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RESEARCH REPORT

Combined effects of some insecticides and different isolates of *Beauveria bassiana* and *Metarhizium anisopliae* on mortality and immune responses of *Chilo suppressalis* Walker (Lepidoptera: Crambidae)

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Abstract

The combined use of microbial agents and chemical insecticides is an effective strategy against insect pests in agroecosystems. This study evaluates the effects of four insecticides-permethrin, fenitrothion, trichlorfon, and tebufenozide-together with four isolates of the entomopathogenic fungus *Beauveria bassiana* and two isolates of *Metarhizium anisopliae* on the fourth instar larvae of *Chilo suppressalis*. In the first experiment, treatment with fungal isolates induced the activities of general esterases and glutathione S-transferases (GST) in the larvae. Field concentrations of insecticides increased larval mortality in both control and fungus-treated groups, except for BBLN2, which showed no significant difference. In the second experiment, conidia cultured in control and insecticide-treated media were exposed to field concentrations of insecticides on *C. suppressalis* larvae. This resulted in a significant decrease in larval mortality when treated with conidia cultured with insecticides. In the third experiment, larvae treated with permethrin exhibited the highest total hemocyte counts among those injected with BBLN1 and BBLN2, whereas larvae treated with fenitrothion and trichlorfon showed similar results after injection with BBAL1. All insecticide and fungal isolate treatments increased phenoloxidase activity compared to control larvae. These results underscore the importance of screening for optimal combinations of insecticides and entomopathogenic fungi to enhance control measures, which depend on the specific fungal isolate and type of insecticide.

Key Words: insecticides; entomopathogenic fungi; interaction; *Chilo suppressalis*; hemocyte; phenoloxidase

Introduction

Food security is a fundamental issue in balancing food supply and demand in every country. While agricultural innovations and proper land use significantly contribute to achieving this balance, they also raise concerns such as changes in land use, severe reductions in biodiversity, climate change, water scarcity, ecological poisoning, and the effects of pesticides on non-target organisms and ecosystems (Pawlak and Kołodziejczak, 2020). A significant threat to food security is insect pests, which can cause up to 40% damage to agricultural products (Food and Agriculture Organization, 2021). Although insecticides are often the first option for reducing pest populations in agroecosystems, there is growing interest in producing healthy yields through integrated pest management (IPM). IPM combines all available pest control techniques to prevent pest population development while keeping insecticides and other interventions at economically justified levels, minimizing environmental risks (Ahmad *et al*., 2019). Historically, chemical insecticides have been the primary choice for pest control. However, concerns about their adverse effects on non-target organisms, environmental pollution, and insect resistance are shifting focus toward biological control methods. Microbial agents, including fungi, bacteria, viruses, and nematodes, along with predators and parasitoids, play a crucial role in biologically suppressing pest populations (Sharma *et al*., 2019). Among these, entomopathogenic fungi, such as *Beauveria bassiana*

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List of abbreviations:
Beauveria bassiana collected from Rasht, BBRR1; *B. Bassiana* collected from Anzali, BBAL1; *B. Bassiana* collected frpm Lahijanl BBLN1 and BBLN2; Metarhizium anisopliae from SomeSara and Anzali MASA and MAAI

