American Journal of Zoology
2022; 5(2): 11-19
http://www.sciencepublishinggroup.com/j/ajz
doi: 10.11648/j.ajz.20220502.11

Evaluation of Ethiopian Entomopathogenic Fungi Isolates Against the Two-Spotted Spider Mite, *Tetranychus urticae* Koch on Tomato, *Solanum lycopersicum* L.

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To cite this article:

Gelana Keno, Belay Habtegebriel, Ferdu Azerefegne. Evaluation of Ethiopian Entomopathogenic Fungi Isolates Against the Two-Spotted Spider Mite, *Tetranychus urticae* Koch on Tomato, *Solanum lycopersicum* L. *American Journal of Zoology*. Vol. 5, No. 2, 2022, pp. 11-19. doi: 10.11648/j.ajz.20220502.11

Received: March 22, 2022; **Accepted**: April 21, 2022; **Published**: April 28, 2022

Abstract: Two spotted spidermite (TSSM), Tetranychus urticae (Koch, 1836) is economically important in greenhouse grown tomato in Ethiopia. This study entomopathogenic fungi (EPF) such as Metarhizium anisopliae (Metschnikoff, 1883) and Beauveria bassiana (Balsamo, 1912) isolates evaluated for the management of the two spotted spider mites under both laboratory and glasshouse controlled environmental conditions. Six fungal newly isolated from the soil and one existing isolate were evaluated and identified in the laboratory for virulence against T. urticae. Spore dilutions were prepared in 0.01% Tween 80. Adults and eggs were sprayed with 1 ml of conidial suspension adjusted to 1×10^8 conidia/ml. The mites those treated were kept under controlled conditions at 25°C and 60–70% RH and the number of dead mites was recorded daily on tomato leaf. All the isolates tested were infected and killed adults and eggs of spider mites at a concentration of 1×10^8 conidia ml^{-1} after eight and seven days respectively, but had varying virulence on adults ranged from 33% to 93% and on egg ranged from 32% to 87%. Two B. bassiana isolates, GF3 and 9604, which resulted in 93% and 85% mortality of adults 8 days post inoculation, respectively were categorized as highly virulent whereas M. anisopliae MF3 and AF5 which resulted in 64% and 54% mortality were considered as moderately virulent isolates. Similarly, B. bassiana AF2, BF4 and KF3 which resulted in mortality of 47%, 36% and 33%, respectively, were considered as weak isolates against the two spotted spider mites. The highly virulent isolates were further evaluated under glasshouse condition on potted tomato plants for their virulence against T. urticae. Isolate GF3 caused the highest mortality (78%) followed by 9604 which caused 70% mortality after seven days of inoculation. From this study the native M. anisopliae and B. bassiana isolates have potential to kill the TSSM both eggs and adults' stages of the pest. Thus why, EPF isolates can be used as an alternative to chemical treatments.

Keywords: Beauveria Bassiana, LT_{50}, Metarhizium Anisopliae, Pathogenicity, Mortality

1. Introduction

Back Ground of the Study

Tomato (*Solanum lycopersicum* L.) is one of the vital horticultural crops with an estimated global production of 170.8 million tons of productivity [7]. It can be grown under greenhouse conditions as well as in open fields for consumption as fresh produce or processed into many products. It has become an important cash crop among vegetables in Ethiopia. Major limiting factor for tomato productions and productivity are several biotic and abiotic stresses. Biotic stresses: arthropod pest, early blight, late blight, anthracnose, fusarium wilt, bacterial blight, bacterial canker and weeds while abiotic factor are water quality, deficit irrigation, salinity, climate variability and soil factor. From arthropod pests Spider mites (Acari: Tetranychidae) are the most important phytophagous mite pests of agricultural greenhouse crops worldwide, the population outbreaks of which can cause serious damage and yield losses. Reduction

of total leaf area could result in a significant yield loss ranged from 50 to 100% [19]. Two-spotted spider mite (*Tetranychus urticae)* is great economically significant pest among spider mite species [10]. *T. urticae* is a member of the family Tetranychidae that are harmful species to the plant [21]. *T. urticae* was first described by Koch in 1836 [24] and is believed to have originated in temperate climates agroecology. Around 1200 species of plants hosts for *T. urticae*, of which more than 150 are economically hosts for the pest [7, 10]. Several predators are commercially available from bio control suppliers. Microorganisms play great role in regulating population size of spider mites. Pathogenssuch as Entomopathogenic fungi, bacteria and viruses are important to control spider mites [23, 33]. Entomopathogenic fungi are frequently tested in the laboratory against spider mites [5]. The entomopathogenic fungi of *Beauvaria bassiana* and *Metarhizium anisopliae* have been suggested to cause mortality in several mite species including the two-spotted spider mite, *T. urticae* [2]. This study, therefore, aims at investigating the potentials of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* to control the two-spotted spider mite, *T. urticae* on tomato *with specific objectives of*

i. To isolate and identify entomopathogenic fungi from soil of major tomato producing areas of Ethiopia and evaluate their performance against the two-spotted spider mite under laboratory conditions,
ii. To evaluate the efficacy of entomopathogenic fungi isolates under glasshouse condition.

