Original Article

Possible Synergistic Effect of Combined *Metarhizium anisopliae* and Fenitrothion for Control of German Cockroach (*Blattella germanica*) as a Novel Approach

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Abstract

Background: Cockroaches are one of the most common pests in many residential areas. In this study, the simultaneous effects of fungi, *Metarhizium anisopliae* and fenitrothion-coated baits on the mortality rate of the German cockroach nymphs were investigated.

Methods: To determine the lethal level of fenitrothion insecticide, a bioassay test was performed on the last instar nymphs of the German cockroach reared at insectarium conditions. Various toxic concentrations of fenitrothion (0.1%, 0.3%, 0.5%, 0.7%, 0.9%, 1.5%, and 2%) were used. Different concentrations of *M. anisopliae* (1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 Conidia/ml) were also applied to nymphs. Eventually, we combined the effective dose of fenitrothion (0.93%) with the effective concentration of *M. anisopliae* (6.6×10^6 Conidia/ml) to provide the fungus-coated bait to attract insects. Mortality was recorded 24–96 hours after exposure to the toxic bait. The resulting data were subjected to Probit analysis.

Results: The results of applying *M. anisopliae* spores with fenitrothion composition showed that the mortality rate of German cockroach nymphs was significant. Therefore, the optimal dose of fenitrothion used in combination with *M. anisopliae* seems essential to reduce the German cockroach nymphs.

Conclusion: The results of this study can be considered a suitable method as a mixture with low cost and minimal damage to the environment and other organisms.

Keywords: *Blattella germanica*; *Metarhizium anisopliae*; Fenitrothion; Synergism

Introduction

The German cockroach, (*Blattella germanica*), is considered one of the most annoying pests in the world because of its widespread distribution in residential areas (1-2). Of four major cockroaches (American, Oriental, German, and brown-

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banded cockroach), the German cockroach (*B. germanica*) is the most common and most frequently reported cockroach that has developed insecticide resistance (3). Thus, their control is very important because they are capable of carrying a variety of pathogenic bacteria, viruses and fungi that can be dangerous to human health (2, 4-5).

Currently, the German cockroach is resistant to many common insecticides because it has been exposed to pesticides continuously and for a long time; therefore, further exposure to pesticides cannot seriously harm the German cockroach (5-7). Thus, regarding some adverse consequences of using insecticides, such as the persistence of toxins in food, development of potential cancers, exposure of vertebrates and non-target insects, environmental pollution and development of insect resistance, it is essential to applicate more effective pesticides to ensure safety and efficiency(8-9). It seems that in order to prevent insecticide resistance forcing us to use more doses, first, we need to use insecticides that have more effects on pests, and secondly, we can enhance the effects of other factors resulting in a lower dose of insecticides (10). The result is that, in addition to reducing insecticide consumption and reducing costs, we can reduce the adverse effects of insecticides on the ecosystem and human health. In line with other parts of the world, insecticides are being used in Iran to control the German cockroach (5). Cockroaches collected from homes, dorms, restaurants and hospitals have shown varying degrees of resistance to insecticides from organochlorines, organophosphates, carbamate and pyrethroids insecticides groups. The highest level of environmental pollution is related to organochlorine, organophosphate and carbamate insecticides and the least of it is related to pyrethroids (9, 11). Thus, since heavy use of these compounds has harmful impacts on the life of living organisms in the environment as well as humans, it is necessary to look for agents or factors that are less hazardous to the environment in addition to pest disposal. There are methods for the biological control of insects using fungi insecticides which can be very effective in terms of pest control and high reliability (12).