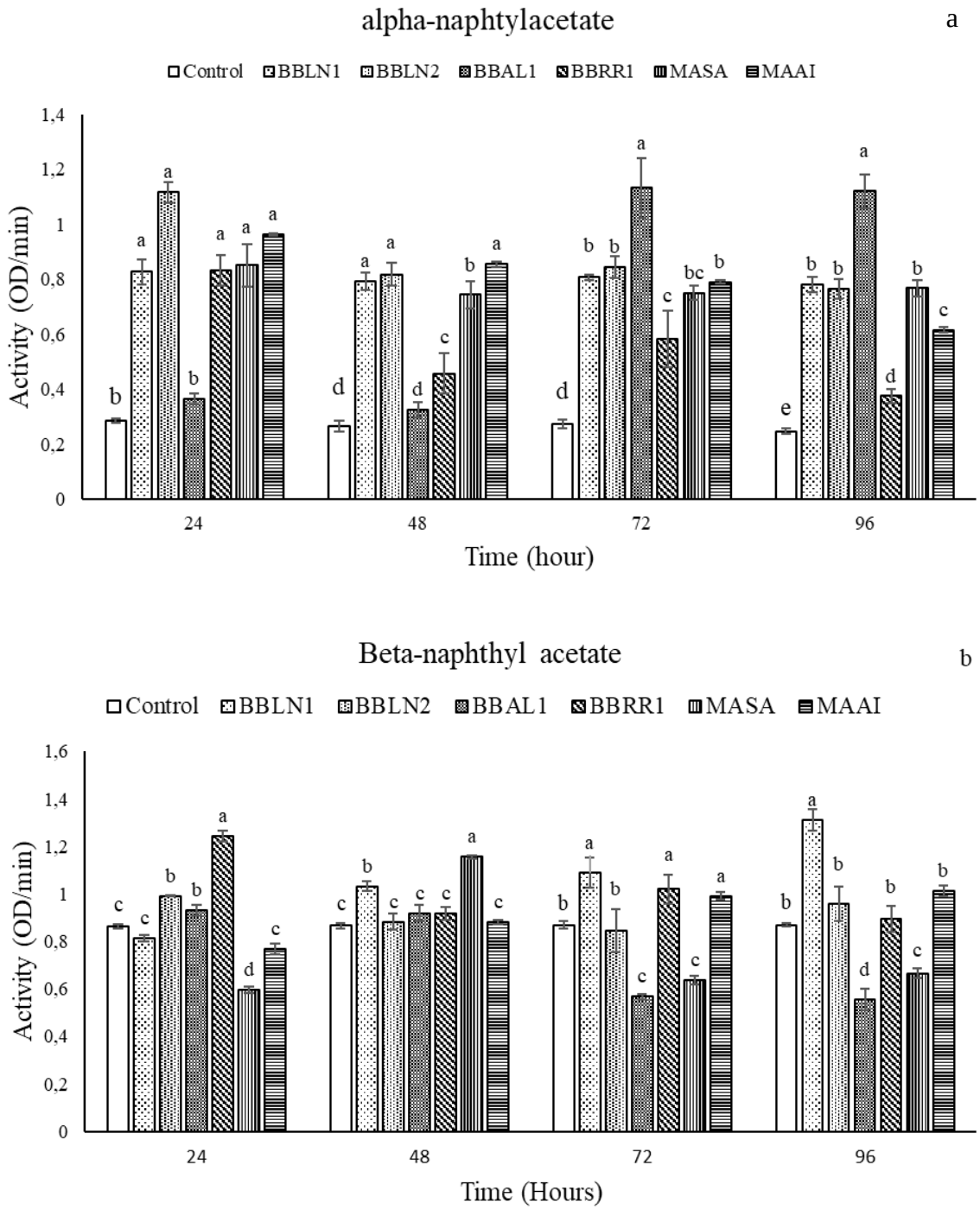

Fig. 1 Activity of general esterases in *C. suppressalis* larvae treated by different isolates of *B. bassiana* and *M. anisopliae*. Statistical differences have been shown by different letters (Tukey test, $p \leq 0.05$)

and *Metarhizium anisopliae*, are noteworthy due to their unique mode of action, widespread habitat, and safety for humans and non-target organisms. These fungi are recognized for their diverse host range, ease of mass rearing and formulation, persistence in agroecosystems, and effectiveness against host insects.

Despite the benefits of using entomopathogenic fungi in agriculture for producing healthy crops, their slower mode of action compared to chemical pesticides limits their acceptability for widespread field use. Additionally, agricultural ecosystems are frequently exposed to chemical compounds such as fertilizers, fungicides, and insecticides, which can

negatively affect the survival of entomopathogenic fungi. Therefore, an integrated pest management strategy worth considering is the simultaneous use of entomopathogenic fungi and chemical pesticides (Wu *et al*., 2020). This approach necessitates proper screening of the effects of various chemical compounds on entomopathogenic fungal isolates and their combined effects on pests. Our previous study investigated the interaction of permethrin, fenitrothion, tebufenozide, and trichlorfon on the germination, mycelial growth, and spore production of *B. bassiana* (BBRR1, BBAL1, BBLN1, BBLN2) and *M. anisopliae* (MASA, MAAI). Results indicated a significant decrease in spore production following treatment with insecticides, except for permethrin and fenitrothion on BBAL1 and MASA. Similarly, the germination rate decreased significantly after treatment with insecticides, except for MAAI. These findings suggest that BBAL1 is an isolate to consider when using insecticides in conjunction with entomopathogenic fungi (Firouzbakht *et al*., unpublished data).

