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Alterations in the germination, spore production, vegetative growth and microbial enzymes of Beauveria bassiana and Metarhizium anisopliae isolates following treatments by some insecticides

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ABSTRACT

Insecticides have influences on survival, growth and pathogenic pathways of entomopathogenic fungi (EF). Screening of EFs compatibility to insecticides is important to achieve an efficient pest control in integrated pest management. In the current investigation, potential effects of permethrin, fenitrothion, tebufenozide and trichlorfen were investigated on germination, mycelial growth, spore production and enzymatic activities of the native isolates of Beauveria bassiana (BBRR1, BBAL1, BBLN1, BBLN2) and Metarhizium anisopliae (MASA, MAAI). Spore production of the isolates significantly decreased following treatment with insecticides, mainly at the field dose concentration, except for permethrin and fenitrothion on BBAL1 and MASA. Germination rate significantly decreased after fungal isolates spores treatments with the insecticides except for MAAI. Treatment of the fungal isolates by insecticides reduced colony radial growth rate, especially after field dose treatments. Chitinase activity significantly decreased in the treated isolates, although no statistical differences were recorded on BBAL1 following treatment by fenitrothion, tebufenozide and trichlorfen. Finally, protease activity was also affected in the treated fungal isolates by insecticides. The current results showed that permethrin, fenitrothion, tebufenozide and trichlorfen negatively affected growth, development and spore production in addition to microbial enzymes involved in the pathogenic process. Some exceptions were observed mainly on BBAL1, which should be considered in the joint use of insecticides with the entomopathogenic fungi.

Keywords: Chemical, Entomopathogenic fungi, Native isolate, Growth, Enzyme activity.
Article type: Research Article.

INTRODUCTION

Entomopathogenic fungi are pathogens of arthropods that have a significant ability to regulate pests' populations. In fact, entomopathogenic fungi naturally keep insect populations at a balanced level and therefore have a favorable potential as microbial insecticides (Kikankie *et al.* 2010; Araujo & Hughes 2016). Entomopathogenic fungi are found across the Oomycota, Microsporidia, Chytridiomycota, Zygomycota, Basidiomycota and Ascomycota, most species belong to the Zygomycota and the Entomophthorales orders (Humber 2012; Araujo & Hughes 2016). These species have been mass produced and formulated against several pests of agroecosystems and forests because of easy culture on several rearing media, safety for humans and livestock, production of non-sexual spores on hosts and resistance to environmental extremes by producing inactive spores (Maina *et al.* 2018). Pesticides may inflict entomopathogenic fungi by inhibiting conidial production and germination, hence compatibility of entomopathogenic fungi and synthetic insecticides should be assessed by determination of vegetative growth, conidia production and germination, and before any decision for simultaneous field application (Mietkiewski & Gorski 1995; Gupta *et al.* 1999). The percentage of conidial germination should be considered as a main parameter to determine compatibility between entomopathogenic fungi and insecticides, because

germination of conidia and subsequent penetration into the host body is essential for infection (Hirose *et al.* 2001; Neves *et al.* 2001; Oliveria *et al.* 2003). Vegetative growth is another important factor to demonstrate the colonizing potential of entomopathogenic fungi that may be limited by synthetic insecticides once they are sprayed on plants and soil surfaces (Zibaee 2019). Entomopathogenic fungi employ two main enzymes to penetrate through insect integument. Chitinases are complex enzymes in biological systems that hydrolyze b-(1,4)-linked polymer of N-acetyl-D-glucosamine of chitin. In addition to the role of chitinases during the infection processes of entomopathogenic fungi, they are also involved in several functions of fungal biology such as conidial germination, hyphal growth and morphogenesis (Dias *et al.* 2008). Because proteins are the other main molecules in the insect integument, fungi utilize two main groups of proteases to hydrolase these molecules. The first one is subtilisin-like serine protease (Pr1) that is synthesized in the host cuticle during the early stages of penetration. The second is trypsin-like enzyme (Pr2) that is critical in degrading extracellular proteins complementary to that of Pr1 (Dias *et al.* 2008). One of the effective and appropriate methods in pest control is to simultaneously target pests with pesticides and biological control agents. This measure, referred to as a "dual attack", can increase mortality in insect pests and slow down resistance to insecticides (Hiromori & Nishigaki 1993; Bitsadze *et al.* 2001). This requires an understanding of ecological, molecular and physiological interactions between pesticides and entomopathogens. Our previous study reported six entomopathogenic fungal isolates, four *Beauveria bassiana* and two *Metarhizium anisopliae*, as highly virulent against the larvae of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) under laboratory conditions (Shahriari *et al.* 2021a). Since insecticide spray is a main control procedure in paddy fields of Northern Iran, support and recommendation of any mycoinsecticide requires evaluating compatibility to the conventional used insecticides. So, the current investigation was designed to determine the effects of field, semi-field and quarter-field doses of permethrin, fenitrothion, tebufenozide and trichlorfen on mycelial growth, spore production and spore germination in *B. bassiana* (BBRR1, BBLN1, BBLN2, BBAL1) and *M. anisopliae* (MASA and MAAI). Afterward, the field dose effect of the aforementioned insecticides was evaluated on chitinase and protease activities of the fungal isolates. Results of the current study will reveal whether these insecticides are compatible with the most virulent isolates of *B. bassiana* and *M. anisopliae* to consider other steps of formulation and field application. There is no similar data to find effects of insecticides on EFs collected from paddy fields while determination of insecticide-treated conidia on insects are the novel aspect of our study.

MATERIALS AND METHODS

Entomopathogenic fungal cultures

The native isolates of *B. bassiana* (BBRR1, BBAL1, BBLN1 and BBLN2) and *M. anisopliae* (MASA and MAAI) were selected based on virulence and immunological studies on rice striped stem borer larvae (Shahriari *et al.* 2021a, b). In order to prepare the sufficient amounts of fungal culture, these isolates were separately cultured on Potato Dextrose Agar, then kept for three weeks at 22 ± 2 °C and humidity of 70% (Shahriari *et al.* 2021a).

