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# Pathogenicity of two fungal pathogens on larvae of the elm leaf beetle, *Xanthogaleruca luteola* Muller (Coleoptera: Chrysomelidae)

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## Abstract

**Background:** The elm leaf beetle (ELB), *Xanthogaleruca luteola* Muller (Coleoptera: Chrysomelidae), is one of the most important insect pests that cause damage and physiological stress for the trees. In the present research, pathogenicity of both entomopathogenic fungi (EPF), *Beauveria bassiana* and *Metarhizium anisopliae* on the 1st, 2nd, and 3rd instar larval of ELB at the concentrations of $10^5$, $10^6$, $10^7$, and $10^8$ conidia per ml, using immersion and spray methods and were evaluated.

**Results:** The results showed that $LC_{50}$ of *B. bassiana* in immersion and spray methods were $3.88 \times 10^2$ and $7.52 \times 10^2$ conidia/ml, while $LC_{50}$ of *M. anisopliae* in mentioned methods were $7.05 \times 10^2$ and $11.85 \times 10^2$ conidia/ml, respectively. The highest mortality rate in both EPF was observed in the 1st instar larvae that were significantly different than the control treatment. Moreover, the highest and lowest mortality in both EPF, using immersion and spray methods, were observed at the concentrations of $10^8$ and $10^5$ conidia/ml, respectively. Therefore, susceptibility of 1st and 2nd larval instars was more than 1st instar larvae. Based on the findings of the present study, there was no mortality during the first 24 h, while after 2 days, the mortality was recorded and increased over time in case of both pathogens. Accordingly, *B. bassiana* and *M. anisopliae* were pathogenic on larvae of ELB.

**Conclusions:** The outputs of the study demonstrated that applications of these eco-friendly fungal pathogens with other non-chemical methods are recommended. Of course, under natural conditions, their efficiency may change; thus, more comprehensive studies are necessary.

**Keywords:** Biological control, Fungal pathogens, *Xanthogaleruca luteola*, Mortality, Virulence

## Background

The elm leaf beetle (ELB), *Xanthogaleruca luteola* Muller (Coleoptera: Chrysomelidae), is a native insect pest to Europe that only attacks elm trees, *Ulmus* sp., that was introduced into the other parts of the world. Damaged leaves cause physiological stress to the trees and are an aesthetic problem (Arbab et al. 2001; Alford 2012). It has been progressively introduced into North America, Argentina, Chile, Central Asia, the Middle East, North Africa (Algeria), Siberia, and South Africa (Aslan et al. 2000). Also, this pest is one of the most important monophagous and leaf-chewing pest elm ornamental trees in urban areas (Alford 2012). In addition to direct damage, it reduces the resistance of elm trees to the Dutch elm disease caused by elm bark beetles. In most parts of the world, the damage due to ELB has been reported (Gregory 2001). Both adult and larval stages are phyllophagous and feed on leaves' parenchyma that leads to weakness in the tree (Alford 2012). Tree flaccidity causes problems such as attack of xylophagous

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and bark beetles, tree contamination to pathogens, the deformation of plant canopy, the reduction of photosynthesis, and physiological disorders (Gillespie et al. 2000). The use of synthetic insecticides is a tool to control this pest, but its serious consequences include poisoning humans and animals; pollution of water, air, and soil; a residue of toxin in food; resistance to pests; and effects on beneficial insects (Huerta et al. 2010). The EPF are a group of insect pathogens commonly found in forest soil more than farms' soil. Insect pathogenic fungi which cause diseases in nature are often an important regulator of insect populations (Ezz 2012). Insecticides based on EPF are remarkable as a biological control agent and suitable alternates of chemical insecticides. Both EPF, *Metarhizium anisopliae* Sorokin and *Beauveria bassiana* Vuill are useful biocontrol agents that are commonly utilized against insect pests. These microbial agents are used in biological control of various agricultural pests including whiteflies, beetles, grasshopper, and aphids (De La Rosa et al. 2000; Brownbridge et al. 2001). Despite other pathogens of insects, the EPF infect insects with complex mechanisms using direct penetration through their cuticle (Charnley 1989).

Due to the presence of elm trees in green spaces and urban areas, non-biological control with ELB pest leads to many unpleasant effects. Therefore, eco-friendly compounds can be utilized to control the pest. In the present study, efficacy of the EPF, *M. anisopliae* and *B. bassiana*, on 1st, 2nd, and 3rd larval instar of ELB were evaluated under laboratory conditions.