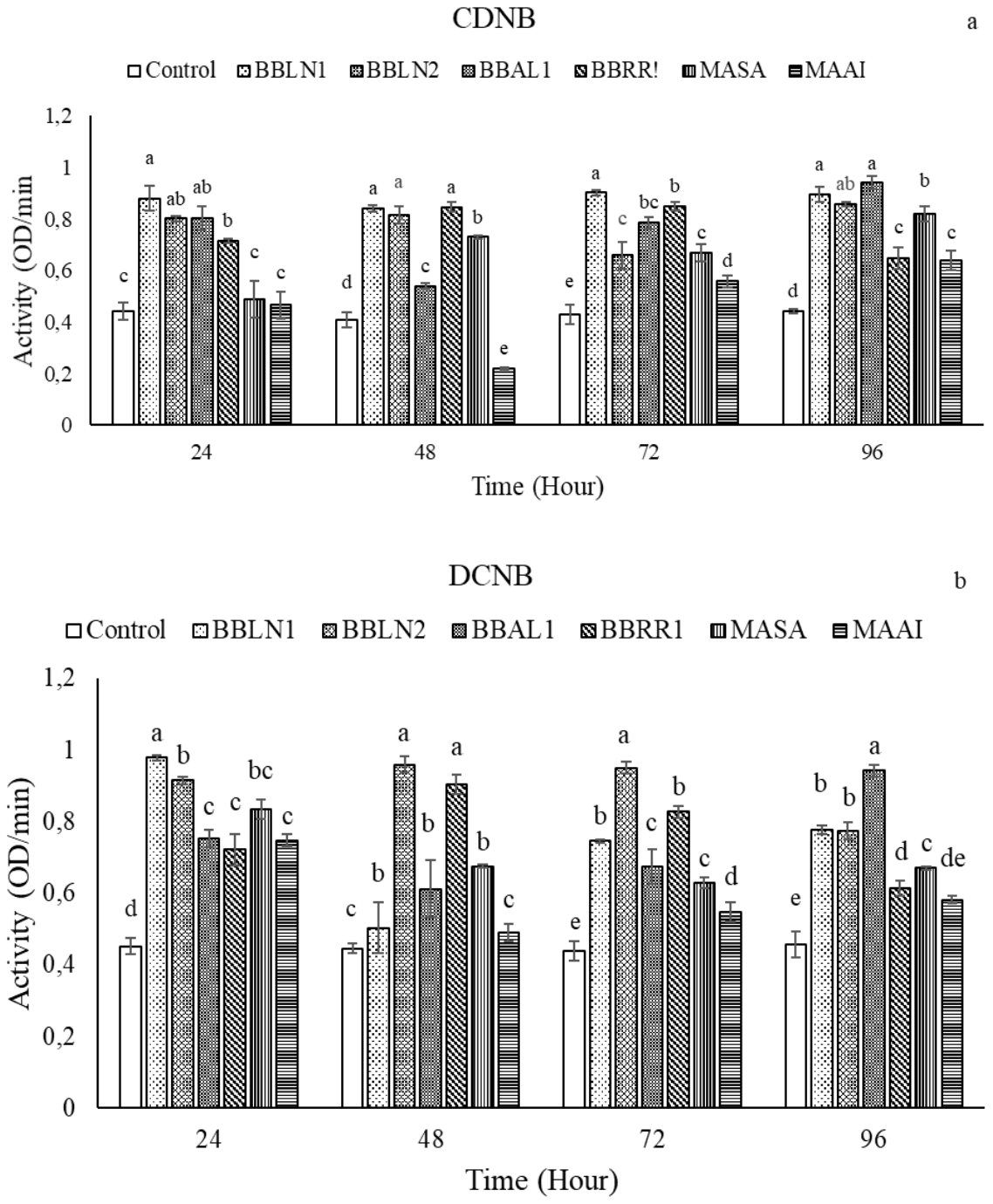

Fig. 2 Activity of glutathione *S*-transferase in *C. suppressalis* larvae treated by different isolates of *B. bassiana* and *M. anisopliae*. Statistical differences have been shown by different letters (Tukey test, $p \leq 0.05$)

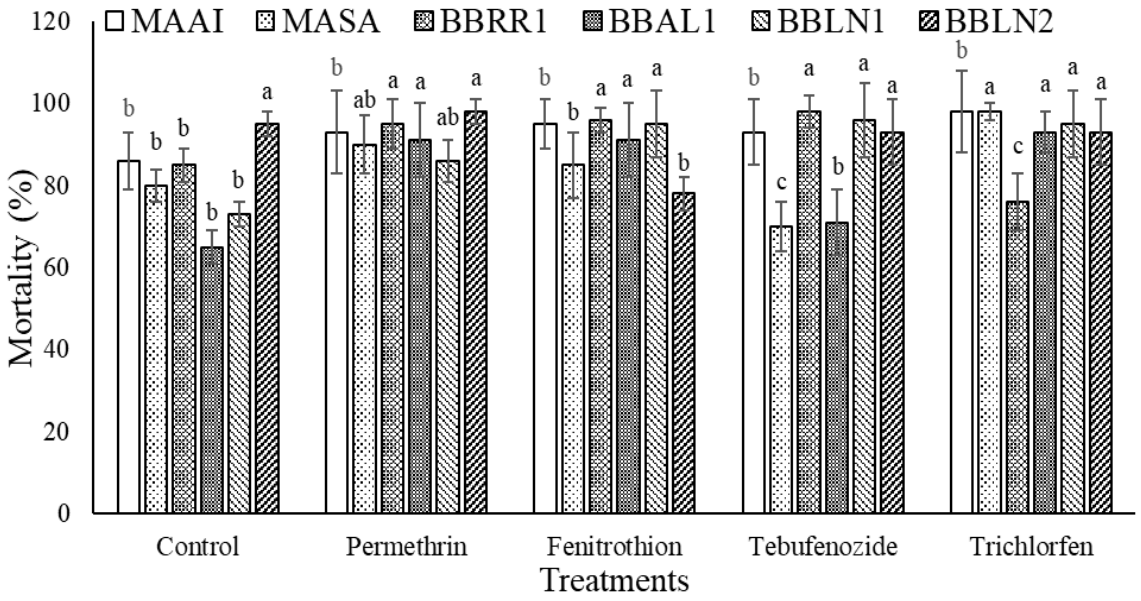

Fig. 3 Comparison of Permethrin, Fenitrothion, Tebufenozide and Trichlorfen toxicity on the control and the fungal treated larvae of *C. suppressalis*. Different letters show statistical differences (Tukey test, $p \leq 0.05$)