To fight against insects, more than 700 species of fungi have been identified as entomopathogenic agents (13). The entomopathogenic fungi (EPF) found in nature are readily digestible and non-pathogenic to livestock and plants (14). It has also been observed that the results of exposure to *Metarhizium anisopliae* fungus in reducing the number of the German cockroachs is evident, and these results include *M. anisopliae* species and their impact on killing the German cockroach (15). There are several reports on the use of *M. anisopliae* in combination with inorganic insecticides as solid constituents in the prey of the German cockroach. Also, there has been no well-documented study of the use of fungal pathogens in beetle prey in Iran (16). The assessment of conidia-dust formulation of *M. anisopliae* strain Iran 437 has shown that this insecticide is highly effective in reducing the number of German cockroaches which have been recently widely distributed in Ahwaz city, Khuzestan Province, southwestern Iran, especially in wards and hospitals (8, 17). In 2007, Hartelt and colleagues investigated the effects of entomopathogenic fungi at different nymphal stages of *Ixodes ricinus* ticks and observed that all the tested fungi were effective on *I. ricinus*. In another study, it was reported that *M. anisopliae* strain 97 was the most effective type of fungus (18). Various studies have been conducted on different pest-producing products reporting that some species of entomopathogenic fungi have desirable insecticidal effects (19). In a study conducted on the relationship between cone germination growth and fungal growth with nine conventional pesticides, it was observed that the efficiency of pesticides was compatible with entomopathogenic fungi because some pesticides had the potential to inhibit the germination of entomopathogenic fungi in vitro. If used at low concentrations with the fungus, their

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effect on the germination of the fungus is negligible, which occurs only in vitro; in the in vivo environment, there is little effect on their virulence against the target insect, so they can be successfully used for various pest-control purposes (20-22). In this study, the synchronous effects of entomopathogenic fungus (*M. anisopliae*) and fenitrothion-coated baits on the mortality rate of the German cockroach (*Blattella germanica*) were investigated.

Materials and Methods

Rearing German cockroaches

The German cockroach nymphs were reared under insectarium conditions at the Department of Public Health, Faculty of Health at Qom University of Medical Sciences. In this study, the last instar nymphs of the German cockroach were used for the experiments. Plastic boxes were used to hold cockroaches with food as prey, including 10.5 g of rice bran, wheat bran, flour, sugar and rodent food. The environmental condition was 27±2 °C, 60±10% relative humidity and a photoperiod of 12:12h (L: D). Nymphs were anesthetized with carbon dioxide gas and sealed in a container with a lace.

Fungi resource

Entomopathogenic fungus (*M. anisopliae*, Iranian strain V245) was obtained from the stock of the Regional Center for the Collection of Industrial Fungi and Bacteria of Iran with the code number of PTCC 2881. The fungi were cultured on PDA (Potato Dextrose Agar) medium and kept in relative humidity of 70±5% and 28 °C± 2 for two weeks. The conidia were scraped of 14-day-old culture with a sterile metal loop and a suspension of conidia was then diluted as the determinate concentration in Conidia/ml by using Tween 80 solution 0.01% in Physiological serum. The resulting mixture was stirred for 10 minutes with a magnetic stirrer. The concentrations of conidia suspension were performed by Neobar Lam while the direct count by using a hemocytometer (Neubauer chamber) was down (23-24).

Chemicals and pesticides

Fenitrothion insecticide in the form of technical grade (95%) was used for toxicity testing and was purchased from MOSHKFAM FARS CHEMICAL CO (MFC), Iran.

Preparing the baits

The baits were prepared to trap live German cockroaches (25). In order to prepare baits, a mixture of rice bran, wheat flour, rodent food and sugar was blended proportionally and gradually distilled with water to obtain a consistent semi-solid food in various weights up to about 10.5 grams. Subsequently, the same baits were exposed to different concentrations of fenitrothion solution according to the doses prescribed in the experiments. Baits were inoculated with 1ml of the conidia suspension of *M. anisopliae* isolate Iranian strain V245 with intended doses in Conidia/ml (22). Then, the last instar nymphs were placed in the plastic container and remained without a food source for 72 h to be kept in a starvation status. Thereafter, baits containing different doses of insecticide and conidia suspension were given to the cockroaches for 3 hours. After the exposure time, treated baits were replaced with untreated ones. For each bioassay test, concentrations were applied for at least four times. Each test included a control group which fed on untreated bait. Mortality rate was recorded daily until all samples died; this procedure was repeated for two weeks.

