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Virulence and proteolytic activity of entomopathogenic fungi against the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae)

Enas Elhakim[1], Omar Mohamed[1] and Iman Elazouni[2*]

Abstract

The virulence and proteolytic activity of some entomopathogenic fungi isolates, viz., *Metarhizium anisopliae*, *Beauveria bassiana*, *Verticillium lecanii*, and *Trichoderma harzianum*, against the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), were evaluated. Common maize plants (*Zea mays* L.) infested with females of *T. urticae* were treated in vivo by spraying with suspensions of 1×10^8 conidia ml^{-1} concentration of selected isolates. Lethal effects of fungal isolates were assessed as percentages of daily mortalities of mites, compared to the mortality in control. Virulence of the fungi isolates was estimated based on the LC$_{50}$ values calculated by probit analysis for the individuals treated by 1×10^5 conidia ml^{-1} concentration. Proteolytic activity of isolates was assayed on casein substrate to reflect their virulence towards *T. urticae*. The mite mortality rates increased with increasing conidial concentrations as well as days after treatment. The mortality rate caused by *M. anisopliae* isolate varied from 18.75 to 85%, with LC$_{50}$ value of 4.6×10^5 conidia/ml and LC$_{90}$ value of 2.4×10^8 conidia/ml during 7 days, respectively. The isolate of *B. bassiana* caused 15 to 70% mortality, and its LC$_{50}$ and LC$_{90}$ values estimated 3.3×10^6 and 7.8×10^9 conidia/ml, respectively. However, *V. lecanii* isolate caused 11.25 to 72.50% mortality with LC$_{50}$ of 5.2×10^6 conidia/ml, while *T. harzianum* was potentially less virulent than other isolates causing 8.75 to 63.75% mortality rate to *T. urticae* with LC$_{50}$ of 9.4×10^6 conidia/ml. *M. anisopliae* showed the highest proteolytic activity at all concentrations, followed by *B. bassiana* in 3rd, 5th, and 7th day post treatment. These findings recommend the selection of virulent fungal isolates for use as natural and environmentally safe agents in biological control programs to combat mite pests.

Keywords: Virulence, Proteolytic activity, Entomopathogenic fungi, *Tetranychus urticae*, Mortality

1. The two-spotted spider mites *T. urticae* were sensitive and affected by entomopathogenic fungi.
2. *M. anisopliae* isolate had the most lethal effect, but the isolates *V. lecanii*, *B. bassiana* and *T. harzianum* were less lethal.
3. *M. anisopliae* was the highest virulent isolate against *T. urticae* than the other isolates with LC$_{50}$ 4.6×10^5 ppm.
4. Protease activities of fungal isolates were increased with increased conidia concentrations from 1×10^5 and up to 1×10^8 conidia ml^{-1}.
5. *M. anisopliae* gave the highest activities at all concentrations, compared with other isolates, followed by *B. bassiana* which exceeded *V. lecanii* and *T. harzianum* in its proteolytic activities.

* Correspondence: ielazouni@yahoo.com
[2]Botany and Microbiology Department, Faculty of Science, Zagazig University, Zagazig 44591, Egypt
Full list of author information is available at the end of the article

Background

Spider mites (Acari: Prostigmata: Tetranychidae) are obligated predatory plant mites, which are unique in possessing highly elongated movable stylets used to puncture the individual cells of their host plants (Walter et al., 2009). Pests of known economic significance belonging to this group are the two-spotted mites, *Tetranychus urticae* Koch, which are considered as violent pest that affects vegetables, orchards, medicinal, and ornamental plants (Naher et al., 2006). The conventional control of this pest includes acaricides that have been widely used for mite control in greenhouses, orchards, and many other cropping systems (Van Leeuwen et al., 2005), but it can cause undesirable problems like the death of natural enemies such as predators or development of pesticide-resistant races of mites and residue concerns (Draganova and Simova, 2010). Thus, the search for an eligible biocontrol agent is therefore a step in developing new environmentally friendly strategies or improving existing strategies that provide alternatives to conventional pest control. Research and biological control strategies for spider mites have largely focused on preserving natural enemies and releasing predator mites (Zhang, 2003), and nevertheless, an additional spray of acaricides is needed.