In this study, we designed three experiments to evaluate the combined effects of fungal isolates and insecticides on the larvae of *Chilo suppressalis* Walker (Lepidoptera: Crambidae), a major pest of rice in Asia and North Africa (Zibaee *et al*., 2009). The first experiment aimed to determine if exposure to fungal isolates induces larval resistance to the insecticides. The second experiment compared the virulence of fungal isolates grown on insecticide-containing media.

Materials and Methods

Entomopathogenic fungal cultures

The native isolates of *Beauveria bassiana* (BBRR1, BBAL1, BBLN1, and BBLN2) and *Metarhizium anisopliae* (MASA and MAAI) were selected based on their virulence and immunological studies previously conducted on rice striped stem borer larvae (Shahriari *et al*., 2021). To prepare sufficient fungal culture quantities, these isolates were cultured separately on potato dextrose agar and maintained at 22±2 °C for three weeks (Shahriari *et al*., 2021).

Insecticides

Three insecticides-permethrin, tebufenozide, and fenitrothion-were purchased from Kavosh Kimia Kerman, and trichlorfon was obtained from Aria Shimi Company, both located in Iran.

Insect Rearing

Adult *Chilo suppressalis* were collected from rice fields and kept in the laboratory to lay eggs on leaves of the Hashemi rice variety. After hatching, the larvae were transferred to rice stems and kept in sterile containers at 28±2 °C, 80% relative humidity (RH), and a photoperiod of 16 hours light to 8 hours dark (LD 16:8). The larvae were fed fresh stems every two days until they reached the fourth instar. The stock population was maintained for 70 days, and two generations were reared in the laboratory to establish a cohort with minimal environmental stress (Zibaee and Malagoli, 2014). During rearing, the population was not exposed to any chemicals, and the third-generation larvae were used for the experiments.

Experiment I: Effect of Fungal Isolates on Induction of Insecticide Resistance

Bioassay

Initially, 240 fourth instar larvae of the rice striped stem borer (Chilo suppressalis) were treated with a concentration of 10^5 conidia/mL (Shahriari et al., 2021) of different fungal isolates using the immersion method. At intervals of 24, 48, 72, and 96 hours, 30 larvae were randomly selected to determine general esterase and glutathione S-transferase activities. After 96 hours, 120 surviving larvae were divided into four groups of 30 larvae, each to be treated topically with the field dose of permethrin, fenitrothion, trichlorfon, and tebufenozide. Mortality was recorded after 24 hours and compared with 120 control larvae that did not receive initial treatment with fungal isolates (Serebrov *et al*., 2006).

Sample Preparation and Enzyme Assay

Control and treated larvae were randomly selected, weighed, and individually homogenized in phosphate buffer (20 mM, pH 7.1) using a glass pestle. The samples were centrifuged at 20,000g for 20 minutes at 4 °C in 1.5 mL tubes. The supernatant was transferred to new tubes for biochemical experiments.

General Esterase Assay

General esterase was assayed following Han *et al*. (1998) using alpha-naphthyl acetate and beta-naphthyl acetate as substrates. The reaction medium contained 10 µL of each substrate (10 mM), 5 µL of fast blue RR salt (1 mM), and 40 µL of phosphate buffer (20 mM, pH 7). The reaction was initiated by adding 5 µL of enzyme solution, and absorbance was measured at 450 nm after 5 minutes. The assay was performed in triplicate.

Glutathione S-Transferase Activity

Glutathione S-transferase activity was determined using the method reported by Oppenorth (1985). Ten microliters of CDNB (1-chloro, 2,4-dinitrobenzene; 20 mM) or DCNB (2,4-dinitrochlorobenzene; 20 mM) were pipetted separately into microplate wells containing 40 µL of phosphate buffer (20 mM, pH 7.1). Then, 20 µL of reduced glutathione (20 mM) and 5 µL of enzyme solution were added and incubated for 5 minutes before measuring absorbance at 340 nm. The experiment was performed in triplicate.

Experiment II: Changes in Mortality of Larvae by Control and Treated Conidia of Fungal Isolates

This experiment aimed to determine whether culturing fungal isolates on media containing the field-dose concentration of each insecticide affected their virulence against *C. suppressalis* larvae. Thirty larvae (in three replicates of 10) were immersed separately in solutions containing 10^5 spores/mL of the fungal isolates grown on PDA media with field-dose concentrations of each insecticide and those grown on control media. The larvae were immersed in each solution for 15 seconds and then transferred to rearing containers (20×15 cm) covered with Whatman No. 1 filter paper and provided with rice stems. The larvae were kept in a growth chamber for 10-15 days (depending on the observation of mortality) at 25±2 °C, relative humidity of 70±5%, and a 16L:8D photoperiod. Fresh rice stems were provided daily, and mortality was recorded.

