) and kept at 25 °C for 10 min. Activity of CAT was recorded as the ΔA at 240 nm/min/mg protein.

Peroxidase (POD)

As described by Addy and Goodman (1972), a reaction mixture was prepared containing buffered pyrogallol [80 µL, 50 mM pyrogallol in 100 mM phosphate buffer (pH 7.0)], solution of 1 % of H_2O_2 (80 µL) and enzyme solution (20 µL). Alteration in absorbance was read every 30 seconds at 430 nm for 2 min and reported as ΔA 430 nm/min/mg protein.

Ascorbate peroxidase (APOX)

The APOX activity was assayed based on the procedure of Asada (1984). The reaction mixture was

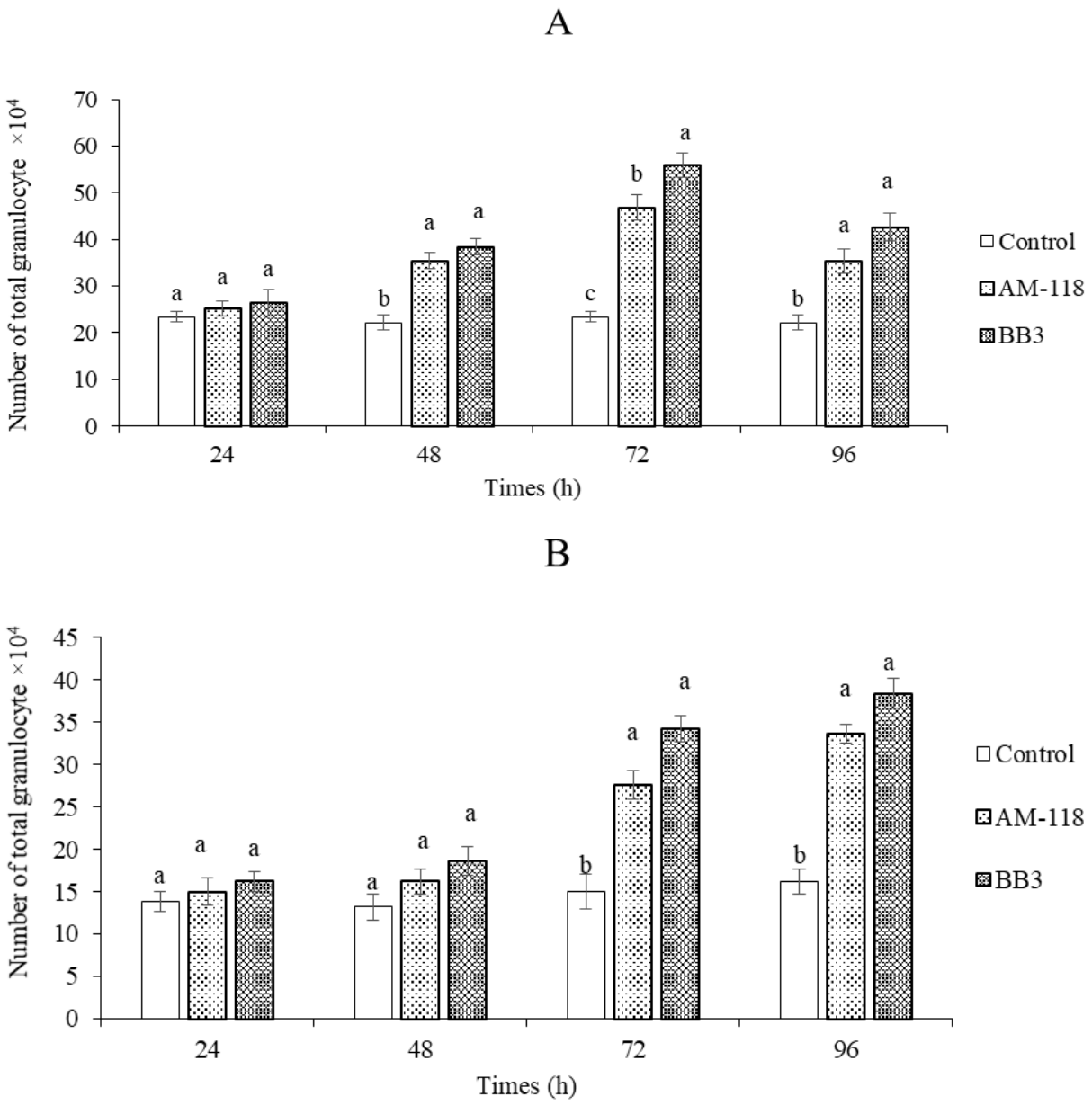

Fig. 2 Changes of granulocyte counts in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

200 µL of H_2O_2 (30 mM), 50 µL of enzyme solution, 70 µL of ascorbic acid (2.5 mM) and 150 µL of phosphate buffer (100 mM, pH 7.0). The absorbance was continually recorded for 5 min at 290 nm and reported as ΔA at 290 nm/min/mg protein.

Glucose-6-phosphate dehydrogenase (GPDH)

GPDH was assayed based on the method of Balinsky and Bernstein (1963) which 15 µL of $MgCl_2$ (100 mM), 70 µL of Tris-HCl (100 mM, pH 8) and 30 µL of NADP (0.2 mM) were poured together as the stock mixture. Then, 70 µL of GPDH (6 mM), 20 µL of distilled water and 20 µL of the enzyme were mixed with stock mixture before reading the absorbance changes at 340 nm. Activity was reported as ΔA at 340nm/min/mg protein.

Malondialdehyde content (MDA)

The amount of MDA was assayed based on the method of Bar-Or *et al*. (2001). A solution containing 20 µL of the sample solution and 80 µL of trichloroacetic acid (20 %) was mixed and centrifuged at 15000 g for 10 min at 4 °C. Afterward, supernatant was carefully added into 100 µL of 2-thiobarbituric acid solution (TBA, 0.8 %) and maintained for 60 min at 100 °C. Absorbance of the samples was measured at 535 nm. The molar extinction coefficient was 1.56×105 M^{-1} cm^{-1} to determine MDA concentration per mg protein.