Insecticides

Permethrin, tebufenozide and fenitrothion were purchased from Kavosh Kimia Kerman, and trichlorfen was purchased from Aria Shimi Company, both in Iran.

Effects of insecticides on mycelial growth, spore production and germination

First, PDA culture media were prepared separately in a volume of 5 mL within petri dishes. Then, three different concentrations of permethrin, tebufenozide, fenitrothion and trichlorfen (1 mL = field dose, ½ and ¼ field doses) were separately added to the media, although the control culture media were treated with 2 mL of distilled water. Each medium was inoculated by a concentration of 10^7 conidia/mL (2 mL solution volume). Culture media were incubated for three days at 22 ± 2 °C, then the number of produced spores was counted by adding one mL of sterile distilled water solution containing 0.02% Tween-80 using a hemocytometer. Five petri dishes including each fungal isolates were considered for control and insecticide doses (Wu *et al.* 2019). In order to investigate the effect of insecticides on the mycelial growth of fungal isolates, culture media-containing field, semi field and quarter field doses of insecticides were prepared and incubated overnight at 25 ± 2 °C. Thereafter, a 5-mm plug was gently taken from the standard culture media containing newly-grown fungal isolates and placed in the center

of the culture media containing insecticides (10 plates were used for each treatment even control). After 7 days of incubation at 22 ± 2 °C, the mycelial growth was measured under an optic microscope (Olympus; Wu *et al.* 2019). Germination rate of the fungal isolates was evaluated by adding the field, semi-field and quarter-field doses of insecticides (prepared at 0.02% Tween-80 solution) to 50 mL of PDA culture media. Then, 1 mL of 10^7 conidia/ml of each isolate was separately prepared and slowly spread on petri dishes. After incubation for 24 hours at 25 ± 2 °C in darkness, the germination rate was determined under a stereomicroscope, so that the length of the germ tube was greater than the diameter of the conidia (Marcuzzo & Eli 2016).

Effects of insecticides on chitinases and proteases of fungal isolates

Liquid culture for enzyme production

Field dose concentration of each insecticide prepared in 0.02% Tween 80 solution and separately added to the liquid culture medium, considering control media and all inoculated with 2 mL of 10^7 conidia/mL. The liquid medium contained $CaCl_2$ (0.01%), Na_2HPO_4 (0.02%), KH_2PO_4 (0.02%), $MgCl_2$ (0.01%) and $ZnCl_2$ (0.01%) added into distilled water and enriched by 1% of yeast and 5% larval cuticle of *C. suppressalis*. The resulting medium was autoclaved for two hours before adding insecticides and conidia. A 100 mL of liquid medium was prepared for each fungal isolate and treatment. The flasks containing culture medium and conidia were shaken for 8 days at 25 ± 2 °C and darkness. Afterward, samples of each flask were transferred to a 50-mL tubes and centrifuged at 5000 rpm for 30 min at laboratory temperature. The supernatant was discarded, and the grown mycelia were washed with Tris-HCl (25 mM, pH 7) and homogenized in the same buffer solution. The samples were centrifuged at 13000 rpm and 4 °C for 30 min (Hajji *et al.* 2007).

Enzymatic assay

Exochitinase assay

The activity of exochitinase was assayed by the method of Miller (1959) using p-nitrophenyl-Nacetyl-β-D-glucosaminide (pNPg) substrate. The reaction mixture contained 25 µL sample (control and treatment separately), 200 µL substrate (50 mM) and 500 µL Tris-HCl buffer (25 mM, pH 7) was incubated for 20 hours at 30 °C. Then, the mixture was centrifuged at 13,000 rpm and 4 °C for 30 min, and the supernatant was added to a new 1.5-mL Eppendorf containing 200 µL Tris-HCl buffer (125 mM, pH 10). Finally, 200 µL of the latter mixture was added to the well of the microplate reader and read at 405 nm. Based on the following formula, the enzyme activity was measured:

Volume activity (U/mL) = [ΔOD(ODtest-ODblank) × Vt × df]/(18.5 × t × 1.0 × Vs)

where Vt is the total reaction volume, Vs is the sample volume, 18.5 is extinction coefficient based on mM, 1.0 is the constant of the formula, t is the reaction time, and Df is the dilution coefficient. The negative control included all components of the biochemical reaction except for the enzyme, which was replaced by distilled water.

Endochitinase assay

The reaction mixture contained 20 µL of enzyme, 50 µL of 0.5% colloidal chitin and 100 µL of Tris-HCl buffer (20 mM, pH 7). The mixture was kept in a water bath at 30 °C for 60 min, then 100 µL of dinitrosalicylic acid solution was added and incubated for 10 min at boiling water. Finally, the absorbance was read at 545 nm. The tubes containing the negative control were placed in a boiling water bath for 15 min to destroy the enzyme activity and then cooled prior to being added to the substrate. Other assay steps were done as described earlier (Miller 1959).

Protease assay

Subtilisin-like and trypsin-like activities were assayed using succinyl-(alanine) 2-proline phenylalanine-p-nitroanilide and benzoyl-phenylalanine-valinearginine-p-nitroanilide as substrates, respectively. The reaction mixture consisted of 30 µL substrate (5 mM), 100 µL Tris-HCl buffer (20 mM, pH 8) and 20 µL enzyme. The mixture was incubated for 10 min at 25 °C, then the reaction was terminated by adding 100 µL of 30% trichloroacetic acid. Finally, absorbance was read at 405 nm (Dias *et al.* 2009).

Protein assay

Protein content in each enzymatic sample was assayed and 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 and t-test when significant differences were found at a probability less than 0.05 (SAS 9.4). All experiments were done in 10 replicates.