2. Materials and Methods

2.1. Description of Study Area

The study was conducted under Laboratory and glasshouse condition at Ambo Plant Protection Research Center (APPRC) from November to May of 2017/18.

2.2. Isolation of Entomopathogenic Fungi (EPF)

2.2.1. Collection of Soil Samples

The samples of soils were collected with a garden spade to a depth of about 10 cm after removal of surface litter or unwanted materials from different fields in November, 2017. It was collected from Meki, Ada'a, Koka of East Shoa and Guder and Bako of West Shoa zones of the Oromia regional state of Ethiopia, which are major tomato production areas (Table 1). From each area five farms were visited and six random samples of one kg soil were taken. A composite sample was prepared to take representative sample by mixing the samples and a one kg sample was drawn and used from each farm and placed in clear plastic bags and sealed. Each sample bag containing the soils was labeled with the site of collection, date and then taken to the laboratory in Ambo Plant Protection Research Center (APPRC).

Table 1. *Description of sampling sites.*

Sites	Altitude (m.a.s.l)	Rain fall (mm)	Soil type	Temperature (°C)
Ada'a	1878	1000-1600	loamy	8-28
Bako	1743	920-1200	sandy	14-28
Guder	2101	1100-1300	loamy	8-24
Koka	939	800-1000	loamy	10-25
Meki	1636	800-950	sandy	11-34

2.2.2. Rearing of Galleria mellonella

EPF were isolated from the sample soil by using the bait Galleria method [37]. Larvae of the moth were multiplied within the laboratory at Ambo plant protection research institute. To get larvae of moth, the adults were placed in 500 ml flasks capacity. When the moth laid eggs, the tissue paper was taken from the flasks and put in plastic rearing boxes containing 180, 50 and 180g of honey, wheat bran and glycerol, respectively, as feed for the larvae. The boxes were incubated within the dark at 20°C for four weeks. The resulting fourth to fifth instar larvae (approximately 30 days after hatching) were used as baits [37].

2.2.3. Insect Bait Technique and Conidia Preparation

Insect bait method used as described by Inglis *et al*., [17]. The soils collected from various places were placed in clear glass jars of one liter capacity. Glass jars size measured 20 cm height and 10 cm diameter was used. In each jar one kg of soil from each sample site were placed. There were twenty-five clear glass jars with one kilogram of each of collected soil samples. The soils were dried up to appropriate moistness content, optimal for fungal growth and limiting the development of nematodes. Optimum moisture shows the degree of moisture in soil at which the soil could be compacted to its greatest density. Suitable moisture level fungal growth related to temperature between 25 to 30°C which may result in prolific mold growth. Deposition of large numbers of virulent conidia in a dry period, disease progression prevented or reversed because the insect is able to elevate its body temperature to 40°C. In the other hand, under cool conditions, when insects were incapable of elevating their body temperature, the pathogen's inoculum threshold may drop too low to result in disease initiation. The soil should not be too wet and not leave too much condensation on the inside of the container. Ten larvae (4^{th} to 5^{th} instar) of *G. mellonella* were placed in to each jar that contain soil sample and the jars were incubated in the dark at room temperature.

The jars were inverted every day to ensure the movement of the larvae across the soil and to increase contact between insects and soil particles. The soil was checked for dead larvae after three days and then every two days up to day 15.

Dead larvae were taken out and surface sterilized in 1% sodium hypochlorite solution for 3 minutes followed by three washes in sterile, distilled water. Surface-sterilized larvae were placed on moistened filter paper in sterile plastic Petri dishes (90cm) sealed with Para film to reduce dehydration and maintain adequate moisture levels for fungal growth and incubated at room temperature. The larvae were inspected daily to check for sporulation and to confirm death due to fungal infection. When fungal sporulation was visible on dead larvae, inoculating needles were flamed over the burning Bunsen burner. Then small portion of sporulated fungi on dead larvae was transferred into SDA culture media under safety cabinet and incubated at 25°C. Fungal growths were once re-isolated and pure cultures prepared. This was carried out by picking from the growing edge of fungi with sterilized metal spatula and inoculating fresh sterile petri dishes containing sabouraud dextrose agar. The plates with cultured fungi were incubated at 25°C and about 75% RH. Fungal isolates obtained in this study were used for further experiment or identification.