The entomopathogenic fungi (EPF), developed as mycoacaricides, can naturally manage mite populations and can be used in a useful control strategy as stand-alone to replace synthetic acaricides already used, or as an integrated component for mite management (Maniania et al., 2008). The fungus penetrates the pest cuticle with the help of hyphae protrusion germinated from the conidia, as soon as contact to the host (Revathi et al., 2011). The EPF rely on their enzymatic capacity to degrade the integument of insects or mites, consisting of lipases, proteases, and chitinases, furthermore, glutaminase, β-galactosidase, and catalase as well (Mondal et al., 2016). Fungal proteases seem to be particularly important in the penetrating process since the insect and mite cuticle comprises up to 70% protein (Charnley, 2003). The virulence of the EPF against their hosts can be verified through their production of cuticle-degrading enzymes (Pinto et al., 2002). The successful use of EPF as agents for biological control of mites ultimately depends upon the extent of selected fungal strains (Maniania et al., 2008).

The goal of this study was to determine the activity of protease and the virulence of 4 EPF isolates against the two-spotted spider mite, *T. urticae*.

Material and methods

Isolation of fungi, culture conditions, purification, and identification

The fungi, *Metarhizium anisopliae*, *Beauveria bassiana*, *Verticillium lecanii*, and Trichoderma harzianum were collected from dead cadavers of the two-spotted spider mite individuals on leaf surface of maize plants (*Zea mays* L.). Samples containing fungi, wrapped in bags, were transferred to the Biological and Integrated Pest Control Laboratory, Plant Protection Institute, Zagazig, Egypt. Serial dilution method was used to isolate EPF according to the method of Goettel (1996). One ml of each soil dilution was poured onto Petri dishes containing Sabouraud's dextrose agar (SDAY) medium (Merck, Germany) containing 1% yeast extract. To isolate the fungi grown on the surface of the mite body, the mites were superficially sterilized with 70% ethanol for 1 min and 5% sodium hypochlorite for 3 min and rinsed three times with sterile distilled water; then, the fungi were transferred to SDAY medium. The dishes were incubated at 25 °C for 15 days until the colonies appeared. Then, the colonies were selected, picked up, and maintained on PDA (potato dextrose agar) medium until identification. Fungal species were identified using the lacto-phenol cotton blue staining method according to Humber (2005). Single spore fungus was carried out according to Ho and Ko (1997). For preparation of the bioassay suspensions, the conidia of 10-day-old cultures, which were previously developed on SDAY medium, were harvested by washing with sterile distilled water containing 0.02% Tween 80, followed by decimal dilutions of the conidial suspensions obtained and their concentrations estimated using a hemocytometer under light microscope (Zeiss) (× 40 magnification).

Mite culture

Females of *T. urticae* were obtained from the Biological and Integrated Pest Control Laboratory, Plant Protection Institute, Zagazig, Egypt, and transferred to caged maize plants for subsequent culturing. A stock colony was established in ventilated jars (26 × 20 × 10 cm) at 32 ± 2 °C, 55% R.H., and 16:8 (L:D) photoperiod. To avoid the excessive accumulation of excrements and exuviae of larvae, the growing media were updated three times weekly.

Protease assay

Casein substrate was used to determine protease enzyme activity (Vyas and Deshpande, 1989). A mixture of 2 ml reaction contained an aliquot of an appropriately diluted enzyme solution, 10 mg substrate (casein) in 0.2 mM sodium carbonate buffer, pH 9.7. The enzyme reaction mixture was incubated at 35 °C for 20 min and then 3 ml trichloroacetic acid (TCA) was added (2.6 ml 5% TCA + 0.4 ml 3.3 N HCl). The mixture was centrifuged at 8000 rpm for 5 min, and the resultant was observed at 280 nm (Ultrospec II, LKB Biochrom, UK). One international unit (IU) is defined as an enzyme activity that produced 1 μmole of tyrosine per min.

Virulence test

Bioassays were conducted under laboratory conditions (23 $\pm$ 1 °C, photoperiod 12:12 L:D). Females of mites were taken from the cultured source and gently transferred onto caged maize plants with the help of a fine camel hair brush. *T. urticae* was used as a host for the EPF reared on maize plants in earthen pots. Population of mites was counted on individual plants (having 4–5 leaves) before performing sequential sprays of fungi. The plants were divided into 4 groups (4 isolates), each of which containing 5 plants (4 treatments + control). Five plants in each treated group were sprayed with a selective fungus with recommended concentration (1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}). These concentrations were applied by foliar application with hand atomizers on potted plants. In all cases, 300 µl of each conidial concentration, with Tween 80 solution of 0.02%, were directly sprayed on 10 adult female mites. Control plants received same volume of water containing 0.02% Tween 80. The whole experiment was repeated 3 times. Mite mortality was estimated 3, 5, and 7 days after treatment. Plant leaves carrying dead mites were incubated in a humid chamber to allow the germination of fungus conidia and confirm that the death was due to mycoses. Smears were prepared from dead mites stained with methylene blue, then observed under a light microscope. Fungal lethal effects were assessed as percentages of daily cumulative mortality due to mycoses, compared to mortality in untreated control. Virulence of fungal isolates was calculated by probit analysis (Finney, 1971) depending on the median lethal concentration (LC$_{50}$) of the individuals treated with 10^5 conidia ml^{-1} suspensions. Confidence intervals of varying LC$_{50}$ values were calculated at *P* level < 0.05.

