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Virulent Entomopathogenic Fungi against The Two-Spotted Spider Mite *Tetranychus urticae* and some Associated Predator Mites as Non Target Organisms

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ABSTRACT

Entomopathogenic fungi and predatory mites can independently contribute to control the two-spotted spider mite *Tetranychus urticae* Koch. It is important to assess the risk of possible fungal infections in predators when a combination of them are being considered as a tandem control strategy for *T. urticae* in IPM program. The first part of this study tested 4 *Beauveria bassiana* isolates and 2 *Metarhizium anisopliae* for virulence against *T. urticae*, egg and adult stages. Strains B4 was found to be the most potent toward egg and adult stages, causing 88.5% mortality for the adult stage at a concentration of 10⁸ spores/ml and the LC₅₀ was 6.61x 10⁶. When applied on the egg stage the hatchability was 25.2% compared with the control which reached 99% and the LC₅₀ was 1.14 x10⁷. The second part evaluated the pathogenicity of the most effective isolates B4, three concentrations were applied LC₂₅, LC₅₀ and LC₉₀ against the adult of the two predator mites *Phytoseiulus persimilis* and *Neoseiulus californicus*. The bioassay results indicated that the isolate B4 was harmless against *P. persimilis* and slightly harmful against *N. californicus*.

No viable fungal hyphae were found on predator cadavers. Observations with scanning electron microscopy revealed that conidia were attached to the cuticle of predatory mites within 24 h after spraying with strain B4, and had germinated within 24–48 h. After 48 h, conidia had gradually been shed from the mites, after none of the conidia had penetrated the cuticular surfaces. In contrast, the germinated conidia successfully penetrated the cuticle of *T. urticae*, and within 72 h the fungus colonized the mite’s body. Our study demonstrated that although several *B. bassiana* strains displayed a high virulence in *T. urticae* there was no evident pathogenicity to phytoseiid mites. These findings support the potential use of entomopathogenic fungus in combination with predatory mites in *T. urticae* control programs.
INTRODUCTION

The two-spotted spider mites (TSSM), Tetranychus urticae Koch (Acari: Tetranychidae), are major agricultural pests, which often cause severe damage to a variety of crops (Gotoh et al., 2004). Spider mites become serious pests in a wide range of protected crops across the world. It is one of the pests responsible for yield losses of many horticultural, ornamental and agronomic crops, causing considerable crop damage and economic loss (Puinean et al., 2010). A major problem in the control of T. urticae is its ability to develop rapid resistance to many acaricides. Among arthropods, it has the highest incidence of pesticide resistance (van Leeuwen et al., 2010). Another explanation for this increase in mites populations that widespread insecticide usage has eliminated many of the mites’ natural enemies, resulting in a reduction in predation pressure on the mite (Choi et al., 2004; Prischmann et al., 2005).

Consequently, using biological control agents such as entomopathogenic fungi and predatory mites has been recommended to control insect pests in integrated pest management programs (IPM), because their use is considered to be more environmentally friendly (Jacobson et al., 2001; Zhang, 2003; Maniania et al., 2008).

Microbial control of pests is an important approach to reduce the dependence on chemical pesticides for increased agricultural sustainability. Beauveria bassiana has been tested in the laboratory and applied in the field to control numerous insect pest species (Legaspi et al., 2000) and has shown potential as an effective agent in controlling pest mite species (Maniania et al., 2008; Geroh et al., 2015).

Predatory mites are often used as an alternative to conventional pest management on a variety of plants (Gerson and Weintraub, 2007). The predatory mite, Phytoseiulus persimilis Athias-Henriot (Acari: Phytoseiidae) and Neoseiulus californicus were used in integrated pest management programs for T. urticae suppression (Cote, 2001; Skirvin and Fenlon, 2001; Fitzgerala and Easter, 2003).

In order to increase efficiency in controlling T. urticae, it has been suggested that using predatory mites accompanied by applications of B. bassiana may be an alternative to traditional T. urticae management (Chandler et al., 2005). Considering that there are a lot of insect and mite species that are susceptible to B. bassiana, there is a risk that the fungus may be harmful to predatory mites (Jacobson et al., 2001). Therefore, there is a must to evaluating the compatibility of B. bassiana and predatory mites to the success of potential IPM programs designed to control T. urticae. Several studies have evaluated the effects of pathogens on predators by investigating the predator mortality after exposure to spray residues (Flexner et al., 1986). Previous studies on the interaction between fungal pathogens and predatory mites have focused on the fungal infectivity to predators (Donka et al., 2008; Vergel et al., 2011), or on the sub lethal effects of ingesting pathogen treated prey on predators (Seiedy et al., 2012a; Wu et al., 2015).

The aim of this study is to evaluate the virulence of four B. bassiana and two Metarhizium anisopliae (Metschnikoff) isolates against egg and adult stages of T. urticae. The highly virulent isolate of entomopathogenic fungi on T. urticae was then tested against the adult stage of two predators mites Phytoseiulus persimilis Athias-Henriot (Acari: Phytoseiidae) and Neoseiulus californicus; mortality and fecundity of the predatory mites were tested. Also by using scanning electron microscopy (SEM), a comparison study take place between the infection of T. urticae and the predator mites by entomopathogenic fungi.
MATERIALS AND METHODS

Rearing of *Tetranychus urticae* (Koch):

The original colony of the red spider mites *T. urticae* in this study was supplied from Acarology Laboratory in Plant Protection Research Institute, A.R.C at Dokki. It was reared as a test mite for several generations at 25 ± 1°C and 70± 5 R.H. 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 avoid disc dryness, and to prevent mite escape. Mulberry leaves were changed by fresh one from time to time when necessary (Hassan, 2008; Sewify et al., 2015).

