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Efficacy of Thai isolates of the entomopathogenic fungus, *Beauveria bassiana*, and their combination with indigenous entomopathogenic nematodes against fall armyworm (*Spodoptera frugiperda*)

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ABSTRACT: Fall armyworm (FAW) (*Spodoptera frugiperda*) is a polyphagous insect pest and is reported as one of the most destructive insect pests. Since the time FAW infestation was reported in Thailand, it has caused economic losses. Using synthetic chemicals and insecticides to control this pest may cause undesired consequences. Hence, we tested the effectiveness of entomopathogenic fungus (EPF) *Beauveria bassiana* isolates TBRC 2781 and TBRC 4755 against FAW individually and in combination with an indigenous entomopathogenic nematode (EPN) *Heterorhabditis indica* AUT 13.2 under greenhouse conditions. The second and fifth instar larvae were tested against the two isolates *B. bassiana* (TBRC 2781 and TBRC 4755) at different dosages of fungal spores ($1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, and $1x10^9$ spores/ml). In the laboratory test, the highest mortality of second instar larvae were 72% and 63% at $1x10^8$ spores/ml for the isolates TBRC 2781 and TBRC 4755 respectively. Similarly, the highest mortality of fifth instar larvae was 34% and 24% at $1x10^9$ spores/ml for both the isolates. In the greenhouse second instar larvae were exposed to three concentrations of the fungal suspension i.e., $1x10^8$, $1x10^9$, and $1x10^{10}$ spores/ml. The highest mortality of 35% and 32% were obtained at $1x10^{10}$ spores/ml for both the isolates. In the experiment where we combined EPFs and EPNs, the mortality was increased to 55% and 40%.
Keywords: biological control agents; *Heterorhabditis indica*; insect pest of maize; combinations of biological control agents; additive effect

Introduction

The fall armyworm (FAW) (*Spodoptera frugiperda* (J. E. Smith); Lepidoptera: Noctuidae) is native to the Americas' tropical and subtropical regions (Day et al., 2017). It has never been reported outside of the Americas until it was detected in Africa for the first time in 2016 (Goergen et al., 2016). Thailand, Sri Lanka, Myanmar, Yemen, and China were among the Asian countries where this pest was discovered in 2018. (Chormule et al.,2019). This pest was first spotted in Thailand in December 2018, in a few sub-districts of the Kanchanaburi and Tak provinces, near the Myanmar border (IPPC, 2018). It has now become one of the most devastating insect pests of maize.

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This insect is a polyphagous pest that has been shown to damage approximately 353 plant species in 76 different plant families (Montezano et al., 2018). Crop damage occurs when the insect is in the larval stage. Pesticides are commonly used to reduce the population of fall armyworms, which increases the cost of maize production. Moreover, these chemicals can be seen as a threat to the environment and has a negative impact on human health (Salas et al., 2000). Non-target and beneficial insects are harmed by them (Köhler et al., 2013). In the long run, injudicious application of chemical pesticides will result to the occurrence of an insecticide-resistant population of FAW, making the difficulty in controlling (Yu et al., 2003). Given all these issues, there is a pressing need to identify long-term, environmentally benign, and human-health-friendly alternatives to chemical insecticides. Biocontrol agents such as bacteria, nematodes, fungi, and viruses are regarded as an important choice for the management of a wide range of arthropod species and are an appropriate method for the ecosystem's long-term viability (Charnley and Collins, 2007; Khan et al., 2012). Entomopathogenic fungi (EPFs) are a type of fungus that induces epizootic infection in insects (Ghulam, 2020). The most frequently used EPFs are members of the genera *Aschersonia, Beauveria, Hirsutella, Metarhizium, Nomuraea, Paecilomyces,* and *Verticillium* which belong to the Hyphomycetes class. *Beauveria bassiana* is one of the most common entomopathogenic fungi with over 700 insect species as hosts around the world (Amutha et al., 2010; Shahid et al., 2012). Many researchers have recently reported on the use of entomopathogenic fungi for controlling insect pests in Thailand (Mar et al., 2012; Thaochan et al., 2017). Because EPFs are host-specific pathogens, there is a minimal danger that they will attack non-target species in the environment, particularly beneficial insects. They are commercially produced as a biopesticide for the control of a variety of insect pests (Pell et al., 2001) including FAW (de Faria et al., 2007; Cruz-Avalos et al., 2019). We have considerable isolates of these entomopathogenic fungi in Thailand, and they have been used as a biological control agent against insect pests. Several indigenous entomopathogens can reduce severe insect pests, including the FAW. Here, we conducted laboratory and greenhouse experiments to test the effectiveness of two promising isolates of the entomopathogenic fungus *B. bassiana* (TBRC 2781 and TBRC 4755) native to Thailand against FAW larvae.