Statistical analysis

Data were corrected for control mortality using Abbott's formula (Abbott, 1925). The mortality of each treatment was analyzed by analysis of variance (ANOVA) and means compared by Tukey's HSD test (SPSS 16.0). The LC$_{50}$ and LC$_{90}$ and their 95% fiducial limits were calculated from probit analysis (Finney, 1971) using the Minitab 15 computer software.

Results and discussion

Virulence of entomopathogenic fungi against *T. urticae*

The bioassay revealed that *M. anisopliae*, *B. bassiana*, *V. lecanii*, and *T. harzianum* can be biologically employed to control *T. urticae*. Fungal lethal effects were assessed as percentage of daily cumulative mortality due to mycoses, compared to mortality in control. In general, the mortality of *T. urticae* was found to be the highest with increasing conidial suspensions from 1×10^5 and up to 1×10^8 conidia ml^{-1} (Figs. 1, 2, 3, and 4). Also, data showed that the mortality rate of *T. urticae* females

increased significantly after 7 days more than those after 3 days and/or 5 days of spraying at all concentrations. This trend of deaths over the days after inoculation pointed that the advanced infections and deaths appeared from the third day and continued until the seventh. These results suggested that the experiments with some EPF require a long incubation time before evaluating their results (Amjad et al., 2012). Tamai et al. (2002) reported that all isolates of *M. anisopliae* and *B. bassiana* were pathogenic to *T. urticae* with increased mortality rates after the 3rd day and peaking on the 4th or 5th day post treatment. Thus, the bioassay of some EPF against mites requires a long period of incubation before evaluating the results. This delay may be regarded to the time needed for infectivity process of adhesion, penetration, germination, and fungus growth (Ekesi, 2001).

M. anisopliae isolate had the most lethal effect even at low concentration (1×10^5) reaching 18.75, 23.75, and 36.25% on the 3rd, 5th, and 7th day of spraying, respectively, than other isolates (Fig. 1). After treatment with the highest concentrations (1×10^7 and 1×10^8 conidia ml^{-1}), *M. anisopliae* developed mycosis for *T. urticae* individuals with fast lethal effect caused 40.00 % after 3 days, 51.25% after 5 days, and 85.00% after 7 post-inoculation days.

The isolates *V. lecanii* and *B. bassiana* were found highly virulent to *T. urticae* individuals with mortality rates 72.50 and 70.00, at 1×10^8 conidial ml^{-1} concentration, respectively. This finding was consistent with Wekesa et al. (2005) who tested 2 isolates of *B. bassiana* and 17 isolates of *M. anisopliae* against *T. evansi* and observed 80% mortality rate in mite population at a high concentration (1×10^8 conidia ml^{-1}) caused by the 2 isolates. Also, Shi and Feng (2009) used *B. bassiana*, *Paecilomyces fumosoroseus*, and *M. anisopliae* isolates 10 days after spraying where they caused 73.1, 75.4, and 67.9% mortality rates of mites, respectively. On the reverse, *M. anisopliae* isolate remained least effective against females of *T. urticae* (Amjad et al., 2012), while *V. lecanii* and *P. fumosoroseus* showed more than 75% mortality (76.25 and 79.16%, respectively) at the highest lethal concentration 1×10^8 conidia/ml, 8 days after fungal application. On the other hand, *T. harzianum* was less lethal where it showed potency reaching 63.75% of deaths, 7 days after inoculation. Regarding the virulence of the 4 isolates, there were non-significant differences at *P* level < 0.05.