2.2.4. Media Preparation

One of the most commonly used media for isolation and culture of these fungi is Sabouraud dextrose agar supplemented with 0.2% yeast extract (SDAY) [13].

Sixty-five grams of Sabouraud dextrose agar with yeast extract (SDAY) and 2.5gm of yeast was weighed aseptically on balance and poured into a conical flask. The 100 µg/ml chloramphenicol which used as antibiotics against bacteria was added. One liter of distilled water was added, mixed well and dissolved by heating with frequent agitation and boiled for one minute until completely mixed. It was then covered with aluminum foil and sterilized in an autoclave at 121°c for 15 minutes at 15 Ibs (irritable bowel syndrome) pressure. It was then permitted to cool to a temperature of 40°c and poured into petridishes aseptically. The media were solidified in the plates and used for identification.

2.2.5. Identification of Fungi

The fungi were identified using morphological characteristics of reproductive structures with the aid of taxonomic references of Disease symptoms, spore shape and size, colony character: pigmentation and growth rate [13, 16, 20]. Feng *et al.* [12] reported that no difference exists in the morphology and infectivity of conidia produced on solid media and those produced on the surface of insect cadavers. Initially, the fungal cultures were examined for morphological traits (conidia and colony) using compound microscope at 100 times. *Metarhizium anisopliae* is easily identified because it forms green chains of cylindrical conidia that are densely compact on the infected host, causing green muscardine disease. Fungal colonies are initially white or creamy mycelium, becoming shades of green/yellow to shades of dark green during sporulation. Conidiophores are simple or double- branched with elongated shape. The surface of the colony of *Beauveria bassiana* is white to cream and fluffy to powdery. The fungal hyphae are tubular, narrow, septate fragile filaments. It is usually white becoming slightly yellowish over time. Conidia was single-celled and globose or ovate in shape.

2.2.6. Conidial Germination

The viability of conidia for suspensions was assessed on SDAY medium following a Modified protocol of that described by Goettel and Inglis [13]. Conidial germination was assessed by adjusting stock suspension to 3×10^6 conidia/ml using an improved neubaour heamocytometer. A spore suspension of 100µl was then spread on SDAY plates and plates were incubated at room temperature. After 24hr percentage germination was determined by dividing germinated conidia over total conidia counted on specified place. Spores were counted by placing the microscope cover-slip on each plate and each plate was replicated three times and germination percentage were calculated [31].

2.3. Rearing of the Two-Spotted Spider Mite (TSSM)

The two-spotted spider mites (TSSM) colonies were collected from different plants such as cucumber, weeds, tomato and reared on haricot bean (*Phaseolus vulgaris*) because pulse crop contain high protein which increase developmental rate of mite. Haricot plants were grown in pots of cages with 1×1×1.3m size for rearing. When plants start to die because of heavy infestations, new colonies were initiated on newly grown haricot bean plants. For experiment the adult TSSM - maintained on haricot bean plants were transferred to tomato plants in a glasshouse by placing three infested haricot bean leaves on each tomato plants per pot. Haricot bean leaflets were removed after confirmation of their transfer to the leaves of the tomato plants. The plants were watered regularly and observed daily for population establishment of mites. Rearing process was maintained at temperature 28-32°C, photoperiod of 12:12h light and dark and a relative humidity of approximately 60-70%. Hand lens and simple microscope using a stereo microscope were used to identify two-spotted spider mites throughout the experimental period. Reared mites were used as stock to carry out experiments.

2.4. Laboratory Experiment

2.4.1. Evaluation of Entomopathogenic Fungi for Pathogenicity Against the Adults of Two-Spotted Spider Mite

Two isolates of *M. anisopliae* (MF_3 and AF_5) and five isolates of *B. bassiana* (9604, GF_3, BF_4, KF_3 and AF_2) were evaluated for their pathogenecity against the two-spotted spider mites. Experiments were conducted under laboratory conditions with an average temperature of 24-26°C and 70% relative humidity. Three-week-old culture harvested by metal spatula and spore concentration of 1×10^8 ml^{-1} (Chandier *et al*., 2005) were prepared by adjusting the concentration based on the counts of the conidia, in 1 ml of suspensions using improved Neubauer haemocytometer. Each of the seven isolates was used as treatments and was replicated 4 times including the control. Experiment was arranged in

completely randomized design (CRD). Fresh tomato leaves with petiole taken from glasshouse were surface sterilized using 70% alcohol for about 10 seconds to discourage surface contaminants. The fresh leaves were then air-dried under laminar flow cabinet for 10 minutes. Petiole of fresh leaves inserted in to cotton wool on dampened filter paper within a sterilized Petri dish (9cm diameter) to hold moisture. Thirty to forty uniform larger sized adults of *T. urticae* were counted from stock culture of haricot beans *(P. vulgaris*) in order to calculate their average and transferred on to each treated inverted tomato fresh leaves by soft camel hairbrush. Uniform larger sized mites were obtained by adult mites collected in petridish contain leaf of tomato and removed the adults after 24hr, then when the larvae developed to adult after six day used for experiment.