Entomopathogenic nematodes (EPNs), on the other hand, are considered insect pathogens because they can produce symbiotic bacteria that kill insect hosts (Zimmermann, 2007; Lacey & Georgis, 2012; Vashisth, Chandel, & Sharma, 2013). *Steinernema* and *Heterorhabditis*, two EPN genera that belong to the Steinernematidae and Heterorhabditidae families, are widely used as biological control agents. *Steinernema* nematode is associated with the symbiotic bacteria *Xenorhabdus*, whereas *Heterorhabditis* nematode species are with *Photorhabdus* bacteria (Boemare et al., 1993). These bacteria offer primarily two functions: 1) provide food for nematodes and indirectly supply nutrients through the decomposition of insect carcasses (Mohan, 2015; Stock & Blair, 2008); 2) major cause of insect death (Mohan, 2015; Stock & Blair, 2008). Entomopathogen efficacy varies from genus to genus, species to species, and strain to strain (Molina Ochoa et al., 1996; Tavassoli et al., 2008). In a recent study, two indigenous entomopathogenic nematodes (*Heterorhabditis indica* isolate AUT 13.2 and *S. siamkayai* isolate APL 12.3) were evaluated in the laboratory and greenhouse against the second instar of the FAW (*S. frugiperda*). *H. indica* (isolate AUT 13.2) gave the highest mortality in both conditions, 67.50 % and 57.50 %, respectively, at a density of 50,000 IJs/ml (Wattanachaiyingcharoen et al., 2021).

Besides using a single promising biological control agent to control insect pests, combinations of biological control agents have been considered a more successful controlling strategy. This strategy provides either additive

or synergistic effects that can enhance the effectiveness of biological control agents. The amplification of the synergistic interaction may limit the tolerance of the host insects. Later, the second agent can infect them virulently and effectively kill them. It has been reported that either two combined entomopathogenic pathogens or pathogens paired with EPNs have been used. For example, the combination of an EPF, *Metharhizium anisopliae*, with a bacterium, *Serratia entomophila*, increased the mortality of the grass grub, *Costelytra zealandica* (Choo et al., 2002; Acevedo et al., 2007). Furthermore, when the bacterial pathogen *Bacillus thuringiensis* Berliner (Buibui strain) was combined with the EPNs, the control of white grubs was increased (Thurston et al, 1994; Koppenhfer and Kaya, 1997). In this study, we also determined the efficiency of combining the EPFs (*B. bassiana*) and the effective indigenous EPNs from our previous study (*H. indica* AUT 13.2) in greenhouse conditions.

Materials and methods

Collection and rearing of the fall armyworm

Fall armyworms were collected from maize fields in Phitsanulok, Sukhothai, and Uttaradit provinces of Thailand. Identification and confirmation of the larvae were carried out as per identification guidelines provided by Visser (2017). Larvae were put in 20 ml plastic containers and fed with the leaves of sweet corn variety 'Supersweet" which was grown without any chemical pesticides. The pupae were collected and put in a plastic container, which was then placed inside a rearing cage (30 cm in length, 30 cm in height, and 30 cm in width). Adults were given a 10% sugar solution. Eggs were collected, and after hatching, newly hatched larvae were fed with young maize leaves, and subsequent larvae were used for experiments.

Culture of the Entomopathogenic Fungus (*Beauveria bassiana*)

The entomopathogenic fungus (*B. bassiana*) employed in this study was obtained from the Thailand Bioresource Research Center (TBRC). The pure fungus culture of two isolates of *B. bassiana*, TBRC 2781 and TBRC 4755, was employed in this study. The isolates were cultured and maintained on Potato dextrose agar (PDA) in a petri dish (9 cm diameter) and incubated at the temperature of 25 ºC for a 14:10 hours light: dark photoperiod.