Experiment III: Effects of Insecticides on Immunity of the Larvae Against Fungal Isolates

Initially, sixty-fourth instar larvae of *C. suppressalis* were treated topically with 2 µL of ½ the field concentration of each insecticide, separately dissolved in acetone. After 2 hours, a concentration of 10^4 spores/mL of each fungal isolate was injected into the third larval segment. After 20 hours, the third prolegs of the larvae were cut, and the hemolymph was collected in 1.5 mL tubes containing anticoagulant solution [0.1 M glucose, 0.01 M ethylenediaminetetraacetic acid, 0.026 M citric acid, 0.062 M NaCl, pH 4.6; (Azambuja *et al*., 1991)]. The samples were loaded separately into a Neubauer hemocytometer (Chemkind Co. China) to count total and differential hemocytes (plasmatocytes and granulocytes).

Phenoloxidase Activity Assay

Phenoloxidase activity was determined using the hemocyte lysate method based on Leonard *et al*. (1985). In this method, the hemolymph of each larva was collected and centrifuged at 20,000g for 8 minutes at 4 °C. The supernatant was removed, and the pellet was washed twice with phosphate buffer (20 mM, pH 7.1). Finally, 100 µL of phosphate buffer was added to the pellet, which was then homogenized with a glass pestle and centrifuged again at 20,000g for 8 minutes at 4 °C. The enzyme reaction mixture contained 100 µL of phosphate buffer, 50 µL of 10 mM dihydroxyphenylalanine as substrate, and 20 µL of sample. The mixture was incubated for 5 minutes before measuring absorbance at 490 nm. Three replicates were used to determine the average phenoloxidase activity in all experiments.

Protein Assay

The protein content of each enzyme sample was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad) as a standard.

Statistical Analysis

All data were analyzed by one-way analysis of variance followed by Tukey's post hoc test when significant differences were found with a probability of less than 0.05 (SAS 9.4). All experiments were performed in five replicates.

Results

Experiment I: Effect of fungal isolates on induction of larval insecticide resistance

Treatment of larvae with different isolates of *B. bassiana* and *M. anisopliae* induced activities of general esterase and glutathione S-transferase compared to control larvae at different time intervals (Figures 1 and 2). The assay of general esterase with alpha-naphthyl acetate showed the higher enzymatic activity in larvae treated with fungal isolates compared to the control, except for those treated with BBAL1 at 24 and 48 h (F=24.37, Pr>F=0.0001; df=6, 14; Figure 1a). When beta-naphthyl acetate was used as substrate, only larvae treated with BBRR1 at 24 h, MASA at 48 h, BBLN1, BBRR1 and MAAI at 72 h and BBLN1 at 96 h showed the highest activity (F=61.23, Pr>F=0.0001; df=6, 14; Figure 1b).

When CDNB was used as a reagent, the activity of glutathione S-transferase increased in all larvae treated with fungal isolates compared to the control at four time intervals, except for MASA and MAAI at 24 h and MAAI at 48 h (F=39.45, Pr>F=0.0002; df=6, 14; Figure 2a). Except for the larvae treated with MAAI at 48 h, the other treated larvae showed the higher activity of glutathione S-transferase compared to the control using DCNB as a reagent (F=33.29, Pr>F=0.0001; df=6, 14; Figure 2b).

Similar to BBLN2, there was no statistical difference in mortality of MAAI treated larvae by different insecticides (F=1.89, Pr>F=0.246; df=6, 14; Figure 3). In contrast, larvae already treated with BBRR1, BBAL1 and BBLN2 had the highest mortality following insecticide treatment (F=52.84, Pr>F=0.0001; df=6, 14; Figure 3). In the case of MASA treated larvae, treatment with permethrin and trichlorfen increased larval mortality compared to the control, while those treated with fenitrothion