It was also shown that *B. bassiana* exceeded both *V. lecanii* and *T. harzianum* in mortality percentages at low concentrations (1×10^5 and 1×10^6 conidia ml^{-1}) (Figs. 2, 3, and 4), while *V. lecanii* exceeded at the highest concentration (1×10^8 conidia ml^{-1}) during inoculation days (Fig. 3). The mortality rates in the variants treated with 1×10^5 and 1×10^6 conidial suspensions of *B. bassiana* strain were as follows: 15.00, 18.75, and
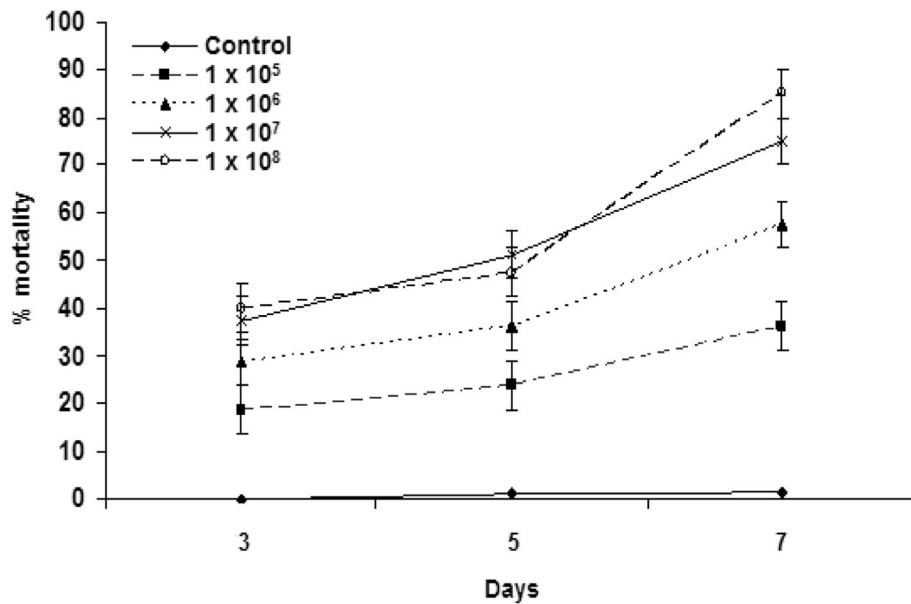
Fig. 1 Lethal effect of *Metarhizium anisopliae* isolate to *Tetranychus urticae* after treatment with conidial suspensions at concentration 1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia ml^{-1} (control variant, 0 conidia) Error bars: SD

28.75; 23.75, 27.50, and 43.75% at the 3rd, 5th, and 7th day post treatment, respectively. Meanwhile, a high lethal effect recorded 43.75, 51.25, and 72.50% was obtained with *V. lecanii* at the 1×10^8 conidia ml^{-1} concentration on the 3rd, 5th, and 7th day after treatment, respectively.

Virulence data demonstrated the efficacy of *M. anisopliae* strain in controlling mite individuals where they had the lowest LC$_{50}$ value of 4.6×10^5 conidia/ml, followed by *B. bassiana* which was less virulent to *T. urticae* having LC$_{50}$ of 3.3×10^6 conidia/ml (Table 1). The efficacy trials carried out in the field are largely supported by laboratory tests. While searching for microbial control agents in laboratory experiments, several workers have found that the mobile stages of *T. urticae* were sensitive and influenced to a large extent by the
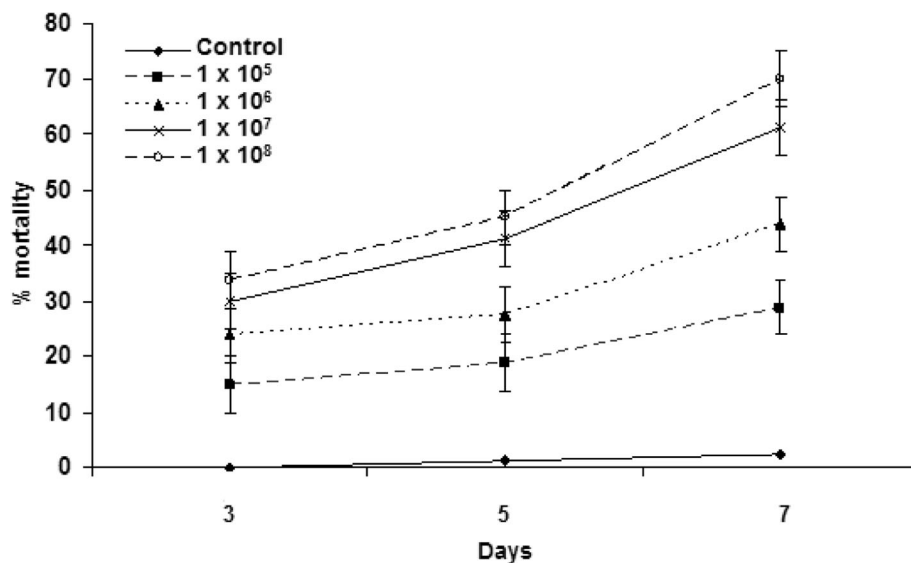