Preparation of the spore suspension

Spores were harvested from the three-week-old culture with a sterile spatula in a laminar flow chamber. Spores were carefully scraped from the PDA and were suspended in sterilized distilled water. The suspension was vortexed to get a homogenous state and was strained through a double-layered muslin cloth to separate spores from other fungal parts. The final volume of spore suspension was made to 500 ml (stock). One milliliter of fungal suspension was taken from the stock suspension and diluted with sterilized distilled water in 1:10 dilution, afterwards, tween 20 was added to the suspension for the determination of the spore concentration. Spore concentration was estimated using a Hemacytometer (Neubauer chamber) under a 40x magnification in a compound microscope. Five distinct fungal suspensions were made from stock, with spore densities of $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, and $1x10^9$ spores/ml of sterilized distilled water. The spore suspension was sealed and refrigerated at 4 ºC until use.

The efficacy of *Beauveria bassiana* isolates TBRC 2781 and TBRC 4755 against the fall armyworm in the laboratory conditions

The effectiveness of *B. bassiana* isolates TBRC 2781 and TBRC 4755 was determined in the laboratory conditions with set temperature at 25±2°C and RH at 60±5%. The test was carried using larval bioassay of the second and fifth instar larvae of the FAW. A completely randomized design (CRD) was used for this experiment. For each isolate, six treatments were replicated four times. The treatments consisted of five distinct fungal suspensions with varying spore densities per milliliter (ml) of sterilized distilled water, i.e., $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, and $1x10^9$, and the same volume of sterilized distilled water without fungal spore was used as control. When the FAW larvae reached the second and fifth instar stages, they were removed from the rearing container, and each larva was placed in a Petri dish (5.5cm diameter) with a detached maize leaf as food. Using a micropipette, one milliliter of fungal suspension was applied topically to cover the larva and the leaf. The treated larvae were incubated at 25 °C under a 14:10 (light: dark) photoperiod with a relative humidity of 60±5%, and the food was changed every 24 hours. Forty larvae (10 larvae/replication) of each second instar and fifth instar were treated with the abovementioned concentrations.

Assessment of mortality in the laboratory

The assessment for the mortality was carried out 48 hours after inoculation. When the larvae did not respond to the forceps' contact, they were recorded as dead. Dead larvae were kept in a clean Petri dish containing moist filter paper to observe for sporulation. Only those larvae that showed sporulation was recorded as the one killed by the fungus. For each treatment, the number of dead larvae was counted every 24 hours for a period of 10 days.

The mortality of the tested sample was calculated by summing the number of deceased larvae across all exposure replicates and then expressing the result as a percentage of the total number of exposed larvae. The mortality obtained was corrected using Abbott's formula. (Abbott, 1925)

$$\text{Mortality percentage} = \frac{\text{Total number of dead larvae}}{\text{Total number of tested larvae}} \times 100$$

The efficacy of the EPFs and the combined application of the EPFs and the EPNs again the FAW in the greenhouse conditions

Approximately two weeks after emergence, when the seedling had four completely developed leaves, ten-second instar larvae of the FAW were placed into each pot for the tests. To keep the larvae from escaping, the pots were covered with insect mesh vertically and from the top. Each test was conducted twenty-four hours after the release of the FAW larvae. Two sets of experiments were conducted in the greenhouse with the temperature ranged from 28-32 °C and approximate RH 80%. Both sets of studies were designed using a Randomized Complete Block Design (RCBD). The following components were repeated eight times in each experiment.

The first set of experiments was assigned to evaluate the efficacy of EPFs against the second instar larvae of FAW. Three dosages of *B. bassiana* isolates TBRC 2781 and isolate TBRC 4755, at densities of 1×10^8, $1x10^9$, and 1×10^{10} spores/ml were tested by spraying 100 ml of fungal suspension with a hand sprayer on tested plants. As a control treatment, an equal volume of sterilized distilled water free of fungal spores was employed. Each treatment

was carried out three times: the first spray was done 24 hours after the larvae were released, and the second and third sprays were performed 48 and 96 hours after the first spray, respectively. For a period of ten days, the mortality of larvae was measured daily.

In the second set of experiments, EPF isolate *B. bassiana* TBRC 2781 at spore density of $1x10^{10}$ spores/ml and the EPN (*H. indica* AUT 13.2) at 50,000 IJs/ml were applied in combination at the amount of 100 ml of suspension. The experiment was designed using RCBD with three treatments including control. The first treatment consisted of spraying EPN, which was sequentially sprayed by EPF and EPN, namely *H. indica* AUT 13.2 followed by *B. bassiana* TBRC 2781 and *H. indica* AUT 13.2. The second treatment consisted of spraying EPF isolates sequentially followed by EPN and EPF, i.e., *B. bassiana* TBRC 2781 followed by *H. indica* AUT 13.2 and *B. bassiana* TBRC 2781. An equal amount of sterilized distilled water was applied as a control.