Bait bioassay

The German cockroach insectary strain, kept at insectarium conditions and clean containers, was used. Seven concentrations of insecticides, including 0.1%, 0.3%, 0.5%, 0.7%, 0.9%, 1.5%, and 2%, were tested by control dish with four replicates. The control group was designed as follows: one group consisting of 10 nymphs that received water with the prey dry composition. For each target concentration of insecticide as well as control tests, 10 cockroach nymphs were exposed four times by contact exposure test; thus, a total of 320 cockroaches were required for the insecticide resistance test. For entomopathogenic fungi bioassay, five different concentrations of the aqueous suspensions of *M. anisopliae*, including 1×10^4, 1×10^5, 1×10^6, 1×10^7 to 1×10^8 Conidia/ml, were regulated. For each concentration, 10 cockroach nymphs were exposed to inoculated bait containing one ml of the fungal suspension for each repetition and then were placed in plastic containers containing filter paper. The control group was designed as follows: one group consisting of 10 nymphs that received water with the prey dry composition. For each target concentration of fungi, 10 cockroach nymphs were exposed four times by contact exposure test; thus, a total of 240 cockroaches were required for fungi resistance test.

Synergism effect of fungi

In this section, the effective dose of fenitrothion and fungal conidia included sublethal doses that had the best and highest lethality effects on the target insects, obtained through statistical analysis which included the effective dose of fenitrothion (0.93%) and *M. anisopliae* fungi (6.6×10^6 Conidia/ml). Cockroach nymphs were applied to test the insecticide and fungi bioassay for 10 cockroaches per container with four replications. The control group at this stage included one group comprising 10 nymphs that received water with the prey dry composition. Also, a total of 80 cockroaches were required for the synergistic effect of fungi. Finally, 640 German cockroaches were needed to complete all the bioassay tests.

Statistical analysis

After the conversion of mortality data to percentages, the mean and standard error mortality rates for the intended concentrations of fenitrothion insecticide and fungal spores per each experiment were assessed from the first day to the fourth day after the exposure to the poisonous bait. By using the data obtained, the Probit regression relationship was plotted as an equation among the death of German cockroach nymphs, insecticide concentrations and fungal spores by SPSS version 16. The significance level was set at $P < 0.05$ for all the analyses.

Results

The results of the mortality rate of the German cockroach nymphs resulting from the exposure to fenitrothion insecticide are presented in Table 1. It was observed that the mortality rate increased in accordance with the increase in the concentrations of insecticide as the mortality rate of nymphs ranged from 5% to 70% in the minimum and maximum concentrations in the first day of exposure. The highest mortality rate was observed on the fourth day ranging from 22.5% to 92.5% ($P < 0.001$). Therefore, there was a significant relationship between the increase in mortality due to the increase in insecticide concentration and the exposure

Table 1. The percentage of deaths of the German cockroach nymphs* based on insecticidal concentration

The percentage of cockroaches wasted (Mean±SE)				Insecticidal concentration (%)
First day	**Second day**	**Third day**	**Fourth day**	
5.0±5.8	15.0±5.8	20.0±8.2	22.5±9.6	0.1
20.0±8.2	27.5±9.6	32.5±18.9	37.5±23.6	0.3
25.5±10.0	37.5±5.0	52.5±9.6	52.5±9.6	0.5
37.0±23.6	50.0±21.6	55.5±17.3	57.5±17.1	0.7
45.0±19.1	50.0±28.3	60.0±21.6	62.5±18.9	0.9
55.0±30.0	60.0±25.8	67.5±26.3	72.5±26.3	1.5
70.0±25.8	75.0±25.2	87.5±9.6	92.5±9.6	2.0
0.002	0.006	< 0.001	< 0.001	P-value

*****Each treatment contained 10 German cockroach nymphs replicated four times. Treatments were significantly effective at $P < 0.05$.

time from the first day to the fourth day. The best time to investigate mortality was 48 to 72 h after the treatment. The values of LC_{50} and LC_{90} were 1.29 mg/L and 2.76 mg/L respectively on the first day, decreasing to 0.61 mg/L and 2.08 mg/L with 95% confidence interval on the fourth day (Table 2). The lethal dose values showed a significant decrease over time from the day of exposure as the deceasing amount of LC_{50} had a lower percentage of toxicity after exposure compared to LC_{90}; thus, it can be concluded that this gradual decrease in insecticide concentration dose was very significant.

