

# Effects on detoxification enzymes of *Helicoverpa armigera* (Lepidoptera: Noctuidae) infected by *Beauveria bassiana* spores and detection of its infection by PCR

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**ABSTRACT:** This research aimed to investigate virulence of *Beauveria bassiana* 36 and its pathogenicity on the infected larvae of *Helicoverpa armigera*. The virulence was measured by LD<sub>50</sub> values and pathogenicity was monitored from detoxification enzymatic changes and pathogenic detection using polymerase chain reaction (PCR). LD<sub>50</sub> of *B. bassiana* against *H. armigera* larvae was highly effective at a very low dose of  $7.2 \times 10^8$  spores/ml. Phenoloxidase (PO), glutathione S-transferase (GST), esterase (EST), and glucose oxidase (GOX) activities were found from the hemolymph of the infected *H. armigera* larvae. PO and GST activities from the infected larval sample increased from  $53.72 \pm 1.10$  to  $79.03 \pm 1.22$  U/mg protein and from  $3.81 \pm 0.17$  to  $5.77 \pm 0.09$  U/mg protein, respectively, from 0–48 h and then decreased from  $61.75 \pm 1.12$  to  $37.46 \pm 1.06$  U/mg protein and from  $4.45 \pm 0.09$  to  $2.61 \pm 0.12$  U/mg protein from 72–120 h, respectively. EST and GOX activities from the infected larval sample increased from  $10.24 \pm 0.44$  to  $21.84 \pm 0.64$  U/mg protein and from  $3.38 \pm 0.30$  to  $10.76 \pm 0.34$  U/mg protein, respectively, from 0–72 h, but decreased slightly from  $21.72 \pm 0.76$  to  $16.00 \pm 0.89$  U/mg protein and from  $4.33 \pm 0.55$  to  $4.02 \pm 0.41$  U/mg protein from 96–120 h, respectively. The presence of *B. bassiana* in the infected larvae was detected at post-inoculation using PCR with the specific primers (P1–P3). *B. bassiana* was successfully and rapidly detected and identified within 72–120 h with molecular techniques.

**KEYWORDS:** *Beauveria bassiana*, detoxification enzyme, *Helicoverpa armigera*, lethal dose, polymerase chain reaction

## INTRODUCTION

*Helicoverpa armigera* (Lepidoptera, Noctuidae) has been recorded as the most serious pest of 181 plant species belonging to 45 families<sup>1</sup> with an annual damage cost estimated at over 5 billion US dollars. It has developed resistance up to 54% of chemical insecticides including pyrethroid, cyclodiene, organophosphate, and carbamates<sup>2</sup>. Integrated control is thought to be an effective alternative control. *Beauveria bassiana* is an entomopathogenic fungus that causes a natural mortality on insects that is environmentally safe. Therefore, there is worldwide interest in using *B. bassiana* for integrated pest management. After the fungal spores attach to the insect's cuticle, germ tubes develop and penetrate into the cuticle and form blastospores in

the hemocoel. Host death is often due to a combination of the action of fungal toxin, the physical obstruction of blood circulation, nutrient depletion, and invasion of the organs<sup>3</sup>. In an attempt to resist the fungus, the immune system of the insect attempts to resist the fungus using the innate immunity by forming a cuticle or peritrophic envelope and using the mechanisms of cellular immunity that include encapsulation, phagocytosis, antimicrobial peptides, and detoxification enzymes. The detoxification enzymes include the main enzymes such as esterase (EST), glutathione S-transferase (GST), and phenoloxidase (PO)<sup>4</sup>. These are the most common enzymes involved in detoxification of xenobiotics and increase in activity of these enzymes is the key mechanism of insect resistance to insecticides<sup>5</sup>. It is known that the attack process of a fungus on an