Assessment of Mortality in the greenhouse conditions

In both experiments, the pots were assessed daily for dead larvae. When dead larvae were discovered, the death of the larvae was verified by the sporulation of fungus or the emergence of entomopathogenic nematodes via the White trap technique. For ten days, the number of dead larvae was counted every 24 hours. The mortality percentage was calculated as above as laboratory condition.

$$\text{Mortality percentage} = \frac{\text{Total number of dead larvae}}{\text{Total number of tested larvae}} \times 100$$

The results were disregarded if the death rate for the control treatment was greater than 20%. When control mortality was less than 20%, Abbott's formula was performed to correct observed mortality, as follows. (Abbott, 1925)

$$\text{Corrected mortality percentage} = \frac{\text{\% tested mortality - \% control mortality}}{\text{Total number of tested larvae}} \times 100$$

Data Analysis

The corrected mortality of dead larvae in different treatments (dosage) by each isolate of the EPF from the laboratory conditions experiment were subjected to statistical analysis of variance (ANOVA) ($p<0.05$). Duncan multiple range tests (DMRT) were used to compare the means of each treatment to evaluate if there was a significant difference between treatments**.** The number of dead larvae recorded from both greenhouse experiments was subjected to statistical analysis of variance (ANOVA) ($p\leq0.05$). The mean was compared using the Tukey test to detect a significant difference between treatments.

Results and discussion

The efficacy of *Beauveria bassiana* isolates TBRC 2781 and TBRC 4755 against the Fall armyworm in the laboratory conditions

The effectiveness of two isolates of *B. bassiana*, TBRC 2781 and TBRC 4755, against the second and fifth instar larvae of the fall armyworm was evaluated. The mortality of second instar larvae treated with a fungal suspension of *B. bassiana* (isolates TBRC 2781 and TBRC 4755) was statistically significant between treatments as determined by one-way ANOVA ($F(5,18) = 64.34$, $p \leq 0.05$) and ($F(5,18) = 36.34$, $p \leq 0.05$), respectively. At substantially all spore concentrations, *B. bassiana* isolate TBRC 2781 demonstrated higher mortality (**Table 1**). The highest mortality of 72.23% and 63.89% was obtained at a spore density of $1x10^8$ spore/ml for TBRC 2781 and TBRC 4755, respectively

Table 1 Effect of different dosages of *B. bassiana* isolate TBRC 2781 and TBRC 4755 on mortality of the second instar larvae of fall armyworm after 10 days

Larval stage	Treatments (spores/ml)	*B. bassiana* TBRC2781 Mean±SD (%)	*B. bassiana* TBRC4755 Mean±SD (%)
Second instar	Control	10.00±8.16a	15.00±5.77a
	$1x10^5$	30.55±5.56b	22.22±9.07b
	$1x10^6$	47.22±5.56c	30.55±5.56b
	$1x10^7$	58.34±5.55d	55.56±9.06c
	$1x10^8$	72.23±6.41e	63.89±5.56c
	$1x10^9$	72.23±5.05e	61.12±6.41c
	F value	49.45	28.53
	%CV	14.08	18.74

The mean followed by a different letter differs considerably in each column. Mean separated by Duncan Multiple Range Test at $p \leq 0.05$

Similarly, both fungal isolates were capable of invading and killing fifth instar FAW larvae. According to one-way ANOVA, mortality was statistically significant among treatments for isolates TBRC 2781 and TBRC 4755 ($F(5,18) = 17.86$, $p \leq 0.05$) and ($F(5,18) = 6.14$, $p \leq 0.05$), respectively. The mortality of fifth instar larvae was likewise affected by spore concentrations (**Table 2**). The isolate TBRC 2781 caused higher mortality of the fifth instar larvae than the isolate TBRC 4755. The greatest death rates, 34.82 %, and 24.62 % were obtained with TBRC 2781 and TBRC 4755 at $1x10^9$ spore density, respectively.