To obtain adult *T. urticae* of uniform age, 25 adult females were taken from the mite culture and put on leaf discs placed on wet cotton in Petri dishes (20cm in diameter), and allowed to lay eggs for 24h. After which the females were removed and the eggs remained till adult (Seiedy et al., 2012).

Rearing of the predaceous mites:

The predaceous mites, *Phytoseiulus persimilis* and *Neselius californicus* were obtained from rearing lab, Kaha Research Station, Agriculture Research Center. The predatory mites were maintained at 25± 1°C and 70 ± 5% R.H with a photoperiod of 16:8h (L:D) and away from any pesticide contamination. The predatory mites were 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 avoid disc dryness, and to prevent mite escape. The colony of the predator was fed on a mixture of various stages of *T. urticae* day-to-day. Also Mulberry leaves were changed by fresh one from time to time when necessary (Nadimi et al., 2008; Seiedy et al., 2012).

To obtain females of the same age, about five gravid females from the stock colony were transferred to *T. urticae* infested mulberry leaf in each of a series of Petri dishes. The females were allowed to lay eggs for 24h and then removed. Newly emerged predators were of the same age (Seiedy et al., 2012).

Fungal culture and preparation of conidial suspension:

Six isolates of entomopathogenic fungi were used in this study, four isolates of *Beauveria bassiana* (Balsamo) (B1- B2- B3- B4) and two isolates of *Metarhizium anisopliae* (Metschnikoff) (M1-M2). They were obtained from the soil. Virulence of all isolates against *T. urticae* was studied. All the six tested entomopathogenic fungi were cultured on autoclaved sabouraud dextrose agar with yeast extract (SDAY) media (4 % Dextrose , 0.1 % peptone + 1.5 % Agar + 0.2 % yeast extract dissolved in 1L of Distilled Water). The media were then incubated at 25 ± 1°C, 60 –70 % RH and in darkness for 10–14 days to obtain conidia (Uma Devi et al., 2005; Sewify et al., 2015).

The susceptibility of egg and adult female *T. urticae* to the entomopathogenic fungi *B. bassiana* and *M. anisopliae*

Spores of the six incubated entomopathogenic fungi isolates were harvested from the culture medium by rinsing with sterile distilled water containing 0.01% Tween 80, then scrubbing the surface with a glass bar, and filtered the conidia suspension through sterile layer of cheesecloth to remove conidial clumps and mycelial debris. The spores were counted in the suspension using a hemocytometer (Neubauer improved HBG, Germany 0.100 mm² x 0.0025 mm²) under a phase-contrast microscope. Five concentrations of each isolate were prepared: 10⁶, 5 x 10⁶, 10⁷, 5 x 10⁷, and 10⁸ spore/ml, as well as the control (Distilled Water of 0.01%
Tween 80) and were used on the same day after preparation and shaken before use (Hassan, 2008; Sewify et al., 2015).

**Treatment of eggs:**
Ten vigorous adult females taken from the population were transferred to a mulberry leaf disks (2.5cm in diameter) and kept on moist cotton wool in fiber dishes with cotton around each disk in circle way to prevent mite escaping. Each dish contained 5 disks as replicates. Adult female were allowed to lay eggs freely for 24 hours to deposit egg then adults were removed from the disks and deposited egg were counted. The eggs were sprayed using direct spray technique (Abo-Shabana, 1980; Hassan, 2008; Sewify et al., 2015) by a glass atomizer at 30cm high with 2ml spore suspension for each treatment and 2ml sterilized distilled water of 0.01% Tween 80 as control.

Eggs were incubated at 25 ± 1°C to favor conidial germination, and the number of hatched and non-hatched eggs were daily examined for 7 days of oviposition. The unhatched eggs were transferred to moist Petri dishes for observing fungal out growths. Final egg mortalities in all treatments were computed based on the last-day counts of the hatched and non-hatched eggs. The percentage of mortality was determined and corrected by Abbott’s formula (1925) as follows:

\[
\text{Percentage of mortality} = \frac{\text{tested mortality - } \% \text{ control mortality} \times 100}{100 - \% \text{ control mortality}}
\]

LC$_{50}$, LC$_{90}$ and slope values were calculated according to Finney (1971), using "Ldp line" software by (Bakr, 2000).

**Treatment of adult females:**
Ten fertilized adult female of *T. urticae* were placed on a single leaf-disk of mulberry (2.5cm in diameter) and were kept on moist cotton wool in fiber dishes; each dish contained 5 disks as replicates. The direct spray technique was applied directly to the body surface of the mites according to Abo-Shabana (1980) and Sewify et al. (2015).

