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RESEARCH REPORT

Enhanced activities of acetylcholinesterase, acid and alkaline phosphatases in *Helicoverpa armigera* after exposure to entomopathogenic fungi

M Bilal, S Freed, M Zubair Ashraf, S Muhammad

Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan

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Abstract

The toxicity of entomopathogenic fungi, *Metarhizium anisopliae* (isolates Ma-11.1 and Ma-4.1), *Isaria fumosorosea* (isolates If-02 and If-2.3) and *Beauveria bassiana* (isolates Bb-01 and Bb-08) to *Helicoverpa armigera* was assessed by measuring the activity of acetylcholinesterase (AChE) and acid/alkaline phosphatases after 48 h of exposure till 168 h. The results showed high AChE activity in the hemolymph, intestine and fat bodies samples of *H. armigera* exposed to higher concentrations of I*. fumosorosea* (If-02 and If-2.3), *M. anisopliae* (Ma-4.1), *B. bassiana* (Bb-08) between 72 -168h at 7×10^8 spores/mL concentration, while *M. anisopliae* (Ma-4.1) exhibited maximum acid phosphatases (ACP) activity at similar concentration in the hemolymph and intestine samples with similar duration of exposure. On the other hand, isolates If-2.3, Ma-4.1 and Bb-08 showed maximum ACP activity during the same exposure duration. The results showed similar high alkaline phosphatases (ALP) activity in all body samples of *H. armigera* when treated with Ma-4.1 (7×10^8 spores/mL), while, isolate Bb-08 at similar concentration showed maximum ALP activity after 72 and 120 h of exposure. The increased production of these detoxification enzymes in this economic insect pest can possibly lead to the development of resistance against entomopathogenic fungi.

Key Words: acetylcholinesterase; acid phosphatases; alkaline phosphatases; body tissues; *Helicoverpa armigera*; hemolymph

Introduction

Helicoverpa armigera (Lepidoptera: Noctuidae) generally known as American bollworm or cotton bollworm is a serious noctuid pest which causes severe economic losses to agricultural crops (*Talekar et al.,* 2006). About 180 crop species including sorghum (*Sorghum bicolor*), tomato (*Lycopersicon esculentum*), corn (*Zea mays*), cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*), sunflower (*Helianthus annuus*) and soybean (*Glycine max*) are attacked by this pest (Czepak *et al.,* 2013). The infestation of *H. armigera* is mainly controlled by the use of insecticides and about 30 % of chemicals used all over the world are being applied against *H. armigera*, which becomes the major cause of resistance to insecticides and *Bacillus thuringiensis* endotoxins(Ahmad *et al.,* 1995; Akhurst *et al.,* 2003; Gao *et al.,* 2009).

Corresponding author:
Shoaib Freed
Department of Entomology
Faculty of Agricultural Sciences and Technology
Bahauddin Zakariya University
Multan, Pakistan
E-mail: sfareed@bzu.edu.pk

Entomopathogenic fungi (EPF) are mostly used for the control of several insect pests such as *Galleria mellonella* and *Myzus persicae* (Yang *et al.,* 2014). Approximately 1000 species of insect pathogenic fungi are known to cause death in pests. Most common EPF are *Metarhizium anisopliae*, *Beauveria brongniartii*, *Isaria fumosorosea*, and *Beauveria bassiana* (de Faria and Wraight, 2007). These fungi are the environment friendly alternatives to majority of insecticides being applied in the field against insect pests (Federici *et al.,* 2008). Insects normally defend themselves from xenobiotics by producing certain detoxification enzymes such glutathione S-transferases (GST), esterase, acid and alkaline phosphatases which are commonly present in the insects for their defense (Serebrov *et al.,* 2006; Zibaee *et al.,* 2009).

In insects acetylcholinesterase (AChE) which catalyzes the hydrolysis of acetylcholine, a neurotransmitter conducts messages in the nerve impulses throughout the synapses (Wang *et al.,* 2004). In many insect species the alteration of AChE is also a cause of resistance against insecticides belonging to carbamates and organophosphates. The information about these enzymes in evaluating the

comprehensive effect of insect pathogenic during exposure is not much explored. The objective of the current work was to evaluate the effect of entomopathogenic fungi on detoxification enzymes activities in insects as well as the role of these enzymes in the development of resistance to EPF.

Materials and Methods

Helicoverpa armigera rearing

Helicoverpa armigera larvae collected from fields were reared on fresh *Brassica oleracea* var. *botrytis* leaves individually in (5cm) petri dishes. Uneaten leaves and feces were cleaned and leaves were changed daily. The pupae were placed in plastic cages (30×30×30cm) for the emergence of moths. The newly emerged male and female adults 5:5 were shifted in plastic jars for egg laying with provision of 10 % sugar solution soaked in cotton swab. The 3rd instar larvae of F_1 generation were used for the treatment.

Entomopathogenic fungi

The already maintained laboratory culture of entomopathogenic fungi *i.e.*, *B. bassiana* (Bb-01, 08), *M. anisopliae* (Ma-11.1, 4.1) and *I. fumosorosea* (If-02, 2.3) were further propagated by the method of Freed *et al.* (2012). The spores of all isolates were individually collected in 0.1 % Tween-80 solution and final concentration was determined by hemocytometer. The stock solutions of different isolates were serially diluted to obtain the desired concentrations for bioassay.

Insect pathogenic fungi exposure

Experiments with the selected EPF consisted of six treatments including a control. There were four replicates for each treatment. Third instar larvae of *H. armigera* were immersed individually in various mycological concentrations 7×10^8 spores/mL (highest concentration) followed by 6×10^8, 5×10^8, 4×10^8, 3×10^8 and 2×10^8 spores/mL (lowest concentration) for twenty seconds. Each fungal isolate was used in same high and low concentration to observe the efficiency against *H. armigera*. Larvae of control set were treated with distilled water containing 0.1 % Tween 80 only. The extra moisture of treated larvae was soaked on tissue paper. The individual larva was placed in plastic petri dish having cauliflower leaves. The treated larvae were kept under temperature 26 ± 2 ºC and humidity 75 ± 5 % maintained for complete treatment. Forty larvae were used in each treatment and half of the alive larvae from each treatment were sampled for the enzyme activity analysis.