Spores harvested from three-week-old culture surfaces of media using sterile metal spatula and added to test tubes containing 10 ml of sterilized Tween 80 (0.01%) to make a stock suspension. The stock conidia suspension was then vortexed to break spore chain to get single spores. After vigorous shaking in vortex mixer, the suspension was standardized at a concentration of 1×10^8 conidia ml^{-1} by counting the spores in a Neubauer haemocytometer chamber. The mites were sprayed with 1ml of a suspension of fungal conidia, using hand held sprayer of Bottle Quick mist HDPE 16 OZ. Petri dishes were sealed with parafilm and small holes were created on the lid with a hot needle for aeration and incubated at 25°C. Petiole of fresh leaves inserted in to cotton wool in Petri dish were watered in two days interval using a syringe which prevented the leaf from desiccation and confined the mites. The control was treated with solution of Tween 80 (0.01%). Mites which did not move when touched with a dissecting needle were considered as dead under a dissecting microscope [6]. The numbers of dead mites were counted daily for 8 days. Died bodies were removed and placed onto damp filter paper within Petri dishes sealed with parafilm. Died bodies with visible fungal growth on their body surface were considered to have died as a result of fungal infection. Mortality data were corrected for the corresponding control mortality by the formula:

$$\%CM = \frac{\%T-\%C}{100-\%C} \times 100$$

Where CM is corrected mortality, T is mortality in treated insects and C is mortality in untreated insects [1].

2.4.2. Evaluation of Entomopathogenic Fungi for Pathogenicity Against the Eggs of Two-Spotted Spider Mite

Twenty-five to thirty large sized adults of the two-spotted spider mites were taken from bean plants, placed on clean inverted tomato leaflets on damp filter paper in Petri dishes and incubated at room temperature to allow oviposition for 24 h. All the adults were removed from the leaf, leaving eggs to spray with fungal suspension. Conidial suspensions of new *B. bassiana* isolates of GF_3, BF_4, AF_2, KF_3 and *M. anisopliae* isolates of MF_3 and AF_5 with existing *B. bassiana* isolates of 9604 were prepared using 0.01% Tween 80. Conidia dose of 1×10^8 ml^{-1} [6] Chandler was used for inoculation and 0.01% Tween 80 was used as control. Fifty eggs were counted and left in each tomato leaf and sprayed with 1 ml of conidial suspension and arranged in n Completely Randomized Design with four replications. The number of hatched and un-hatched eggs in each treatment was counted every day starting from 24 hours after inoculation and continued for one week because no more eggs hutched after one week. Unhatched eggs were considered as dead those shows fungal out growth from their body.

2.5. Glasshouse Evaluation of Selected EPF Against the Adults of Two-Spotted Spider Mite

The experiment was conducted on potted tomato plants (*Solanum lycopersicum*) in a glasshouse. Tomato seeds were sown in pots. When they attained five-centimeter height the seedlings were transplanted in to plastic pots of two liters capacity filled with sand soil, loam soil and compost at a ratio of 2:1:1 respectively. A total of 12 pots four treatment with three replications were used for this trial. Pot size measured 25 cm height and 20 cm diameter was used. The mite population was established by placing three heavily infested leaflets of haricot bean on each potted tomato leaf plant and allowing the mites to establish for one week. Three random samples of infested tomato leaves from each pot were tagged and the number of adult *T. urticae* on each side of the tomato leaves was counted on the same day before treatment application. Each treatment was applied to the plant and three marked leaves per plant, at the top, middle and bottom of the plant were used for data collection. There were a total of 12 leaves per treatment in the glasshouse including control. Conidial suspensions of isolates (GF_3, 9604 and MF_3) selected from the laboratory bioassays due to their high pathogenicity were used as treatments. These fungal isolates were grown on artificial media and conidia were harvested using sterile metal spatula. Conidial suspensions were prepared using Tween 80 (0.01%) solution. The concentrations of the suspensions were adjusted to 1×10^8 conidia ml^{-1} and sprayed on leaves and a treatment with 0.01% Tween 80 was used for the control groups.

Spraying was conducted in the late afternoon by using a hand-held sprayer of Bottle Quick mist HDPE 16 OZ until run off as described by Cote [8]. The reason spraying in the late afternoon because the fungal strains sensitive to the light in mid-day. After treatment application the pots were placed in the glasshouse by making some space among them to prevent migration of mites within the pots and replicated four times including control. The number of spider mites on both sides of each tagged tomato leaf was counted daily for seven consecutive days after application. After seven days all tagged leaves of tomato were removed and the mortality of spider mite due to fungal species were recorded under microscope.

