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Isolation of entomopathogenic fungi and efficacy as a biological control agent on red spider mite *Tetranychus urticae* (Acari: Tetranychidae)

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Abstract:

Entomopathogenic fungi and non entomopathogens were isolated from Egyptian soil using two isolation methods, from soil by *Galleria* bait method and insect. The sample were identified using the conventional methods. The virulence of four entomopathogenic fungi isolates were tested on red spider mites *Tetranychus urticae* (Koch) (Acari: Tetranychidae), *Beauveria bassiana* (B1 and B2) and *Metarhizium anisopliae* (M1 and M2). Results proved that (B2) was more effective against adult compared with all other isolates. When studying the toxicity of fungal metabolites the results showed that, highest effective toxin was of the M1, followed by B2, M2 then B1, the mortality percentages at seventh day in concentration 100% of crude were 85.13, 76.12, 73.8 and 61.97 for M1, B2, M2 and B1, respectively.

Introduction

Tetranychus urticae (Koch)(Acari: Tetranychidae) attacks a wide range of plants, which often cause severe damage to a variety of crops, it frequently occurs in glasshouse and outdoor crops (Gotoh *et al.*, 2004). It is one of the most serious pests responsible for yield losses of many horticultural, ornamental and agronomic crops, causing considerable crop damage and economic loss (Puinean *et al*., 2010). This pest is commonly controlled by pesticides. However, resistance to pesticides has guided research to find new methods intended to control *Tetranychus* sp. Additionally, the indiscriminate use of different synthetic acaricides to avoid serious health hazards for mammalians. There is an essential and urgent need to develop new acaricides which can be termed green pesticides or new combinations of allelochemicals to gain effective control of this pest.

Biological control agents such as entomopathogenic fungi can be used as a component of integrated pest management. Entomopathogenic fungi can be isolated from the soil (Korosi *et al.*, 2019). Under natural conditions, fungal pathogens are frequent and often cause natural mortalities to the insect populations. Many fungal species such as *Metarhizium anisopliae*, *Lecanicillium lecanii*, *Isaria fumosoroseus* and *Beauveria bassiana* are used as biocontrol agents for controlling various insect pests including termites, black vine weevil, whiteflies,

aphids, corn borers, colons and other insects (Ravensberg, 2011). Various researchers in different parts of the world conducted bioassays on different crops and found one or two isolates of entomopathogenic fungi (EPF) to be highly effective against mites like *Paecilomycis fumosoroseus*, *M. anisopliae* and *B. bassiana* etc. (Draganova and Simova, 2010).

Most published reports reveal that multiple toxic substances were derived from entomopathogenic fungi, which is an attractive approach for identifying important bioactive compounds from EPF. Based on this approach, few studies on EPF have been directed at elucidating the relevant virulence factors of insecticidal metabolites, antifeedants. EPF produce secondary metabolites that disable several immune mechanisms allowing the fungus to overcome and then kill its host. This characteristic makes *B. bassiana* and *M. anisopliae* promising model for biological control of insect pests (Zibaee *et al.*, 2011). The insecticidal activities of secondary metabolites against many pests have been described in several reports (Ríos-Moreno *et al.*, 2017).The traditional bait insect is the highly susceptible larvae of the wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) but also mealworm larvae, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), are suitable. Baiting soil samples with larvae of *G. mellonella* is a widely applied tool to screen for indigenous species of entomopathogenic fungi (Meyling and Eilenberg, 2006). Keller *et al*. (2003) showed that galleria bait method was sensitive than traditional plating on media and was therefore useful for isolation and identification of the entomopathogenic fungi indigenously present in soils. The aim of the present work is to study the isolation entomopathogenic fungi and its effect against the red spider mite *T. urticae*

Materials and methods

1. Isolation of fungi:

1.1. From soil by *Galleria* bait method:

The soil samples were collected from random locations under different crops (Table, 1). About 5 cm depth were removed first in the site of collected soil, then the sample was collected from the next 10 cm depth into clean sterile bags and brought back to the laboratory. Three samples at least were taken randomly from each locality. Samples from each place were mixed to make homogeneity and coarse debris removed (Ali-Shtayeh *et al*., 2002). Avoid sample drying or exposure to high temperatures during the mixing process.