Table 2 Effect of different dosages of *B. bassiana* isolate TBRC 2781 and TBRC 4755 on mortality of the fifth instar larvae of Fall armyworm after 10 days

Larval stage	Treatments (spores/ml)	*B. bassiana* TBRC2781 Mean±SD (%)	*B. bassiana* TBRC 4755 Mean±SD (%)
Fifth instar	Control	2.50±5.00a	5.00±5.77a
	$1x10^5$	17.30±4.88b	10.56±8.20a
	$1x10^6$	22.32±4.65b	15.56±5.13a
	$1x10^7$	29.85±7.91c	13.33±5.95a
	$1x10^8$	32.32±5.13c	21.11±1.28b
	$1x10^9$	34.82±5.98c	24.62±5.82b
	F value	12.97	6.79
	%CV	27	35.9

The average of four replications. In each column, the mean followed by a different letter differs significantly. Mean separated by Duncan Multiple Range Test at $p \leq 0.05$

The efficacy of *Beauveria bassiana* TBRC 2781 and TBRC 4755 in the greenhouse conditions

The impact of two isolates of *B. bassiana*, TBRC 2781 and TBRC 4755, on mortality of FAW larvae under greenhouse circumstances, was tested by spraying the second instar larvae of FAW with three different fungus doses, namely 1×10^8, 1×10^9, and 1×10^{10} spores/ml. Each dosage's effectiveness was studied independently. Both isolates were capable of infecting and killing FAW larvae at all three doses of fungal suspension. The mortality caused by the two isolates was statistically significant (**Table 3**). At all three spore densities, isolate TBRC 2781 caused greater mortality (21.25, 25.00, and 35.00 %, respectively) than isolate TBRC 4755.

Table 3 Effect of *B. bassiana* isolates on mortality of fall armyworm under greenhouse conditions at the end of 10 days

Treatments	Spore density	Mean mortality ± SD (%)
B. bassiana TBRC 2781	1 x 10^8	21.25±8.35a
B. bassiana TBRC 4755	1 x 10^8	18.76±6.41a
Control	Distilled water	0±0.00b
F value		29.24
%CV		36.9
B. bassiana TBRC 2781	1 x 10^9	25.00±5.35a
B. bassiana TBRC 4755	1 x 10^9	20.00±7.56a
Control	Distilled water	0±0.00b
F value		49
%CV		28
B. bassiana TBRC 2781	1 x 10^{10}	35.00±5.35a
B. bassiana TBRC 4755	1 x 10^{10}	32.50±7.07a
Control	Distilled water	0±00b
F value		116.45
%CV		18.54

Treatments means followed by the same letter do not differ significantly (ANOVA and Tukey test p≤0.05)

According to our findings, the two EPF isolates (TBRC 2781 and TBRC 4755) varied in their potential to infect and kill FAW larvae. Even though the efficacy of both spore density (1 x 10^8 and 1 x 10^9 spores/ml) of both isolates were not significantly different, there were slightly higher mortality percentage when the spore suspension of the 1 x 10^9 spores/ml was applied. This may be due to the optimal virulence of the fungal isolate that can infect and kill the insect host. Even higher density may cause similar mortality percentage. Hence, we choose the density of 1 x 10^8, 1 x 10^9 and 1 x 10^{10} spores/ml for further test in the greenhouse. It was obvious that younger larvae (second instar) were more vulnerable to EPFs than older larvae (fifth instar). This can be related to the advancement of the insect in term of their cuticle. It is reported that chemical constituents of insect cuticle changes with advancement in age (Amer et al., 2008). Chemical changes that occur in insect cuticle increased hormonal immune to the microbial infections (Boman, 1980). Thus, younger larvae of FAW are more vulnerable to EPFs. This was also reported by Zalucki et al. (1986) that mortality was greater in the younger stage of Lepidopteran insects when they were infected with entomopathogens. Many factors influence an entomopathogen's potential to infect and kill the host, particularly the pathogen's virulence (Sengonca et al., 2006). In the case of entomopathogenic fungi, pathogenicity is determined by their ability to produce a variety of enzymes involved in insect infection (Feng et al., 1994). Many publications have demonstrated the variations in virulence across different isolates. Ramanujam et al. (2020) reported various levels of FAW mortality among ten different fungal strains, with *M. anisopliae* ICAR-NBAIR Ma-35 causing the highest mortality (67.8 %), followed by *B. bassiana* ICAR-NBAIR Bb-45 (64.3 %) and Bb-11 (57.1 %), and the remaining strains showing 10.7–28.6 % mortality. Although these potential fungal isolates were efficient against