The mortality rate of German cockroach nymphs with increased doses of fungal spores in four consecutive days is shown in Table 3. The mortality rate of nymphs exposed to the lowest dose (10%) on the first day increased three-fold (30%) until the fourth day of exposure. Moreover, the mortality rate of nymphs exposed to the highest dose of fungal spores 1×10^8 Conidia/ml on the first day was 30%, increasing three-fold on the fourth day. The values of LD_{50} and LD_{90} on the first day were 5.4×10^8 Conidia/ml and 3.6×10^{11} Conidia/ml, respectively, decreasing to 2.3×10^5 Conidia/ml and 1.5×10^9 Conidia/ml on the fourth day (Table 4). The lethal dose values showed a significant decrease over time from the first day of exposure, and the decreasing amount of LD_{50} showed a lower percentage of toxicity after the exposure day,

Table 2. The LC_{50} and LC_{90} concentrations of insecticide in the Probit model of the death of the German cockroach nymphs on different days

Post exposure day	LC_{90} (95% CI)	LC_{50} (95% CI)	P-value
1	2.76 (2.46, 3.14)	1.29 (1.11, 1.49)	< 0.001
2	2.48 (2.21, 2.82)	1.01 (0.83, 1.19)	< 0.001
3	2.19 (1.94, 2.51)	0.72 (0.54, 0.90)	< 0.001
4	2.08 (1.84, 2.39)	0.61 (0.43, 0.79)	< 0.001

Table 3. The deaths percentage of German cockroach nymphs* based on different doses of fungal spores

The percentage of cockroaches wasted (Mean ± SE)				**Dose of fungal spores (spore/ml)**
first day	Second day	Third day	Fourth day	
10.0±8/2	12.5±9.6	22.5±5.0	30.0±8/2	1×10^4
12.5±12.6	17.5±15.0	35.0±23.8	37.5±22.2	1×10^5
22.5±12.6	37.5±17.1	47.5±5.0	60.0±18.3	1×10^6
25.0±10.0	42.5±9.6	47.5±34.0	67.5±9.6	1×10^7
30.0±8.2	55.0±5.8	75.5±12.9	92.5±9.6	1×10^8
0.037	0.001	0.023	< 0.001	P-value

*****Each treatment contained 10 German cockroach nymphs replicated four times. Treatments were significantly effective at P < 0.05.

Table 4. The LD_{50} and LD_{90} values of fungal spore dose in the Probit model against the German cockroach nymphs on the post-exposure day

Post exposure day	LD_{90} (95% CI)	LD_{50} (95% CI)	P-value
1	$(1.9\times10^{10}–5.4\times10^{12})$ 3.6×10^{11}	$(1.1\times10^8 – 4.4\times10^9)$ 5.4×10^8	0.001
2	$(2.3\times10^{11} –2.0\times10^{14})$ 1.9×10^{12}	$(7.7\times10–1.4\times10^8)$ 2.9×10^7	0.001
3	$(1.3\times10^9 –1.3\times10^{11})$ 8.7×10^9	$(3.8\times10^5–4.8\times10^6)$ 1.3×10^6	0.001
4	$(2.6\times10^8– 1.7\times10^{10})$ 1.5×10^9	$(5.8\times10^4 –8.0\times10^5)$ 2.3×10^5	0.001

compared with $LD_{90.}$ However, according to the Probit model used in the data analysis, it can be concluded that this decrease was quite significant in terms of fungal spore concentration.

The results of applying fungus (*M. anisopliae*)sporescombinedwithfenitrothion insecticide composition, performed by the bait method, is demonstrated in Table 5. In all samples, the mortality trend significantly increased and reached 100% on the fourth day of exposure. Nymph mortality of German cockroaches was significantly increased with incremental concentrations of insecticides and fungi separately on the first day of exposure (Tables 1 and 3).

Significant differences on the first to the fourth days indicate that the best time for insect mortality with the selected dose of fungus and the selected concentration of insects initiated from the second day onward. The results of the analysis of the data obtained from the seven-dose fenitrothion-covered baits on mortality rate in terms of percent (%) for four days are depicted in Fig. 1. The mortality rate of cockroach nymphs rose with an increase in concentration levels, and the chosen concentration of 0.93% between the second and third days of average chart data was obtained for LC_{50}% concentration determination.

The results of the analysis of the data

Table 5. The results of the mortality rate of German cockroach nymphs* due to exposure to the effective combination of insecticide and fungal spores based on the rising trend of mortality in exposure days

Days of exposure	Day1	Day2	Day3	Day4	P-value
	70%	80%	90%	100%	0.001
Mortality rate	70%	80%	100%	100%	0.001
	80%	100%	100%	100%	0.001
	30%	60%	90%	100%	0.001

*****Each treatment contained 10 German cockroach nymphs replicated four times. Treatments were significantly effective at P < 0.001.