Sample preparation for enzyme activity analysis

The activities of detoxification enzymes in *H. armigera* samples were evaluated according to methods of Serebrov *et al.* (2006) with slight changes. The hemolymph was collected into Eppendorf tubes by making a small cut on the mid abdominal prolegs and few crystals of ascorbic acid were added to avoid coagulation. In about 100 µL of 0.15 M NaCl, intestine and fat bodies free from debris were put in Eppendorf tubes for crushing and later the final volume was adjusted to 400 µL per sample for centrifugation. The body tissue samples were centrifuged at 3,000 and 1,500 rpm for 10 min (5 ± 1 °C). The supernatants were used for determining enzyme activity and the samples were made for three durations (72, 120 and 168 h) after 48 h of treatment to allow EPF infection in the treated *H. armigera* larvae.

Determination of protein concentrations in samples

Bradford (1976) assay was used for the determination of protein in hemolymph and body tissues homogenates by using bovine serum albumin as standard on a spectrophotometer (UV3000, O.R.I. Germany).

Acetylcholinesterase (AChE) assay

The activity of acetylcholinesterase was measured as explained by Ellman *et al.* (1961) using 0.075M acetylcholine iodide as substrate. The variation in absorbance at λ of 412 nm was recorded for 4 min.

Alkaline and acid phosphatases assay

The alkaline and acid phosphatases activities were determined by the method of Serebrov *et al.* (2006), while 0.23 mM 4-Nitrophenyl disodium orthophosphate was used as substrate. At 30 ºC the samples were incubated for 2 h and 400 µL of 0.05 M NaOH was added for color development. The change in absorbance at λ of 410nm was noted.

Statistical analysis

All statistical analyses were performed with the statistical software Statistix (version 8.1) and Microsoft Excel 2010.The activity levels of enzymes were measured and the graphs were plotted, while differences between treatments were compared using Least Significant Difference (LSD) Test.

Results

The exposure of the different concentrations of EPF caused mortalities of *H. armigera* larvae (un published data). In this study it was observed that AChE, ACP and ALP activities in the tested body tissues of *H. armigera* were positively correlated with increasing concentrations and the fungal isolates.

Acetylcholinesterase (AChE) activity

Acetylcholinesterse activity was observed in hemolymph, intestine and fat body samples of *H. armigera* treated with different isolates of EPF on all three days post infection. The results showed that significant AChE activity was observed in hemolymph samples (72-168h), when treated with Ma-4.1 and If-02 at higher concentration of 7×10^8 spores/mL (Figs1a-c) as compared to the control.

The data showed considerable changes in AChE activities in Ma-4.1 and If-2.3 treated intestine samples (72 and 120 h), whereas similar trend was observed in 168 h samples, which depicted isolate Ma-4.1 to enhance the enzyme activity at concentrations 7×10^8 and 5×10^8 spores/mL (Figs 2a-c). As far as, the enzyme activity in the fat bodies treated samples is concerned, statistically significant results were recorded for all three days with different isolates (Figs 3a-c).

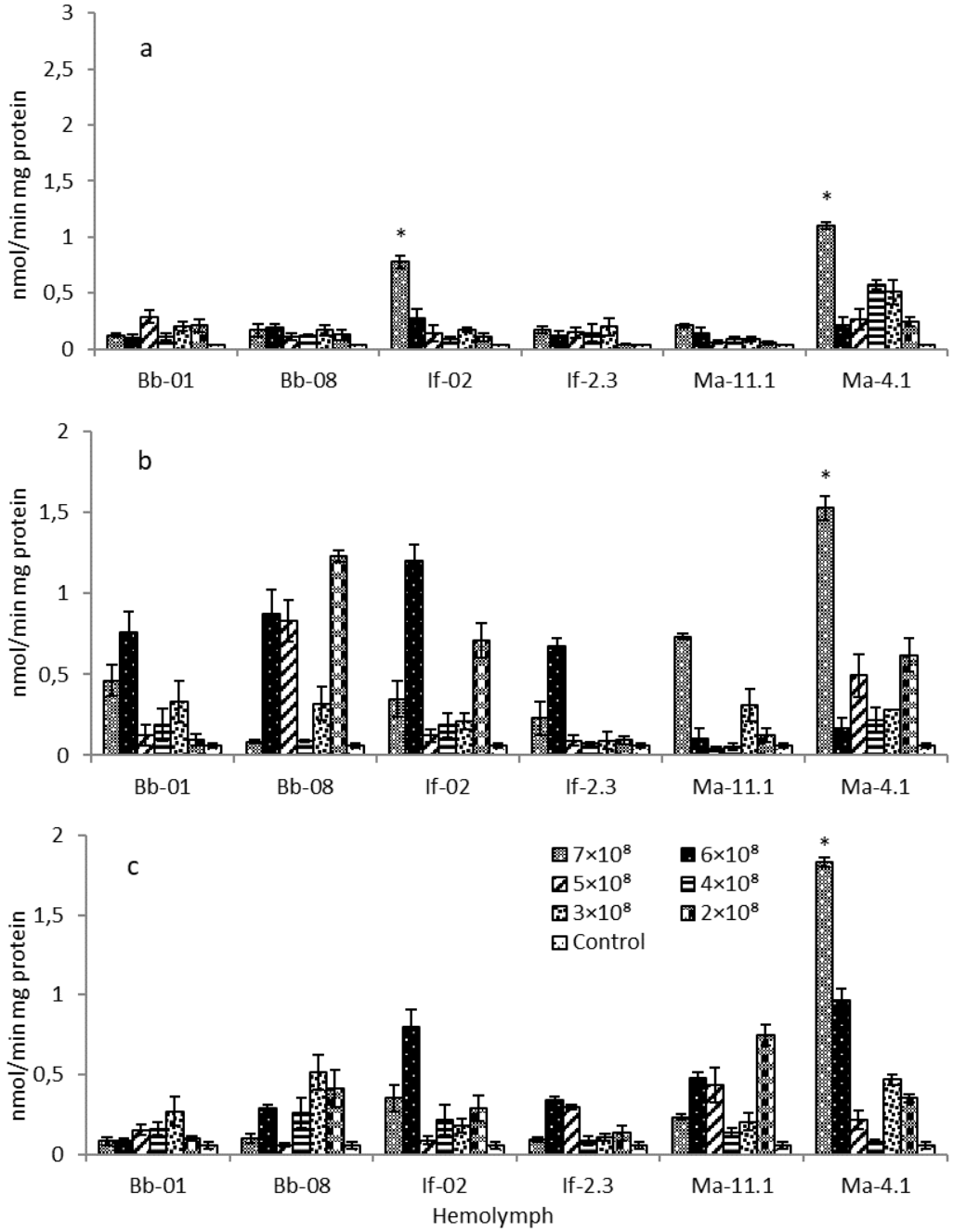

Fig. 1 Activity of AChE enzyme in hemolymph samples of *H. armigera* on (a) 72h (b) 120h (c) 168h after treated with *M. anisopliae* (Ma-11.1, 4.1), *I. fumosorosea* (If-02, 2.3) and *B. bassiana* (Bb-01, 08). *represents significant difference between treatments at 0.05 % level of significance.