insect involves both direct infection and the action of its secondary metabolites that are toxic to the insect<sup>6</sup>. The change in their activities is reflected not only in insect resistance but also to their host plant adaptation as well as in metamorphosis and development<sup>7,8</sup>. In this present study, the glucose oxidase (GOX) enzyme was also analyzed as one parameter of social immunity because this enzyme may act as an antimicrobial agent against insect pathogens<sup>9</sup>. GOX catalyzes the oxidation of  $\beta$ -D-glucose to D-gluconic acid and hydrogen peroxide that are positively correlated with the inhibition of pathogen development<sup>10,11</sup>. Few reports are available on the change in the activities of all four detoxification enzymes of *H. armigera* when infected by *B. bassiana*. In order to develop pest management strategies of *H. armigera*, knowledge is needed on the basic mechanisms of the secondary metabolites of this pest infected by *B. bassiana*. Therefore, we attempted to detect the possible relationships between the natural levels of PO, EST, GST, and GOX activities in *H. armigera* and the insect's tolerance to *B. bassiana*. Moreover, we show that the polymerase chain reaction (PCR) can characterize and detect an insect pathogen that allows identification of *Beauveria* spp. or *B. bassiana* isolates as monitoring systems.

## MATERIALS AND METHODS

### Fungal spore

*B. bassiana* strain 36 was cultured on malt extract agar (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar, all % in w/v) plates at room temperature for 7–10 days. The fungal spores were collected using a funnel and stored in vials. All experiments were performed at the Laboratory of Earth, Environmental and Biological Sciences, Bioscience, Faculty of Science and Engineering, Queensland University of Technology, Australia.

### Ingredients of *H. armigera*'s diet

The composition of the diet for *H. armigera* is shown in Table 1. The wet and dry ingredients of the diet were weighed and kept separately. The agar was suspended in 1.0 l of water and boiled. The mixture was allowed to cool to just below 60 °C with frequent mixing to avoid lumping. Once the mix had cooled to below 60 °C, anti-bacterial and anti-fungal agents were added to the mixture with thorough mixing (modified from Ref. 12).

**Table 1** Ingredients of diets for rearing *H. armigera*.

Ingredients	1000 ml
Wheat germ	75.00 g
Yeast	62.50 g
Soy flour	85.00 g
Boiled water	600 ml
Agar	12.50 g + 300 ml hot tap water
Nipagin	3.75 g
Sorbic acid	1.25 g
Vitamin C	3.75 g
Hydrochloric acid	0.60 ml

### Insect

*H. armigera* larvae were individually reared in 29.5-ml plastic cups containing the prepared diet as a food source. The larvae were then put in trays and kept in a chamber at 25 °C and 70% RH until pupation. The pupae were sterilized with 0.025% (v/v) bleach for 5 min and they were then transferred to new containers and kept in buckets containing a 10% (v/v) honey solution as the food source. After the individual adults emerged, they were fed with 10% honey solution. The female adults laid their eggs on a layer of cloth (15 × 30 cm). The eggs were sterilized with 0.025% bleach for 8 min. All of the liquid was removed using a Buchner funnel with filter paper connected to a vacuum pump. The eggs were then placed into a plastic bag and secured with a rubber band until they hatched. After the eggs hatched as F<sub>1</sub> larvae, the larvae of 3rd instar (10 days old) were used for all experiments<sup>13</sup>.

### Bioassay test

The spore suspension of *B. bassiana* strain 36 was obtained by 0.05% Tween 80 (v/v) adding sterile distilled water and then mixed thoroughly using a vortex mixer and diluted to obtain a suspension of 10<sup>5</sup>–10<sup>10</sup> spores/ml for the bioassay test compared with a control. The larvae samples (315 samples: 15 samples per replication, 3 replications) of 3rd instar (10 days old) of *H. armigera* were dipped for 5 s in the spore suspension described above. The controls were maintained by applying 0.05% Tween 80 and then kept in 29.5-ml plastic cups containing the diet as a food source in the chamber at 25 °C and 70% RH. Mortality was measured and recorded until surviving larvae in each treatment had either pupated or were in the pre-pupal stage. The highest concentration causing 50% mortality was prepared as the standard suspension for enzyme assay as well as PCR detection.