RESULTS

Conidia production in *B. bassiana* and *M. anisopliae* isolates was significantly influenced by all three concentrations of permethrin, fenitrothion, tebufenozide and trichlorfen. Conidia production significantly decreased in the BBRR1, BBLN1 and BBLN2 isolates after insecticidal treatments except for ¼ field dose of fenitrothion and permethrin (Fig. 1). In BBLA1, permethrin and fenitrothion showed no significant effects on conidia production in all treated doses, while two other insecticides reduced conidia production except for ¼ field dose of trichlorfen (Fig. 1). All used insecticides also decreased conidia production in *M. anisopliae* isolates, while trichlorfen (¼ field dose), permethrin and tebufenozide had no significant effects on conidia production in the MASA and MAAI isolates (Fig. 2).

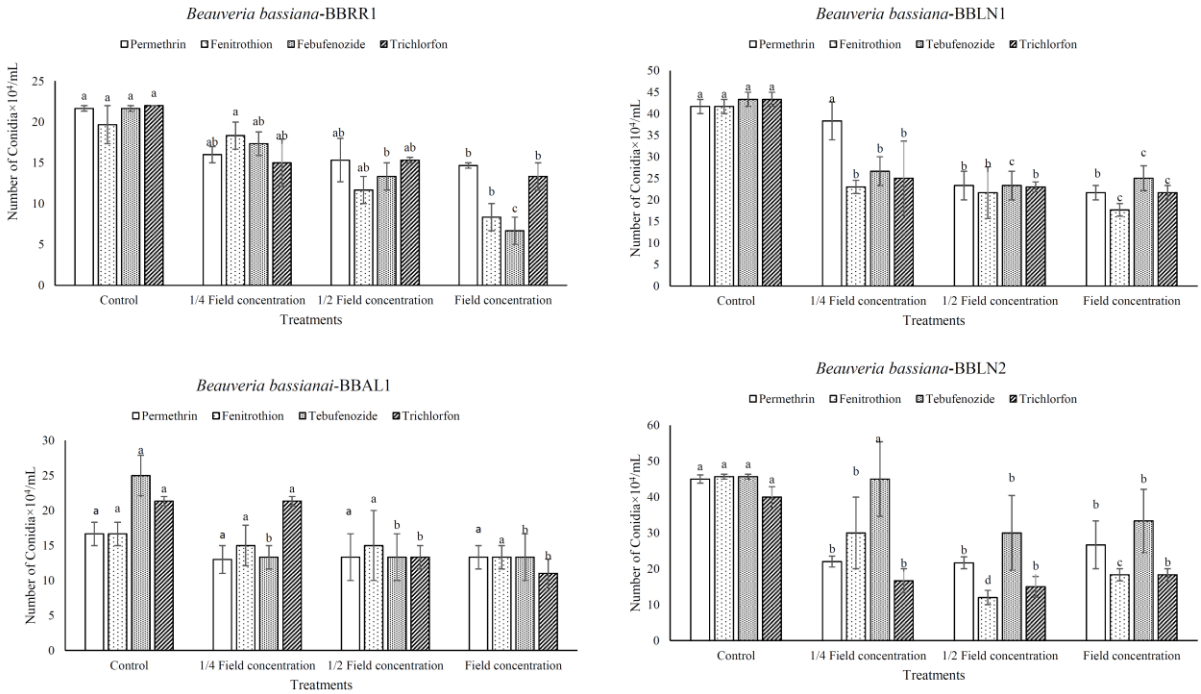

Fig. 1. Quantity of conidia production in the four isolates of *Beauveria bassiana* following treatment with the three concentrations of insecticides. Means ± SE with different letters show statistical differences at the probability less than 5% (Tukey's test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Treatments of permethrin, fenitrothion, tebufenozide and trichlorfen significantly affected radial growth in the *B. bassiana* and *M. anisopliae* isolates (Table 1). Permethrin significantly reduced colony diameter in all fungal isolates except for the ¼ and ½ field dose in BBLN2 that no significant differences were recorded compared to the control (Table 1). Fenitrothion had no significant effects on radial growth in the BBRR1, BBLN1 and MASA isolates. Similarly, the ¼ and ½ field doses of fenitrothion resulted in no significant differences in colony diameter in the BBAL1 and MAAI isoaltes compared to control (Table 1). Tebufenozide treatment resulted in no significant differences in colony diameter in the BBLN1 and BBLN2 isolates, but it significantly reduced colony diameter of other fungal isolates (Table 1). Except for BBAL1 and MAAI, trichlorfen demonstrated no significant effects on radial growth in fungal isolates compared to control (Table 1).

Germination rate (%) significantly decreased in the fungal isolates treated by the field dose concentrations of permethrin, fenitrothion, tebufenozide and trichlorfen compared to control (Fig. 3). Among the treatments, tebufenozide led to the least germination rate in BBLN2 and BBAL1 (Fig. 3), while no significant differences were recorded between control and all insecticides treatments in MAAI (Fig. 4).

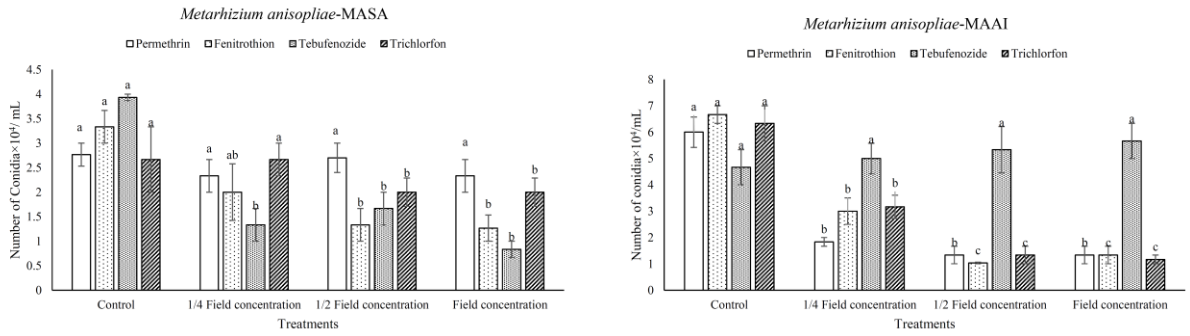

Fig. 2. Quantity of conidia production in the isolates of *Metarhizium anisopliae* following treatment with the three concentrations of insecticides. Means±SE with different letters show statistical differences at ethe probability less than 5% (Tukey's test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Table 1. Effects of insecticides on the colony diameter (mm) of *Beauveria bassiana* and *Metarhizium anisopliae*.