2.6. Data Collection

Data were collected on the number of positive soil samples that show how many larvae dead due to EPF, mortality of *G. mellonella* per all soil samples, Fungal isolates, levels of Pathogenecity of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* isolates on eggs and adults of the two-spotted spider mites under laboratory and glasshouse conditions.

2.7. Data Analysis

The recorded percent mortality data was arcsine transformed to check normality before analysis after Abbott corrected formula. Data were analyzed using ANOVA procedure of SAS software version 9.2 [27] followed by mean separation with Student Newman Kuel (SNK) test. Lethal time of conidia required to kill 50% of the treated mite population (LT_{50}) was determined by probit analysis using SPSS software version 20 followed by ANOVA and means were separated with LSD test.

3. Results and Discussion

3.1. Laboratory Experiment

3.1.1. Evaluation Mortality of Galleria mellonella

The insect bait method using the larvae of *G. mellonella* showed that there were dead *G. mellonella* larvae in all the soil samples collected from tomato growing farms of Bako, Guder, Ada'a, Koka and Meki of Ethiopia, although the extent of mortality varied (Table 2). According to Tuininga [32] Galleria bait method is a more common, simple, efficient and sensitive method for isolating entomopathogenic fungi. Percent mortality of *G. mellonella* in the soil samples ranged from 20 to 100% (Table 2). However, mortality because of entomopathogenic fungi was observed only from six of the 25 (24%) soil samples (figure 1), and no entomopathogenic fungi were isolated from the remaining 19 of the soil samples (76%). Deaths on the remaining 19 soil samples were because other factors and did not show any sporulation of entomopathogenic fungi. Those fungi have less enzyme mode of action that used to penetrate insect cuticle.

Table 2. *Extent of Galleria mellonella larvae mortality 15 days after introduction to soil samples collected from tomato growing areas of East and West Showa, Ethiopia.*

District	Site	Geographic position	% Number of soil samples with larvae	% Total larval mortality	% Larvae mortality due to entomopathogenic fungi
Ada'a	1		100	70	0
	2	$8^0$4'44"N, $39^0$71"E	100	60	50
	3		100	20	0
	4		100	40	0
	5		100	90	70
Bako	1		100	30	0
	2	$9^0$8'N, $37^0$3'E	100	50	0
	3		100	60	0
	4		100	70	70
	5		100	60	0
Guder	1		100	70	0
	2	$8^0$58'N, $37^0$46'E	100	50	0
	3		100	100	90
	4		100	40	0
	5		100	40	0
Koka	1		100	50	0
	2	$8^0$23'N, $39^0$5'E	100	60	0
	3		100	80	60
	4		100	60	0
	5		100	40	0
Meki	1		100	40	0
	2	$8^0$9'N, $38^0$49'E	100	30	0
	3		100	90	70
	4		100	40	0
	5		100	30	0

Six indigenous isolates belonging to two entomopathogenic fungal species of *B. bassiana* (GF3, AF2, KF3 and BF4) and two *M. anisopliae* (MF3 and AF5) (Table 3) were obtained from sampled soils using the insect bait technique. The number of *B. bassiana* in the soil samples was higher than *M. anisopliae*. The isolated fungi were evaluated in the subsequent experiments.

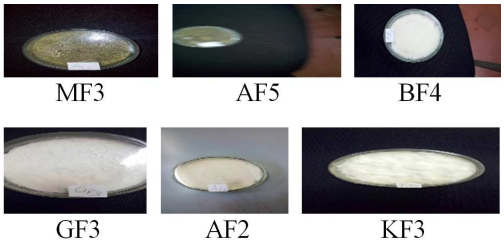

Figure 1. *Isolates of EPF from Died of G. mellonella.*

Table 3. *Lists of isolated fungi from soil samples of tomato growing areas.*

List of fungal species	Isolate code	Location	
		Site	Geographic location
Beauveria bassiana	AF_2	Ada'a	1878 m.a.s.l
Beauveria bassiana	BF_4	Bako	1743 m.a.s.l
Beauveria bassiana	GF_3	Guder	2101 m.a.s.l
Beauveria bassiana	KF_3	Koka	939 m.a.s.l
Metarhizium anisopliae	AF5	Ada'a	1878 m.a.s.l
Metarhizium anisopliae	MF3	Maki	1636 m.a.s.l