Each larva from non-infected larvae (control) and infected larvae (*B. bassiana* 36) was observed as live or dead. Thirty larvae that were still alive after inoculation were collected at 0, 24, 48, 72, 96, and 120 h for enzyme analysis in their hemolymph and detection of the infection using PCR.

### Enzyme assays

The infected larvae were surface-sterilized with 95% ethanol and rinsed twice in sterile distilled water. The cleaned larvae were individually cut at the third proleg with a surgical knife. Twenty microliters of hemolymph were collected into the tip of a pipette. The hemolymph collections were centrifuged at  $10\,000 \times g$  for 15 min at 4 °C and the supernatants were used as the enzyme source. The enzyme source was used to determine the enzyme activities of GST, EST, PO, and the protein content<sup>14</sup>. The heads of the individual larvae were separated and ground with phosphate buffer pH 7.0 for GOX analysis. The whole body of larvae was stored at -20 °C for DNA extraction. All 4 enzyme tests were done with 3 replications.

GST was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as the substrate<sup>15</sup>. The assay mixture consisted of 50 mM CDNB in 95% ethanol, 50 mM GSH and 10–20 µg of enzyme sample in 3 ml of 50 mM phosphate buffer (pH 7.5). Enzyme activity is expressed in absorbance units at 340 nm.

Esterase activity in the samples was determined spectrophotometrically with minor modifications<sup>16</sup>. The incubation mixture contained 1 ml of 0.54 mM 1-naphthyl acetate in phosphate buffer and 20 µl of the sample. The concentration of 1-naphthyl produced during the reaction was measured spectrophotometrically by absorbance at 620 nm.

Phenoloxidase activity was measured using 5 µl of fresh whole hemolymph dropped into 95 µl of phosphate buffer (pH 6.9). After centrifugation ( $10\,000 \times g$ , at 4 °C for 5 min), 40 µl of the supernatant was pipetted into a well of a microtiter plate and 160 µl of dopamine (3 mg/ml phosphate buffer) added as substrate (instead of the L-dopa used previously) because of its high solubility in aqueous media. Phenoloxidase activity was assessed by determining the initial linear increase in absorbance at 492 nm over 30 min using a Lab systems Multiskan Bichromatic plate reader. Enzyme activity is expressed in absorbance units at 492 nm<sup>17</sup>.

GOX activity was determined by measuring the change in absorbance of the reaction mixture at

460 nm on a spectrophotometer. The reaction mixture contained 1.0 ml of 0.31 mM o-dianisidine in 1.5 ml of 0.1 M citrate sodium phosphate (pH 4.5), 0.3 ml of 1.0 M D-glucose, 0.1 ml of 2.0 mg/ml horse radish peroxidase<sup>18</sup> and 0.1 ml of the sample.

Protein concentration in the samples was determined using bovine serum albumin as standard to make a calibration curve<sup>19</sup>.

### DNA extraction

The individual larva that showed the highest enzyme activities of each enzyme in their hemolymph was selected to be representative of the whole body to detect the infection using PCR. Four whole bodies of larvae from each time point (0–120 h) were selected from individual larvae that showed the highest enzyme activity. The DNA extraction was done from the whole body of larvae using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's recommendations. The extracted DNA was stored at -20 °C until use.

### PCR detection of fungus within the infected larvae of *H. armigera*

The *B. bassiana*-specific primers used for detection of *B. bassiana* amplification within the infected larvae of *H. armigera* were P1 (5'AAGCTTCGACATGGTCTG) and P3 (5'GGAGGTGGTGGGTTCTGTT)<sup>20</sup>. PCR reactions were performed in a final volume of 25 µl, containing 5 µl of 5X buffer, 1 µl of 25 mM MgCl<sub>2</sub>, 200 µM dNTPs mix, 0.4 mM of each primer, 1 U of Taq DNA polymerase, and 1 µl of DNA template. PCR condition were as follow: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min. PCRs were analyzed on gels.