Galleria mellonella L., greater wax moth was reared in Bio-Insecticides Production Unit, Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt. Each five larvae of *G. mellonella* were placed into a small plastic box filled with soil. The boxes were shaken and incubated at (27±1°C) . The larvae were examined on days 3, 4, 5, 7, until 14 days. The surface of dead larvae was sterilized by alcohol (70%) for 10 sec. and then rinsed with sterile distilled water then the larvae were placed in Petri dishes on moistened filter paper to germination of fungal spores on the cuticle of the insect. The Petri dishes were covered with parafilm to maintain suitable relative humidity and incubated in darkness at 70 ± 5 % relative humidity (R.H.) and 27 ±1°C. Larvae were observed until mycelium appeared. Infected larvae placed into plates of Czapeck's Dox's agar medium. The fungi were identified using traditional procedures, morphological characteristics with the help relevant taxonomic literature.

1.2. from insect:

Desert locust *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) cadaver was coated with fungal spores. It was taken and purified as follow:

Infected cadavers of larvae placed into Czapeck's Dox's agar medium (NouriAiin *et al*., 2014). The isolates were incubated at 27± 1°C for 8–14 days. The

plates were daily examined for up 14 days or more, the differently looking fungal colonies developed on each plate. After fungal hyphae emergence and spores, they were sub cultured by placed into a new Dox's agar medium and incubated at 27ºC±1.The pure developed fungal colonies were identified to the species level These fungi identified by using the conventional methods (Sahar and Moharram , 2014). The identification was done at the Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. The identification of entomopathogenic fungi was made with the help of the following universally accepted keys for identification of different isolated fungi (Domsh *et al.*, 1980; Nelson *et al*., 1983 and Barnett and Hunter, 1987).

3. Virulence test on *Tetranychus urticae* :

3.1. Rearing of mites:

The original colony of the red spider mites *T. urticae* in this study was supplied from Acarology Laboratory in Plant Protection Research Institute, Agricultural Research Center at Dokki, Egypt. *T. urticae* was reared for several generations at 25 ± 0.5ºC away from any pesticide contamination. *T. urticae* was maintained on detached mulberry leaves with the lower surface upwards placed on moist cotton wool pads in fiber-dishes (20cm in diameter). The cotton pads were moistened daily to prevent mite escape and to avoid dryness leaves of mulberry were replaced by fresh one when necessary (Hassan, 2018).

3.2. Bioassay by conidiospores:

Entomopathogenic fungi conidiospores (aerobic spores produced asexually by a fungus) were harvested by distilled water mixed with 0.05. % Tween 80 to obtain the stock spore suspension. The suspension was vortexed well and then filtered through cheesecloth to reduce mycelium clumping. Heamocytometer (Neubauer improved HBG, Germany) was used to counted the spores in the suspension (Lozano-Tovar *et al*., 2013). Three concentrations of each isolate were prepared: 1×10^6, 1×10^7, and 1×10^8 spores/ml. Twenty adult mites were placed on a single leaf-disk of mulberry (2.5 cm in diameter) and kept on moist cotton wool in fiber-dishes with cotton around each disk in a circle way to prevent mite escaping. Each dish contained five replicate (disks). The direct spray technique was applied by a glass atomizer at 30cm away from the treated surface with 1ml spore suspension for each treatment and 1ml sterilized distilled water of 0.05% Tween 80 as a control (Abo-Shabana, 1980 and Hassan, 2018). The treated adult was incubated at 27 ± 1°C. Mortality was assessed daily. The mortality percentage corrected by Abbott's formula (1925) .

3.3. Production of fungal metabolites:

One ml of spores suspensions contented of 1 x 10^7 spore/ ml from Entomopathogenic fungi isolates were prepared then poured into flask with 100 mL Czapek-Dox broth medium , and incubated for 21 days at 240 r/min and 27± 1°C. Each isolate was replicated 4 times. Each flask was filtered twice time through Whatman 1 filter paper to remove the spores and mycelia (El Basyouni and Vining ,1966; Kershaw *et al*., 1999; Hsiao and Ko, 2001 and Hu *et al.*, 2006 and Ting-yan *et al*., 2016).