The treated adult females were incubated at 25 ± 1°C. Mortality was assessed daily for 7 days (Ayoub, 1984). Cadavers were transferred into moist Petri dishes and considered as "mycotised" if fungal out growths were visible after three days of incubation at same laboratory conditions. The percentage of mortality was determined and corrected by Abbott’s formula (1925). LC$_{50}$, LC$_{90}$ and slope values were calculated according to Finney (1971), using "Ldp line" software by (Bakr, 2000).

**The susceptibility of predaceous mites, Phytoseiulus persimilis and Neoseiulus californicus to the selective strain of entomopathogenic fungi B. bassiana**

This part of the investigation was undertaken to evaluate the side effect of using the selected entomopathogenic fungus *B. bassiana* (B4) on mortality and fecundity of the predatory mites, *P. persimilis* and *N. californicus*. The prey used in the present study was adults of *T. urticae*.

Two factors were our reason to choose adults' stage, because of their relatively big size, disease diagnosis in adults of *T. urticae* is easier in contrast with the other stages. Also, the adults of two predators aggressively attack to adults of *T. urticae*. (Cote, 2001; Seiedy et al., 2014).
Experimental Procedures:

The most effective isolate of the previous tested entomopathogenic fungi was *B. bassiana* (B4). Three concentrations of the fungus were tested (LC₂₅, LC₅₀ and LC₉₀) against the adult stage of both predaceous mites *P. persimilis* and *N. californicus*. By using direct spray technique (Abo-Shabana, 1980; Hassan, 2008; Sewify et al., 2015). A glass atomizer at 30cm high with 2ml spore suspension for each treatment and 2ml sterilized distilled water of 0.01% Tween 80 as control. The sprayed predators were transferred by the aid of fine brush to small circular leaf disc (2 inch in diameter) punched from mulberry leaves were placed in Petri dishes (12 cm diameter) lined with water saturated cotton wool. Ten dishes for each concentration, each dish contains two adult females of the tested predator as a replicate.

After spraying the treatments were kept under constant conditions of 25 ± 1°C and 70 ± 5% relative humidity with a photoperiod of 16:8 (L:D). The predators were fed on a mixture of various stages of uninfected *T. urticae* each day. Mortality of predators recorded daily for 7 days until no more mortality could be observed and reproduction per female during the first 7 days of the adult stage were assessed. All dead and living mites were counted, and dead mites were removed daily. Mites were considered dead when they failed to move after repeated gentle prodding with a brush. Cadavers were transferred into moist Petri dishes and incubated at same laboratory conditions to observe any fungal out growths. Predator eggs were counted and removed daily for 7 days after spraying (Nadimi et al., 2008; Gaber, 2016).

Statistical analysis:

Based on total effects, rating of toxicity of the entomopathogenic fungi was evaluated according to International Organization for Biological Control (IOBC) guideline (Blumel and Hausdorf, 2002).

\[ Er = \frac{Rt}{Rc} \]

Where: \(Er\) = Effect on reproduction
\(Rt\) = Reproduction in treatment
\(Rc\) = Reproduction in control

Subsequently effect on survival and effect on reproduction were combined using the following formula (Overmeer and van Zon, 1982):

\[ E = 100\% - (100\% - Ma) \times Er \]

Where: \(Ma\) = Mortality corrected according to Abbott
\(E\) = Total effect

Class 1: \(E < 30\%\) (harmless)
Class 2: \(30 < E < 80\) (slightly harmful)
Class 3: \(80 < E < 99\) (moderately harmful)
Class 4: \(E > 99\%\) (harmful)

Decreasing of total eggs/females =

\[ \frac{(\text{total eggs in treatment} - \text{total eggs in control})}{\text{total eggs in control}} \times 100 \]

Selectivity ratio = LC₅₀ of the compound against the predator/ LC₅₀ of the compound against the spider mite (El- Adawy et al., 2000; Gaber, 2016).

Scanning electron microscope:

This work carried out in the electronic microscope unite, central laboratory, National Research Centre. The adults of *T. urticae* and *P. persimilis* were killed by chloroform solvent, cleaning manually and freezing. Freezing of the sample very quickly was instead of fixing it. This technique providing the sample stays cold enough, this ‘locks up’ the water and prevents it from evaporating inside the microscope. After that the adults were coated by gold. Coating of samples with gold
is required in the field of electron microscopy to enable or improve the imaging of samples. Creating a conductive layer of metal on the sample inhibits charging, reduces thermal damage and improves the secondary electron signal required for topographic examination in the SEM. All images were taken under low vacuum scanning electron microscope. (Jeol-JSM-5600LV in SEM). Ragaei and Sabry (2017)

RESULTS AND DISCUSSION

The present results show the efficiency of six entomopathogenic fungi four isolates of *Beauveria bassiana* (Balsamo) (B1-B2-B3-B4) and two isolates of *Metarhizium anisopliae* (Metschnikoff) (M1-M2), against egg and adult stages of *T. urticae* in laboratory experiments.

Also the effect of using the selected isolates of entomopathogenic fungus *B. bassiana* (B4) on mortality and fecundity of the predatory mites, *P. persimilis* and *N. californicus* were tested. A SEM was used to observe and document the micro morphological processes that occurred due to fungal conidial inoculation in each of the *T. urticae* and the predatory mite species.