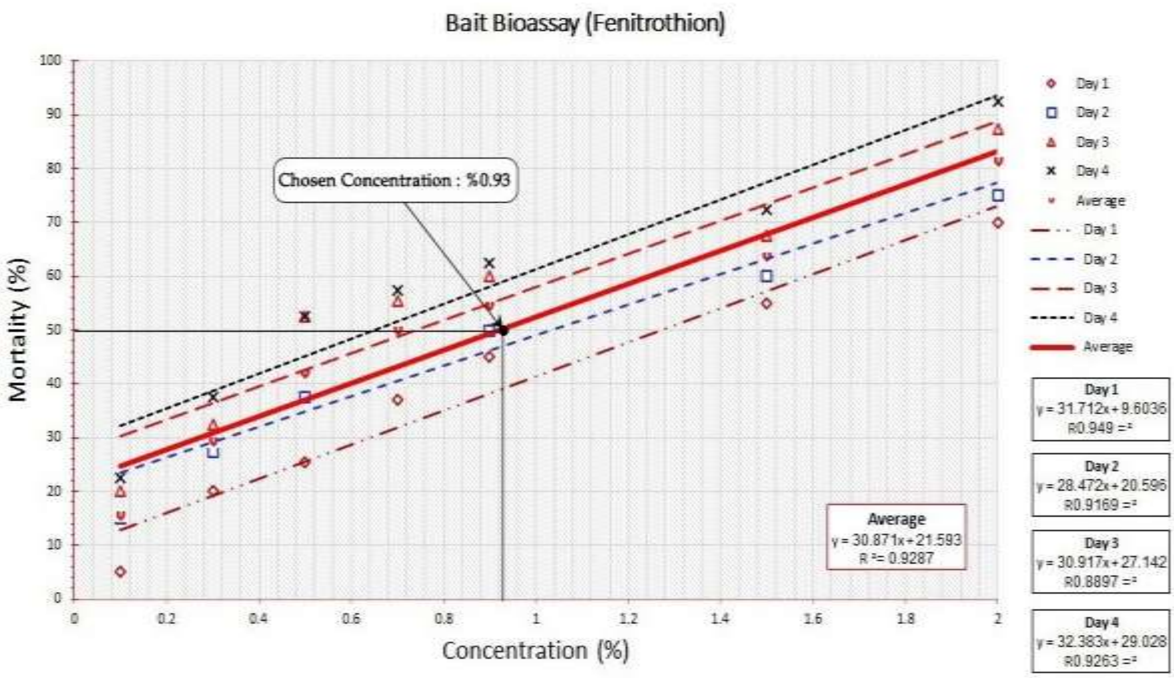

Fig. 1. Regression line from the mortality test of the German cockroach nymphs using bait methods on the first day to the fourth day after the application of fenitrothion