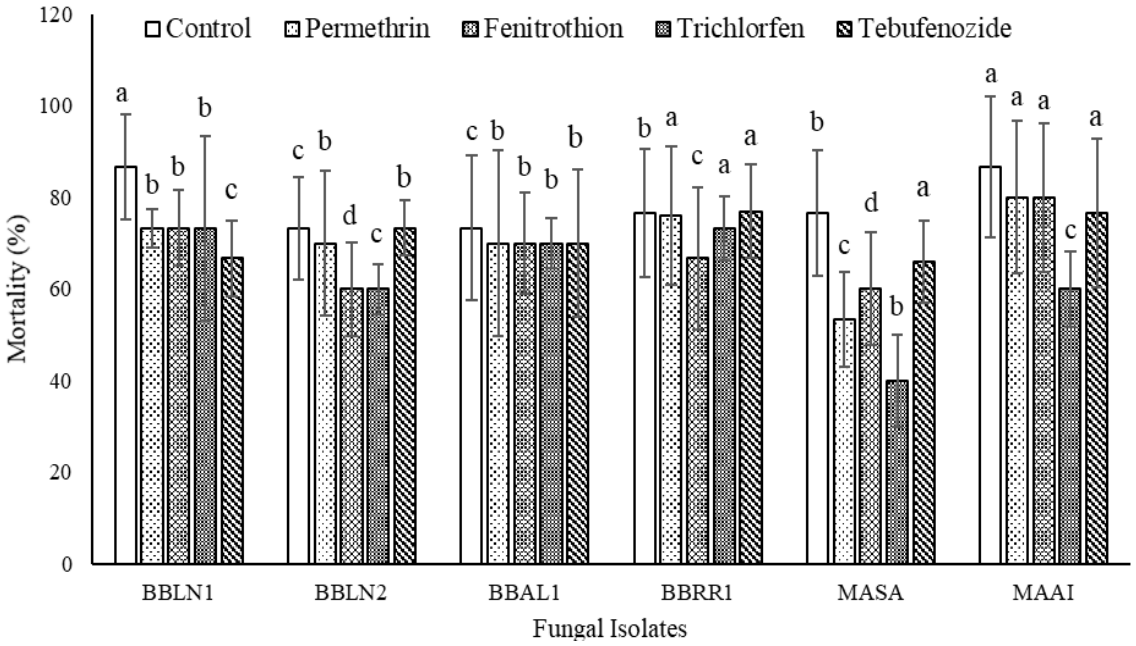

Fig. 4 Effects of control and treated conidia of *B. bassiana* and *M. anisopliae* isolates combined with different insecticides on mortality of the larvae of *C. suppressalis*. Different letters show statistical differences (Tukey test, $p \leq 0.05$)

showed no significant differences and those treated with tebufenozide showed the lower mortality compared to the control (F=34.28, Pr>F=0.0042; df=6, 14; Figure 3).

Experiment II; Changes in mortality of larvae by the control and treated conidia of fungal isolates

Conidia of BBLN1 cultured in control media caused the highest mortality of *C. suppressalis* larvae, while addition of insecticides to fungal culture media decreased larval mortality compared to control, with the lowest mortality caused by tebufenozide (F=55.21, Pr>F=0.0003; df=6, 14; Figure 4). There were no statistical differences in larval mortality between control conidia of BBLN2 and those cultured in media containing tebufenozide, while other insecticides decreased mortality compared to control (F=0.82, Pr>F=0.359; df=6, 14; Figure 4). The conidia of BBAL1 cultured in the media containing insecticides caused lower mortality against the larvae compared to the control without any significant differences (F=18.34, Pr>F=0.0023; df=6, 14; Figure 4).

No significant differences were observed in the mortality of *C. suppressalis* larvae treated by conidia cultured in control, permethrin and tebufenozide-containing media (F=1.18, Pr>F=0.581; df=6, 14), while fenitrothion and trichlorfen caused lower mortality compared to the control (F=55.29, Pr>F=0.0031; df=6, 14; Figure 4). The addition of the insecticides to the culture media of M. anisopliae isolates resulted in lower mortality of *C. suppressalis* larvae compared to the control, with trichlorfen treatment causing the least mortality compared to the control (F=29.75, Pr>F=0.0002; df=6, 14; Figure 4).

Experiment III, Effects of insecticides on immunity of the larvae to fungal isolates

Total haemocyte counts showed no statistical differences in control and insecticide-treated *C. suppressalis* larvae after BBLN1 conidia treatment, except for permethrin, which increased significantly compared to control (F=3.48, Pr>F=0.064; df=6, 14; Figure 5a). Although the number of haemocytes increased in the permethrin-treated larvae against BBLN2, the other treatments showed lower total haemocyte counts except for the tebufenozide-treated larvae (F=76.38, Pr>F=0.0001; df=6, 14; Figure 5a). The insecticide-treated larvae showed the lowest number of total haemocyte counts against BBAL1, BBRR1 and MAAI compared to the control with the lowest number in the tebufenozide treatment (F=35.67, Pr>F=0.0002; df=6, 14; Figure 5a). The number of total haemocytes in treated larvae by insecticides decreased after MASA treatment compared to control, except for trichlorfen which increased significantly compared to control (F=18.27, Pr>F=0.0025; df=6, 14; Figure 5a).