Insecticides*	**Conc.**	***Beauveria bassiana***				***Metarhizium anisopliae***	
Isolates		BBRR1	BBLN1	BBLN2	BBAL1	MASA	MAAI
Permethrin	Control	2.69 ± 0.017^{a}	3.19 ± 0.029^{a}	2.70 ± 0.029^{a}	3.45 ± 0.032^{a}	2.69 ± 0.017^{a}	4.26 ± 0.033^{a}
	¼ Field	1.46 ± 0.033^{b}	2.93 ± 0.38^{ab}	2.58 ± 0.21^{a}	2.83 ± 0.088^{ab}	1.46 ± 0.033^{b}	3.20 ± 0.17^{b}
	½ Field	146 ± 0.013^{b}	2 ± 0.057^{bc}	2.66 ± 0.22^{a}	2.35 ± 0.20^{b}	1.46 ± 0.033^{b}	2.15 ± 0.13^{c}
	Field	1.46 ± 0.12^{b}	1.23 ± 0.14^{c}	1.36 ± 0.088^{b}	2.40 ± 0.20^{b}	1.46 ± 0.12^{b}	1.91 ± 0.22^{c}
Fenitrothion	Control	2.56 ± 0.13^{a}	2.60 ± 0.30^{a}	5.68 ± 0.046^{a}	4.26 ± 0.033^{a}	2.54 ± 0.13^{a}	4.26 ± 0.033^{a}
	¼ Field	2.23 ± 0.12^{a}	2.56 ± 0.18^{a}	3.26 ± 0.088^{ab}	4.13 ± 0.066^{a}	2.23 ± 0.29^{a}	4.13 ± 0.066^{a}
	½ Field	2.16 ± 0.29^{a}	2.53 ± 0.033^{a}	3.63 ± 0.26^{ab}	4.06 ± 0.88^{a}	2.16 ± 0.12^{a}	4.06 ± 0.088^{a}
	Field	2.23 ± 0.64^{a}	2.66 ± 0.33^{a}	2.78 ± 0.30^{b}	2.95 ± 0.37^{b}	2.23 ± 0.64^{a}	2.95 ± 0.37^{b}
Tebufenozide	Control	2.69 ± 0.017^{a}	3.28 ± 0.061^{a}	3.70 ± 0.029^{a}	4.26 ± 0.033^{a}	2.69 ± 0.017^{a}	4.26 ± 0.033^{a}
	¼ Field	2.75 ± 0.25^{a}	3.30 ± 0.35^{a}	3.81 ± 0.37^{a}	4.28 ± 0.11^{b}	2.75 ± 0.25^{a}	4.28 ± 0.11^{b}
	½ Field	1.93 ± 0.066^{b}	3.36 ± 0.32^{a}	3.56 ± 0.088^{a}	3.66 ± 0.066^{bc}	1.93 ± 0.066^{b}	3.66 ± 0.066^{bc}
	Field	1.66 ± 0.066^{b}	3.00 ± 0.11^{a}	3.51 ± 0.25^{a}	3.06 ± 0.033^{c}	1.66 ± 0.066^{b}	3.06 ± 0.033^{c}
Trichlorfen	Control	2.69 ± 0.017^{a}	3.19 ± 0.029^{a}	2.70 ± 0.029^{a}	5.26 ± 0.033^{a}	2.69 ± 0.017^{a}	5.26 ± 0.033^{a}
	¼ Field	2.53 ± 0.033^{a}	2.80 ± 0.40^{a}	2.50 ± 0.15^{a}	5.50 ± 0.28^{a}	2.53 ± 0.033^{a}	5.50 ± 0.28^{a}
	½ Field	2.79 ± 0.14^{a}	2.83 ± 0.083^{a}	2.50 ± 0.25^{a}	3.91 ± 0.033^{ab}	2.75 ± 0.033^{a}	3.91 ± 0.033^{ab}
	Field	2.73 ± 0.14^{a}	2.83 ± 0.16^{a}	2.41 ± 0.16^{a}	3.41 ± 0.083^{b}	2.73 ± 0.14^{a}	3.41 ± 0.083^{b}

Note *. Means ± SE with asterisks show statistical differences at the probability of less than 5% (Tukey's test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Field dose of insecticides significantly decreased chitinase activity of *B. bassiana* isolates except for exochitinase after treatment by fenitrothion and trichlorfen (Table 2). Also, exochitinase activity showed no significant difference between control and treated BBAL1 by fenitrothion, tebufenozide and trichlorfen (Table 2). Moreover, chitinase activity decreased in the fungal isolates treated by insecticides except for endochitinase activity in MASA treated by permethrin and fenitrothion as well as exochitinase activity in both *M. anisopliae* isolates treated by trichlorfen (Table 3). The trypsin-like protease activity decreased in *B. bassiana* isolates, as BBRR1 treated by permethrin, BBLN1 treated by fenitrothion, tebufenozide and trichlorfen, BBLN2 treated by permethrin and fenitrothion as well as BBAL1 treated by fenitrothion, while other treatments showed no significant differences compared to control (Table 4). The subtilisin-like protease activity significantly decreased in *B. bassiana* isolates treated by insecticides except for BBLN2 treated by trichlorfen (Table 4). Activity of trypsin-like protease

significantly decreased in *M. anisopliae* isolates treated by insecticides except for MASA treatment by trichlorfen (Table 5). Similar results were recorded in the case of subtilisin-like protease except for MASA isolate treated by permethrin and trichlorfen that showed no significant differences compared to control treatment (Table 5).