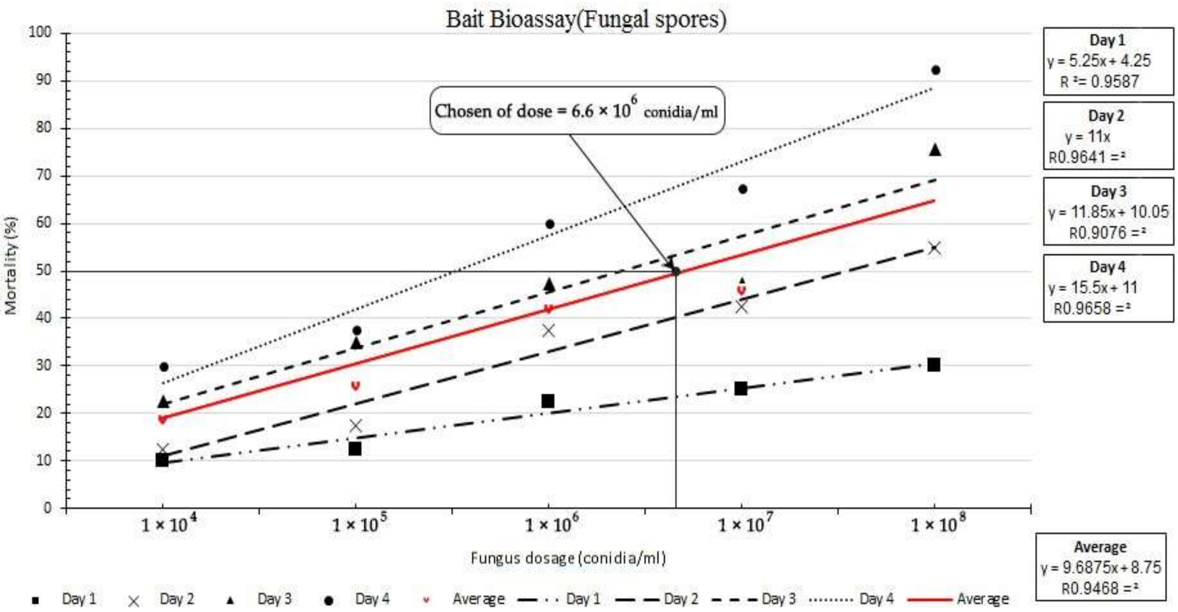

Fig. 2. The regression line of the mortality test of the German cockroach nymphs using bait methods on the first day to the fourth day after the application of fungal spores

obtained from the five-dose *M. anisopliae* concentration logarithm in terms of Conidia/ml for four days are shown in Fig. 2. As observed, the mortality rate of cockroach nymphs increased as the concentrations moved up, and a chosen concentration of 6.6×10^6 Conidia/m between the second and third days of average chart data were obtained for the $LD_{50\%}$ concentration determination. In terms of forms, the lower the slope of the graph, the lower the strain of insects and the greater the impact of insecticide and fungi on insects. Moreover, it was observed that the more heterogeneous the population, the harder it is to cope with the homogeneous population.

Discussion

The German cockroach has been known to be the effective transmitter of various infectious agents such as *Aspergillus* fungi, yeast, *Candida* and bacteria such as *Enterobacter*, *Klebsiella* spp. and so on. The majority of these agents have been proven to have resistance to antibiotics (5, 26). A recent study on the resistance of the German cockroach in Iran has reported that although adult cockroaches have been abundantly investigated, further studies on the susceptibility of nymphs are needed (27). Since German cockroaches are more resistant to controlling agents than other species are, such as brown-banded cockroaches (28), it is logical to fight them with combined chemical and biological controlling methods in minimal doses and a shorter period. Cockroaches collected from homes, dorms, restaurants and hospitals have shown varying degrees of resistance to insecticides made from carbamate, phosphorus and pyrethroids. Studies on the German cockroach indicate that they are often resistant to insecticides which are usually expected to have a marked and lethal effect. The results included organophosphorus chlorpyrifos, pirimiphos-methyl and malathion toxins, carbamates such as propoxur, bendiocarb, carbaryl and pyrethroid such as permethrin, deltamethrin and cypermethrin (9, 11). The study on the effects of actellic, ficam, diazinon, fenitrothion and coopex on cockroaches in Tehran, Iran, demonstrated that their sensitivity to pesticides was relatively low; therefore, they suggested

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that other compounds and methods be used to control cockroaches (29). The mechanisms of insecticide resistance may include changes in insecticide target sites, the regulation of degradative enzymes and increased insecticide excretion (29-30). The inappropriate and unscientific application of insecticides by non-specialists may not only result in gradual resistance but also increase economic costs and damage the environment.