3.4. Toxicity of fungal metabolites :

Four concentrations of each fungal metabolites were prepared as follow: (100%) metabolites as it is, (75%) were added 3 parts of metabolites to 1part of distal water, (50 %) were added 2 parts of metabolites to 2 parts of distal water and (25%) were added 1 part of metabolites to 3 parts of distal water, each treatment had five replicates. Sterilized distilled water were a control to each concentration. Mortality was assessed daily. The percentage of mortality was determined and corrected by Abbott's formula (1925).

4. Statistical analysis:

LC_{50}, LC_{90} and slope values were calculated according to Finney (1971), using "Ldp Line" software (Bakr, 2000). All

experiments contained. 3-5 replicates and data were analyzed by one – way analysis of variance (ANOVA) using SPSS 17.0 statistical software. When the ANOVA statistics were significant (P <0.05), means were compared by the Duncan's multiple range test.

Table (1): Location of soil samples.

Governorate	Location	Crop
Giza	Malaut	Okra, rocca and corn.
Qilyubia	Qaha	Orang, corn and strawberries
Bani-Sweif	Bani-Sweif	Taro and banana
Kafr EL Sheikh	Desouq	Banana

Results and discussion

The study revealed the presence of entomopathogenic and non entomopathogenic fungi isolates. Four isolates of entomopathogenic fungi *i.e.*, two isolates of *B.bassiana* (B1 and B2) and another two isolates of *M. anisopliae* (M1 and M2) were isolated, 3 isolates from soil at random location and one from insect. Data given in Table (2) showed entomopathogenic isolates and non-entomopathogenic fungi isolates.

Table (2): The fungal isolates

Isolate	From	Crops	Location	Pathogenicity*
***Paecilomyces lilacinus*(Thom) Samson** ***Aspergillus flavus va columnaris* Raper** ***Aspergillus tamarii* Kita** ***Fusarium semitectum* Berkeley**	Soil	Orang	Qilyubia	NP PP PP PP
***Gliocladium roseum* Bainier.** ***Mucor hiemalis* Wehmer**	Soil	Strawberries	Qilyubia	PP, NP and FP PP
***Beauveria bassiana* (Balsamo) Vuillemin**	Soil	Vegetable crops	Giza	EP
Cunninghamella echinulata* (Thaxter)** ***Aspergillus tamari	Soil	Taro	Bani-Sweif	SF PP
Beauveria bassiana ***Fusarium oxysporum* Schlechtendal**	Soil	Banana	Kafr ELSheikh	EP PP
Metarhizium anisopliae* (Metschnikow) Sorokin** ***Fusarium semitectum	Soil	Watercress plant	Giza	EP PP
Metarhizium anisopliae	Insect (Desert locust)	Alfalfa	Baharyia Oasis	EP

Pathogenicity* = (NP) Nematode Pathogen; (PP) Plant Pathogen; (EP) Entomo pathogen;(SF) Saprophytic Fungi or (FP) Fungi Pathogen.

B. bassiana and *M. anisopliae* are a common entomopathogenic fungi in soil. Several studies in Egypt demonstrated isolation of entomopathogenic fungi (Nada, 1999 ; Sahar and Moharram, 2014; Hussein, 2015 and Lokma, 2016).

1.Virulence of entomopathogenic isolates:

The efficiency of four entomopathogenic isolates, two isolates of *B. bassiana* (B1 and B2) and two isolates of *M. anisopliae* (M1 and M2), against adult *T. urticae* in laboratory experiments. Total mortality percentage values after exposing to series of concentrations of 1×10^6, 1×10^7 and 1×10^8 spores/ml were shown for seven days after

treatment in Table (3). While the highest concentration of 1×10^8 spores/ml revealed 39.2, 78.65, 21.15 and 43.8 % for B1, B2, M1 and M2, respectively after the same consecutive days. LC_{50} and LC_{90} values were tabulated with their corresponding slopes after treatment in The LC_{50} for B1, B2, M1, and M2 were 4.90×10^8 2.86×10^7, 2.75×10^9, and 4.58×10^8spores/ml, respectively. Results proved that (B2) was more effective against adult compared with all other isolates (Table, 3).