**Susceptibility of *T. urticae* egg and adult stages to entomopathogenic fungi *B. bassiana* and *M. anisopliae***

**Egg stage:**

The obtained results in Table (1) showed susceptibility of *T. urticae* eggs to the six isolates of entomopathogenic fungus *B. bassiana* and *M. anisopliae* after exposing to series of concentrations of $10^6$, $5 \times 10^6$, $10^7$, $5 \times 10^7$ and $10^8$ spores/ml. The hatchability gradually decreased along with increasing spore concentration. The lowest concentration ($10^6$ spores/ml) revealed (93.29%, 81.67%, 85.93%, 80.20 %, 92.2 % and 82.5 %) for (B1, B2, B3, B4, M1 and M2) respectively, 7 days after treatment. While hatching at highest concentration ($10^8$ spores/ml), decreased to reach (68.07%, 33.9 %, 56.66%, 25.20%, 63.3% and 46.2%) for (B1, B2, B3, B4, M1 and M2) respectively, 7 days after treatment. Compared with the control which reached to 97.34, 96.58, 97.78, 99.08, 96.2 and 96.4 respectively.

Table (1): Effect of entomopathogenic fungus *B. bassiana* and *M. anisopliae* treatment on egg hatchability of *T. urticae* at different concentrations.

| Hatchability percentage of all tested isolates at 7th day |
|---|---|---|---|---|---|---|
| B1 | B2 | B3 | B4 | M1 | M2 |
| 7th day | 7th day | 7th day | 7th day | 7th day | 7th day |
| con | H% | H% | H% | H% | H% | H% |
| 0 | 97.34 | 96.58 | 97.78 | 99.08 | 96.2 | 96.4 |
| $10^6$ | 93.29 | 81.67 | 85.93 | 80.2 | 92.2 | 82.5 |
| $5 \times 10^6$ | 92.36 | 72.31 | 83.79 | 68.18 | 90.1 | 75.8 |
| $10^7$ | 87.26 | 58.75 | 71.94 | 36.38 | 85.2 | 62.2 |
| $5 \times 10^7$ | 80.99 | 46.3 | 68.85 | 33.5 | 74.1 | 57.2 |
| $10^8$ | 68.07 | 33.9 | 56.66 | 25.2 | 63.3 | 46.2 |
Results in Table (2), Fig (1) proved that *B. bassiana* (B4) was more effective against *T. urticae* eggs compared with all other isolates. The LC<sub>50</sub> value of B4 was 1.14 x 10<sup>7</sup> spores/ml while (B2, M2, B3, M1 and B1) revealed greater LC<sub>50</sub> value (3.33 x 10<sup>7</sup>, 9.33 x 10<sup>7</sup>, 3.82 x 10<sup>8</sup>, 4.57 x 10<sup>8</sup> and 9.82 x 10<sup>8</sup> spores/ml) respectively.

Table (2): Comparison of the pathogenicity among six isolates of *entomopathogenic fungi B. bassiana and M. anisopliae* against *T. urticae* egg stage.

<table>
<thead>
<tr>
<th>No.</th>
<th>Line</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (Limits)</th>
<th>Slope</th>
<th>LC&lt;sub&gt;90&lt;/sub&gt; ( Limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B4</td>
<td>1.14 x 10&lt;sup&gt;7&lt;/sup&gt; (4.33 x 10&lt;sup&gt;6&lt;/sup&gt; - 5.67 x 10&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>0.77</td>
<td>5.24 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>B2</td>
<td>3.33 x 10&lt;sup&gt;7&lt;/sup&gt; (2.23 x 10&lt;sup&gt;7&lt;/sup&gt; - 5.53 x 10&lt;sup&gt;7&lt;/sup&gt;)</td>
<td>0.69</td>
<td>2.36 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>M2</td>
<td>9.33 x 10&lt;sup&gt;7&lt;/sup&gt; (4.98 x 10&lt;sup&gt;7&lt;/sup&gt; - 2.62 x 10&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>0.54</td>
<td>2.24 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>B3</td>
<td>3.82 x 10&lt;sup&gt;8&lt;/sup&gt; (1.40 x 10&lt;sup&gt;8&lt;/sup&gt; - 2.93 x 10&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>0.48</td>
<td>1.86 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>M1</td>
<td>4.57 x 10&lt;sup&gt;9&lt;/sup&gt; (2.02 x 10&lt;sup&gt;8&lt;/sup&gt; - 1.98 x 10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>0.71</td>
<td>2.85 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>B1</td>
<td>9.82 x 10&lt;sup&gt;8&lt;/sup&gt; (3.30 x 10&lt;sup&gt;8&lt;/sup&gt; - 8.86 x 10&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>0.64</td>
<td>9.86 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Fig. (1) Percentage Mortality regression lines of six isolates of *entomopathogenic fungi B. bassiana and M. anisopliae* against *T. urticae* egg stage.
Adult stage:

The susceptibility of adult females of *T. urticae* to entomopathogenic fungi *B. bassiana* and *M. anisopliae* were conducted. The percentage of mortality values after exposing to series of concentrations of 10^6, 5x10^6, 10^7, 5x10^7 and 10^8 spores/ml were tabulated for 7 days after treatment in Table (3).