## Methods

### Rearing of *X. luteola*

The initial population of adult *X. luteola* was collected from elm trees around Pakdasht county, Tehran province, Iran. The ELB was reared on elm leaves in container cages (5 × 10 × 5 cm) covered with cloth mesh. New plants were provided to the culture when necessary. The cages were maintained under laboratory conditions of 25 ± 1 °C, 75 ± 5% RH and a photoperiod of 14: 10 h (L: D). After getting larval instars, main experiments were carried out on the 1st, 2nd, and 3rd larval instars.

### Preparation of spore suspension

The entomopathogenic fungi, *M. anisopliae* and *B. bassiana*, were collected from agricultural soil. To prepare the spore suspension, the surface of fungi colonies were shaved by a sterile scalpel into sterilized distilled water plus two drops of Tween 80 (0.5%) was added into Falcon tubes. To separate spores from the spore's chain, they were stirred by a shaker for 1 min, and then a uniform suspension of each pathogen was prepared. Counting of spores and preparation of spore density per unit volume were carried out by an improved Neubauer (Hemocytometer) and optical microscope (× 40). To perform the experiments, concentrations of $10^5$, $10^6$, $10^7$, and $10^8$ conidia/ml from *M. anisopliae* and *B. bassiana* pathogens were prepared separately. In order to increase the confidence level of concentrations, the count of spores was repeated 3 times.

### Viability test of isolates

To evaluate the viability of fungi, spores' germination test was done. About 24 h before the bioassay test, dilute suspensions of isolates were prepared in Tween 80 (0.5%) solutions. Then 100 μl of suspension was poured into the PDA medium in Petri dishes and maintained at 25 °C. After 18 h, 3 parts of the Petri dishes were specified, and randomly, 100 spores were selected from each part, and it was counted by optical microscope (× 40). When the germ tube length reached more than one half the diameter of spore, it was considered as a germinated spore (Greenfield et al. 2016). The germination rate, more than 85%, was selected for bioassay. The percentage of spore germination was calculated using the formula of Benslim et al. (2016):

$$\text{Spore germination percentage} = \frac{\text{Number of germinated spore}}{\text{Number of total spores}} \times 100$$

### Pathogenicity of fungal isolates

Each larval instars of ELB were exposed separately to the different fungal conidial concentrations ($10^5$, $10^6$, $10^7$, and $10^8$ conidia/ml) of *M. anisopliae* and *B. bassiana* at 4 replicates with control treatment. Sterilized distilled water plus two drops of Tween 80 (0.5%) was used as control treatment. For each replicate from the concentrations, 20 larvae from each of the 1st, 2nd, and 3rd instars of ELB were utilized. To eliminate saprophytes from the body surface of ELB, larvae were immersed in a sodium hypochlorite 1% for 20 s and washed in sterilized distilled water for 20 s. Eventually, to remove excess water, they were placed on a Whatman's filter paper for 1 min.

Inoculation of ELB larvae with fungal pathogen was made by spraying and immersion methods. To ensure that each insect of each treatment receives the same concentration, all same instar larvae were immersed and/or sprayed simultaneously. A volume of 5 ml suspensions of each concentration was poured into Petri dishes. Then, suspension was got out, and additional suspension was removed using a Whatman's filter paper. Within each Petri dish, a moist sterile cotton ball was added to provide moisture. In both spray and immersion methods, 5 ml of each concentration of the 2 fungi were utilized separately on the 1st, 2nd, and 3rd larval instars

**Table 1** Estimated lethal concentrations for *Beauveria bassiana* and *Metarhizium anisopliae* on 1st instar larvae of *Xanthogaleruca luteola* using immersion and spraying methods

| Fungal isolates | Methods | df | $LC_{50}$ (spore/ml) 95% CL | $LC_{90}$ (spore/ml) 95% CL | Slope ± SE | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|
| *B. bassiana* | Immersion | 3 | $3.88 \times 10^2$ (3.47–4.46) | $14.04 \times 10^2$ (10.49–21.84) | 2.29 ± 0.08 | 4.81 | 0.89 |
| | Spraying | 3 | $7.52 \times 10^2$ (6.01–10.75) | $50.91 \times 10^2$ (28.15–131.14) | 1.54 ± 0.07 | 3.20 | 0.92 |
| *M. anisopliae* | Immersion | 3 | $7.05 \times 10^2$ (5.79–9.54) | $35.23 \times 10^2$ (20.26–93.21) | 2.74 ± 0.07 | 1.53 | 0.91 |
| | Spraying | 3 | $11.85 \times 10^2$ (8.37–22.27) | $99.28 \times 10^2$ (43.35–465.61) | 1.38 ± 0.81 | 3.12 | 0.88 |