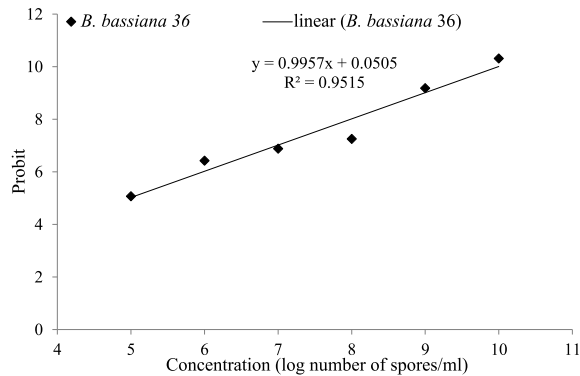
### Statistical analysis

The median lethal insecticide dose (LD<sub>50</sub>) that killed 50% of the insects was determined by probit analysis. Enzyme activities were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test. The enzyme responses obtained are presented as mean and standard error (SE). The level of significance was taken as  $p \leq 0.001$  and all tests were undertaken using Genstat.

## RESULTS AND DISCUSSION

### Bioassay

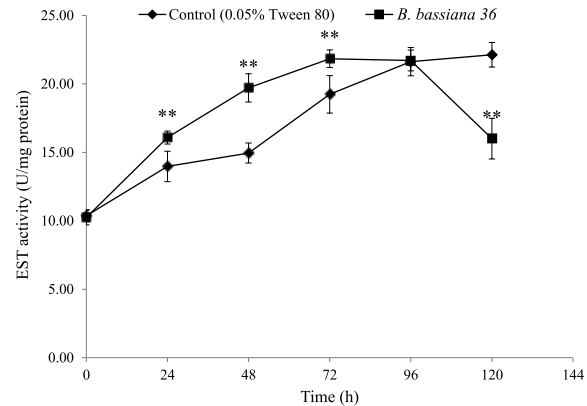
After the 3rd instar *H. armigera* larvae were inoculated by serial concentrations (10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup>,



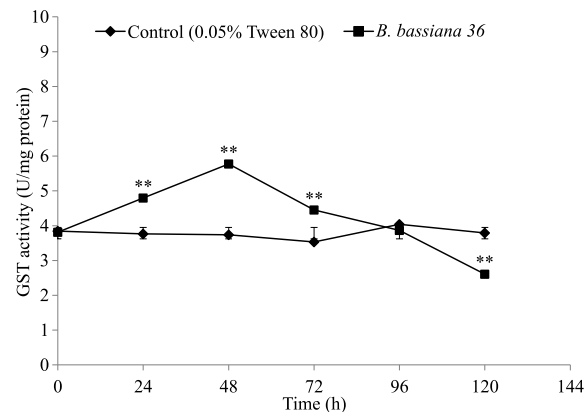
**Fig. 1** Effect of *B. bassiana* on larvae of *H. armigera*, death-rate is converted to probit.

$10^9$ , and  $10^{10}$  spores/ml) of the fungus *B. bassiana*. The mortality was observed as a linear relationship between probit mortality and dose concentration. *H. armigera* larvae mortality due to spore concentration was different among all concentrations and varied from 5.07–10.31 probits. These results showed that there was a correlation between increasing spore and enhancement of mortality in *H. armigera* larvae (Fig. 1). The analysis of variance results showed significant differences in the mortality of *H. armigera* larvae treated with spores analyzed by line regression analysis (Fig. 1). Treatment of the larvae using the highest spore concentration of  $10^{10}$  spores/ml resulted in complete mortality (100%).  $LD_{50}$  (the effective concentration of fungal spore or 50% mortality, calculated by bioassay tests) was at a concentration of  $7.2 \times 10^8$  spores/ml after 5 days post-inoculation. During 0–5 days post-inoculation of 3rd instar larvae of *H. armigera* infected with *B. bassiana* ( $10^7$  spores/ml), Ref. 21 reported larval mortality ranged from 29.69–41.89%. The percentage mortality in our experiment was slightly higher than Ref. 21. This was possibly due to a higher dose inoculation. Earlier study reported that 84% mortality in 3rd instar larvae of *H. armigera* when sprayed with *B. bassiana* at  $10^5$  spores/ml<sup>22</sup>. So, the isolates of different fungi could result in different rates of pathogenicity.