Acid phosphatases (ACP) activity

Acid phosphatases activity was noted in hemolymph and body homogenates samples of *H. armigera* on all three days post infection. At higher concentration of 7×10^8 spores/mL, maximum significant activity was observed in Ma-4.1 treated 72 - 168 h samples, while statistically significant results were recorded in hemolymph samples at concentration of 4×10^8 and 2×10^8 spores/mL after treated with Bb-08 (Figs 4a, b) however, no significant activity was observed on 7th day (Fig. 4c).

In case of intestine samples, the maximum changes in ACP activity were observed in all three samples (72 - 168 h) of Ma-4.1 at concentrations 7×10^8, 5×10^8 and 2×10^8 spores/mL, while significant results were also noted in Bb-08 (7×10^8 and 2×10^8 spores/mL) treated insect samples after 72 and 120 h of infection (Figs 5a-c). Similarly, statistically significant variations in ACP activities were also recorded for all sampling durations after treatment with various isolates as compared to the control (Figs 6a-c).

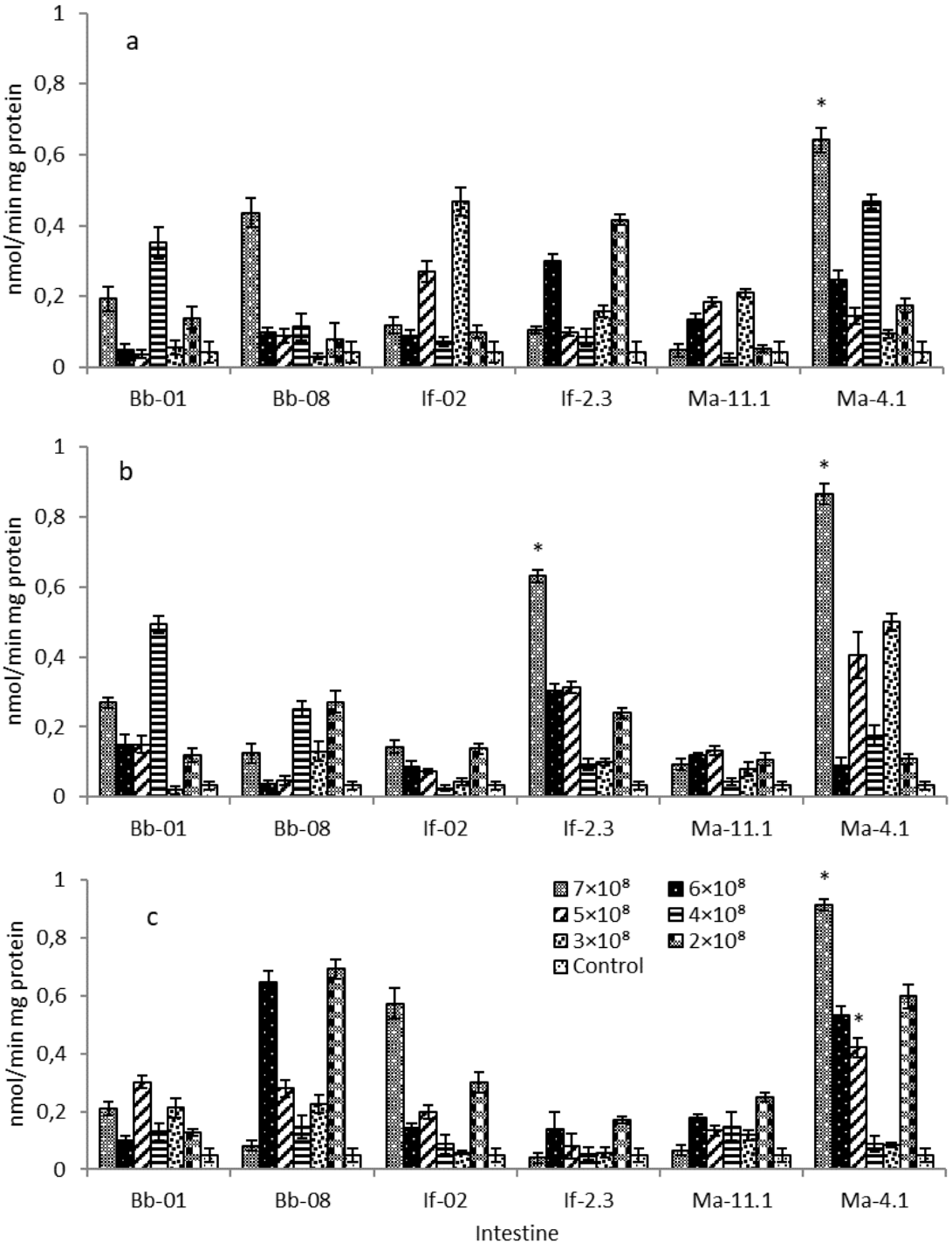

Fig. 2 Activity of AChE enzyme in intestine samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1, 4.1), *I. fumosorosea* (If-02, 2.3) and *B. bassiana* (Bb-01, 08). *represents significant difference between treatments at 0.05 % level of significance.

Alkaline phosphatases (ALP) activity

In the current investigations statistically significant change in ALP activities were observed in hemolymph samples treated with Ma-4.1 at various concentrations (4-7×10^8 spores/mL) as compared to the control (Figs 7a-c), while similar trend was observed in case of intestine samples (Figs 8a-c). On the other hand, as far as the fat bodies treated samples are concerned, maximum change in ALP was noted only in 168h treated samples of Ma-4.1 at concentration of 7×10^8 spores/mL (Fig.9c).