One way ANOVA statistical analysis indicated that significant levels of effect between fungal isolates B1, B2, M1 and M2, on adult *T. urticae* were presented in Table (3). The obtained results showed that there was significant effect ($F_{3,16}$= 22.98, P≤0.05) between four isolates were mean of mortalities were 78.54, 42.44, 38.49 and 21.27% for B2, M2, B1 and M1, respectively. The most effective isolate was B2. The LT_{50} value of the four isolates of entomopathogenic fungi (B1, B2) and (M1, M2) in Figure (1) showed that isolate B2 caused high mortality in shortest time, LT_{50} value was 3.57 days. While for the other isolates M2, B1 and M1 the LT_{50} values were 9.24, 10.16 and 23.17 days, respectively.

Table (3): virulence of entomopathogenic fungi isolates on *Tetranychus urticae* after seven days post treatment.

Isolate	Total mortality%	LC_{50}	Slope	LC_{90}	Index	Mean ±SE
B1	39.2	4.90×10^8	0.768	2.28×10^{10}	5.835	38.49±1.1b
B2	78.65	2.86×10^7	0.565	5.33×10^9	100	78.54±0.95a
M1	21.15	2.75×10^9	0.724	1.62×10^{11}	1.038	21.27±0.37c
M2	43.8	4.58×10^8	0.357	1.77×10^{12}	6.245	42.44±1.6b

B1 , B2 = ***Beauveria bassiana*** **M1, M2=** ***Metarhizium anisopliae***

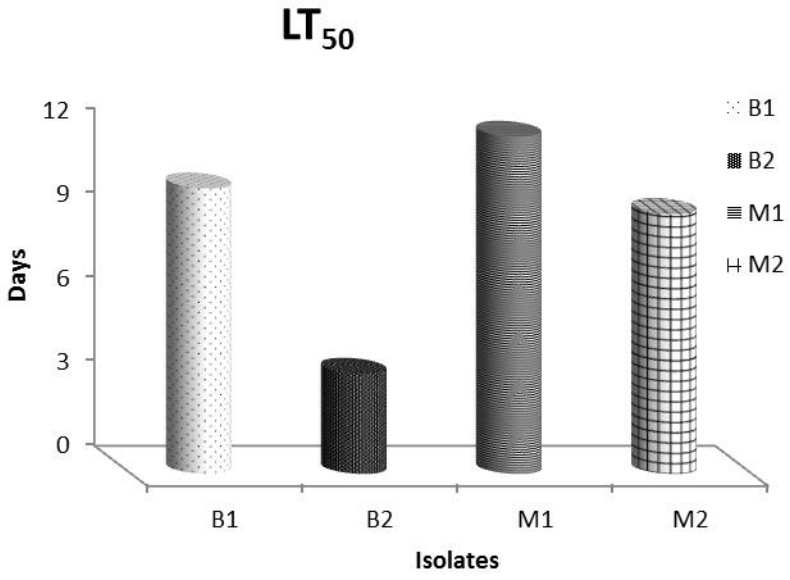

Figure (1): Comparison LT_{50} between four entomopathogenic isolates against adult *Tetranychus urticae.*

Virulence of entomopathogenic isolates against spider mites In the light of the results obtained during the present experimental work, it was clear that entomopathogenic fungi; *B. bassiana* (B1, B2) and *M. anisopliae* (M1, M2) were pathogenic to adult *T. urticae*. Pathogenity of fungus increased with the increase of concentration and time. Death results due to a severe damage in the tissues., toxicosis, cell dehydration, loss of nutrient intake, and finally the hyphae emerge from the insect body sporulates and starts a new infection cycle (Perez *et al.*, 2014). The results compatible with Various researchers in different parts of the world conducted bioassays on different crops and found one or two isolates of entomopathogenic fungi (EPF) to be highly effective against mites like *M. anisopliae*, *B. bassiana* and *Paecilomycis fumosoroseus*, etc. (Nugroho and Ibrahim, 2004 and Draganova and Simova, 2010). These findings in agreement with Chandler *et al.* (2000) they studied pathogenity a commercial isolate of entomopathogenic fungi *B. bassiana* (Naturalis - L) against *T. urticae* on tomato leaflets under laboratory conditions and glasshouse experiments. they found that (Naturalis – L) was high pathogenic against *T. urticae*. Also, Hassan *et al.*, 2017 tested two *M. anisopliae* and four *B. bassiana* isolates for virulence against *T. urticae*, egg and adult stages. B4 isolate was found to be the most potent, causing 88.5% mortality for adult stage at a concentration 10^8 spores/ml. The LC_{50} was 6.61 $x10^6$.