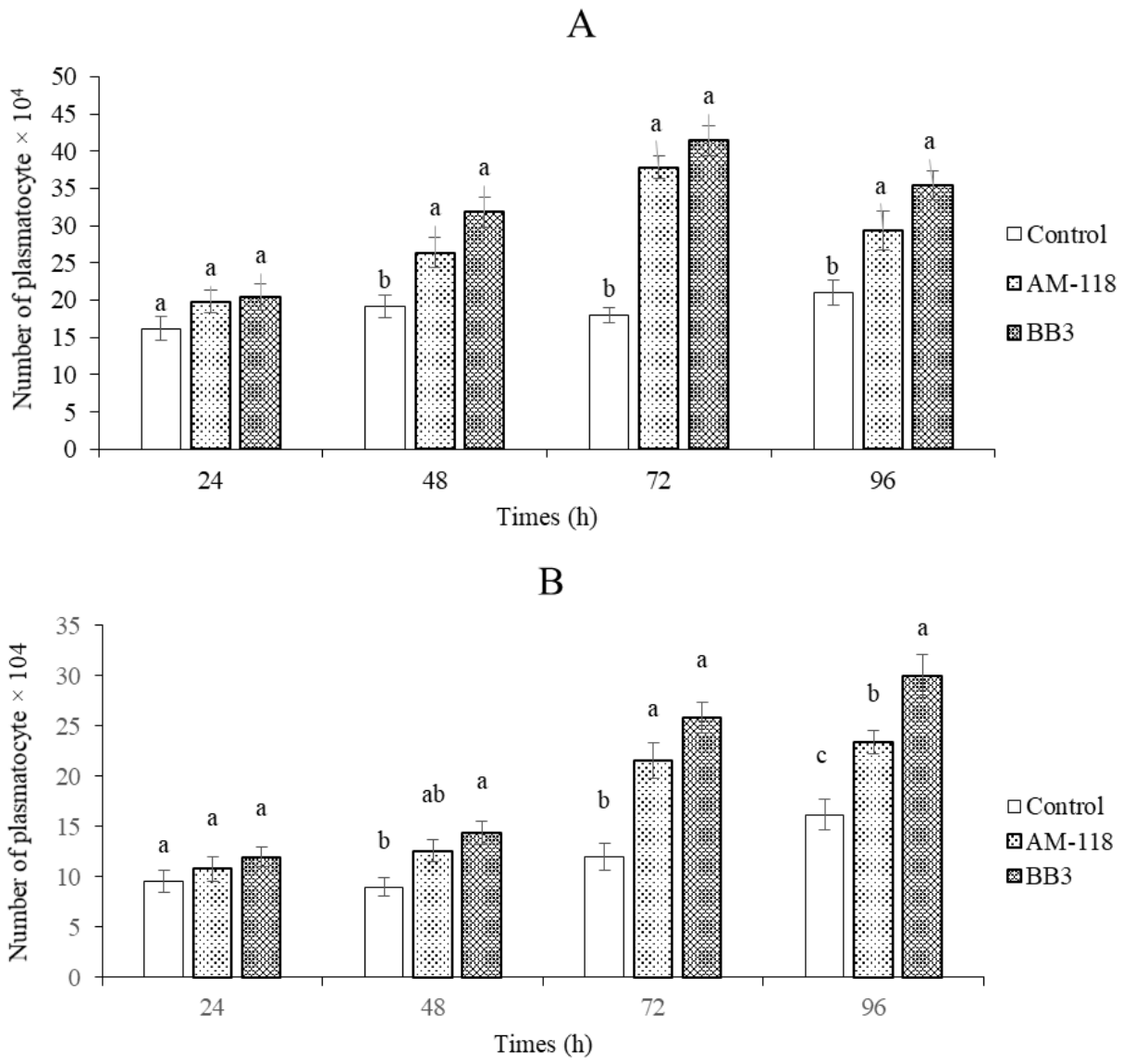

Fig. 3 Changes of plasmatocyte counts in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

Total protein content

The content of protein in the samples was determined by the Lowry *et al*. (1951) procedure. The reaction contained 20 µL of the sample mixed with 100 µL of reagent. The incubation was done for 30 min prior to read the absorbance at 545 nm.

Statistical analysis

Probit analysis calculated LC_{30}, LC_{50} and LT_{50} values at the corresponding 95 % confidence interval (CI) values using POLO-Plus software. A one-way analysis of variance (ANOVA) followed by Tukey's test was used to compare biochemical and immunological data. The statistical differences were marked by different letters at a probability less than 5 %.

Results

Bioassay

The effects of AM-118 and BB3 against the third instar larvae and the adults of *C. montrouzieri* have been shown in Tables 1 and 2. The LC_{30} and LC_{50} concentrations after larval treatment were 4×10^4 and 6.4×10^5 conidia/mL for AM-118 as well as 2×10^4 and 2.2×10^5 conidia/mL for BB3, respectively (Table 1). Moreover, the LC_{30} and LC_{50} values on the adults were obtained 1.3×10^5 and 2.6×10^6 conidia/mL for AM-118 as well as 8.1×10^4 and 1.2×10^6 conidia/mL for BB3, respectively (Table 1). The LT_{50} values of AM-118 and BB3 isolates were calculated 8.12 and 8.23 days on the larvae (Table 2) as well as 9.90 and 9.72 days on the adults, respectively (Table 2).