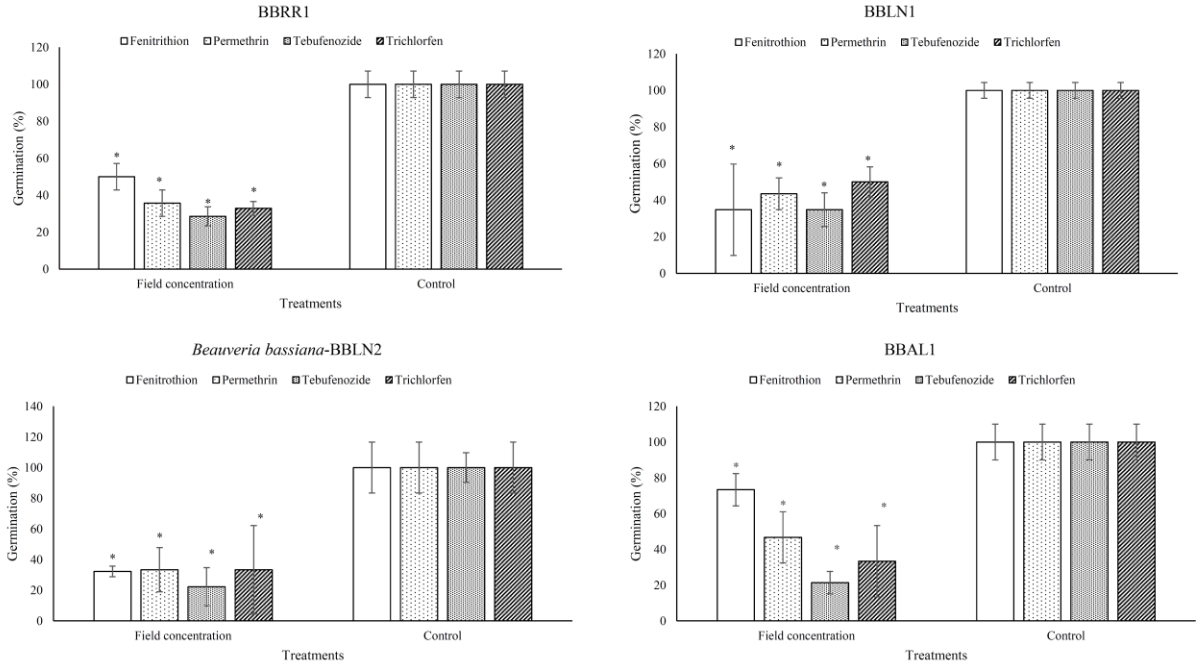

Fig. 3. Germination rate (%) in the isolates of *Beauveria bassiana* following treatment with the insecticides. Means±SE with asterisks show statistical differences at ethe probability less than 5% (Tukey's test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

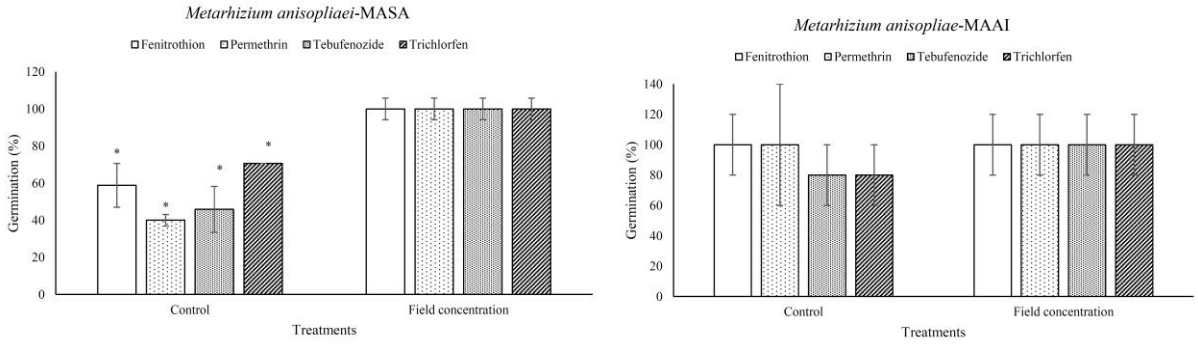

Fig. 4. Germination rate (%) in the isolates of *Metarhizium anisopliae* following treatment with the insecticides. Means ± SE with asterisks show statistical differences at ethe probability less than 5% (Tukey's test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Table 2. Effects of insecticides on the chitinase activity (U/mg protein) of *Beauveria bassiana*.

Insecticides*	**Concentrations**	**Endochitinase**				**Exochitinase**			
Isolates		BBRR1	BBLN1	BBLN2	BBAL1	BBRR1	BBLN1	BBLN2	BBAL1
Permethrin	Control	0.17 ± 0.047*	0.16 ± 0.002*	0.15 ± 0.003*	0.18 ± 0.008*	0.37 ± 0.006*	0.34 ± 0.03*	0.34 ± 0.041*	0.32 ± 0.06*
	Field	0.15 ± 0.006	0.14 ± 0.007	0.14 ± 0.003	0.16 ± 0.008	0.30 ± 0.042	0.30 ± 0.005	0.30 ± 0.009	0.27 ± 0.026
Fenitrothion	Control	0.16 ± 0.002*	0.18 ± 0.004*	0.16 ± 0.007*	0.18 ± 0.008*	0.33 ± 0.013	0.41 ± 0.006*	0.34 ± 0.028*	0.35 ± 0.013
	Field	0.13 ± 0.012	0.13 ± 0.008	0.15 ± 0.01	0.18 ± 0.008	0.32 ± 0.024	0.30 ± 0.045	0.30 ± 0.009	0.34 ± 0.028

Tebufenozide	Control	0.17 ± 0.002*	0.17 ± 0.004*	0.17 ± 0.012*	0.19 ± 0.002*	0.34 ± 0.002*	0.33 ± 0.012	0.35 ± 0.008*	0.34 ± 0.004
	Field	0.15 ± 0.005	0.15 ± 0.009	0.14 ± 0.003	0.15 ± 0.011	0.31 ± 0.010	0.33 ± 0.002	0.33 ± 0.013	0.33 ± 0.005
Trichlorfen	Control	0.18 ± 0.004*	0.18 ± 0.005*	0.17 ± 0.013*	0.19 ± 0.003*	0.34 ± 0.005	0.38 ± 0.019*	0.33 ± 0.005*	0.35 ± 0.033
	Field	0.15 ± 0.003	0.15 ± 0.002	0.15 ± 0.003	0.15 ± 0.008	0.34 ± 0.01	0.32 ± 0.007	0.30 ± 0.002	0.34 ± 0.008

Note *. Means ± SE with asterisks show statistical differences at the probability of less than 5% (t-test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Table 3. Effects of insecticides on the chitinase activity (U/mg protein) of *Metarhizium anisopliae*.