Simultaneous testing of insecticides and fungal isolates on *C. suppressalis* larvae also caused changes in the number of plasmatocytes and granulocytes (Figure 5b, c). The number of plasmatocytes increased after BBAN treatment with permethrin and trichlorfen except for fenitrothion and tebufenozide (F=112.23, Pr>F=0.0001; df=6, 14; Figure 5b). A similar finding was found with BBLN2 treatment, although the number of plasmatocytes decreased after trichlorfen and tebufenozide treatments (F=86.23, Pr>F=0.0001; df=6, 14; Figure 5b). The number of plasmatocytes increased in all concurrent treatments of BBAL1 and insecticides compared to the control, while BBRR1,

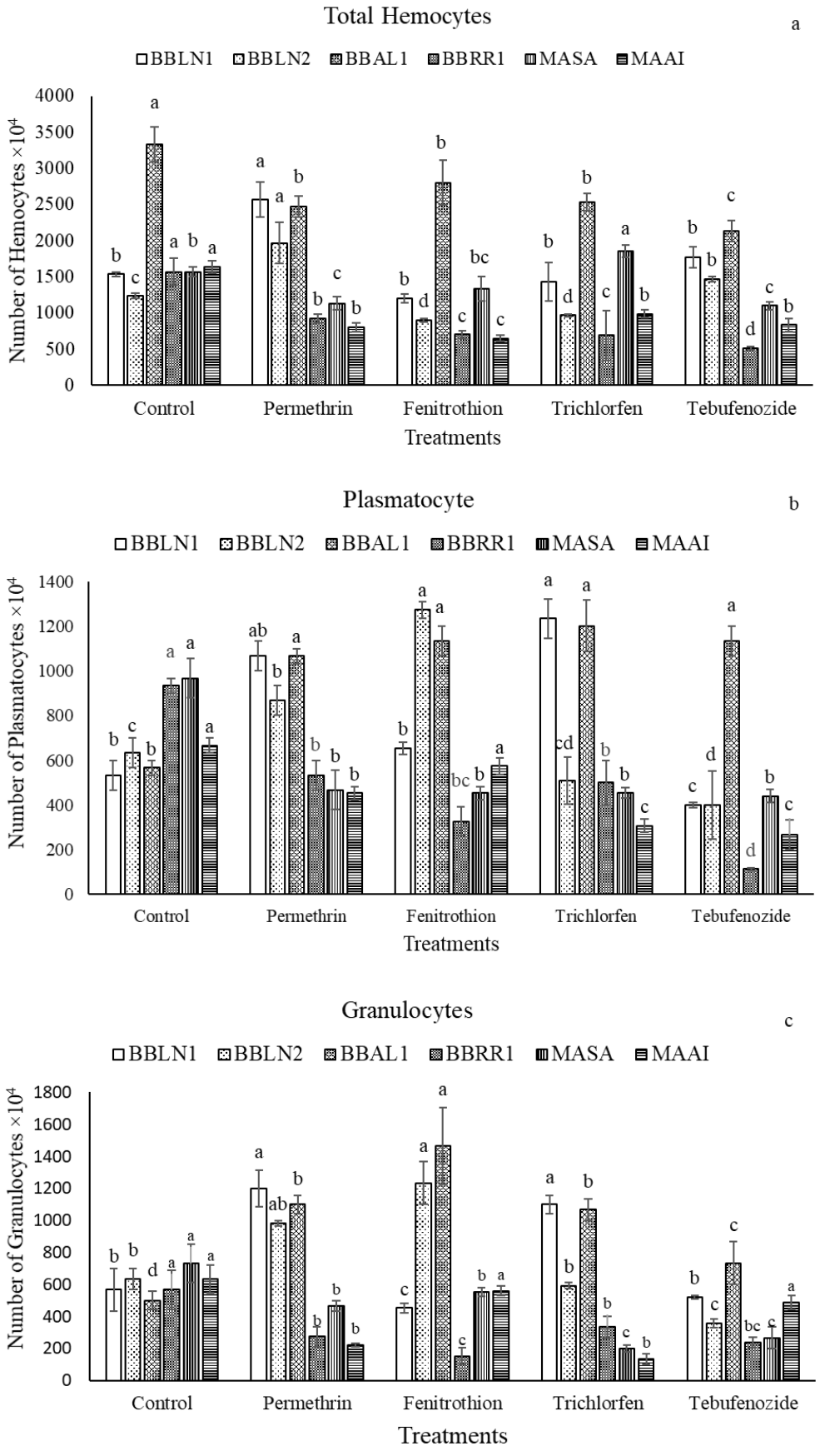

Fig. 5 Effects of *B. bassiana* and *M. anisopliae* isolated on total and differentiate hemocyte counts of the control and treated larvae of *Chilo suppressalis* by Permethrin, Fenitrothion, Trichlorfen and Tebufenozide. Different letters show statistical differences (Tukey test, $p \leq 0.05$)