The percentage of mortality gradually increased along with spores concentrations. The Lowest concentration of 10^6 spores/ml revealed (29.89, 40.23, 36.67, 45.45, 40.04 and 46 %) for (B1, B2, B3, B4, M1 and M2) respectively 7 days after treatment. While the highest concentration of 10^8 spores/ml revealed (65.63, 56.00, 62.79, 88.52, 54.00 and 80.85%) for (B1, B2, B3, B4, M1 and M2) when mortality was assessed after the same consecutive days, respectively.

Table (3): Percentage mortality of *T. urticae* adult female treated with series concentrations of *B. bassiana* and *M. anisopliae* after seven days.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>7th day</td>
<td>Mortality %</td>
<td>Mortality %</td>
<td>Mortality %</td>
<td>Mortality %</td>
<td>Mortality %</td>
<td>Mortality %</td>
</tr>
<tr>
<td>0</td>
<td>27.38</td>
<td>26.12</td>
<td>22.89</td>
<td>24.64</td>
<td>27.2</td>
<td>24.00</td>
</tr>
<tr>
<td>10^6</td>
<td>29.89</td>
<td>40.95</td>
<td>36.67</td>
<td>45.45</td>
<td>40.04</td>
<td>46.00</td>
</tr>
<tr>
<td>5x 10^6</td>
<td>54.05</td>
<td>46.30</td>
<td>47.73</td>
<td>60.00</td>
<td>45.01</td>
<td>50.00</td>
</tr>
<tr>
<td>10^7</td>
<td>55.95</td>
<td>49.15</td>
<td>51.95</td>
<td>64.00</td>
<td>48.03</td>
<td>55.77</td>
</tr>
<tr>
<td>5x 10^7</td>
<td>64.38</td>
<td>50.00</td>
<td>53.33</td>
<td>80.36</td>
<td>50.02</td>
<td>72.00</td>
</tr>
<tr>
<td>10^8</td>
<td>65.63</td>
<td>56.00</td>
<td>62.79</td>
<td>88.52</td>
<td>54.00</td>
<td>80.85</td>
</tr>
</tbody>
</table>

Results in Table (4), Fig (2) proved that *B. bassiana* (B4) was more effective against *T. urticae* adult females compared with all other isolates. The LC50 value of B4 was 6.57 x 10^6 spores/ml while (M2, B1, B3, B2 and M1) revealed greater LC50 value (1.39 x 10^7, 4.43 x 10^7, 1.01 x 10^8, 1.20 x 10^9 and 1.92 x 10^9 spores/ml) respectively.

Table (4): Comparison of pathogenicity among six isolates of enomopathogenic fungi *B. bassiana* and *M. anisopliae* against *T. urticae* adult stage.

<table>
<thead>
<tr>
<th>No.</th>
<th>Line name</th>
<th>LC50 (Limits)</th>
<th>Slope</th>
<th>LC90 (Limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B4</td>
<td>6.57 x 10^6</td>
<td>0.78</td>
<td>2.88 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.42 x 10^5 – 9.30 x 10^7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M2</td>
<td>1.39 x 10^7</td>
<td>0.62</td>
<td>1.54 x 10^9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9.04 x 10^6 – 2.16 x 10^8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B1</td>
<td>4.43 x 10^8</td>
<td>0.69</td>
<td>3.17 x 10^9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.39 x 10^7 – 2.25 x 10^9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B3</td>
<td>1.01 x 10^9</td>
<td>0.41</td>
<td>1.34 x 10^11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.56 x 10^8 – 4.77 x 10^9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
<td>1.20 x 10^10</td>
<td>0.26</td>
<td>9.31 x 10^13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.78 x 10^9 – 2.85 x 10^12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M1</td>
<td>1.92 x 10^11</td>
<td>0.27</td>
<td>9.72 x 10^13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.50 x 10^10 – 6.13 x 10^12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Virulent entomopathogenic fungi against the two-spotted spider mite

The LT$_{50}$ and LT$_{90}$ values of the six isolates entomopathogenic fungi $B$. bassiana and $M$. anisopliae were tabulated with their corresponding slopes for 7 days after treatment in Table (5).

The obtained results in Table (5) and Fig. (3) showed the mortality time regression line at concentration of $10^8$ spores/ml. The data showed that isolate B4 caused high mortality in shortest time, LT$_{50}$ value was 3.26 days. While for the other isolates (M2, B1, B3, B2 and M1) the LT$_{50}$ values were (3.55, 3.90, 5.16, 5.93 and 6.00) respectively.

Table (5): Comparison of the mortality time among six isolates of entomopathogenic fungi $B$. bassiana and $M$. anisopliae against $T$. urticae adult stage.