Observations of the host defensive reaction were conducted after two days post-inoculation. Response to the fungal attack showed dark spots of various shapes and sizes that appeared on the cuticle surface of the infected larvae. The larvae died after developing various morphological deformities. These abnormalities ranged from blackening of the infected skin which formed a fragile skin that



**Fig. 2** EST activity (U/mg) affected by *B. bassiana* strain 36, bar represents mean  $\pm$  SE ( $n = 30$ ) and \*\* shows significant differences in activities versus control ( $p < 0.001$ ).



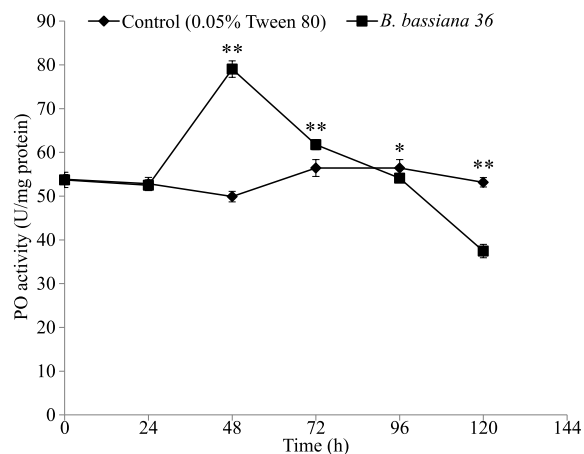
**Fig. 3** GST activity (U/mg) affected by *B. bassiana* strain 36, bar represents mean  $\pm$  SE ( $n = 30$ ) and \*\* shows significant differences in activities versus control ( $p < 0.001$ ).

developed abnormal body parts. The larvae became very weak and their death from mycosis started 3 days after the infection and ended on day 4 or 5.

### Detoxification enzyme activities

The effects of detoxification on the activities of the enzymes (EST, GST, PO) of *H. armigera* larvae infected with fungal spores of *B. bassiana* are shown in Figs. 2–4. Most of the enzyme activities were significantly higher than the control during the 24–72 h post-inoculation period.

The EST activity in the hemolymph of the *H. armigera* larvae treated with fungal spores was significantly induced. The EST activities of the infected larvae at 24 h ( $F = 91.7$ ;  $df = 59$ ;  $p < 0.001$ ),



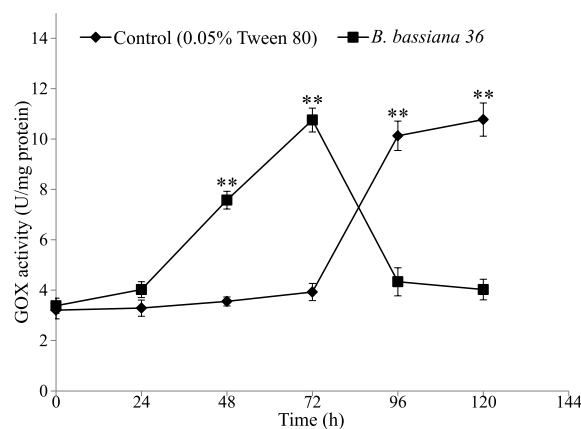
**Fig. 4** PO activity (U/mg) affected by *B. bassiana* strain 36, bar represents mean  $\pm$  SE ( $n = 30$ ) and asterisks show significant differences in activities versus control (\*\*,  $p < 0.001$  and \*,  $p < 0.005$ ).

48 h ( $F = 425.3$ ;  $df = 59$ ;  $p < 0.001$ ), and 72 h ( $F = 88.4$ ;  $df = 59$ ;  $p < 0.001$ ) were significantly higher than the control (Fig. 2). The highest activity was observed at 72 h post-inoculation (21.84 U/mg protein) and then it decreased. At 120 h post-inoculation, the EST activity was significantly lower than the control ( $F = 371.6$ ;  $df = 59$ ;  $p < 0.001$ ). The activity of nonspecific esterase increased due to induction of additional isoenzymes<sup>23</sup>.