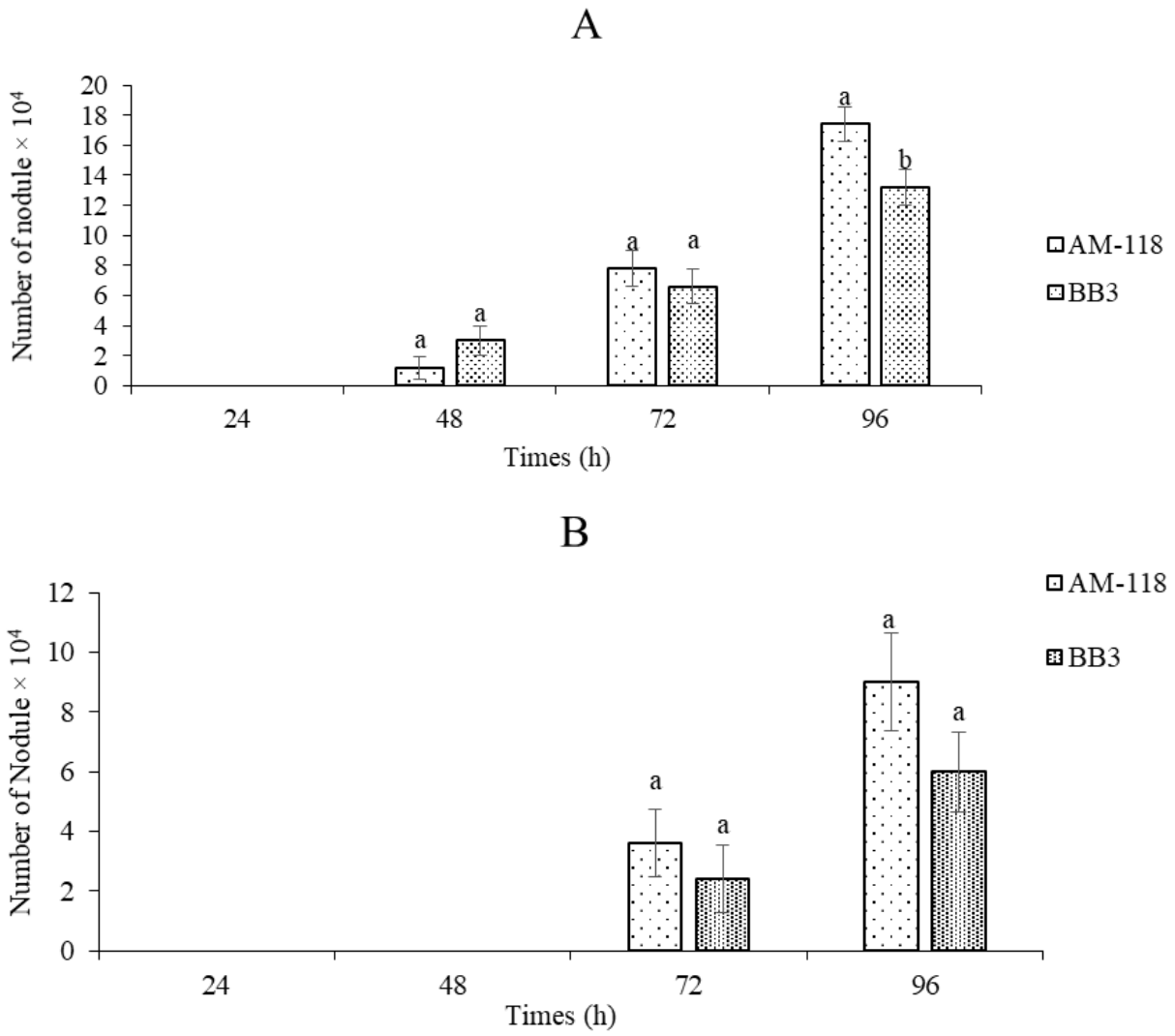

Fig. 4 Changes of nodule counts in *C. montrouzieri* infected by 1 × 10^5 conidium/mL of two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

Cellular immunity

The two native isolates of *B. bassiana* considerably increased the total and differential hemocyte counts, nodule formation and PO activity in the third instar larvae and the adults of *C. montrouzieri*. The total hemocyte count in the larvae were significantly enhanced at 48, 72 and 96 h of post-treatment (F = 25.38, dF = 4, P: 0.0001; F = 64.66, df = 4, P: 0.0001; F = 25.83, df = 4, P: 0.0001) while no significant difference were recorded at 24 h post-treatment (F = 2.34, dF = 4, P: 0.139) (Figure 1). The highest hemocyte counts were recorded after 72 and 96 h by treating BB3 conidia (Figure 1). Moreover, the number of total hemocytes in the adults significantly increased at 72 and 96 h post-treatment (F = 35.69, df = 4, P: 0.0001; F = 62.65, df = 4, P: 0.0001) although no significant different was observed between AM-118 and BB3 isolates (Figure 1). A considerable increase of granulocytes counts was observed following larval treatment after 48, 72 and 96 h (F = 26.34, dF = 4, P: 0.0001; F = 52.98, dF = 4, P: 0.0001; F = 18.39, dF = 4, P: 0.0001) (Figure 2). After 72 and 96 h, the numbers of granulocytes significantly elevated in the treated adults compared to control (F = 28.84, dF = 4, P: 0.0001; F = 61.46, dF = 4, P: 0.0001) (Figure 2). After 48, 72 and 96 h of exposures, the number of plasmatocytes at all treated larvae significantly increased compared to control (F = 11.48, dF = 4, P: 0.002; F = 66.15, dF = 4, P: 0.0001; F = 11.78, dF = 4, P: 0.001) but the number of plasmatocytes significantly increased after 72 and 96 h for the adults (F = 20.85, dF = 4, P: 0.0001; F = 17.64, dF = 4, P: 0.0001) (Figure 3). The larval treatment by AM-118 and BB3 significantly increased the number of nodules after 48, 72, and 96 h (F = 4.75, dF = 2, P: 0.030; F = 16.60, dF = 2, P: 0.0001; F = 91.60, dF = 2, Pr>F: 0.0001) although the adults showed the highest number of nodules for all time intervals (F = 4.0, dF = 2, P: 0.047; F = 14.0, dF = 2, P: 0.001) (Figure 4). In both larvae and adults, the highest number of nodules was recorded after 96 h following fungal treatments (Figure 4).

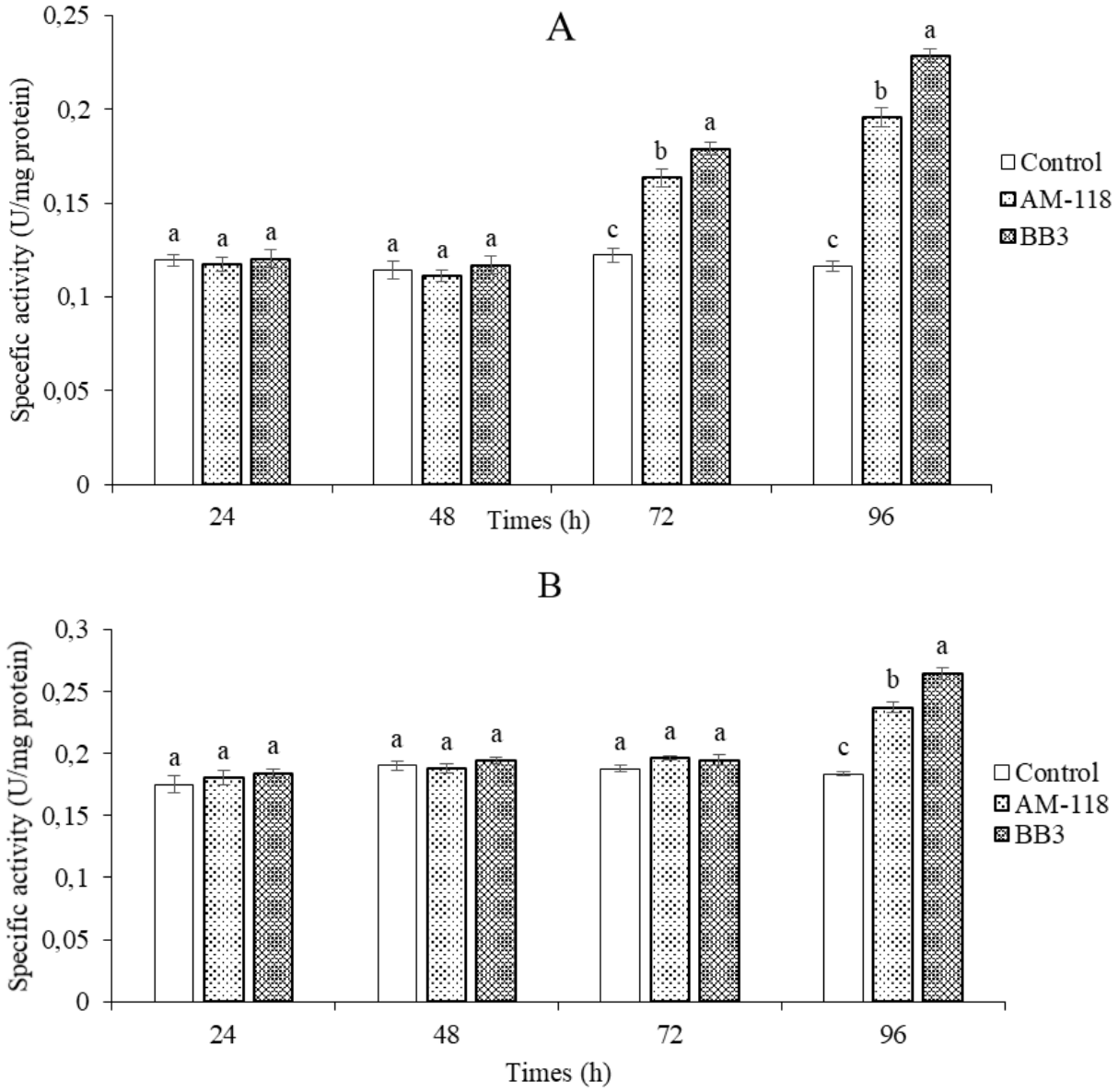

Fig. 5 Changes of phenoloxidase activity in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

Phenoloxidase activity

The activity of PO in the larvae treated by both isolates significantly increased at 72 and 96 h of post-treatment with the highest activity by BB3 treatment after 96 h (F = 54.74, dF = 4, P: 0.0001; F = 215.21, dF = 4, P: 0.0001) (Figure 5). Similarly, the treated adults by both isolates demonstrated the highest PO activity after 96 h (F = 124.61, dF = 4, P: 0.0001) (Figure 5).