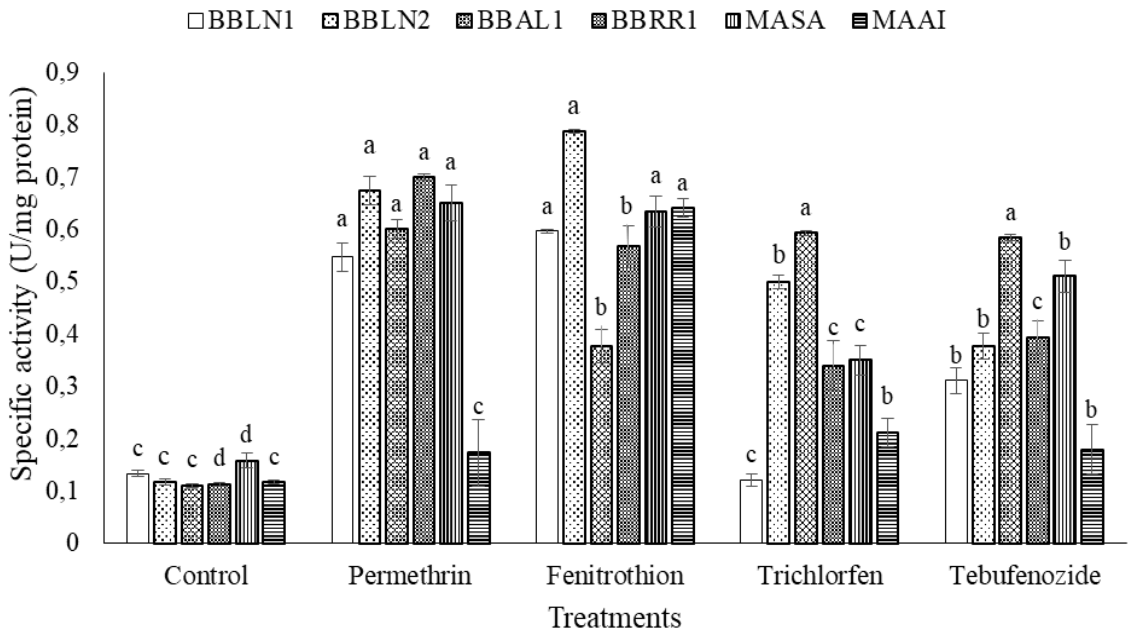

Fig. 6 Effects of *B. bassiana* and *M. anisopliae* isolated on phenoloxidase activity of the control and treated larvae of *C. suppressalis* by Permethrin, Fenitrothion, Trichlorfen and Tebufenozide. Different letters show statistical differences (Tukey test, $p \leq 0.05$)

MASA and MAAI with insecticidal treatments significantly decreased the number of plasmatocytes in *C. suppressalis* larvae (F=61.44, Pr>F=0.0001; df=6, 14; Figure 5b). Although the number of granulocytes increased in the simultaneous assay of BBLN1 with permethrin and trichlorfen, it significantly decreased in the other two treatments (F=53.26, Pr>F=0.0001; df=6, 14; Figure 5c).

Discussion

The simultaneous use of multiple control methods to reduce pest populations and damage is one of the most efficient strategies in pest management. However, it is essential to conduct various ecological and physiological evaluations before combining these methods to ensure their effectiveness and understand their consequences. In this study, we aimed to determine the impact of applying entomopathogenic fungi on the potential resistance of *C. suppressalis* to chemical pesticides through the induction of detoxifying agents. Additionally, we evaluated the combined effects of insecticides and fungal isolates on the larval immune system to assess the effectiveness of microbial control.

Cytochrome P450, general esterases, and glutathione S-transferases are the three primary enzymes involved in the insect detoxification system. Cytochrome P450 plays a dual role in insects by detoxifying xenobiotics and increasing the toxicity of certain pesticides, such as organophosphates (Levi *et al*., 1988). General esterases hydrolyze a wide range of aliphatic and aromatic esters, choline esters, and other compounds. Due to their high substrate activity, these enzymes play a crucial role in the detoxification of all xenobiotics, including phytochemicals, chemical insecticides, and some microbial components (Hemingway & Karunatne, 1998). Glutathione S-transferases are a multigenic family of multifunctional proteins that conjugate molecules containing an electrophilic site to reduce glutathione. These enzymes typically convert reactive lipophilic molecules into water-soluble, non-reactive forms that are easily excreted (Habig *et al*., 1974). Our results indicated that treatment of larvae with different isolates of *B. bassiana* and *M. anisopliae* induced the activities of general esterase and glutathione S-transferase compared to control larvae at various time intervals, with some strain-specific exceptions.

Conclusions

The present study found that the initial application of entomopathogenic fungi to *C. suppressalis* larvae induced the detoxification system but did not affect larval mortality following insecticide treatment, except for tebufenozide, which significantly reduced mortality in the cases of MASA and BBAL1. In all treatments, the addition of insecticides to the fungal culture media negatively impacted their virulence against the larvae, except for tebufenozide in the cases of BBLN2 and BBRR1. The results regarding the immune response of *C. suppressalis* larvae to the combined effects of insecticides and fungal isolates were mixed, indicating that each combination should be carefully

selected to enhance the efficiency of fungal isolates in evading larval cellular immunity. The findings of this study expand our understanding of the potential efficacy of microbial and chemical control methods against *C. suppressalis* and should be considered for possible application in rice fields.

Acknowledgement

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