2.Toxicity of fungal metabolites:

The comparison percentage mortality values after exposing to series concentrations of four metabolites extracts at 100%, 75%, 50% and 25% crude/ml were shown in Table (4). Results obtained revealed that the four tested metabolic crudes were toxic to *T. urticae.* The highest effective toxin was of M1, followed by B2, M2 then B1 . The mortality percentages at seventh day and in concentration 100% of crude were 85.13, 76.12, 73.8 and 61.97% for M1, B2, M2 and B1, respectively.

Table (4): Percentage total mortality of adult *Tetranychus urticae* treated with four concentrations of *Beauveria bassiana and Metarhizium anisopliae* crudes after seven days.

Concentration	**Mortality%**			
	***Beauveria bassiana* B1**	***Beauveria bassiana* B2**	***Metarhizium anisopliae* M1**	***Metarhizium anisopliae* M2**
25%	24.39	34.48	56.45	58.33
50%	37.17	38.33	63.38	65.85
75%	40.54	63.15	77.02	71.57
100%	61.97	76.12	85.13	73.8

B1 and B2 = M1 and M2=

Results in Table (5) proved that (M1) crude was more effective against adult compared with all other isolates. The LC_{50} value of M1 was 45.67 crude/ml while M2, B2 and B1 revealed greater LC_{50} value, 50.92, 62.47 and 101.65 crude/ml, respectively. Generally, the highest values of mortality were in the M1 treatment. One way ANOVA statistical analysis indicated that were significant levels of effect ($F_{3,16}$=3.19*; $P \leq 0.05$) between different fungal crude M1, M2, B1and B2 on adult *T. urticae* were presented in Table (5). Means of mortalities were 84.4, 74.09, 69.08 and 59.71 % for M1, M2, B1 and B2, respectively. Data indicated M1 was more effective. Figure (2) illustrated

mortality percentages of different curds at within intervals times seven days at 100% crudes/ml. It proves M1 crude was most effective toxin against *T. urticae*.

Table (5): Comparison between four metabolic crude of *Beauveria bassiana* (B1, B2) and *Metarhizium anisopliae* (M1,M2), *Tetranychus urticae* adult stage according to LC_{50} and means of mortality ±SE.

Crude	LC_{50}	Lower limit	Upper limit	Slope	LC_{90}	Index	Mean±SE
B1	101.65	81.94	149.55	1.95	461.67	44.93	59.71±5.36c
B2	62.47	52.69	74.89	2.22	235.77	73.10	69.08±8.41cb
M1	45.67	35.51	54.89	1.91	214.03	100.00	84.4±5.11b
M2	50.92	30.87	71.60	1.00	970.65	89.69	74.09±2.42cb

Index compared with M1* significant at level (P≤0.05). b and c Mean within the same row having different superscripts significantly different (P≤0.05).