Antioxidant components

SOD activity in the larvae and the adults treated by AM-118 and BB3 significantly induced after 72 and 96 h (F = 55.27, dF = 4, P: 0.0001; F = 70.55, dF = 4, P: 0.0001) while no significant difference was recorded among the isolates and the control after 24 and 48 h (F = 0.09, dF = 4, P: 0.917; F = 0.42, dF = 4, P: 0.664) (Figure 6). Similar results obtained for catalase activity (Figure 7). After 72 and 96 h, a significant increase of catalase activity (F = 26.94, dF = 4, P: 0.0001; F = 66.95, dF = 4, P: 0.0001; F = 8.62, dF = 4, P: 0.005; F = 23.84, dF = 4, P: 0.0001) observed in the treated individuals (Figure 7). Moreover, the POD activity of the larvae (F = 49.43, dF = 4, P: 0.0001; F = 39.39, dF = 4, P: 0.0001) and the adults significantly increased after 72 and 96 h although the highest POD activity was recorded in the treated individuals by BB3 (F = 9.93, dF = 4, P: 0.003; F = 25.43, dF = 4, P: 0.0001) (Figure 8).

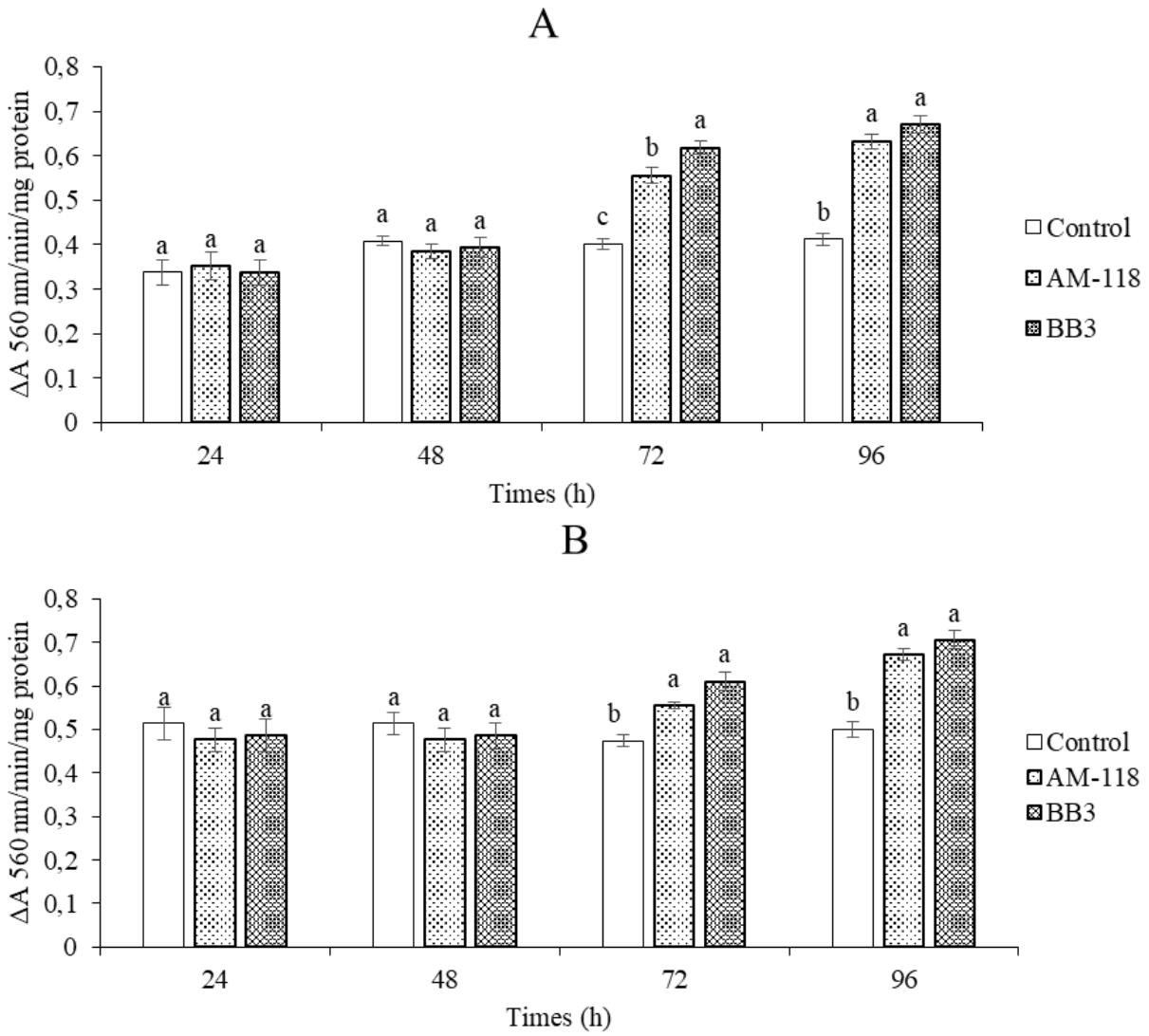

Fig. 6 Changes of superoxide dismutase activity in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

APOD activity in the larvae treated by the isolates significantly increased after 96 h (F = 47.92, dF = 4, P: 0.0001) (Figure 9). Similarly, the adults treated by both isolates showed the highest APOD activity at 96 h post-treatment compared to control (F = 25.43, dF = 4, P: 0.0001) (Figure 9). GPDH demonstrated the highest activity in the treated larvae and adults compared to control after 96 h (F = 215.21, dF = 4, P: 0.0001) (Figure 10) but the statistically highest activity between isolates was recorded in the larvae and the treated by BB3 (F = 122.34, dF = 4, P: 0.0001) (Figure 10). The exposure of AM-118 and BB3 led to the highest content of MDA of the larvae and the adults after 72 and 96 h (F = 47.01, dF = 4, P: 0.0001; F = 132.70, dF = 4, P: 0.0001) (F = 70.74, dF = 4, P: 0.0001; F = 53.53, dF = 4, P: 0.0001), respectively (Figure 11).