3.1.2. Evaluation of Entomopathogenic Fungal Isolates Against the Adults of Two-Spotted Spider Mite

All the six newly isolated entomoathogenic fungi and the existing isolate of 9604 tested were capable of infecting and killing the two-spotted spider mites (Table 4). The infected mites in the sample became dark color when compared to healthy individuals and slowly move before death. Mite cadavers treated with isolates showed outgrowths of the fungus after incubation, confirming that the fungus was the main cause of mite death. The same thing different isolates of both *B. bassiana* and *M. anisopliae* (HH, GG, EE, PPRC-56, FF, PPRC-6 and MM) have been reported to have the potential as control agents in Ethiopia for different pests such as western flower thrips, sorghum chafer, leaf miners, desert locust, and root mealy bugs [3, 22, 29, 30]. However, mortality of *T. urticae* due to the fungal isolates significantly varied among isolates of each of *B. bassiana* and *M. anisopliae* four days after treatment ($F= 9.62$, $DF=7$, $P< 0.001$), six days after treatment ($F= 24.82$, $DF=7$, $P< 0.001$) and eight days after treatment ($F= 46.53$, $DF=7$, $P< 0.001$) (Table 4). Isolates GF3 and 9604 were faster in causing mortality with significantly higher deaths on the fourth, sixth and eight days after treatment (DAT). These two isolates caused the highest corrected mortalities of 93% and 85% respectively, within eight days of application (Table 4). Isolates MF3 and AF5 were considered as moderately virulent, causing the corrected mortality of 64% and 54%, respectively. Isolates AF2, BF4 and KF3 caused 47%, 36% and 33%, respectively, eight days after treatment application and considered as weak in virulence. Mortality caused by the isolates was confirmed based on the visual observation of fungal growth (mycosis) on surface of cadavers.

Table 4. *Pathogenicity of seven isolates of entomopathogenic fungi on T. urticae at the rate of 1×10^8 conidia ml^{-1}, four, six and eight days of post inoculation under Laboratory conditions.*

Fungal Isolates	Mortality ± SE* 4 days after treatment	Mortality ± SE* 6 days after treatment	Mortality ± SE* 8 days after treatment
GF3	37.25 ± 0.93^{a}	56.53 ± 1.78^{a}	93.14 ± 1.30^{a}
9604	39.05 ± 0.91^{a}	51.67 ± 2.12^{ab}	84.76 ± 0.55^{a}
MF3	23.23 ± 0.96^{b}	42.59 ± 2.26^{b}	63.64 ± 2.29^{b}
AF5	9.38 ± 0.79^{d}	30.60 ± 0.52^{c}	54.17 ± 0.60^{c}
AF2	17.14 ± 1.65^{bc}	30.18 ± 2.83^{c}	47.06 ± 1.11^{c}
BF4	13.13 ± 1.17^{c}	32.32 ± 1.34^{c}	36 ± 1.38^{d}
KF3	15.15 ± 0.38^{bc}	29.22 ± 0.90^{c}	33.33 ± 1.38^{d}
Control	0 ± 0^{e}	0 ± 0^{d}	0 ± 0^{e}
CV%	39.84	21.89	19.13

*Values followed by the same letter in the same day do not differ significantly ($P>0.05$) according to Student Newman Kuel (SNK) test.

Previous studies showed variations among isolates in the extent of mortality and time taken to kill the spider mites. For all isolates tested the cumulative mortality increased through time. According to Roza *et al* [26] the effectiveness of isolates varied in the extent of mortality and time taken to kill the spider mites: i.e. isolate 9614 and MM took3.19 and 3.68 days, respectively to cause 50% mortality of *T. urticae.* In this study, isolates of GF3 and 9604 took less time (5.35 and 5.47 days, respectively) to cause 50% mortality at 1×10^8 concentrations. It was found that the moderate virulent isolates MF3 and AF5 had longer LT_{50} of 6.69 and 7.94, respectively while weak virulent isolates AF2, BF4 and KF3 relatively had longest LT_{50} value to cause 50% mortality compared to the highly virulent category. Sinishaw [28] reported that the median lethal time for the highest conidial concentration is low and high mortality of 100% was achieved at 1×10^8 conidia ml^{-1}. In a similar study, Wekesa *et al*. [35] reported that the lethal time to 50% mortality of *Tetranychus evansi* (LT_{50}) of the most active isolates of *B. bassiana* and *M. anisopliae* varied much from weak isolates. Bugeme *et al*. [4] also reported the significant variations in lethal time to 50% mortality (LT_{50}) of adult females of *T. evansi*.

From Table 5 below, it can be seen that those fungal isolates GF3, 9604, MF3, AF5 took 5.35, 5.47, 6.69, 7.94 days respectively to cause 50% mortality of *T. urticae*. The isolates that had low lethal time fifty (LT_{50}) values, indicating rapid infection of TSSM, which is an important feature for selecting fungal isolates as potential biological control means.