*LC* lethal concentrations, *CL* confidence limit, *SE* standard error

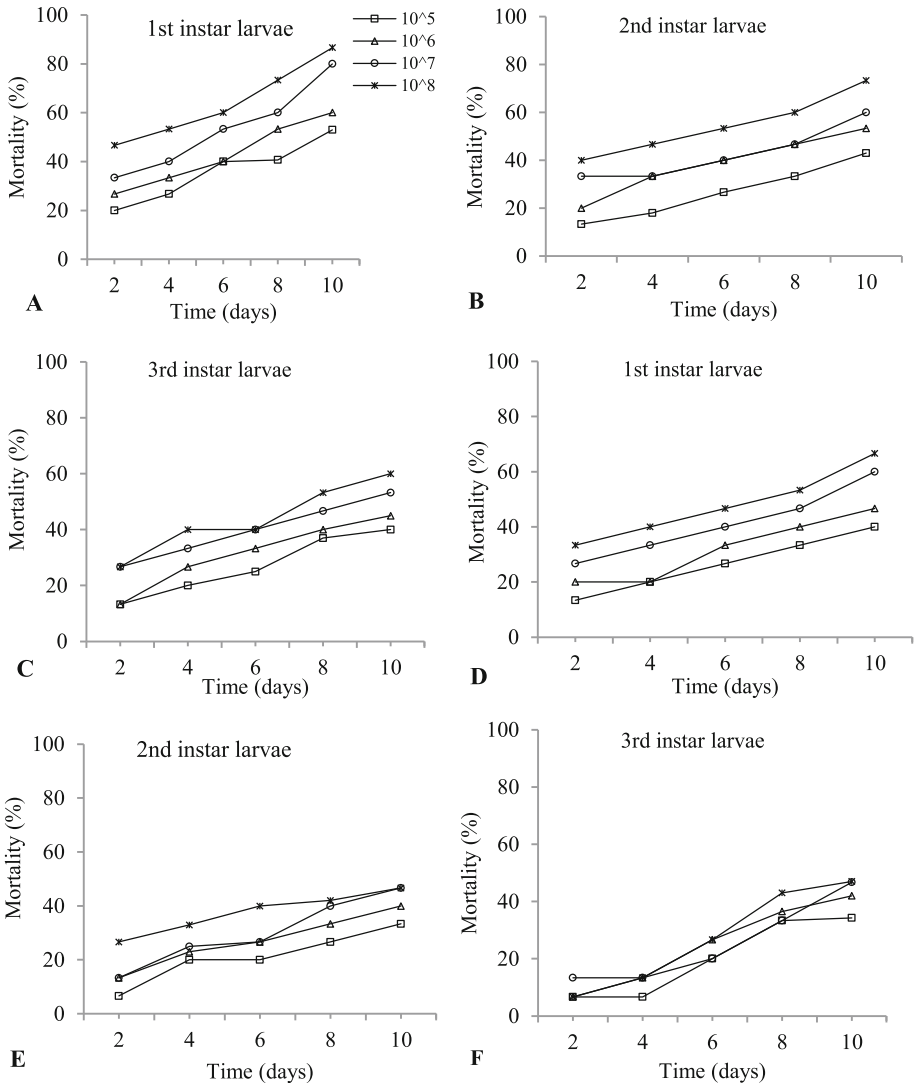


**Fig. 1** The mortality trend of different larval instars of *Xanthogaleruca luteola* treated by *Beauveria bassiana* during different times using immersion (**a, b, c**) and spraying (**d, e, f**) methods