Previous studies on the biological control of mosquito larvae (*Culex pipiens*) have indicated that *M. anisopliae* fungus has a high potentiality in this regard. Studies have also revealed that the combined fungal formulations can have a greater impact on the biological control of brown-banded German cockroach (23, 28). The bulk of insect cuticle contains protein constituents, including hydrophobic and hydrophobic constituents, with long-chain hydrocarbons in the outermost layer or epicuticular layer. These structures are the main target of degradation by the enzymatic function of chitinase and protease of *M. anisopliae* (31). *Metarhizium anisopliae* as an entomopathogenic fungus is a unique arthropod pathogenic fungus whose infection is caused by direct infiltration into the mite cuticle so that its cuticle is pierced by the fungus. In fact, by infecting the cells with growing spores, the fungal structures of the fungus are destroyed by the production of enzymes such as chitinase, protease and lipase (32). A study conducted on German cockroach nymphs on the ninth and 15th days, after exposure to 6.5×10^6 conidia inoculated with fungi, reported over 70% mortality rate (15). Considering the LD_{50} and LD_{90} exposure values of *M. anisopliae* (Iranian strain V245), the mortality rate of cockroach nymphs was 1.3×10^6 and 8.7×10^9 within the third and 2.3×10^5 and 1.5×10^9 within the fourth day, respectively. Compared to the *M. anisopliae* fungus that had a lethality of four days in a relatively lower range, the *Beauveria bassiana* fungus was shown to have a longer effective range of three to seven days resulting from higher fungal efficacy (33). The results of this study showed a higher degree of effectiveness for the *M. anisopliae* fungus. The use of insecticides such as chlorfenapyr, pyridaben and fenpyroximate with fungal *M. anisopliae* sequences was shown to have a significant effect on killing mites in cotton products (21). The synergistic effects between *M. anisopliae* and boric acid as a mineral insecticide to control important agricultural pests was investigated, and it was demonstrated that the effects occurred when these compounds were used as a liquid prey for the German cockroach (16). The interaction between *M. anisopliae* and Spinosad insecticide against the house fly, *Musca domestica*, was observed to have a synergetic effect increasing the house fly mortality and reducing the lethal time (22). In this study, it was found that the dose of the simultaneous use of fenitrothion with *M. anisopliae* to control the German cockroach had no suppressive effects on the viability and growth of conidia. However, accelerating the synergistic virulence of the impact on the German cockroach exposed to fenitrothion-coated baits and *M. anisopliae* was proved to be effective more than other methods in reducing cockroach nymphs. It should be noted that using a combination of *M. anisopliae* and fenitrothion for household or agricultural purposes may not have any adverse effects on mammals if used in an appropriate dose. The results of this study revealed that using the appropriate combination of insecticides and spore-fungi, with a minimal concentration of these compounds, could result in a good chemical and biological control. On the other hand, the presence of insecticides seems to be necessary as well. The reason may be that the fungus itself needs to be used in a higher dose that can damage the living organisms, which in turn can have harmful and toxic effects. The reason behind using fungi as a synergist is to try to reduce the concentration and amount of insecticides used in society to minimize the number of chemicals while minimizing the use of insecticides. Low-risk biological fungi (fungi) are used to enhance the killing rate of insecticides. The findings of this study are in agreement with the idea that the simultaneous use of pesticides and fungal pathogens cause more host vulnerability. The results demonstrated that the lethal dose of fungal spore corresponded to LD_{50} and LD_{90} values and was suitable for killing cockroaches in the range of 1×10^6–1×10^7. After seven days of exposure to the Iranian strain 437C (by the amounts ranging from 2×10^7 up to 4×10^6/cm^2), the mortality rates of brown-banded cockroaches were 97.8% and 93.6%, respectively. The values by which the cockroach nymphs were treated were within the range of LD_{50} and LD_{90}. In another study, it was demonstrated that the amount of time required to kill brown-banded nymphs when exposed to the same fungal spore dose ranged from 1.4 to 2.7 days (28). Similarly, a study on the German cockroach nymphs showed that if the exposure rate to a fungal spore dose was 6.5×10^6 Conidia/cm^2, the life cycle of the cockroaches would only last six days (15). Nevertheless, depending on the type of exposed cockroaches, the dose and the final time after the exposure would vary. In this study, the mortality rate of cockroach nymphs was monitored up to four days after exposure and remained favorable. The mortality rate of the German cockroach nymphs by the fenitrothion in the caddy had a gradual decrease in the concentration required. Thus, the concentration of 0.5 mg/kg insecticide on the third/fourth days and 0.7 on the first day can be considered as the appropriate dose to exterminate cockroach nymphs. Hence, the combination of fungal spore and insecticide doses should be required only in doses that have lethality greater than 50% provided by taking into account the results of lethality studies and post-exposure mortality rates. However, previous results from the concomitant use of pesticides with the fungal pathogen suggest that exposure to both pests can make them more susceptible and result in more pest damage (16, 21).

Conclusion

The results of this study regarding the effects of pesticide and fungal spore on the mortality rate of cockroach nymphs over four days showed that the optimal dose of fenitrothion used in combination with *M. anisopliae* seems essential for controlling of German cockroach nymphs, and it can be considered a suitable method with low cost and minimal damage to the environment and other organisms.

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