Discussion

In the current study, both native isolates of *B. bassiana* demonstrated virulence on the larvae and the adults of *C. montrouzieri*, although the results highlighted the higher virulence BB3 than AM-118 with lower LC_{50} and LT_{50} values. Different virulence of the isolates may be attributed to the initial host and the origin of their collection. BB3 was collected from soil where it was associated with many organisms, while AM-118 was cultured from body mycelium of rice stem stripped borer. Therefore, BB3 may develop more mechanisms to cause pathogenicity on hosts while it seems AM-18 may more specialized to narrow host specificity. Moreover, both isolates were capable to kill the larvae with a lower conidia concentration and in a shorter time compared to the adults. Less

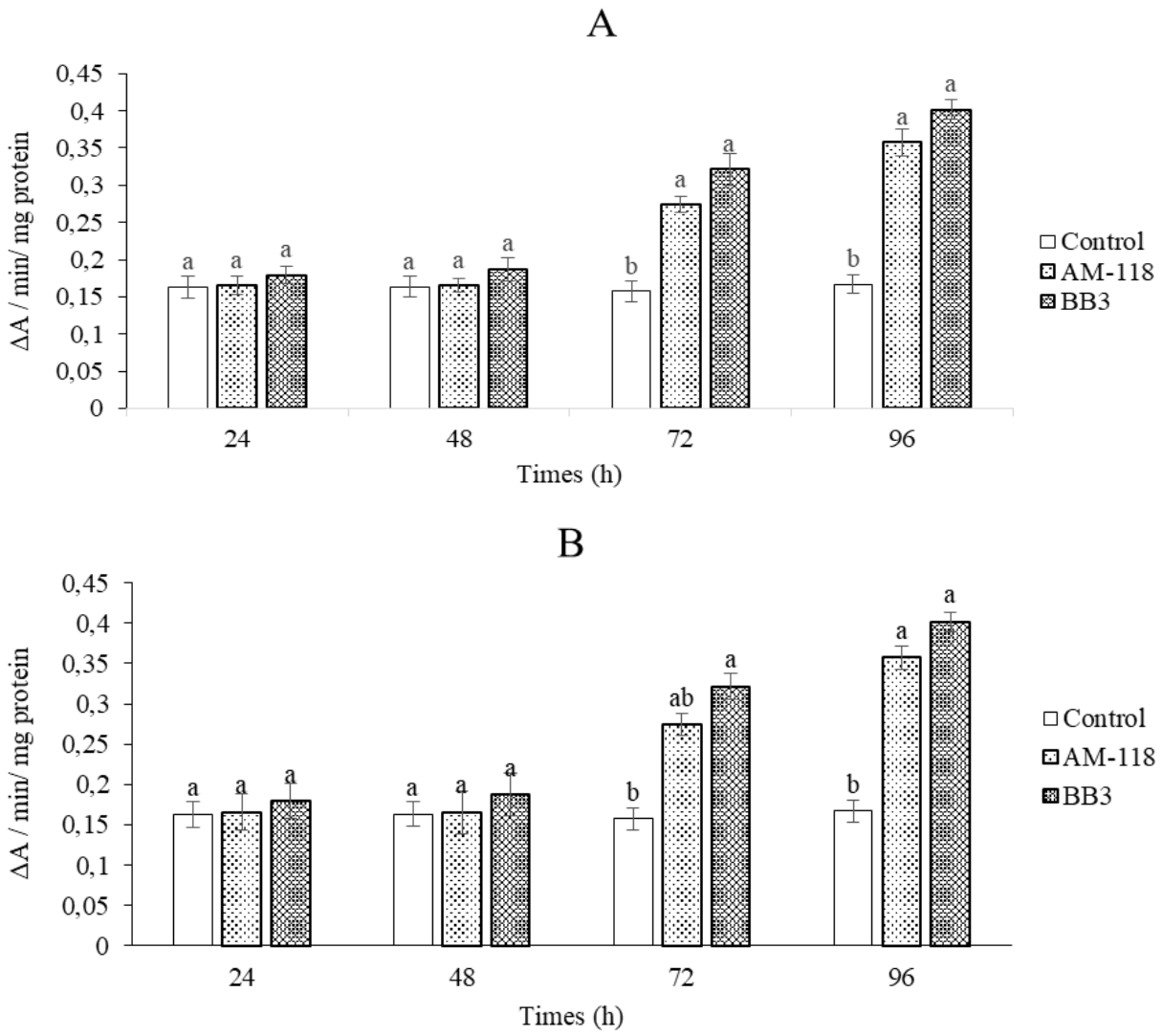

Fig. 7 Changes of catalase activity in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

susceptibility of the adults compared to larvae can be correlated with the hard covering of their cuticles and the slower penetration of conidia into the body. Maqsoudi *et al*. (2018) reported that AM-118 and BB3 were lethal on the tea mealybug, *P. viburni*, by the LC_{50} values of 1.8×10^5 and 2×10^3 conidia/mL, while the LT_{50} of the isolates were obtained to be 6.63 and 3.66 days, respectively. So it may be mentioned BB3 as the more virulent isolate on both tea mealy bug and *C. montrouzieri*. Several studies have shown virulence of the numerous isolates of entomopathogenic fungi against coccinellide predators although the displayed differences in the virulence depend on the lethal concentrations and the time of exposure (Thungrabeab and Tongma, 2007; Er *et al*., 2008; Ibrahim *et al*., 2011; Scorsetti *et al*., 2012; Trizelia *et al*., 2017).

Insect immunity is a fundamental process to protect insects against entomopathogens (Lavine and Strand, 2002). Previous investigations demonstrated that hemocytes have the major roles in immune responses of insects to entomopathogenic fungi (Russo *et al*., 2001; Zibaee *et al*., 2014; Aghaee Pour *et al*., 2021; Shahriari *et al*., 2021b). Cellular immune reactions depend on circulating hemocytes such as prohemocyte, granulocyte and plasmatocyte (Zibaee *et al*., 2014). Granulocytes and plasmatocytes are the two important hemocyte involved in immune reactions through phagocytosis, nodule formation, and encapsulation to ensnare and kill conidia of pathogens (Borges *et al*., 2008). Nodulation occurs rapidly after microbial infection by micro-aggregation of hemocytes and assembling of additional hemocytes (Zibaee *et al*., 2014; Aghaee Pour *et al*., 2021; Shahriari *et al*., 2021b). In the current study, the numbers of total hemocytes, plasmatocytes, granulocytes, and nodules increased in the larvae