Table 5. *LT_{50} of T. urticae eight days after treatment with isolates of M. anisopliae and B. bassiana at the rate of 1×10^8 conidia ml^{-1}.*

Isolate	Mean LT_{50} (days)	95% CI		X^2	Sig. level	Intercept	Slope ± SE
		Lower limit ± SE	Upper limit ± SE				
GF3	5.35^{c}	4.73±0.19c	6.07±0.07^{bc}	10.05^{ab}	0.132	-4.56b^{c}	6.28±0.26^{a}
9604	5.47^{c}	4.76±0.17^{c}	6.35±0.25^{bc}	10.93^{a}	0.095	-4.19b^{c}	5.71±0.40^{a}
MF3	6.69^{bc}	5.93±0.26^{b}	8.00±0.40^{b}	9.29^{abc}	0.215	-4.14b^{c}	5.03±0.34^{ab}
AF5	7.94^{ab}	7.12±0.04^{a}	9.63±0.05^{ab}	4.48^{d}	0.621	-4.84^{c}	5.38±0.72^{ab}
Control	0.00^{d}	0±0^{d}	0±0^{d}	0.00^{e}	0.00d	0.00^{a}	0±0^{d}
CV%	16.54	11.60	42.93	34.46		-18.19	17.13
LSD	1.91	1.15	6.86	3.89		1.16	1.28
P	<0.0001	<0.0001	0.0024	0.0004		<0.0001	<0.001

*Values followed by the same letter in the same column do not differ significantly (P>0.05) according to LSD test.

3.1.3. Pathogenicity of Entomopathogenic Fungal Isolates Against the Eggs of Two-Spotted Spider Mite

All the isolates tested were caused mortality on eggs of *T. urticae* (Table 6). However, there were differences in egg mortality between the isolates applied at the same conidial concentration of 1×10^8 conidia ml^{-1} (F Value = 43.01, DF=7, P<0.001). Un-hatched eggs looked distorted, shrunken and showed fungal outgrowths after observation under a microscope. The highest egg mortality was recorded for isolate *B. bassiana* GF3 (87%) followed by *B. bassiana* 9604 (79%). The remaining five isolates, *M. anisopliae* MF3, *M. anisopliae* AF5, *B. bassiana* AF2, *B. bassiana* BF4, and *B. bassiana* KF3 caused 61%, 50%, 43%, 32%, 33% egg mortality, respectively, at seven days after treatment and no mortality were observed in the Control group treated with only 0.01% Tween 80 (Table 6).

Table 6. *Mortality of two-spotted spider mites eggs treated with isolates of B. bassiana and M. anisopliae within seven days after treatment application.*

Fungal Isolates	Isolate Codes	Mortality ± SE*
B. bassiana	9604	79.33± 1.16^{a}
	AF2	43.33± 0.95^{cd}
	BF4	32.00± 0.91^{d}
	GF3	86.67± 0.77^{a}
	KF3	32.67± 0.88^{d}
M. anisopliae	AF5	50.00± 0.50^{bc}
	MF3	60.67± 1.02^{b}
Control		0±0^{e}
CV%		15.69

Values followed by the same letter in the same column do not differ significantly (P>0.05) according to Student Newman Kuel (SNK) test.

According to Chandler *et al*. [6] poor performance of the fungal strains on eggs compared to adults has been hypothesized to be due to the topography of eggs, which is not suitable for the establishment of conidia. It was concluded that there is a lack of nutrients (lipids) on mite egg shell surfaces and that these nutrients are important for the germination of conidia and subsequent growth of the fungus [6]. The current result also indicated that egg mortality was low compared to adult mortality. Weibin and Mingguang [34] found that both *B. bassiana* and *Paecilomyces fumosoroseus* infections decreased the hatch rates of *Tetranychus cinnanarinus* eggs and the higher the conidial concentrations resulted in greater reduction in the hatch rates. Gouli *et al*. [15] also reported that entomopathogenic fungi, *B. bassiana* and *M. anisopliae* are often potential to infect developmental stages, including eggs. The current study is also similar with Kongchuensin and Takafuji [18] that *M. anisopliae* and *B. bassiana* are virulent or ability to cause disease to the eggs of the pest: i.e. infected (unhatched) eggs conspicuously shrunk, turned orange-brown in egg shape for the *B. bassiana* product or dark-gray for the *M. anisopliae* product.