<table>
<thead>
<tr>
<th>No.</th>
<th>Line name</th>
<th>LT$_{50}$ (Limits)</th>
<th>Slope</th>
<th>LT$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B4</td>
<td>3.26 (2.57–3.94)</td>
<td>2.67</td>
<td>9.86</td>
</tr>
<tr>
<td>2</td>
<td>M2</td>
<td>3.55 (3.28–3.85)</td>
<td>3.31</td>
<td>8.66</td>
</tr>
<tr>
<td>3</td>
<td>B1</td>
<td>3.90 (3.55–4.33)</td>
<td>2.65</td>
<td>11.90</td>
</tr>
<tr>
<td>4</td>
<td>B3</td>
<td>5.16 (4.57–6.04)</td>
<td>2.37</td>
<td>17.91</td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
<td>5.93 (5.22–7.06)</td>
<td>2.62</td>
<td>18.27</td>
</tr>
<tr>
<td>6</td>
<td>M1</td>
<td>6.00 (5.25–7.25)</td>
<td>2.45</td>
<td>20.02</td>
</tr>
</tbody>
</table>
Results revealed that application of entomopathogenic fungi was effective in controlling both egg and adult stages of *T. urticae*. This is in line with the previous study of Nada *et al.* (2012) who recorded that *B. bassiana* and *M. anisopliae* had rapid mortality against mites and correspondent LC$_{50}$ for *B. bassiana* and *M. anisopliae* was 2.98 x $10^6$ and 1.82 x $10^6$ spores/ml, respectively. The LT$_{50}$ of *B. bassiana* and *M. anisopliae* was 4.08 and 2.2 days, respectively. Furthermore, Waked *et al.* (2015) reported that isolates of entomopathogenic fungi (EPF) *B. bassiana* and *Paecilomyces fumosoroseus* were highly effective and virulent against females of the *T. urticae* at four different conidial concentrations. The *P. fumosoroseus* isolate had lower LC$_{50}$ (1.8 x $10^7$ conidia/ml) than *B. bassiana* (2.6 x 10$^7$ conidia/ml).

According to Shi and Feng (2004, 2009), some of the tested isolates of *B. bassiana*, *Paecilomyces fumosoroseus* and *M. anisopliae* have high lethal effect on eggs and females of *T. urticae*. The mite mortality observed was 73.1, 75.4 and 69.9 % for *B. bassiana*, *P. fumosoroseus* and *M. anisopliae*, respectively, after 10 days of spraying whereas the control mortality was 15.5%. Most of the infected females died on days 4-8. The infection by the fungus not only killed *T. urticae* females but greatly reduced their fecundity.

Bugeme *et al.* (2009) assesses the virulence of twenty three isolates of *M. anisopliae* and three isolates of *B. bassiana* against the two spotted spider mites, *T. urticae*. Mean mortality in the control was 11.5% 10 days after treatment. At 26°C, all the fungal isolates were pathogenic to adult female of *T. urticae* causing mortality between 36.5 and 100% and the LT$_{50}$ values ranged from 2.2 to 8.2 days.

According to Draganova and Simova (2010), bioassays of five isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.- Criv.), Vuillemin were conducted under laboratory conditions against the two-spotted spider mite.
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*Tetranychus urticae* Koch. (Acari: Tetranychidae) that was found susceptible to the examined isolates of *B. bassiana*. On the fifth day, lethal effect reached 100% in the variant with 447 Bb, and 84.87 ± 19.17% in the variant with 426 Bb. Mycosis caused by the isolate 417 Bb of *B. bassiana* had a less lethal effect 68.89 ± 7.40% calculated for the fifth post-treatment day. In their study they found that the fast lethal effect of the mycoses to *T. urticae* was due to toxic secondary metabolites and the pigment oosporein produced by fungal isolates. Growing the examined isolates 417 Bb, 426 Bb, 444 Bb, 445 Bb and 447 Bb of *B. bassiana* a red pigmentation of the media was also noticed.

The main characteristic that has an important role in the virulence of entomopathogenic fungi strains from the genera *Beauveria* and *Metarhizium* is the production of enzymes necessary for penetration through arthropod cuticle. The extracellular proteases are considered an important virulence factor in insect disease processes (St. Leger *et al.*, 1986a, 1986b, 1988; Draganova, 1988; Bidochka and Khachatourians, 1990, 1994a, 1994b; Gupta *et al.*, 1992; St. Leger, 1995).

Chandler *et al.* (2005) found in a glasshouse experiment that *B. bassiana* cultured from Naturalis-L (Troy Biosciences, USA) reduced the numbers of *T. urticae* adults, nymphs and eggs (98% reduction in all three cases) on glasshouse tomato crop.

Gatarayiha, *et al.* (2012) screened 62 South African strains of *B. bassiana* for pathogenicity against *T. urticae* in laboratory bioassays on bean leaves, *Phaseolus vulgaris* L., under greenhouse conditions. In the first bioassay, strains of *B. bassiana* were applied at a single concentration of 10^7 conidia/ml. A mortality mite percentage between 4-92.5% was observed, with 40 % of strains causing mortality levels higher than 50%. The LT_{50} ranged between 5.5 - 8.6 days. The six most virulent strains were compared in a second screening, together with the commercial strain PPRI 5339. Five concentrations (2 \times 10^4 to 2 \times 10^8 conidia ml\(^{-1}\)) on female mites and three concentrations (2 \times 10^5, 2 \times 10^6 and 2 \times 10^8 conidia ml\(^{-1}\)) on eggs were used. Mortality of mites assessed indicated that Strain PPRI 7315 and Strain PPRI 7861 performed similarly and were the most efficient, causing mite mortality of > 80%, 9 days after inoculation, at the highest concentration, with LC_{50} concentration of 1.13\times10^6 and 1.22 \times 10^6 conidia ml, respectively. Both strains performed better than the commercial strain (PPRI 5339) in *vitro* and showed good control of *T. urticae* during greenhouse trials.