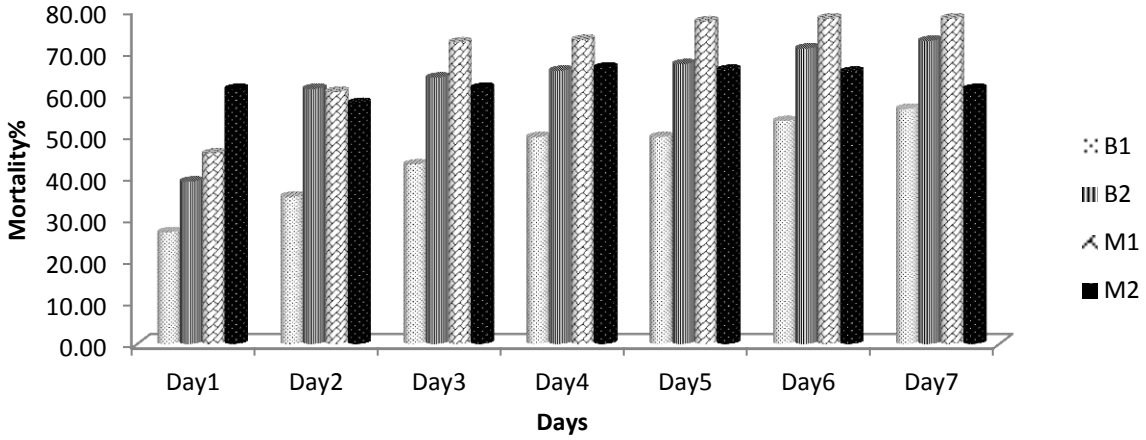

Figure (2): Mortality percentages of adult *Tetranychus urticae* after treated with 100% concentrate of metabolites from four fungal isolates.

Entomopathogenic fungi secrete secondary metabolites which toxic to insect. Destruxins are insecticidal metabolites of a fungus, *M. anisopliae*. These metabolites are usually secreted into the culture medium during growth. The structure of destruxins is classified as being a cyclic hexadepsipeptide. More than 35 different destruxins have been characterized with a wide range of insecticidal activities (Kershaw *et al.* ,1999 and Liu *et al.*, 2004). In the present investigation, the aim was to provide data for enhancement of entomofungal virulence and production of toxic macromolecular insecticidal substance from native entomopathogenic isolates. The present study showed that four native Isolates *B. bassiana* and *M. anisopliae* (B1 and B2 M1 and M2) and its metabolites had toxic effects on *T. urticae* adult, may be due to variations of the composition of the chemical composition of crude. These agree with previous studies carried out by Gaber (2016) who Compared the effect of two extracts (destruxins) from two isolates *M. anisopliae* (Ma1 and Ma2) achieved toxicity against both *T. cucurbitacearum* adult females and deutonymphs three days post treatment. The LC_{50} values of destruxins from isolate (Ma1) were 2.26 and 3.26 gm./100ml against deutonymphs and adult females, respectively. LC_{50} of destruxins from isolate (Ma2) were 2.35 and 3.98 gm./100ml against deutonymphs and adult females, respectively.

Also, agree with Fan *et al.* (2013) they study reviled toxicity effect secondary metabolites of *B. brongniartii* against *Dendrolimus tabulaeformis*. The mortality rate of the larvae in the bioassay positively changed with the concentration of the fungal secondary metabolites. In the groups treated with fungal secondary metabolites at three concentrations, 5.5, 55,and 550 µg /mL, the larval mortalities were 29.33 ± 3.2%, 46.00 ± 1.47%, and 71.33 ±3.07%, respectively. The differences were significant between the groups.

In comparison toxicity of crudes with the conidial infection, it was found that the fungal metabolites were more effective in killing the mite than infection with the conidia. That may be due to conidial infection required more time to complete the series of infection processes, including conidial attachment on the host cuticle, germination, hyphal penetration through the integument, and infection of the internal tissues and organs, whereas the secondary metabolite was more direct attack. Hence, fungal secondary metabolites should be considered for pest management programs in the future. These results in the line with Fan *et al.* (2013) and Zibaee *et al.* (2011), they suggested that the secondary metabolites produced by *B. bassiana* caused problem in several defense mechanisms of *Eurygaster integriceps*, thus helping the fungus to destroy its host. These results agree with those obtained by Lozano-Tovar *et al.* (2015), they investigated the insecticidal activity of the crude extract obtained of *M. brunneum* (Petch EAMb 09/01- Su) strain and its ability to secrete secondary metabolites including destruxins (dtx). Dtx A and A2 were toxic against *Ceratitis capitata* (Wiedemann). The crude extract of seven *Metarhizium* and one *Beauveria* isolates were evaluated against medfly adults. The isolate crude extracts. (EAMb 09/01-Su) resulted in mortality ranging between 95 and 100% at 48 h.

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