Fig. 2 Lethal effect of *Beauveria bassiana* isolate to *Tetranychus urticae* after treatment with conidial suspensions at concentration 1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia ml^{-1} (control variant, 0 conidia) Error bars: SD
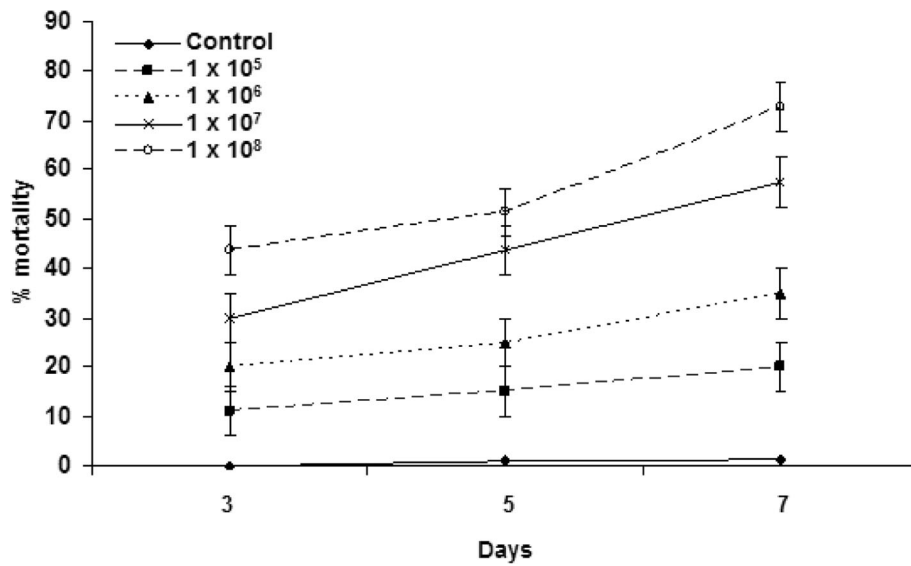
Fig. 3 Lethal effect of *Verticillium lecanii* isolate to *Tetranychus urticae* after treatment with conidial suspensions at concentration 1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia ml^{-1} (control variant, 0 conidia) Error bars: SD

tested isolates of *B. bassiana* and *M. anisopliae* (Tamai et al., 2002; Chandler et al., 2005; Draganova and Simova, 2010; Schapovaloff et al., 2014 and Zare et al., 2014). It was also found that *T. harzianum* was the least virulent fungus with LC$_{50}$ 9.4×10^6 conidia/ml. Considering the toxicity index percentage at LC$_{50}$, *M. anisopliae* was the most toxic fungus with a strong toxicity index 100%, followed by *B. bassiana*, *V. lecanii,* and *T. harzianum* with 13.94, 8.85, and 4.89%, respectively. Given the toxicity levels of LC$_{90}$, *M. anisopliae* was also

the strongest virulent with toxicity index of 100%, followed by *V. lecanii*, *B. bassiana*, and *T. harzianum* with toxicity index 11.43, 3.07, and 0.68%, respectively.