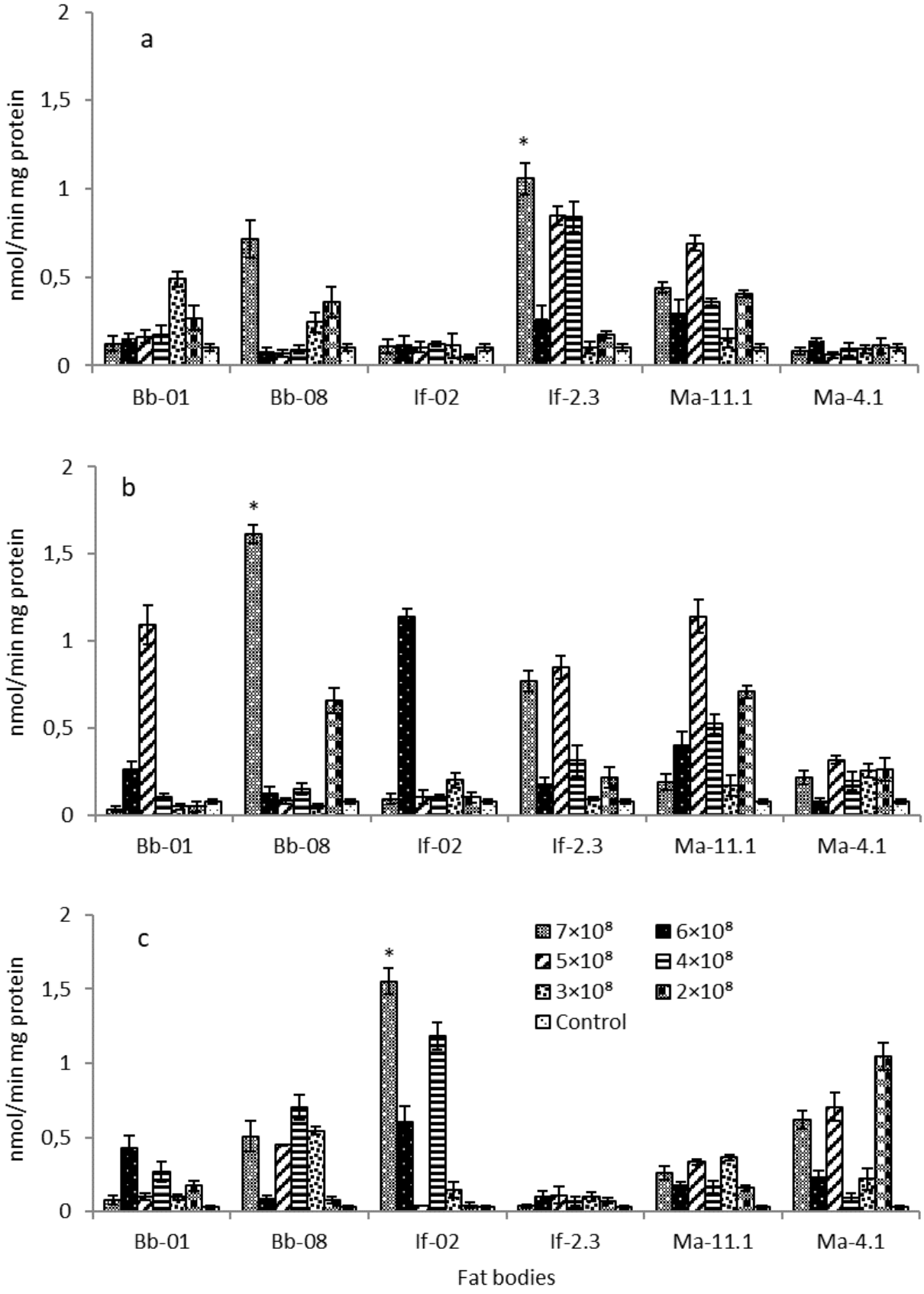

Fig. 3 Activity of AChE enzyme in fat body samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1, 4.1), *I. fumosorosea* (If-02, 2.3) and *B. bassiana* (Bb-01, 08). *represents significant difference between treatments at 0.05 % level of significance.