second instar larvae, they did not infect the eggs. Moreover, Garcia and colleagues (2008) tested eight strains of *B. bassiana* and found that only two isolates (Bb18 and Bb42) were particularly efficient against second instar FAW larvae. Akutse et al. (2019) examined 20 fungal isolates (14 isolates of *M. anisopliae* and six isolates of *B. bassiana*) on FAW larvae. Only ICIPE 281 and ICIPE 676 exhibited a mortality rate of 83.9 % and 53.9 %, respectively, among six isolates of *B. bassiana*, however, they were less efficient against older second instar larvae. These investigations show that when tested against the same stage of host, isolates, strains, genera, and species of fungus varied in their virulence. The differences in mortality found in our investigation can be related to variances in virulence among *B. bassiana* isolates. Furthermore, changes in host larval instars have a considerable impact on the level of susceptibility.

The performance of the fungal isolates in the greenhouse, where environmental circumstances are less than ideal, was predicted to be lower than in the laboratory. When compared to the control, both isolates of *B. bassiana* (TBRC 2781 and TBRC 4755) exhibited favorable results, however, the mortality percentage achieved was considerably lower than what was obtained in the laboratory. The environmental conditions play a significant impact on the capacity of entomopathogens to infect and kill hosts (Zimmermann, 2007; Seid et al., 2019). Because temperature and humidity are important in the growth and development of the fungus, increased temperatures and relative humidity in the greenhouse environment have influenced the fungus's survival (Maina et al., 2018; Seid et al., 2019). Furthermore, exposing the fungal spore to UV light causes the fungal spores to be inactivated (Lovett et al., 2015). In the greenhouse, larvae easily wander from plant to plant, making it difficult for fungus to establish proper host contact. It was also challenging to target larvae with the spray since some larvae remained on the lower surface of the leaves and were untouched. El-Husseini et al. (2008) found a similar finding in which the insect present on the lower surface of sugar beet leaves was unaffected by two formulations of *B. bassiana*. We noticed that second instar larvae quickly establish themselves by traveling to locations with softer plant parts, such as the whorl region of the maize plant. When they reach the whorl, they burrow and conceal themselves in a maize funnel (Day et al., 2017). This habit not only protects larvae from the fungal spray but also allows them to grow quicker by eating in a hidden area of the host plant.

The combined application of the EPFs and the EPNs against the FAW in the greenhouse conditions

Under greenhouse conditions, two combined experiments (EPFs and EPNs) were tested. In the first treatment, EPNs were sprayed first, followed by the EPFs, and lastly sprayed with the EPNs (*H. indica* AUT 13.2 + *B. bassiana* TBRC 2781+ *H. indica* AUT 13.2). In the second treatment, the EPF suspension was sprayed first, followed by the EPN and EPF suspension (*B. bassiana* TBRC 2781 + *H. indica* AUT 13.2 + *B. bassiana* TBRC 2781).

The mortality of second instar larvae obtained in the two treatments was statistically significant ($F(2,21) = 135.80$, $p \leq 0.05$) as verified by one-way ANOVA. The first combination treatment (*H. indica* AUT 13.2 + TBRC 2781 + *H. indica* AUT 13.2) show a higher mortality of FAW larvae (55%) than the second (TBRC 2781 + *H. indica* AUT 13.2 + TBRC 2781) (40 %) **(Table 4)**.

Table 4 Mortality percentage (Mean ± SD) of Fall armyworm larvae using combined treatment under greenhouse conditions

Treatments	Mean±SD (%)
H. indica AUT 13.2 + TBRC 2781 +*H. indica* AUT 13.2	55.00±9.26a
TBRC 2781 + *H. indica* AUT 13.2 + TBRC 2781	40.00±7.56b
Control (Distilled water)	0.00±0.00c
F value	135.8
%CV	17.70

The mean followed by the different letter in the column differs significantly at p≤0.05 according to the Tukey test