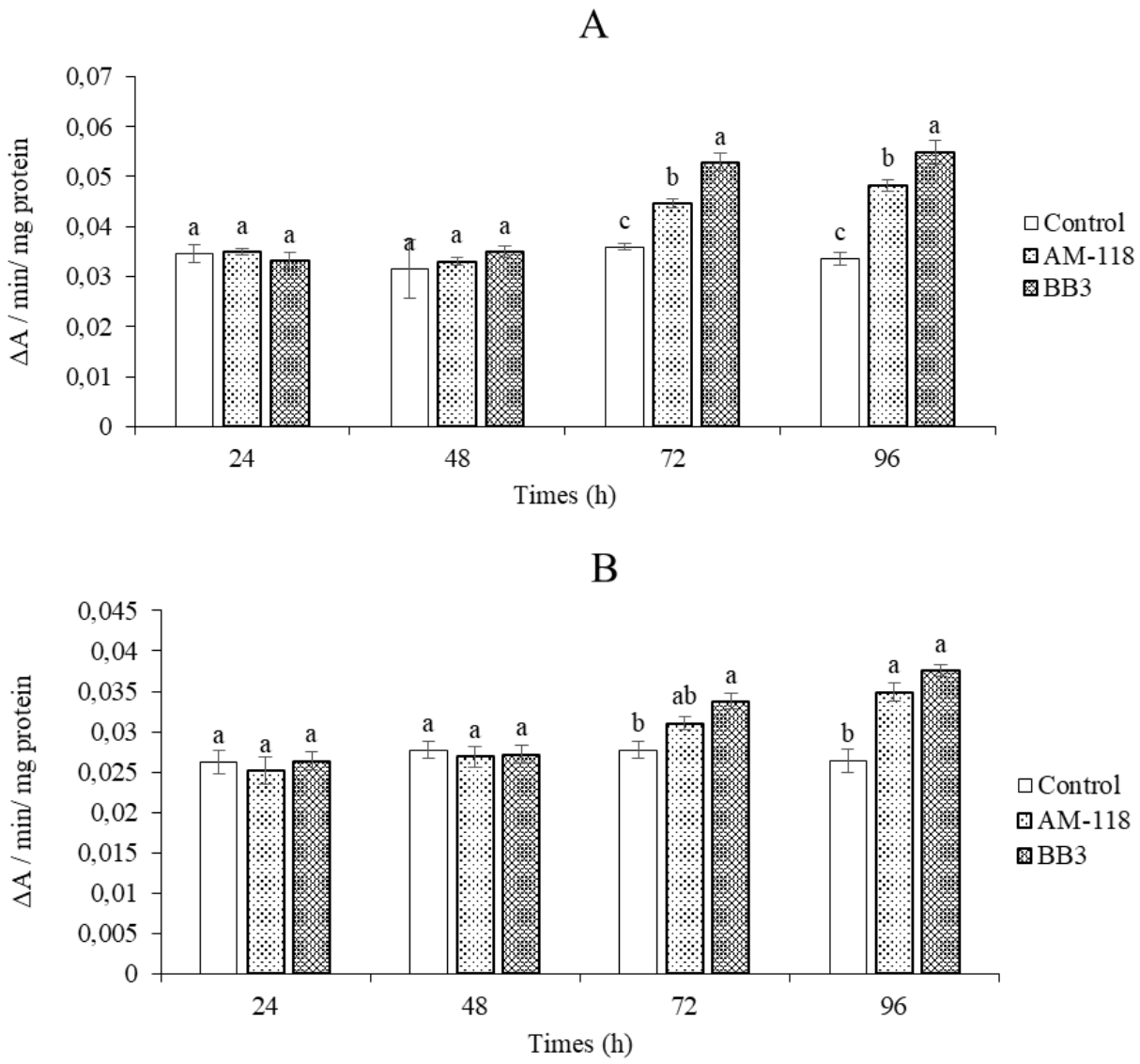

Fig. 8 Changes of proxidase activity in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p<0.05$ in each time interval

and the adults of *C. montrouzieri* after fungal exposure and time intervals of 48, 72 and 96 h, while no significant difference was recorded between treatments and control after 24 h. Although, both isolates caused some fluctuations in the total and differential hemocyte counts, the highest elevation in the hemocyte counts was determined at 72 and 96 h post-treatment in the larvae and the adults, respectively. There were no-significant differences in the hemocyte counts between treatments and control after 24 h which may be attributed to the lack of pathogen entrance into the hemolymph. The subsequent increase in hemocyte counts reported in our study may result from recognizing the conidia in hemolymph, inducing hematopoietic organs to produce more prohemocytes and to trigger the hemocyte adherence on body mass (Zibaee *et al*., 2014; Shahriari *et al*., 2021b). Several studies revealed a direct correlation between entomopathogen infections and number of hemocytes in insects. For example, the increase of hemocyte and nodule counts under mycoses was reported in the *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) when injected by *B. bassiana* and *Metarhizium anisopliae* (Mirhaghparast *et al*., 2013), in addition to *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) infected by *B. bassiana* (AM-118) (Aghaee Pour *et al*., 2021). Meshrif *et al*. (2011) demonstrated no significant alteration in the number of hemocytes after *S. littoralis* larvae treatment by *B. bassiana* until 48 h post treatment although however an increase was observed after 70 h.