Similar results of GST activity were obtained from *H. armigera* larvae after treatment with fungal spores after 0–48 h. GST activities of infected larvae at 24 h ( $F = 400.8$ ;  $df = 59$ ;  $p < 0.001$ ) and 48 h ( $F = 1416$ ;  $df = 59$ ;  $p < 0.001$ ) post-inoculation were significantly higher than the control. The highest activity was observed at 48 h (5.77 U/mg protein) post-inoculation (Fig. 3). The highest PO activity of the infected larvae was 79.03 U/mg protein observed at 48 h post-inoculation and was significantly higher than the control ( $F = 1718$ ;  $df = 59$ ;  $p < 0.001$ ) (Fig. 4).

Apparently, the enhanced levels of the detoxification enzymes resulted from defense mechanisms against the xenobiotics in response to the intoxication due to metabolites of the pathogen or the products of host tissue degradation. GST activity in insects was found to increase the resistant to insecticides<sup>24</sup>.

GST also takes part in the removal of metabolites and the protection of tissues from damage by free radicals<sup>25</sup>. Moreover, GST was found to participate in metabolism and detoxification of or-



**Fig. 5** GOX activity (U/mg) affected by *B. bassiana* strain 36, bar represent mean  $\pm$  SE ( $n = 30$ ) and \*\* shows significant differences in activities versus control ( $p < 0.001$ ).

ganic phosphates, pyrethroids, carbamates, and juvenoids<sup>26</sup>. PO is the key enzyme in the developmental process of insects. PO is responsible for catalyzing the hydroxylation of monophenols and oxidation of o-diphenols<sup>27</sup>, that can spontaneously polymerize to form melanins which are involved in cuticle sclerotization and melanization associated with nodulation, encapsulation, and wound healing. Furthermore, PO might provide cytotoxic quinonoid compounds to kill the opportunistically invading microorganism at this site<sup>28</sup>.

The main factor that increases the activity of detoxification enzymes may be the mechanical damage to the insect's cuticle by hyphae due to the penetration and action of fungal toxins released into the hemocoel<sup>7,23</sup>. Thus, the increase in detoxification enzymes after treatment up to 72 h suggests that these enzyme activities of *H. armigera* larvae may be directed at elimination of fungal metabolites and toxic substances of *B. bassiana* formed by penetration into the hemocoel. Zibae et al<sup>5</sup> also reported that the effects of fungal spores presented a similar trend of changing secondary metabolites of detoxification enzymes of insects.

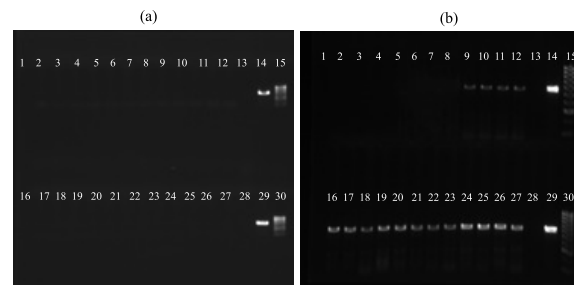
GOX activity of *H. armigera* larvae after treatment with fungal spores significantly increased in *H. armigera* larvae and the highest activity (10.76 U/mg protein) was observed at 72 h post-inoculation (Fig. 5), which was significantly higher than the control ( $F = 1259$ ;  $df = 59$ ;  $p < 0.001$ ). GOX plays an important role in the interaction between the Lepidoptera caterpillar and the host plants<sup>29,30</sup>. GOX activities have been detected in insect species such as *Spodoptera exigua*<sup>31</sup>,

*H. armigera*, and *H. assulta*<sup>32</sup> and recently were extensively investigated in 85 species of Lepidoptera and three species of plant-feeding Hymenoptera<sup>30</sup>. However, few reports showed the secretion of this enzyme in insects infected by entomopathogenic fungi.