Insecticides*	**Concentrations**	**Endochitinase**		**Exochitinase**	
Isolates		MASA	MAAI	MASA	MAAI
Permethrin	Control	0.17 ± 0.010	0.17 ± 0.003*	0.45 ± 0.019*	0.35 ± 0.009*
	Field	0.17 ± 0.005	0.13 ± 0.003	0.35 ± 0.014	0.32 ± 0.009
Fenitrothion	Control	0.14 ± 0.007	0.17 ± 0.005*	0.33 ± 0.012*	0.36 ± 0.032*
	Field	0.13 ± 0.004	0.15 ± 0.005	0.30 ± 0.015	0.31 ± 0.007
Tebufenozide	Control	0.19 ± 0.007*	0.18 ± 0.010*	0.36 ± 0.007*	0.33 ± 0.003*
	Field	0.16 ± 0.007	0.13 ± 0.002	0.33 ± 0.021	0.30 ± 0.021
Trichlorfen	Control	0.18 ± 0.006*	0.19 ± 0.004*	0.32 ± 0.002	0.33 ± 0.004
	Field	0.16 ± 0.003	0.14 ± 0.006	0.31 ± 0.009	0.32 ± 0.004

Note *. Means±SE with asterisks show statistical differences at ethe probability less than 5% (t-test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Table 4. Effects of insecticides on the protease activity (U/mg protein) of *Beauveria bassiana*.

Insecticides*	**Concentrations**	**Trypsin-like**				**Subtilisin-like**			
Isolates		BBRR1	BBLN1	BBLN2	BBAL1	BBRR1	BBLN1	BBLN2	BBAL1
Permethrin	Control	0.20 ± 0.052*	0.15 ± 0.002	0.17 ± 0.001*	0.17 ± 0.005	0.20 ± 0.002*	0.21 ± 0.005	0.24 ± 0.003*	0.17 ± 0.009*
	Field	0.18 ± 0.026	0.14 ± 0.006	0.14 ± 0.020	0.16 ± 0.004	0.18 ± 0.008	0.22 ± 0.009	0.22 ± 0.007	0.15 ± 0.020
Fenitrothion	Control	0.17 ± 0.006	0.19 ± 0.023*	0.20 ± 0.028*	0.17 ± 0.002*	0.23 ± 0.004*	0.19 ± 0.007*	0.25 ± 0.003*	0.17 ± 0.008*
	Field	0.15 ± 0.018	0.14 ± 0.005	0.15 ± 0.011	0.15 ± 0.001	0.16 ± 0.005	0.16 ± 0.018	0.23 ± 0.002	0.13 ± 0.010
Tebufenozide	Control	0.19 ± 0.006	0.19 ± 0.003*	0.17 ± 0.003	0.18 ± 0.005	0.17 ± 0.004*	0.17 ± 0.004*	0.22 ± 0.053*	0.20 ± 0.001*
	Field	0.18 ± 0.015	0.16 ± 0.008	0.16 ± 0.008	0.18 ± 0.012	0.15 ± 0.010	0.13 ± 0.007	0.18 ± 0.002	0.16 ± 0.004
Trichlorfen	Control	0.18 ± 0.012	0.17 ± 0.004*	0.17 ± 0.010	0.17 ± 0.006	0.21 ± 0.008*	0.24 ± 0.005*	0.21 ± 0.003	0.23 ± 0.001*
	Field	0.16 ± 0.041	0.15 ± 0.008	0.17 ± 0.007	0.17 ± 0.007	0.14 ± 0.039	0.17 ± 0.006	0.19 ± 0.007	0.15 ± 0.005

Note *. Means ± SE with asterisks show statistical differences at ethe probability less than 5% (t-test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfen were 0.05%, 0.2%, 0.5% and 0.5% respectively.

Table 5. Effects of insecticides on the protease activity (U/mg protein) of *Metarhizium anisopliae*.

Insecticides*	**Concentrations**	**Trypsin-like**		**Subtilisin-like**	
Isolates		MASA	MAAI	MASA	MAAI
Permethrin	Control	0.17 ± 0.003*	0.19 ± 0.027*	0.18 ± 0.003	0.18 ± 0.010*
	Field	0.15 ± 0.002	0.17 ± 0.023	0.17 ± 0.003	0.14 ± 0.005
Fenitrothion	Control	0.17 ± 0.002*	0.17 ± 0.015*	0.17 ± 0.009*	0.24 ± 0.009*
	Field	0.14 ± 0.008	0.15 ± 0.007	0.16 ± 0.005	0.19 ± 0.025
Tebufenozide	Control	0.21 ± 0.017*	0.16 ± 0.003*	0.18 ± 0.020*	0.22 ± 0.005*
	Field	0.17 ± 0.009	0.14 ± 0.004	0.16 ± 0.007	0.14 ± 0.002
Trichlorfen	Control	0.17 ± 0.002	0.18 ± 0.004*	0.18 ± 0.010	0.23 ± 0.011*
	Field	0.16 ± 0.008	0.16 ± 0.010	0.16 ± 0.006	0.13 ± 0.002

Note*. Means ± SE with asterisks show statistical differences at ethe probability less than 5% (t-test). Field concentrations for permethrin, Fenitrothion, Tebufenozide and Trichlorfn were 0.05%, 0.2%, 0.5% and 0.5% respectively.