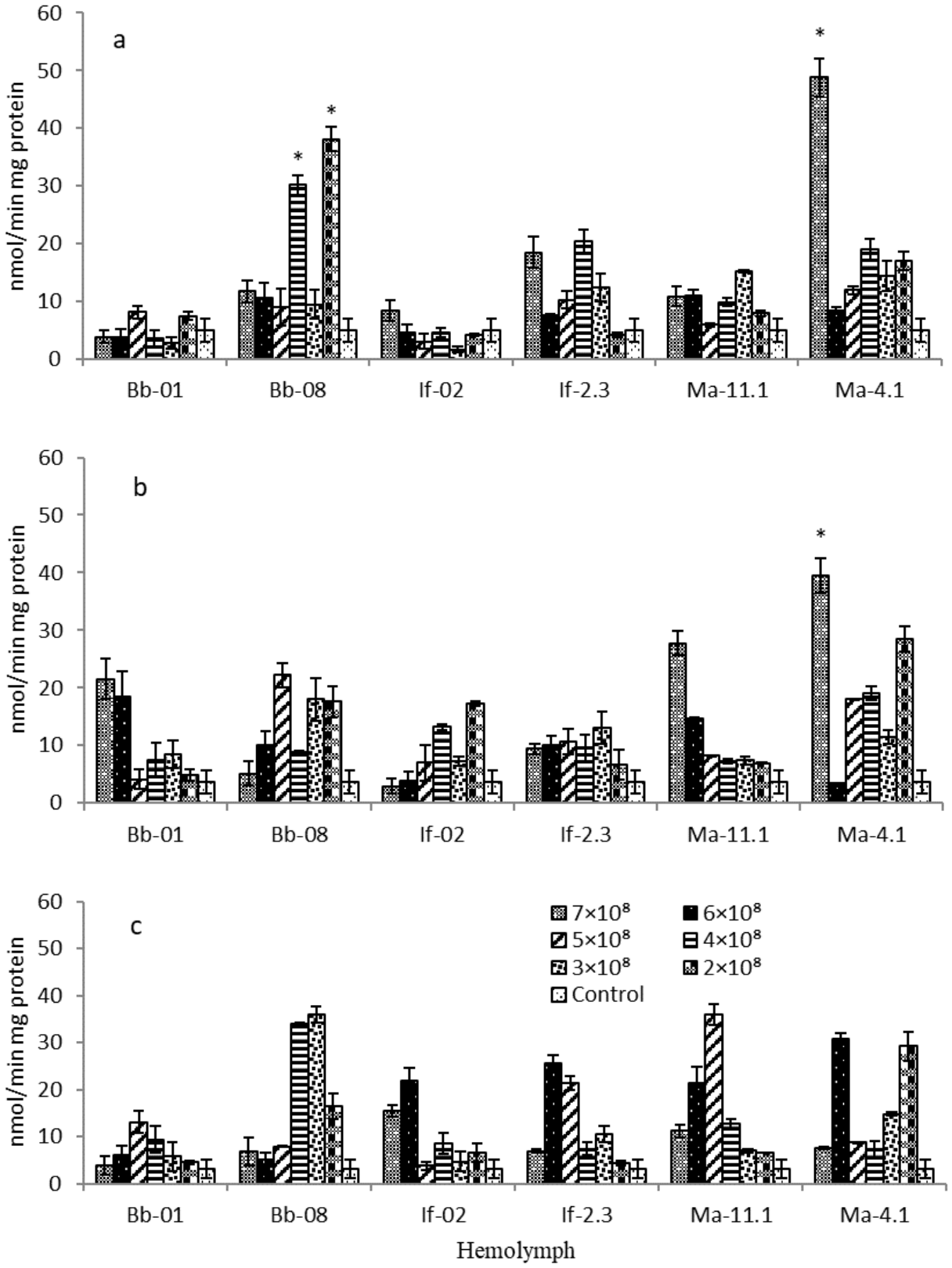

Fig. 4 Activity of acid phosphatases enzyme in hemolymph samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1, 4.1), *I. fumosorosea* (If-02,2.3) and *B. bassiana* (Bb-01, 08). *represents significant difference between treatments at 0.05 % level of significance.

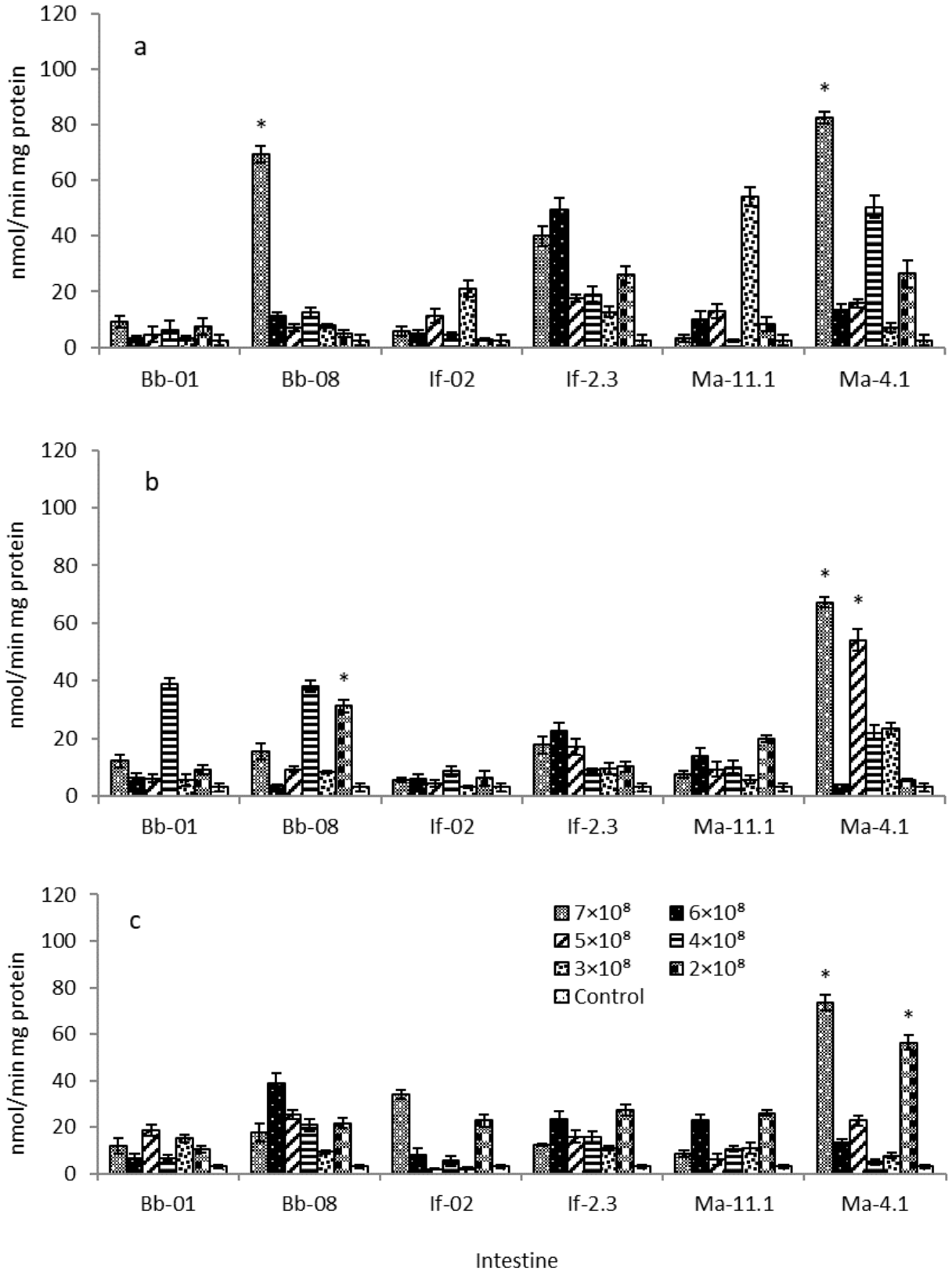

Fig. 5 Activity of acid phosphatase enzyme in intestine samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1, 4.1), *I. fumosorosea* (If-02, 2.3) and *B. bassiana* (Bb-01,08). *represents significant difference between treatments at 0.05 % level of significance.