It has been shown that combining biological control agents greatly improve pest control efficacy (Guetsky et al., 2019; Pal & Gardener, 2006). When evaluated in the greenhouse, the combination treatment of the entomopathogenic fungus and nematode resulted in an increased death percentage of the second instar larvae compared to the fungus application alone. When the larvae were sprayed with *B. bassiana* isolate TBRC 2781, the death rate was the highest (35.00±5.35%). However, when these fungi were combined with EPNs, the mortality rate increased slightly (40.00 ± 7.56% and 55.00 ± 9.26%, respectively) (**Table 4**). Spraying EPNs first, followed by EPFs and EPNs (*H. indica* AUT 13.2 + TBRC 2781 + *H. indica* AUT 13.2) had greater outcomes than spraying EPFs first, followed by EPNs and EPFs. Most of the larvae perished earlier in the first treatment than in the second. The FAW larvae began to die on the third day in the first combination spray, and most larvae were killed between the third and seventh days in the second combination spray, whereas most larvae died later on the fifth to eighth day in the second combination spray. This result can be related to the nature of infection of the two biological control agents used in our study. Most EPNs can infect and kill the host insect within 48 hours whereas EPFs takes about 7 to 14 days for initial attachment to the death of host insect (Ibrahim et al., 2011). When EPNs are applied before EPFs, it might have weakened the larvae which was easily attacked by EPFs. On the other hand, where EPF are applied before EPNs, the larvae remained active until EPNs was applied because the infection process was slower in case of EPFs. The reasons for increasing host mortality have been discussed since both biological control agents may have additive or synergistic effects on the host during concurrent infection. When both biological control agents act liberally from each other, but there is no contact between them, the additive effects occur, which can aid raise or accelerate the mortality of the insect hosts. The negative consequences of synergistic or antagonistic effects resulted in reduced mortality of insect hosts (Koppenhfer and Grewal, 2005). Our results indicated that the biological control agents examined, *B. bassiana*, had additive effects with the nematodes (*H. indica*) in increasing the FAW mortality. When the fungus was used alone, the mortality rate was 35.00 $\pm$ 5.35 %, but it rose to 40.00 $\pm$ 7.56 % and 55.00 $\pm$ 9.26 % when combined with the nematodes. However, the mortality caused by EPNs alone (58%) was higher than combining with EPFs **(**Wattanachaiyingcharoen et al., 2021). Ansari et al. (2004) found additive effects when two entomopathogenic fungi, *M. anisopliae* and *B. bassiana*, were treated one week after the entomopathogenic nematodes *S. glaseri* and *H. megidis*. When treated *S. exigua* larvae with the entomopathogenic fungus (*B. bassiana*) and nematode (*H. bacteriophora*) the additive action was also reported (Barbercheck and Kaya, 1991). Schulte et al. (2009) discovered synergistic effects when *B. bassiana* and *S. carpocapsae* were used to infect a

Lepidopteran insect, *Indarbela dea*. Meanwhile, simultaneous infection of *Curculio caryae* with nematodes and the bacteria *Serratia marcescens*, or with the fungus *Paecilomyces fumosoroseus* and *B. bassiana* resulted in antagonism (Shapiro-Ilan et al., 2004). Using more than two biological control agents to enhance the capability to kill insect pests should take into consideration that both organisms are different in their biology. The capability to infect host insects varies. Moisture, in the form of a water droplet or a water film, is required for both entomopathogenic fungal and nematode infection and survival. As a result, dry conditions may have an impact on the effectiveness of both biological control agents. However, combining at least two biological control agents obviously increases the level of invasion.

In terms of the combined use of nematodes and fungus for better control, understand the biological advantages and disadvantages of the biological control agent involved. Aside from the evidence that entomopathogens are successful in controlling insects, combining both biological control agents provide advantages over their separate usage. These advantages include the ability to infect insects in cryptic habitats, decrease pesticide residues on food, facilitate mass manufacturing, and not threaten the safety of people and several non-target species (Emelianoff et al., 2008; Malan et al., 2011; Shahid et al., 2012).

Conclusion

In both laboratory and greenhouse conditions, Thai indigenous entomopathogenic fungi *B. bassiana* (isolates TBRC 2781 and TBRC 4755) displayed effectiveness against FAW larvae. However, disparities in efficacy were noticed, with the isolates TBRC 2781 performing better, which may be attributable to differences in the virulence of the isolates. The efficacy of these fungi was decreased under the greenhouse conditions, which might be related to the greenhouse's unfavorable environmental conditions. The combination of entomopathogenic fungus and nematodes resulted in enhanced mortality of FAW larvae. This is because those two biological control agents have an additive effect on host survival. As a result, these two *B. bassiana* isolates (TBRC 2781 and TBRC 4755) may be sprayed alone or in combination with Thai indigenous entomopathogenic nematodes to increase the efficiency of enforcing the FAW.

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Animal Ethics

This research was approved for the Ethics of Use Animals for Scientific Work from Naresuan University (Approvement No. 63-01-003).

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