DISCUSSION

Mycelial growth, spore production and germination are the three traits of entomopathogenic fungi implying their potential to create successful epidemy in pest populations. So, suppression of each of these traits strongly influences the efficiency of entomopathogenic fungi (Zibaee 2019). Among these developmental indices, germination is the most important criterion, since isolates with high germination rate may have higher speed and intensity of the vegetative growth which directly affect fungal virulence (Wraight *et al.* 2007). Moreover, germination is also important in secondary infection of entomopathogenic fungi, since after the host's death, the mycelium grows saprophytically and ensures conidial dispersal and circulation among individuals of pest population (Wraight *et al.* 2007). Thus, all these parameters should be evaluated after fungal treatments by insecticides. In our study, different concentrations of used insecticides decreased spore production in fungal isolates except for BBLA1 treated with permethrin and fenitrothion, as well as MASA and MAAI treated by permethrin and tebufenozide. Fenitrothion was the sole insecticide that showed no effect on mycelial growth of fungal isolates, although in some treatments, mainly in concentrations less than field-dose, no negative effects were recorded on fungal isolates. Finally, used insecticides resulted in no negative effect on germination rate in MAAI. Our results revealed that each of four used insecticides has different compatibilities with fungal isolates, so it may be concluded that combined application of insecticide-fungi should be exclusively selected based on compatibility/toxicity results. Different interactions of fungal isolates and insecticides have been reported in several studies which reflects developmental capabilities of fungal isolates and metabolic nature of insecticides. Li & Holdom (1994) reported higher toxicity of organochlorine or organophosphorus insecticides to entomopathogenic fungi than others, so that, carbamates like carbofuran, methoxyl and oxymyl showed moderate toxicity, while chloropyriphos, malathion and tempephos were extremely toxic. Marzieh *et al.* (2010) reported a significant lower germination rate in *M. anisopliae* treated by fipronil, pyriproxyfen and hexaflumuron. Shaabani *et al.* (2015) reported no negative effects of imidacloprid on germination, mycelium growth and sporulation in an Iranian isolate of *M. anisopliae* (001 DEMI). Different observations were recorded on the effects of eight insecticides (fipronil in two formulations, methyl parathion, cypermethrin, thiamethoxam, thiamethoxam+Lambsa-cyhalothrin, lambda-cyhalothrin, methamidophos), four fungicides (difenconazole, propiconazole, trifloxystrobin, azoxystrobin) and five herbicides (glyphosate, 2,4-dichlorophenoxyacetic acid, bentazon, imazapic + imazapyr, pendimethalin) on germination, sporulation and mycelial growth in a Brazilian strain of *M. anisopliae* (CG 168; da Silva et al. 2013). Similarly, Pelizza *et al.* (2018) investigated the effects of five insecticides including Archer PlusTM (gamma-cyhalothrin 15% [w/v]), LambdaTM (lambda-cyhalothrin 25% [w/v]), CoragenTM (rynaxypyr 20% [w/v]), MatchTM (luphenuron 5% [w/v]) and IntrepidTM (methoxyfenozide 24% [w/v]), on different strains of *B. bassiana*, *M. anisopliae* and *M. robertsii*. Both studies

reported lower conidial viability and germination rate following insecticide treatments, although pyrethroids was the most compatible insecticide with the fungal isolates. Sain *et al.* (2019) reported 25-94% reduction of mycelial growth and sporulation in *M. anisopliae* and *Isaria javanica* following treatments by synthetic and botanical insecticides. Entomopathogenic fungi require to pass through a multi-layered integument of insects completing the pathogenic mechanism. Major components of integument are chitin and protein besides some protecting layers of wax and cement (Chapman 2012). All these components serve as physical and biochemical barriers against environmental extremes and microorganisms, mainly entomopathogenic fungi. So penetration of entomopathogenic fungi via integument requires secretion of the two enzymes, chitinases and proteases. In the current study, two types of chitinases and proteases were measured to determine possible effects of insecticides. Our results revealed lower activity of chitinases in the isolates treated by field dose of insecticides, although BBAL1 and MASA were the isolates exhibited compatibility. MASA was the sole isolate which displayed no decreases in proteolytic activity after insecticide treatments. There are no similar reports demonstrating chemical effects on microbial enzymes of entomopathogenic fungi, so our results here is innovative. Since these enzymes are extracellular molecules secreted by hyphal bodies, any suppression of mycelial growth may directly affect enzyme secretion/activity in entomopathogenic fungi. Such a suppression interrupts physiological process of fungi in development and colonization as well as potential of contamination of host insects.

CONCLUSION

Agricultural ecosystems are a rich source of various microorganisms, which is very important to know their potential in the production of high quality and healthy products. Identifying and using native entomopathogenic fungi in each region is one of the desirable options for pest control to decrease pest population density, which should be optimized with other existing methods of pest control. Since the use of synthetic pesticides is one of the main methods of controlling rice pests in Iran, it is necessary to determine the compatibility of these pesticides with native entomopathogenic fungi. In the present study, it was found that the insecticides concentrations lower than the field dose had fewer negative effects on fungal isolates and in some cases even the field dose did not have any negative effect on spore production, vegetative growth and the activity of enzymes engaged in pathogenicity. Therefore, it is recommended that the application of entomopathogenic fungi to be accompanied by reduced doses of synthetic pesticides in order to have a quick effect on alleviating the population density of pests, a sustainable reduction in pest damage and less environmental pollution.

REFERENCES

Araujo, JPM & Hughes, DP 2016, Diversity of entomopathogenic fungi: Which groups conquered the insect body. *Advances in Genetics*, 94: 1-39.

Bitsadze, N, Jaronski S, Khasdan, V, Abashidze, E, Abashidze, M, Latchininsky, A, Samadashvili, D, Sokhadze, I, Rippa, M, Ishaaya, I & Horowitz, AR 2013, Joint action of *Beauveria bassiana* and the insect growth regulators diflubenzuron and novaluron, on the migratory locust, *Locusta migratoria*. *Journal of Pest Science*, 86: 293-300.

Bradford, M 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.