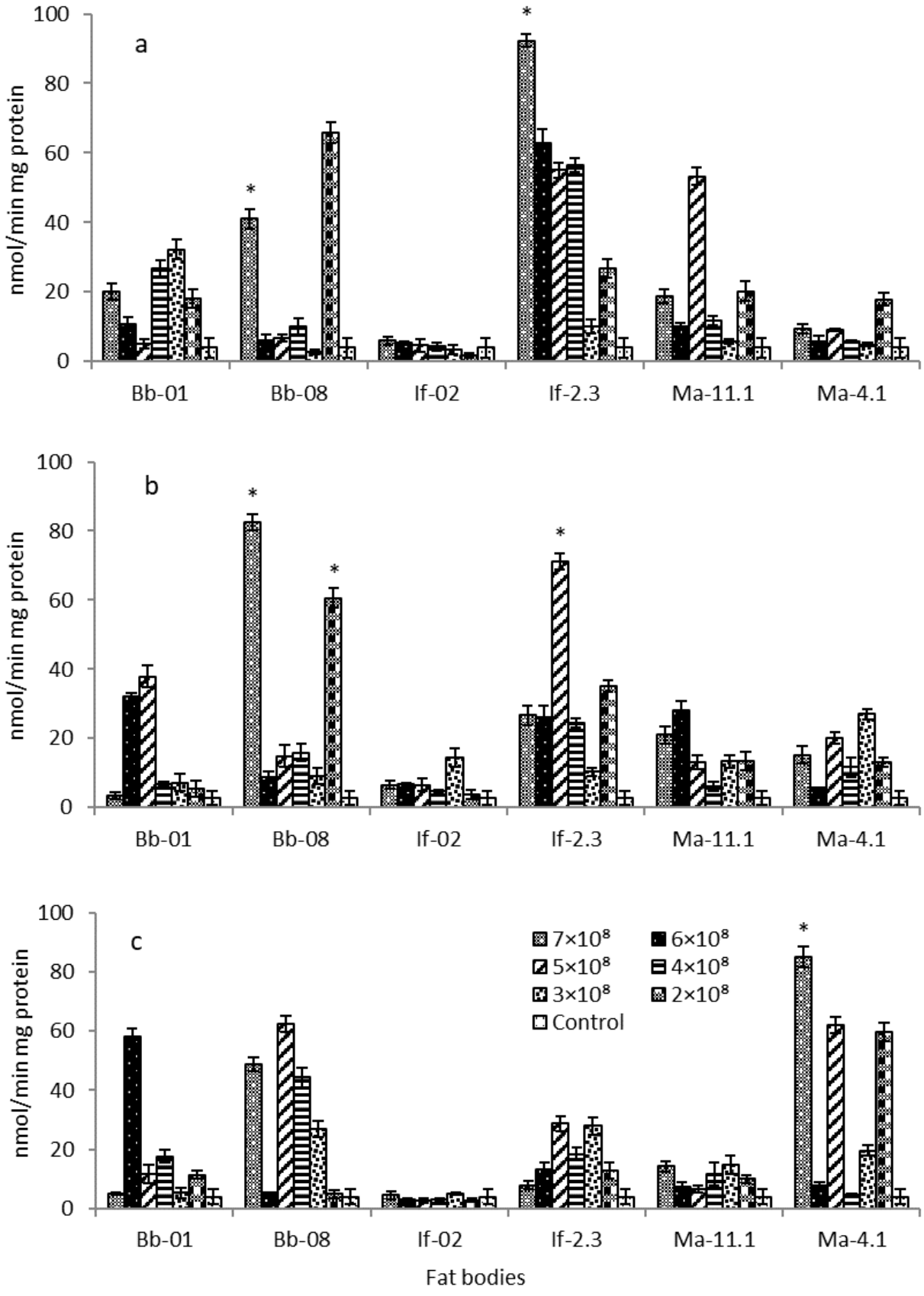

Fig. 6 Activity of acid phosphatases enzyme in fat body samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1,4.1), *I. fumosorosea* (If-02, 2.3) and *B. bassiana* (Bb-01,08). *represents significant difference between treatments at 0.05 % level of significance.

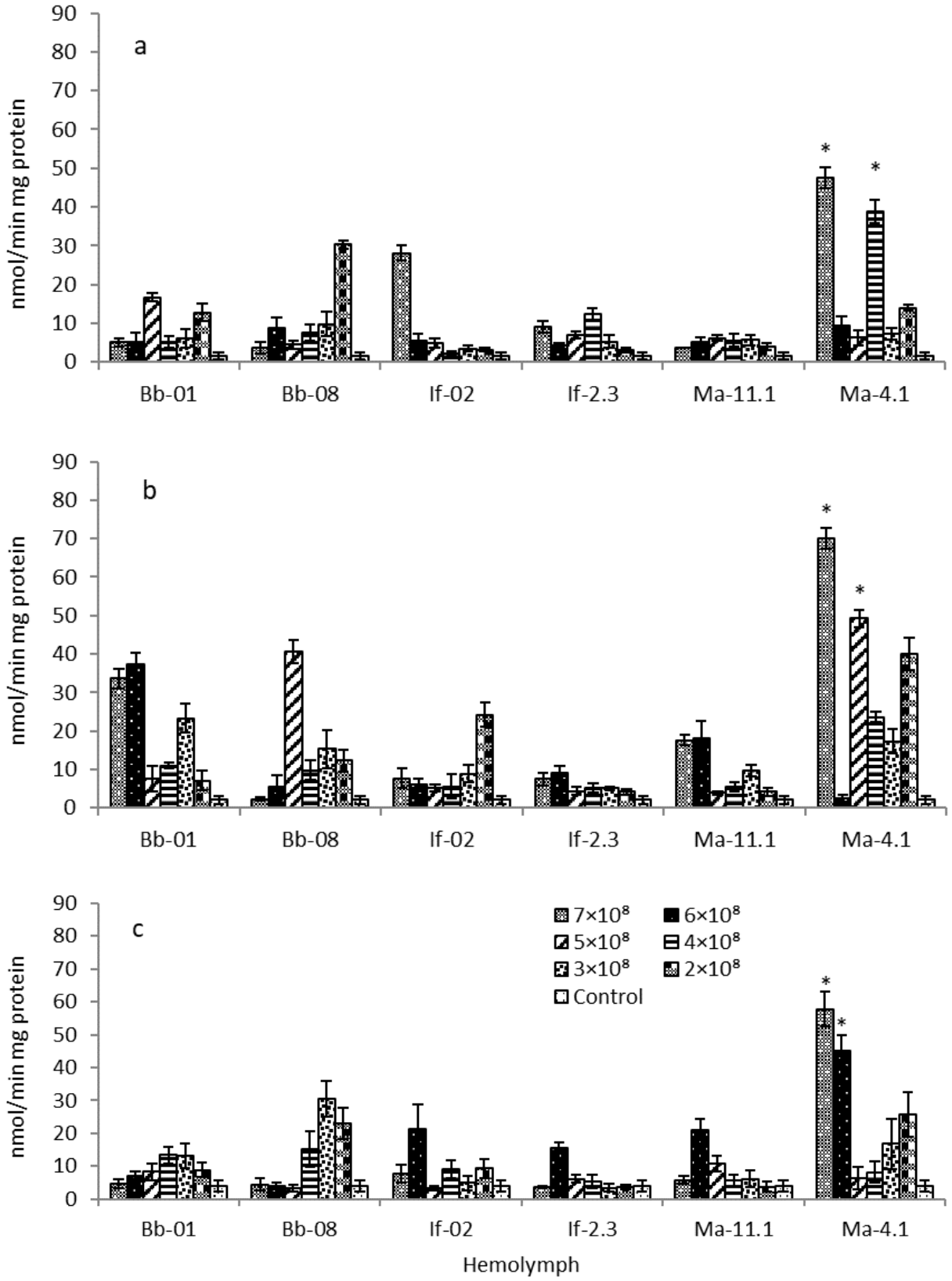

Fig: 7 Activity of alkaline phosphatases enzyme in hemolymph samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168h after treated with *M. anisopliae* (Ma-11.1,4.1), *I. fumosorosea* (If-02,2.3) and *B. bassiana* (Bb-01,08). *represents significant difference between treatments at 0.05 % level of significance.