3.2. Glasshouse Experiment

The three most virulent entomopathogenic fungi GF3, 9604 and MF3 during the laboratory bioassay on adults and eggs of *T. urticae* were further tested on potted tomato plants infested with the mite. All the three isolates reduced (P<0.05) the numbers of motile *T. urticae* compared with the untreated control (Table 7). Chandler *et al*., [6] reported that entomopathogenic fungi of *M. anisopliae* and *B. bassiana* reduced the numbers of motile *T. urticae* populations on tomato leaf in greenhouse compared to the control. Yayan *et al*., [36] also reported that the pathogenicity of entomopathogenic fungal isolates that spray suspension with 1×10^8 conidia ml^{-1} gave superior controls of *Tetranychus kanzawai* on papaya seedlings under greenhouse conditions.

Table 7. *Mean percent mortality of two-spotted spider mites due to three entomopathogenic fungi isolates at the rate of 1×10^8 conidia ml^{-1} on tomato in the glasshouse.*

Fungal Isolates	Mortality (%)			
	Top leaf	Middle leaf	Bottom leaf	Average ± SE
9604	76.19^{a}	68.22^{b}	66.67^{a}	70.36± 1.70^{a}
GF3	77.78^{a}	82.11^{a}	72.86^{a}	77.58± 1.54^{a}
MF3	50.16^{b}	55.71^{c}	64.52^{a}	56.80± 2.41^{b}
Control	0.0^{c}	0.0^{d}	0. 0^{b}	0± 0^{c}
CV%	64.15	33.48	52.79	6.61

Values followed by the same letter in the same column do not differ significantly (P>0.05) according to Student Newman Kuel (SNK) test.

The average mortality of *T. urticae* due to the fungal isolates significantly varied among isolates (F= 3.4816, DF=3, P< 0.001). The two isolates of *B. bassiana,* GF3 and 9604, and one *M. anisopliae,* MF3, reduced the numbers of motile *T. urticae* by 78%, 76% and 50%, at the top, 82%, 68%, 56% at the mid, and 73%, 67%, 65% at the bottom leaf, respectively. The *B. bassiana* isolates GF3 and 9604 did not differ significantly (P>0.05) from each other. However, each

one differed significantly ($P < 0.05$) from *M. anisopliae* MF3. *B. bassiana* GF3 had the greatest effect on the *T. urticae* population on the tomato leaf.

According to Roza *et al*., [25] mortalities of *T. urticae* due to entomopathogenic fungi recorded in the greenhouse experiment were lower than the laboratory. In this study also mortalities recorded in the glasshouse experiment were lower than the laboratory or all the isolates. For example, mortality adults of *T. urticae* by GF3, 9604 and MF3 were 78%, 70% and 57%, respectively, in the glasshouse (Table 7), while there were 93%, 85% and 64%, respectively, in the laboratory experiment (Table 4). This variation could be due to high probability of inoculation of *T. urticae* in case of the laboratory study which was conducted in petridishes. *B. bassiana* isolates of GF3 and 9604 were more virulent in the glasshouse study compared to *M. anisopliae* MF3. This may be due to their ability to withstand the glasshouse conditions better than other.

4. Conclusion

Insect pests are significant constraints to tomato production. Among them, *T. urticae* is a harmful pest of tomato crops due to the continual use of chemical, resistance development among mite population. Again, controlling mites with acaricides cost is extremely high. Entomopathogenic fungi have a potential as alternative successful biological control agents which is a cost-effective, environmentally safe alternative to chemical acaricides for mite control. Evaluated isolates of *B. bassiana* and *M. anisopliae* caused mortality which revealed that they are potential for the management of the two-spotted spider mites. Seven isolates native to Ethiopia, namely two *M. anisopliae* (MF3 and AF5) and five *B. bassiana* isolates (GF3, 9604, AF2, BF4, and KF3) were able to kill the two-spotted spider mites which were significantly different from the control. Isolates of GF3, 9604 and MF3 caused significantly higher levels of mortality compared to the remaining both within the laboratory and glasshouse studies. Within the laboratory, isolates of GF3, 9604 and MF3 caused the mortality of 93%, 85% and 64% while 78%, 70%, and 57% respectively in glasshouse. There were significant differences in the virulence and the time killing among the different entomopathogenic fungi isolates at the identical conidial concentration of 1×10^8 conidia ml^{-1}. The strains of EPF that had low LT_{50} values, indicating rapid infection of TSSM in short periods of time, which is an important for selecting fungal isolates as possible biological control agents. No mortality of mites observed in the control which showed that the conditions of the bioassay protocols were consistent. These isolates can be used for the management of the TSSM and be element of the two-spotted spider mites IPM package. This may help to reduce resistance progress among mite populations. Also, can create a competitive market advantage by meeting the requirements like the levels of Maximum Residue Level (MRL) of synthetic insecticides and reducing the associated miticides costs. Future experiment should focus on collection and testing of different entomopathogenic fungi from various areas of the country is very important with the main purpose of developing highly virulent Ethiopian entomopathogenic fungal isolates which are economical for both production and use.

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