V. lecanii had the highest slope value of (0.489), followed by *M. anisopliae* (0.469), while *T. harzianum* was the lowest one (0.358) and *B. bassiana* had slope of the nearest value of 0.380 (Table 1). The slopes of all listed fungi are considered of low values indicating a certain amount of heterogeneity in the population towards the tested fungi.
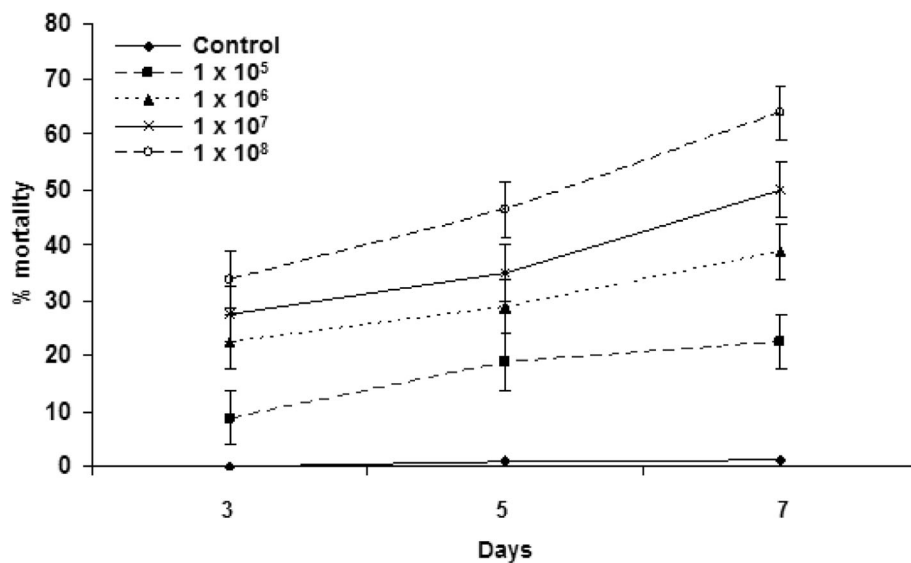

Fig. 4 Lethal effect of Trichoderma harzianum isolate against *Tetranychus urticae* after treatment with conidial suspensions at concentration 1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia ml^{-1} (control variant, 0 conidia) Error bars: SD

Table 1 Virulence of isolates of the *Metarhizium anisopliae, Beauveria bassiana, Verticillium lecanii,* and *Trichoderma harzianum* towards the two-spotted spider mite, *Tetranychus urticae*

Fungal conidial concs/ml	LC$_{50}$ (conidia/ml)	LC$_{90}$ (conidia/ml)	*Toxicity index (%)		Slope	**Confidence intervals of LC$_{50}$ (ppm)	
			LC$_{50}$	LC$_{90}$		Upper	Lower
Metarhizium anisopliae	4.6×10^5	2.4×10^8	100	100	0.469	9.9×10^5	1.6×10^5
Beauveria bassiana	3.3×10^6	7.8×10^9	13.94	3.07	0.380	8.4×10^6	1.3×10^6
Verticillium lecanii	5.2×10^6	2.1×10^9	8.85	11.43	0.489	1.1×10^7	2.6×10^6
Trichoderma harzianum	9.4×10^6	3.5×10^{10}	4.89	0.68	0.358	3.1×10^7	3.7×10^6

*Toxicity index was calculated with respect to *Metarhizium anisopliae* as the most effective fungus
**Confidence intervals were calculated at P level < 0.05

Pathogenicity test

All isolates of *M. anisopliae, B. bassiana, V. lecanii,* and *T. harzianum* infected mite individuals causing mortality (Fig. 5a–d). They demonstrated rapid external hyphal development and sporulation under moist condition. Firstly, filamentous hyphae were evolved from the anal region of the mite cadaver and then quickly covered the cadaver, followed by sporulation within 7 days. Investigation of smears of cadaver of mites confirmed mycosis infectivity as cause of death in individuals treated with conidial suspensions of the fungal isolates. Considering the deadly effect of the tested fungi to *T. urticae* was due to the secondary metabolites toxicity and the dyes they produced (Tamai et al., 2002), a variety of degrading enzymes were produced by EPF worked in a coordinated manner,

popularly known as cuticle degrading enzymes, including chitinases, proteases, and lipases, making their entry through the massive barriers of mite cuticle easier (Krieger de Moraes et al., 2003; Zare et al., 2014).