GOX activity is essential for the production of antiseptic substances<sup>33</sup>. This enzyme catalyzes the oxidation of glucose into hydrogen peroxide and D-glucono- $\delta$ -lactone. GOX is produced by certain species of fungi and insects and displays antibacterial activity when oxygen and glucose are present<sup>34</sup>. Therefore, GOX activity of infected larvae increased rapidly after 24–72 h post-inoculation, which was higher than the control. It was possible that the enzyme in both the larvae and the fungi was presented to oxidize glucose in artificial diet. The control larvae had slightly increased GOX activity after 72 h post-inoculation, probably due to their growth and later developmental stage that leads them to increase food demand. Merx-Jacques and Bede<sup>31</sup> reported that GOX activity is closely correlated with the amount of time larvae spent feeding on artificial diet. GOX activity increased throughout in the later developmental stage of larvae fed with wheat germ-based artificial diet, whole body GOX of *S. exigua* (Lepidoptera: Noctuidae) larvae in 5th instars reared on a wheat germ-based artificial diet was over five-fold higher than 2nd instars<sup>31</sup>. In addition, diet nutritional composition is a major factor influencing the GOX activity of larvae *H. armigera*<sup>35</sup>. The high level of protein in the artificial diet increased GOX activity. Larvae fed on the 24P:17C diet had twice the GOX activity compared to caterpillars fed on the 25P:39C diet<sup>36</sup>. In this study, larvae 3rd instars of *H. armigera* were fed on the 28P:12C diet that might induce GOX secretion. However, GOX activity of the infected larvae decreased rapidly after 72 h post-inoculation which might possibly occur due to the cell death resulting in the loss of all biological activities.

#### Detection of infection by *B. bassiana* within the larvae of *H. armigera*

The ability of the PCR-based system to identify *B. bassiana* infected larvae of *H. armigera* was determined by a comparison with the non-infected larvae (control). An analysis of these results indicated that the PCR product was formed with the extracted DNA at several times after treatment with the P1–P3 primer sets (Fig. 6). Additionally, a product was observed from total DNA from larvae that had been infected with *B. bassiana* and incubated at least 10



**Fig. 6** Gel red agarose gel of P1–P3 PCR products from (a) non-infected larvae of *H. armigera* and (b) infected-larvae of *H. armigera* by *B. bassiana* strain 36 after 0–120 h post inoculation. At 0 h, lanes 1–4; 24 h, lanes 5–8; 48 h, lanes 9–12; lanes 13 and 28, negative PCR product; lanes 14 and 29, positive PCR product; lanes 15 and 30, HyperLadder 1000 kb; 72 h, lanes 16–19; 96 h, lanes 20–23; 120 h, lanes 24–27.

days<sup>20</sup>.

This study proposes to apply the detoxification enzyme to support the investigation on inhibitors of detoxification enzymes. On other hand, ways to block or suppress the activity of insect defense systems would increase their susceptibility to entomopathogenic fungi used for biocontrol. Probably this application could successfully increase the biological control efficiency from biological products as entomopathogenic fungi after combination between biological products and inhibitors of detoxification enzymes.

Insects have efficient immune systems that are able to withstand challenges from the majority of microorganisms present in the different habitats where they live. The innate immune system of insects is highly developed and it relies on humoral and cellular components<sup>37</sup>. Circulating hemocytes have important roles in the immune mechanisms of insects against microorganisms<sup>38</sup>. The ability of the fungus to infect an insect depends on its ability to adhere and penetrate the exoskeleton, resist the insect's hemolymph-borne defenses, and grow rapidly. The spore adheres to the cuticle and germinates for penetration through the exoskeleton with a combination of mechanical pressure and lytic enzymes. Following penetration into the hemolymph, the fungus grows as a yeast-like blastospore from the vegetative hyphae. Insect defense includes encapsulation of the fungus by granulocytes and plasmatocytes and formation of a nodule that may be melanized<sup>39</sup>.

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