**Effect of the fungal spores of *B. bassiana* (B4) on mortality and fecundity of the predatory mites *Phytoseiulus persimilis* and *Neoseiulus californicus*:**

This part of the investigation was undertaken to evaluate the side effect of using the entomopathogenic fungus *B. bassiana* isolate (B4) in spore suspension formula against predatory mites, *P. persimilis* and *N. californicus* the results are tabulated in Tables (6 and 7).

The results showed that the three tested concentrations of the fungal spore suspensions were safe against the predaceous mites, *P. persimilis* and *N. californicus* the results are tabulated in Tables (6 and 7).

The most interesting observation is the fact that the highest concentration LC_{90} caused the highest mortality of the adult females of spider mite, *T. urticae* after 7 day
of exposure, was harmless on *P. persimilis* and slightly harmful on *N. californicus*. This means that the entomopathogenic fungus *B. bassiana* in spore suspension formula could be used without any consideration in IPM programs for controlling spider mite, *T. urticae*. These results are in agreement with those of Ludwig and Oetting (2001), who demonstrated that the predatory mite *Iphiseius degenerans* (Acari: Phytoseiidae) was least susceptible to *M. anisopliae* followed by *V. lecanii* and *B. bassiana*.

### Table (6) Direct effect of the selective strain *Beauveria bassiana* (B4) on *Phytoseiulus persimilis*

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Females mortality</th>
<th>Fecundity</th>
<th>Decreasing of total eggs no. %</th>
<th>(E) Total effect %</th>
<th>IOBC class*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores/ml</td>
<td></td>
<td>Total eggs no./female</td>
<td>Eggs no./female/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28.57</td>
<td>10.19</td>
<td>1.46</td>
<td>-</td>
<td>10.2  1</td>
</tr>
<tr>
<td>LC25</td>
<td>30</td>
<td>9.8</td>
<td>1.4</td>
<td>8.4</td>
<td>17.9  1</td>
</tr>
<tr>
<td>LC50</td>
<td>35</td>
<td>8.65</td>
<td>1.38</td>
<td>9.8</td>
<td>29.7  1</td>
</tr>
<tr>
<td>LC90</td>
<td>42.86</td>
<td>8.95</td>
<td>1.28</td>
<td>12.1</td>
<td></td>
</tr>
</tbody>
</table>

### Table (7) Direct effect of the selective strain *Beauveria bassiana* (B4) on *Neoseiulus californicus*

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Females mortality</th>
<th>Fecundity</th>
<th>Decreasing of total eggs no. %</th>
<th>(E) Total effect %</th>
<th>IOBC class*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores/ml</td>
<td></td>
<td>Total eggs no./female</td>
<td>Eggs no./female/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>8.8</td>
<td>1.26</td>
<td>-</td>
<td>18.33  1</td>
</tr>
<tr>
<td>LC25</td>
<td>30</td>
<td>7.7</td>
<td>1.1</td>
<td>12.5</td>
<td>28.6  1</td>
</tr>
<tr>
<td>LC50</td>
<td>35</td>
<td>7.25</td>
<td>1.04</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>LC90</td>
<td>42.86</td>
<td>6.67</td>
<td>0.95</td>
<td>20.5</td>
<td>39.4  2</td>
</tr>
</tbody>
</table>

**Scanning electron microscope:**

The entomopathogenic fungus *B. bassiana* has been reported as being capable of infecting over 100 species of insects from a wide range of insect orders, although many fungal isolates vary in host range and some isolates have displayed high host specificity (Maurer *et al.*, 1997; McCoy *et al.*, 1988).

For developing entomopathogenic fungi as biocontrol agents, it is crucial to understand their mode of action on their insect pest target. Scanning electron microscopy has been widely used for this purpose (Lopez-Llorca *et al.*, 1999, 2002).

The possible effects of pathogens on natural enemies are particularly relevant to biological control of agricultural pests.

If natural enemies are susceptible to infection by pathogens, applying combinations of these two controls for suppression of pests would not be compatible. In contrast, applying multiple species may act synergistically in reducing a pest population if interference between species is minimal or nonexistent (Roy and Pell, 2000).

Scanning electron microscopy are convenient tools to observe the mode of action of entomopathogenic fungi and to study how *B. bassiana* is able to colonize and infect the predator mites and *T. urticae*. It allowed us to observe adhesion and penetration structures on females of *T. urticae* infected with *B. bassiana*. While it was clearly shown that no such structures were found on *P. persimilis* infected with *B. bassiana*. 
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Fig. (4) Inoculation and attachment of *Beauveria bassiana* isolate B4 conidia on the cuticle of *Phytoseiulus persimilis*, (A) 24h. (X6000); (B) 48 h (X12000); (C) 72(X 6000); (D)72 h(X 10000); (E) 72h (X 20000) after inoculation.
Fig. (5) Germination and infection of *Beauveria bassiana* isolate B4 conidia on the cuticle of *Tetramychus urticae*, (A) and (B) 24h. (X12000); (C) 48 h. (X 12000); (D)72 h (X 1000); (E) 72h (X 6000); (F) 72h (X 12000); (G)72h (X 24000) after inoculation.
Conidia adhered to the cuticle of predatory mites *P. persimilis* within 24–72 h (Figs. 4 A, B, C, D, E). Although conidia germinated within 24–36 h, few conidia were detected on the cuticular surface after 48 h. They apparently did not penetrate the cuticle even after 72h. Figs. (C, D and E) proved that conidia did not penetrate at different magnification scales (X 6000; X 10000 and X 20000).