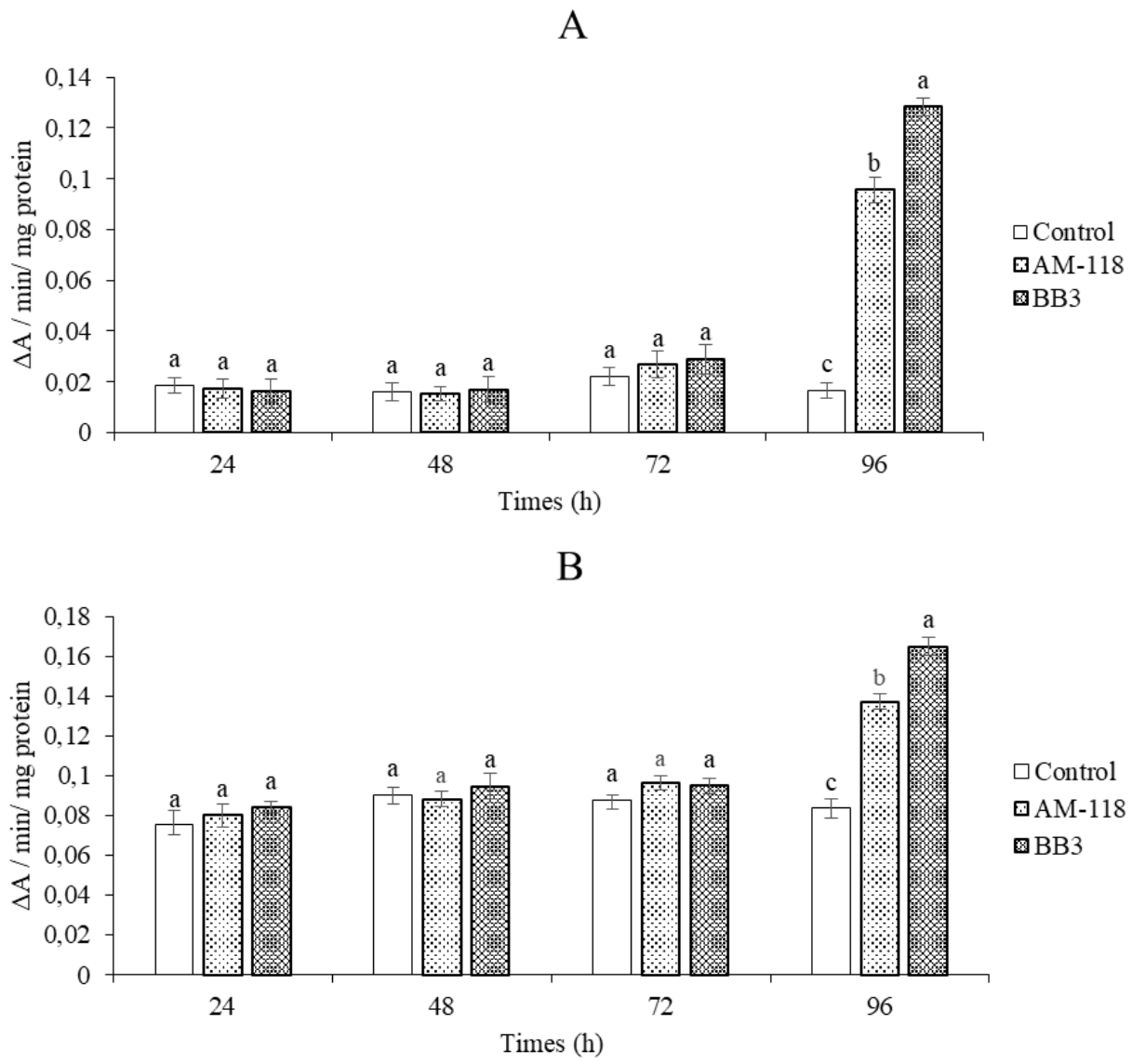

Fig. 9 Changes of glucos-6-phosphate dehydrogenase activity in *C. montrouzieri* infected by 1 × 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

Phenoloxidase is one of the important components in immune system of insects that plays a significant role in the conversion of phenols to quinones, and subsequently to melanin (Gorman *et al*., 2008). In insects, melanin has a key role in cuticle sclerotization, wound healing, coagulation process of hemolymph (Zdybicka-Barabas *et al*., 2014). The higher PO activity was observed in the hemolymph of the individuals treated with both fungal isolates after 72 and 96 h. Since PO is produced and stored within hemocytes (Mak and Saunders, 2006), increased level of PO activity may be attributed to the higher hemocyte counts during fungal treatment as observed in our research. Several studies have been reported an increment of hemocyte counts leading to the higher activity of PO in insects (Gillespie *et al*., 2000; Mirhaghparast *et al*., 2013; Zibaee *et al*., 2014; Shahriari *et al*., 2021b). The higher PO activity increases coagulation and melanization of hemolymph that subsequently causes the higher resistance of insects to entomopathogens mainly due to toxicant effects through generation of the toxic free radical against invading agents (Leger *et al*., 1988).

Secondary metabolites produced by entomopathogens have been recognized as the one of main exogenous resources that lead to production free radicals after tissue afflictions which imposes oxidative stress (Lukasik, 2007; Dubovskiy *et al*., 2008; Jia *et al*., 2016; Karthi *et al*., 2018; Shamakhi *et al*., 2020). Oxidative stress eventually leads to peroxidation of lipid and DNA damage (Dubovskiy *et al*., 2008). CAT, SOD and POD are the major antioxidant enzymes to protect insect

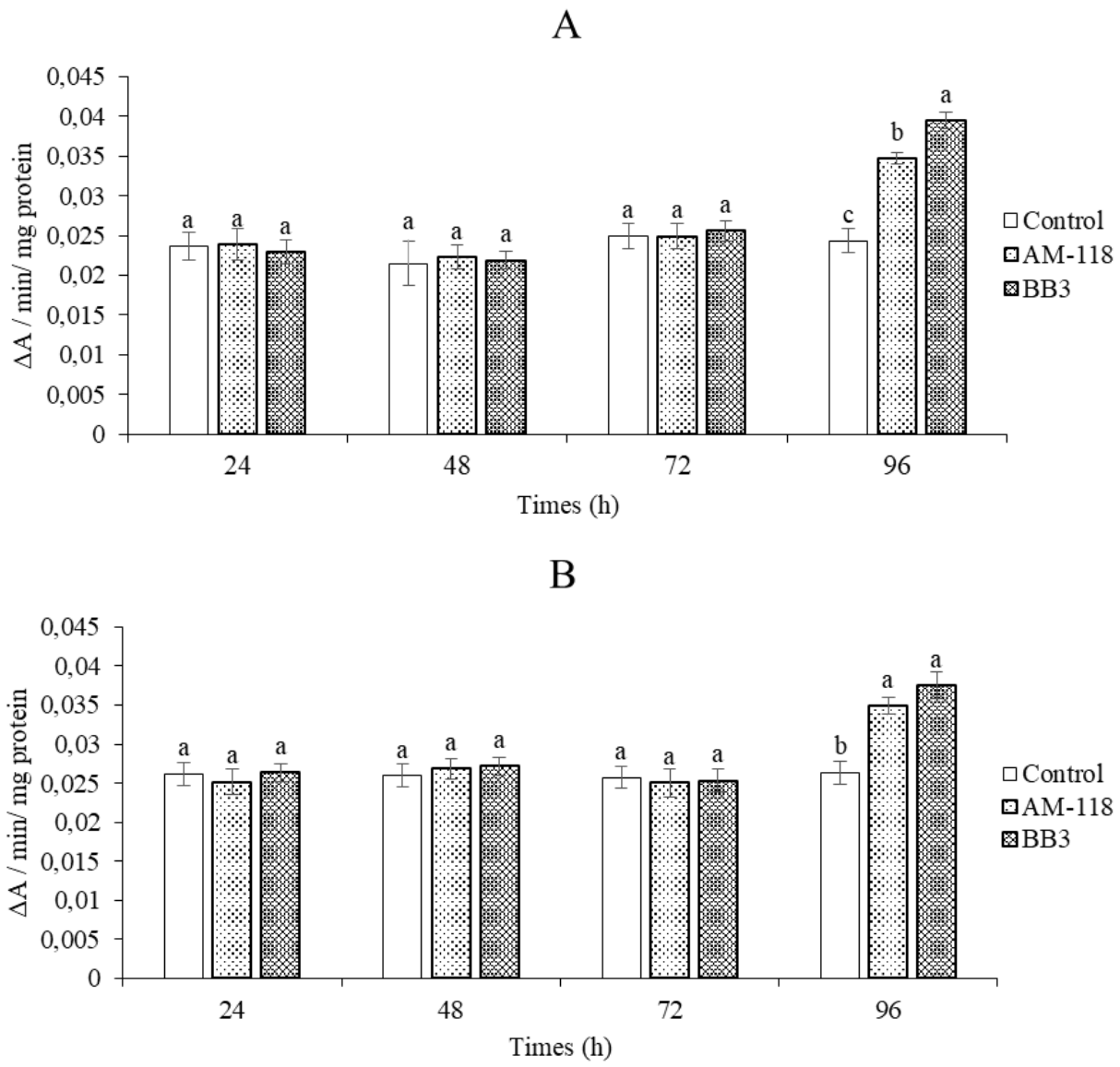

Fig. 10 Changes of ascorbat proxidase activity in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

tissues against oxidative stress. SOD has a key role in catalyzing dismutation of toxic superoxide radicals into hydrogen peroxide and oxygen. Afterward, hydrogen peroxide is converted to H_2O and oxygen by CAT and POD (Dubovskiy *et al*., 2008). In the present study, the activities of SOD, CAT and POD significantly induced in the larvae and the adults treated by AM-118 and BB3 isolates compared to control. Similar results were obtained regarding the effects of *M. anisopliae* on *Locusta migratoria* (Meyen) (Orthoptera: Acrididae) (Jia *et al*., 2016). Shamakhi *et al*. (2020) demonstrated that infection of *C. suppressalis* by *B. bassiana* caused the higher activities of SOD, CAT, and POD. Karthi *et al*. (2018) revealed that *Aspergillus flavus* caused the higher activities of SOD, CAT, and POD in the third instar larvae of *S. litura*. Similarly, our findings revealed the higher activities of SOD, CAT and POD in the larvae and the adults of *C. montrouzieri* infected by the conidia of pathogenic fungi, which may be connected with the higher generation of ROS.