Protease activity

Proteases are considered to be the most important in the infectious process (Mustafa and Kaur, 2009). The dissolved proteins were further degraded into amino acids by exopeptidases and amino peptidases in order to provide nutrients for entomopathogenic fungi (Wang et al., 2002). The present results showed that protease activities of fungal isolates were increased by conidia concentrations from 1×10^5 and up to 1×10^8 conidia ml^{-1}. *M. anisopliae* gave the highest activities at all
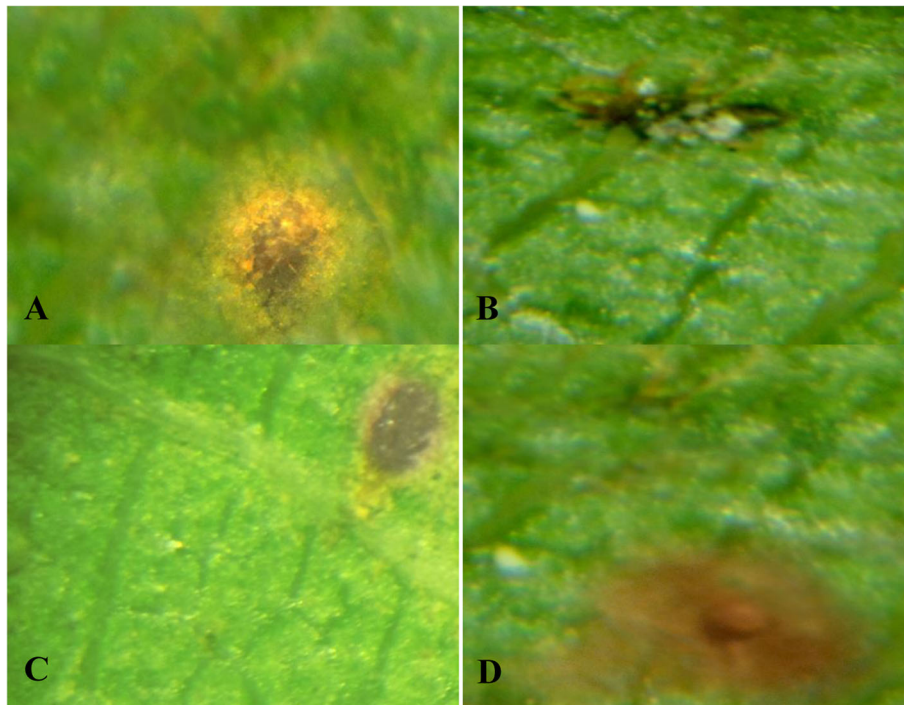

Fig. 5 a. Individuals of *T. urticae* infected with *M. anisopliae* after 7 days treatment. **b.** *T. urticae* individuals infected with *B. bassiana* after 4 days treatment. **c.** *T. urticae* individuals infected with *V. lecanii* after 7 days treatment. **d.** *T. urticae* individuals infected with *T. harzianum* after 7 days treatment (all images were taken with a Leica EZ 4D dissecting microscope at ~ × 16)

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concentrations than other isolates, yielding 1.032, 1.361, 1.977, and 2.111 U/ml at 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1} concentrations, respectively (Fig. 6). This is followed by *B. bassiana*, which exceeded *V. lecanii* and *T. harzianum* in its proteolytic activities at 1×10^5, 1×10^6, and 1×10^7 conidia ml^{-1} concentrations yielding 0.897, 0.964, and 1.121 U/ml, respectively. *V. lecanii* exceeded *B. bassiana* at the highest concentration (1×10^8) yielded 1.356 U/ml enzyme activity. *T. harzianum* was higher than *V. lecanii* at low concentrations (1×10^5 and 1×10^6 conidia ml^{-1}) while lower at higher concentrations (1×10^7 and 1×10^8 conidia ml^{-1}). In this respect, invasive fungi produce large quantities of serine-protease (Pr1), which degrades protein substances, after the epicuticle breakdown by lipases. The subtilisin-like serine-protease (Pr1) and trypsin-like protease (Pr2) are the most common studied protein decomposition enzymes. Pr1 and Pr2 activities have been detected in *M. anisopliae*, *M. flavoviride*, *B. bassiana*, *Nomuraea rileyi*, and *Lecanicillium lecanni* (Liu et al., 2007). Furthermore, the finding of protease Pr1 in EPF was considered as an indicator of virulence (Castellanos-Moguel et al., 2007).

Recent evidence indicated that different EPF isolates may show some degrees of variety in cuticle degrading proteases production (Pinto et al., 2002; Revathi et al., 2011 and Zare et al., 2014). These varieties may reflect differences observed in the virulence of isolates and species such that, isolates with high protease activity are expected to represent a high virulence towards their host. These findings were in accordance with obtained findings, which revealed a wide variation in the activities of proteases among the studied isolates. A positive correlation between proteases activity and virulence of isolates was found, so that the highest the protease activity of a particular isolate, the highest the virulence of that isolate against *T. urticae*. The present findings were also coincided with those obtained by Zare et al. (2014) who showed a wide diversity in proteolytic activity and virulence of studied isolates. Additionally, they found a positive correlation between the proteolytic activity on casein substrate and virulence of the isolates against the Khapra beetle *Trogoderma granarium*.

The present results revealed that the 2 virulent isolates *M. anisopliae* and *B. bassiana* showed the highest protease activities. It is well known that both the tested EPF produce higher enzymes of chitinases, proteases, and lipases than other fungi species (Revathi et al., 2011). Besides, *M. anisopliae*, *B. bassiana*, and *V. lecanii* secreted a diversity of extracellular hydrolytic enzymes in liquid cultures that contain locust cuticle as sole carbon source (St Leger et al., 1986).