Similarly, conidia adhered to the cuticle of *T. urticae* within 24–72 h (Fig. a) and germinated within 48 h (Fig. 4 B). However, Germ tubes of conidia successfully penetrated the mite cuticle within 48 h (Fig. 4 C).

In contrast, *B. bassiana* strain B4 conidia penetrated *T. urticae* cuticle soon after germination. These results do raise questions why *B. bassiana* is so virulent to *T. urticae* but shows no apparent infectivity in predatory mite *P. persimilis*.

In this study, although conidia were able to germinate, they apparently were not able to penetrate the cuticle of the predatory mite species (Fig. 5), but were able to penetrate the cuticle of *T. urticae*.

Evaluating the compatibility of entomopathogenic fungi and predatory mites is a critical issue for the successful uses of IPM programs to control pest mite species (Vergel et al., 2011). This study tested the *B. bassiana* strains (B4) that were virulent to *T. urticae*, but were found to be non infective to the predatory mites *P. persimilis* under laboratory bioassay and SEM observation. The findings here support the potential use of *B. bassiana* in combination with predatory mites to control *T. urticae*. The defence mechanism present in the cuticle of predatory mites that is responsible for this apparent immunity to fungal penetration deserves further study.

The ability of fungal conidia to attach to the insect cuticle is strongly correlated with virulence (Altre et al., 1999). Insect infection occurs following germination of conidia on the cuticle, with subsequent penetration of the cuticle by specialized infection structures (Butt, 1990). Insect cuticle constitutes a defensive barrier to fungal penetration (Samuels and Paterson, 1995).

This result comes in parallel with (Wu et al., 2016) who evaluated the pathogenicity of five strains of *B. bassiana* in five species of predatory phytoseiid mites. The bioassay results indicated that no viable fungal hyphae were found on predatory cadavers. Observations with scanning electron microscopy revealed that conidia were attached to the cuticle of predatory mites within 2–12 h after spraying, and had germinated within 24–36 h. After 48 h, conidia had gradually been shed from the mites, after none of the conidia had penetrated the cuticular surfaces. In contrast, the germinated conidia successfully penetrated the cuticle of *T. urticae*, and within 60 h the fungus colonized the mite’s body. The study added that although several *B. bassiana* strains displayed a high virulence in *T. urticae*. There was no evident pathogenicity to phytoseiid mites. These findings support the potential use of entomopathogenic fungus in combination with predatory mites in *T. urticae* control.

**REFERENCES**


Virulent entomopathogenic fungi against the two-spotted spider mite


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ARABIC SUMMARY

توضيح: بعض الفطريات المرضية ضد بعض المفترسات الأكاروسية المصاحبة Tetranychus urticae

َداًيا محمد أحمد حسن - مارجريت علوي زيز - حسن محمد صبحي - وفاني زكي عازر

بعض الفطرية تستخدم الأكاروسية المفترسات والممرضة كوسيلة للمكافحة البيولوجية للعنكبوت Tetranychus urticae Koch. ذُو البقعتين.

وعزلتين من Metarhizium anisopliae، حيث أحدثت 88.5% نسبة موت للطور البالغ عند التركيز المرتفع 10^5 spores/ml ونسبة الفقس في طور البيضة انخفضت إلى 25.2% بالمقارنة بالإفراد الغير معاملة، التي وصل بها نسبة الفقس إلى 99%.

الجزء الثاني من هذه الدراسة يتضمن دراسة لعائمة أربع عزلات من B. bassiana على مرضية الفطر الفطرية على ورق السجق وتستثمر في programs of biological control.

وبدراسة وتحليل العينات المصابة بواسطة الميكروسكوب الحقيقي لم تظهر البدايات على المفترس الأكاروسى، وضح الميكروسكوب الإلكتروني أن كونيديا العنقود تلتقي بالكيوتيكل في غضون 24 ساعة بعد العزلة B4، وتحدث الأعراض في غضون 48-48 ساعة، ولكن بعد ذلك لم يحدث اختراق لكيوتيكل البالغة، وتتطلب أيضًا الاستخدام المحتمل للمفاهيم الضارة مع المفترسات الأكاروسية في برامج مكافحة T. urticae.

وتوضح هذه الدراسة أن على الرغم من أن العديد من السلالات B. bassiana أظهرت مرضية عالية على العنكبوت الأحمر. ولكن لم تظهر أي اعراض مرضاة واضحة على العنكبوت الأحمر. تدعم الاستخدام المحتمل للفطريات المرضية للحشرات مع المفترسات الأكاروسية في برامج مكافحة T. urticae الأحمر.