Glucose-6-phosphate dehydrogenase (GPDH) and ascorbate peroxide (APOD) are the two antioxidant components which involved in detoxification of pro-oxidant compounds in different tissues (Asada, 1984). APOD removes hydrogen peroxide in cytoplasm, chloroplasts, and mitochondria, while GPDH engaged in eliminating oxidant compounds within cytosol through transduction of NADPH to NADP+ (Asada, 1984; Nation, 2008). In our study, the higher activities of

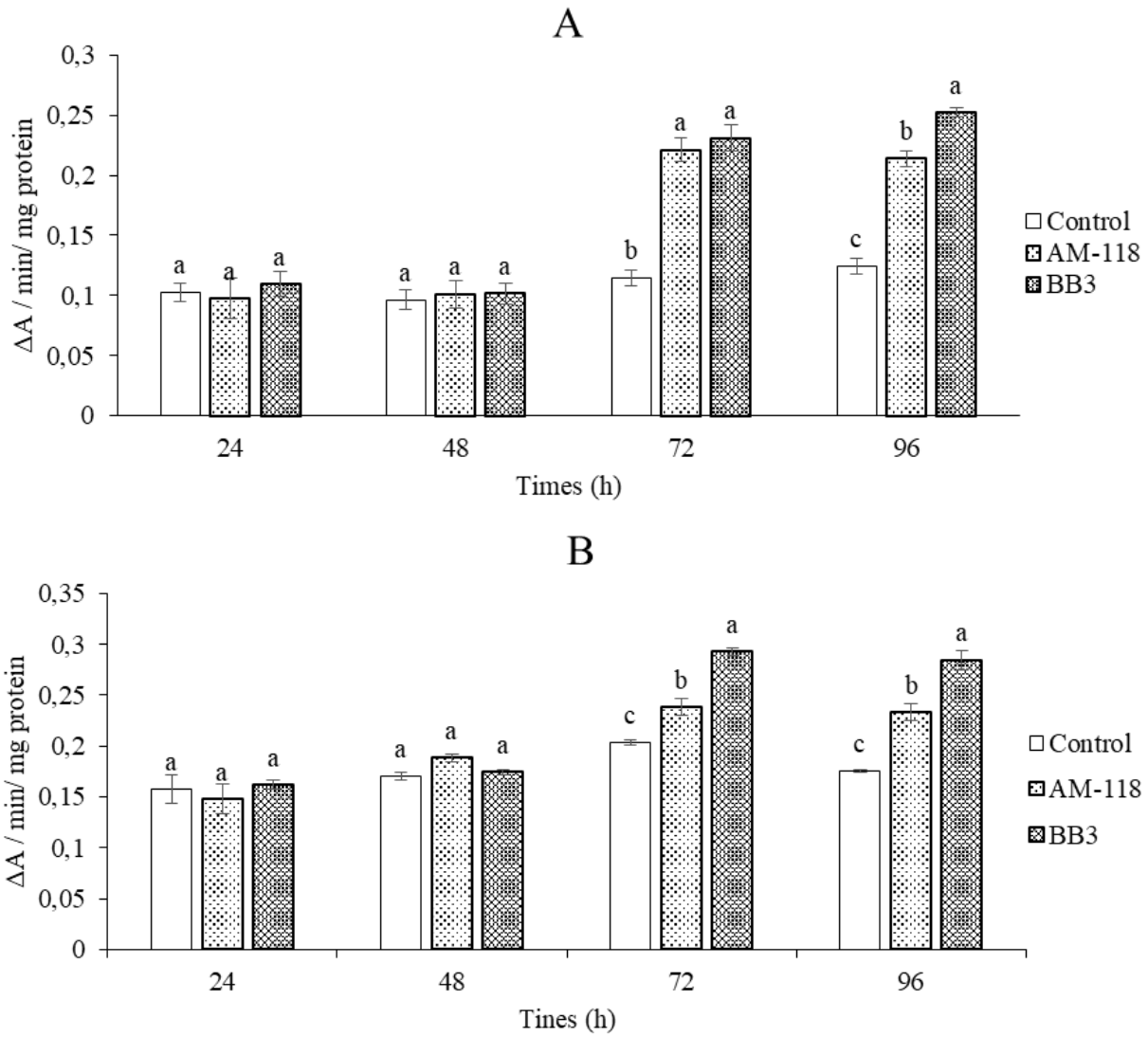

Fig. 11 Changes of malondialdehyde content in *C. montrouzieri* infected by 10^5 conidium/mL of the two isolates of *B. bassiana* (AM-118 and BB3), larvae (A), adults (B). Different letters indicate significance at $p < 0.05$ in each time interval

APOX and GPDH found in the larvae and the adults exposed to conidia of AM-118 and BB3 isolates. A similar result was observed in APOX and GPDH activities when *C. suppressalis* were injected by *B. bassiana* conidia so fungi may act as stress factors to induce generation of ROSs in the treated insects (Shamakhi *et al*., 2020). Dubovskiy *et al*. (2008) reported the significant higher activities of SOD and CAT in *G. mellonella* exposed to *Bacillus thuringiensis*. The higher GPDH activity caused enhancement of NADPH generation to eliminate products of activity of APOX. Moreover, NADPH decreases possible virulent effects through shifting electrons to free radicals (Barbehenn, 2002; Dubovskiy *et al*., 2008). Lipid peroxidation is another marker indicating ROSs damages to cellular membranes. MDA have been known as a significant by-product of lipid peroxidation so its content demonstrates destruction and elevation of cell membrane dissociation (Dubovskiy *et al*., 2008). Our results indicated that lipid peroxidation was higher in the larvae and the adults exposed to AM-118 and BB3 than in control. Our results are similar to previous findings like *S. littura* infection to *A. flavus* (Karthi *et al*., 2018) and *C. suppressalis* to *B. bassiana* (Shamakhi *et al*., 2020).

Conclusions

In a successful biocontrol program, it is very crucial to recognize the indirect effects of biopesticides on natural enemies like predators because such a program requires biopesticides with low toxicity to natural enemies. The current study revealed the direct and indirect effects of the two isolates of entomopathogenic fungi, *B. bassiana* (AM-118 and BB3) on *C. montrouzieri* through mortality on the larvae and the adults as well as on

some physiological processes. Overall, bioassay results demonstrated that AM-118 and BB3 isolates were less virulent to *C. montrouzieri* than to mealybugs and more specifically, AM-118 has a lesser virulence than BB3. Moreover, both larvae and adults were able to induce immune and antioxidant systems to protect themselves against infective conidia. Therefore, it may be concluded that AM-118 may be a better candidate to be used along with *C. montrouzieri* in integrative management of tea mealybug.

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