Chapman, RF 2012, The Insects: Structure and function. 5th ed., SJ, Simpson & AE, Douglas (Eds), Cambridge University Press, New York, USA, 929 p.

Da Silva, RA, Quintela, ED, Mascarin, G M, Barrigossi, JAF & Lião, LM 2013, Compatibility of conventional agrochemicals used in rice crops with the entomopathogenic fungus *Metarhizium anisopliae*. *Science of Agriculture*, 70: 152-160.

Dias, BA, Neves, PMOJ, Furlaneto-Maia, L & Furlaneto, MC 2008, Cuticle-degrading proteases produced by the entomopathogenic fungus *Beuveria bassiana* in the presence of coffee berry borer cuticle. *Microbiology*, 39: 301-306.

Gupta, P, Paul, MS & Sharma, SN 1999, Studies on compatibility of white muscardine fungus *Beauveria bassinia* with neem products. *Indian Phytopathology*, 52: 278-280.

Hajji, M, Kanoun, S, Nasri, M & Gharsallah N 2007, Purification and Characterization of an Alkaline Serine-protease Produced by a New Isolated *Aspergillus clavatus* ES1. *Process Biochemistry*, 42: 791-797.

Hiromori, H & Nishigaki, J 2001, Factor analysis of synergistic effect between the entomopathogenic fungus *Metarhizium anisopliae* and synthetic insecticides. *Applied Entomology Zoology*, 36: 231-236.

Hirose, E, Neves, PMOJ, Zequi, JAC, Martins, LH, Peralta, CH & Moino, JA 2001, Effect of biofertilizers and Neem oil on the entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. *Brazilian Archives of Biological Technology*, 44: 419-423.

Humber, RA 2012, Entomophthoromycota: a new phylum and reclassification for entomophthoroid fungi. *Mycotaxon*, 120: 477-492.

Kikankie, CK, Brooke, BD, Knols, BGJ, Koekemoer, LL, Farenhorst, M, Hunt, RH, Thomas, MB & Coetzee, M 2010, The infectivity of the entomopathogenic fungus *Beauveria bassiana* to insecticide-resistant and susceptible *Anopheles arabiensis* mosquitoes at two different temperatures. *Malaria Journal*, 9: 71-80.

Li, DP & Holdom, DG 1994, Effects of pesticides on growth and sporulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycets). *Journal of Invertebrate Pathology*, 63: 209-211.

Maina, UM, Galadima, IB, Gambo, FM & Zakaria, D 2018, A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *Journal of Entomological and Zoological Studies*, 6: 27-32.

Marcuzzo, LL & Eli, K 2016, Effect of temperature and photoperiod on the in vitro germination of conidia of *Botrytis squamosa*, the causal agent of Botrytis leaf blight of onion. *Summa Phytopathology*, 42: 261-263.

Marzieh, R, Ahmad, B, Aziz, S, Hamid-Reza, P & Mehran, G 2010, Compatibility of *Metarhizium anisopliae* (Ascomycota: Hypocreales) with several insecticides. *Journal of Plant Protection Research*, 50: 22-27.

Mietkiewski, R & Gorski, R 1995, Growth of selected entomopathogenic fungi species and isolates on media containing insecticides. *Acta Mycologica*, 30: 27-33.

Miller, GL 1959, Use of dinitrosalicylic acid reagent for deter- mination of reducing sugars. *Analytical Chemistry*, 31: 426-428.

Neves, PMOJ, Hirose, E, Techujo, PT & Moino, JA 2001, Compatibility of entomopathogenic fungi with neonicotinoid insecticides. *Neotropical Entomology*, 3: 263-268.

Oliveira, RCD & Neves, PM 2004, Compatibility of *Beauveria bassiana* with acaricides. *Neotropical Entomology*, 33: 353-358.

Pelizza, SA, Schalamuk, S, Simón, MR, Stenglein, SA, Pacheco-Marino, SG & Scorsetti, AC 2018, Compatibility of chemical insecticides and entomopathogenic fungi for control of soybean defoliating pest, *Rachiplusia nu*. *Revista Argentina Microbiology*, 50: 189-201.

Sain, SK, Monga, D, Kumar, R, Nagrale, DT, Hiremani, NS & Kranthi, S 2019, Compatibility of entomopathogenic fungi with insecticides and their efficacy for IPM of *Bemisia tabaci* in cotton. *Journal of Pesticide Science*, 44: 97-105.

Shaabani, M, Habibpour, B & Mossadegh, MS 2015, Compatibility of the entomopathogenic fungus *Metarhizium anisopliae* senso latowith imidacloprid for control of *Microcerotermes diversus* Silvestri (Iso.: Termitidae) in laboratory conditions. *Plant Pests Research*, 5: 27-36 (In Persian).

Shahriari, M, Zibaee, A, Khodaparast, SA & Fazeli-Dinan, M 2020, Screening and Virulence of the Entomopathogenic Fungi Associated with *Chilo suppressalis* Walker. *Journal of Fungi*, 7: 34.

Shapiro-Ilan, D, Hazir, S & Glazer, I 2017, Basic and applied research: Entomopathogenic nematodes. In: LA Lacey (Ed.), Microbial control of insect and mite pests from theory to practice. Oxford: Academic Press. London, UK, 380 p.

Wraight, SP, Inglis, GD & Goettel, MS 2007, Fungi. In: LA, Lacey & HK, Kaya, (Eds), Field manual of techniques in invertebrate pathology, 2nd edn. Springer, Dordrecht, Amsterdam, Netherland, 469 p.

Wu, JH, Yu, X, Wang, XS, Tang, LD & Ali, S 2019. Matrine enhances the pathogenicity of *Beauveria brongniartii* against Spodoptera litura (Lepidoptera: Noctuidae). *Frontiers in Microbiology*, 1: 387-1812.

Zibaee, A 2019, Entomopathogen and synthetic chemical insecticide: Synergist and antagonist. In: MA, Khan & W, Ahmad (Eds), Microbes for sustainable insect pest management, sustainability in plant and crop protection. Springer Nature, Lozan, Switzerland, 489 p.