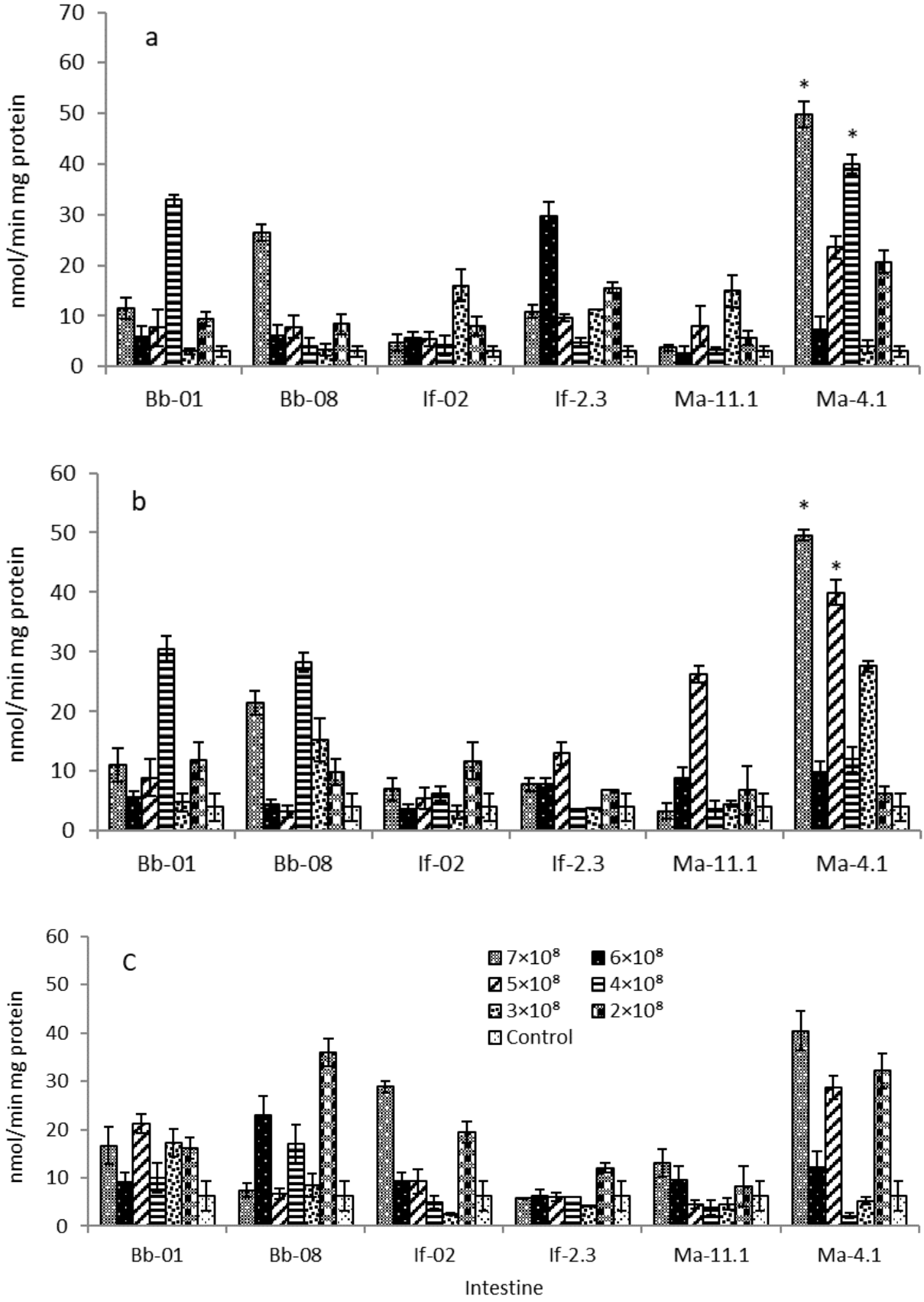

Fig. 8 Activity of alkaline phosphatase enzyme in intestine samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1,4.1), *I. fumosorosea* (If-02,2.3) and *B. bassiana* (Bb-01,08). *represents significant difference between treatments at 0.05 % level of significance.