Conclusion
Degrading enzymes might regulate many infectious processes and function as control agents. The finding has given biotechnology researchers an

Fig. 6 Mean (± SE) proteolytic activity (U/ml) of different EPF isolates on casein substrate
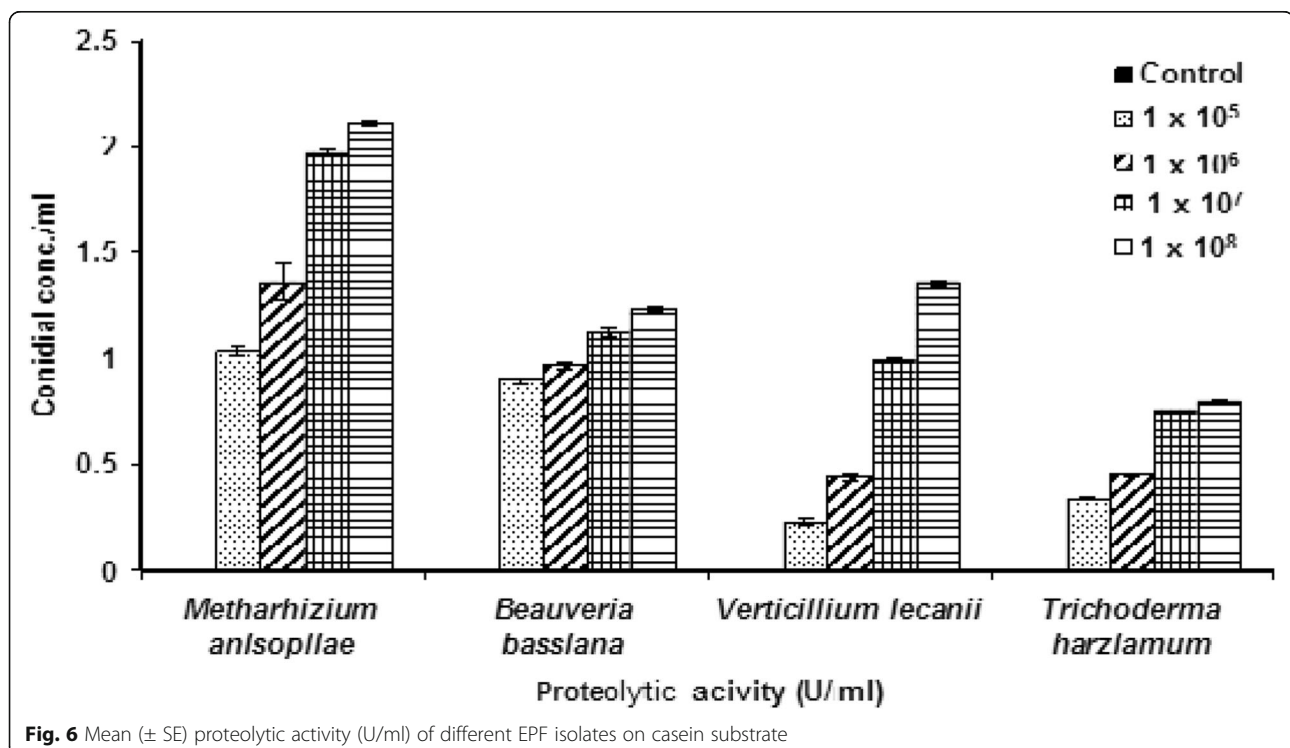
opportunity to implement bio-process engineering to produce these enzymes on a large scale, along with strategies to create applications for the enzymatic extracts, as well as potential applications such as mycoacaricides in a green technology-based environment.

Abbreviations
EPF: Entomopathogenic fungi; IU: One international unit; PDA: Potato dextrose agar; R.H.: Relative humidity; SDAY: Sabouraud's dextrose agar plus yeast extract; TCA: Trichloroacetic acid

Availability of data and materials
All data are available in the manuscript, and the materials used in this work are of high transparency and grade.

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with human participants or animals.

Consent for publication
The manuscript has not been published in completely or in part elsewhere. Informed consent was obtained from all individual participants included in the study.

Competing interests
The author declares that they have no competing interests.

Author details
[1]Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. [2]Botany and Microbiology Department, Faculty of Science, Zagazig University, Zagazig 44591, Egypt.

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