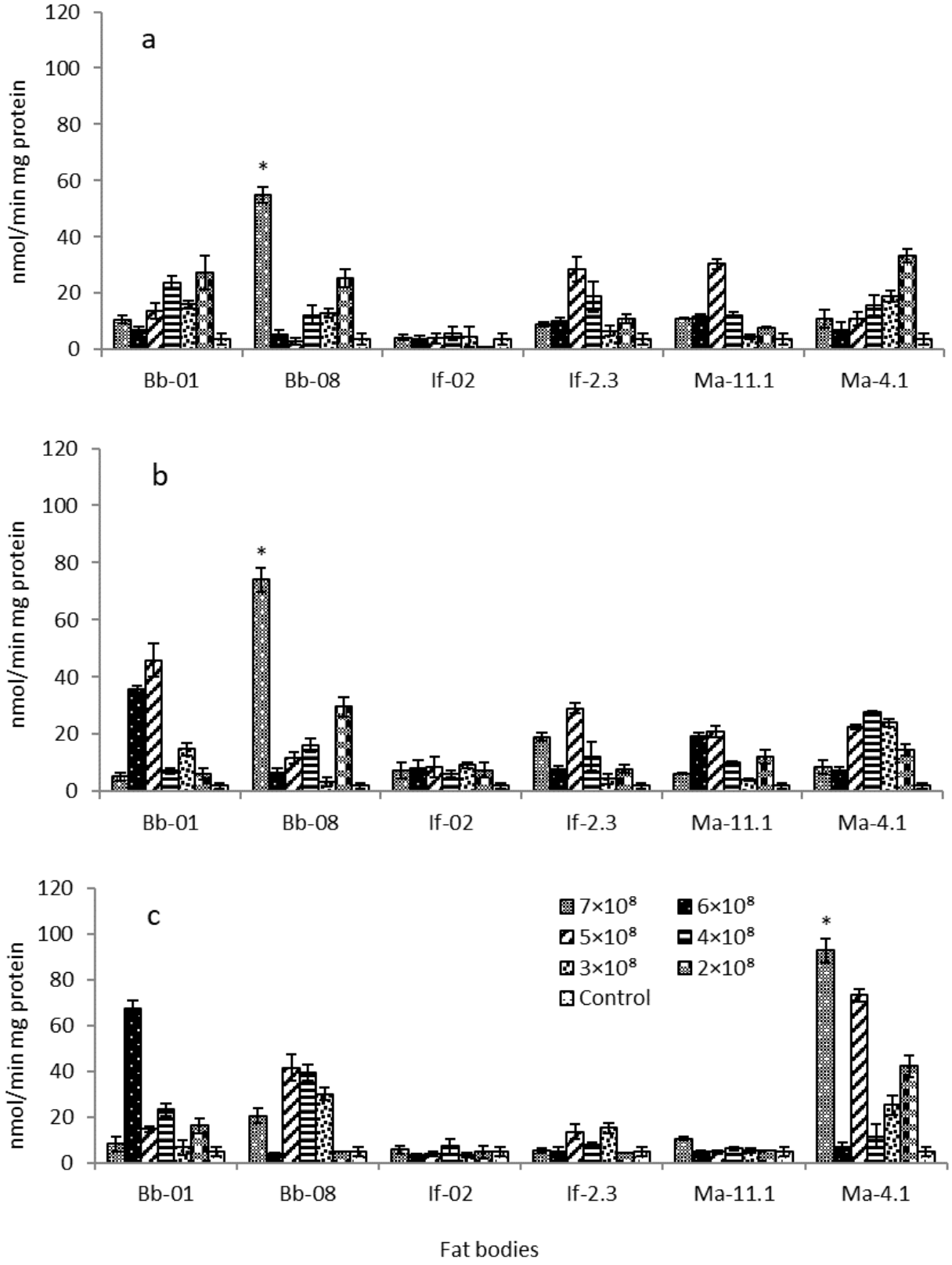

Fig. 9 Activity of alkaline phosphatase enzyme in fat body samples of *H. armigera* on (a) 72 h (b) 120 h (c) 168 h after treated with *M. anisopliae* (Ma-11.1,4.1), *I. fumosorosea* (If-02,2.3) and *B.bassiana* (Bb-01, 08). *represents significant difference between treatments at 0.05 % level of significance.

Discussion

In insects, development of resistance against many microorganisms is mainly by the removal of disease causing microbes from the body and escaping their attack (Lord and Howard, 2004). Outcomes of recent work support the findings of previous research on the effect of EPF on detoxifying enzymes of insects (Serebro *et al.,* 2001). The previous studies illustrate that infectious diseases play a pivotal role in detoxification of pathogens with the help of enzymatic actions in insects. The enzymes such as AChE, ACP and ALP detoxify the toxic metabolites, depict the insect's response to body intoxication and development of resistance (Serebro *et al.,* 2006). AChE an important enzyme, hydrolyses acetylcholine (ACh), a neurotransmitter present in the nervous system of insects (Wang *et al.,* 2004). Inhibition of AChE results in increase level of ACh at the synapses causing the post-synaptic membrane in a state of long lasting stimulation, ultimately leading to ataxia and insect death (Singh and Singh, 2004). In our research, the treatment of *H. armigera* larvae by EPF changed the activities of AChE, ACP and ALP in hemolymph, intestine and fat bodies homogenates. These results (increased activity of detoxification enzymes) coincide with prior research work. Previously, it revealed that AChE activity decreased in Sunn pest, *Eurygaster integriceps* after the treatment of *B. bassiana* (Zibaee *et al.,* 2009). According to Shafeek *et al.* (2004) AChE activity was observed in various parts of the nervous system such as brain, terminal ganglion, nerve cord and muscles of *Periplaneta americana* (Linnaeus) after exposure to azadirachtin, while there was no significant activity of AChE as compared to the control. Similarly, application of neem extracts inhibited the AChE activity in *Blatella germania* and *Musca domestica* (Khan *et al.,* 2003), while increased AChE activity was also observed when *Tribolium castaneum* was treated with Ambush and Dimilin(Saleem and Shakoori, 1987). In the current study the highest AChE activity was observed in hemolymph samples (120h) treated with *B. bassiana* (Bb-08), while lowest was noted in *I. fumosorosea* (If-2.3) treated samples for all three durations, whereas,*M. anisopliae* (Ma-4.1) and *I. fumosorosea* (If-2.3)treated intestine and fat bodies samples showed the highest AChE activity.

Less infectious EPF strains are commonly present in natural insect populations that not necessarily kill the insects. Hence, detoxification enzymes are generally affected by EPF as well as other pathogenic microbes. Moreover, detoxification enzymes have several functions and can facilitate repair procedures, metabolism of biologically active compounds and intoxication of toxic products. The variations in activities of enzymes can develop resistance in insects and may have impact on the insect's body that makes them adaptable to environment (Serebro *et al.,* 2001, 2006).

In the previous studies, the treatment of *Spodoptera exigua* larvae with nuclear polyhedrosis virus showed high activity of ACP in fat bodies with respect to days and high activity was observed on third day until the death of the larvae, while ALP activity was three times less than ACP in the fat bodies samples (Sujak *et al.,* 1978). Earlier, maximum ACP activity was observed in the hemolymph of desert locust, *Schistocerca gregaria* on the third day after exposure to *M. anisopliae* (Xia *et al.,* 2000). Parallel activities of ACP and ALP in hemolymph, intestine and fat bodies samples treated with *M. anisopliae* (Ma-4.1), *B. bassiana* (Bb-08) and *I. fumosorosea* (If-2.3) for different days were observed in the current study.

Enzymes activities sharply increase degradation in infected insects after exposure with xenobiotics. This enhances the adaptation ability of insect body and decreases their sensitivity against insecticides. Inhibition of detoxification enzymes abruptly increases the insect death from fungal contamination, which ultimately confirms the participation of detoxification enzymes in insect resistance development to EPF and opens novel opportunities for the development of proficient biological products on the basis of enzyme inhibitors and EPF.

Acknowledgements

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