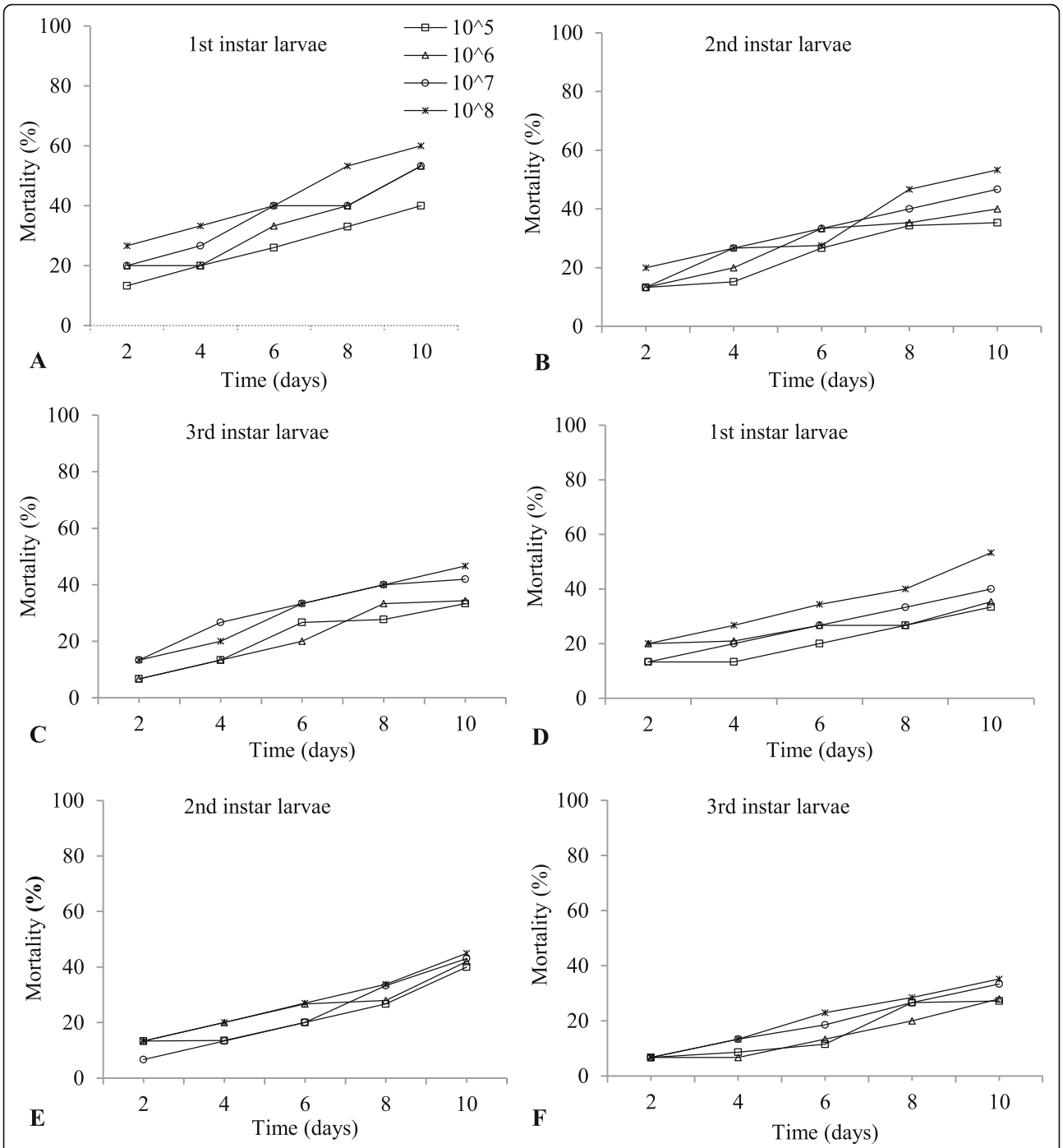


**Fig. 2** The mortality trend of different larval instars of *Xanthogaleruca luteola* treated by *Metarhizium anisopliae* during different times using immersion (**a, b, c**) and spraying (**d, e, f**) methods

of ELB. The Petri dishes were covered by parafilm and then placed in an incubator at 25 ± 1 °C and 75 ± 5% for 24 h. After 24 h, dead insects and wet cotton were removed. In the following, sterilized new elm leaves were placed at disposal of the larvae, and Petri dishes were covered with a net lid. Sterilized elm leaves were provided daily to the larvae. Petri dishes were maintained in an incubator at controllable conditions of 25 ± 1 °C, 75 ± 5 RH and a photoperiod of 14:10 h (L: D). Eventually, mortality means of both EPF were counted and recorded in both spraying and immersion methods for 10 days at 48-h intervals.

### Statistical analysis

The percentage of mortality was corrected using Abbott's formula (1925). Probit analysis was used to determine the values of lethal concentrations ($LC_{50}$ and $LC_{90}$). Arcsine transformation was applied to mortality data and then subjected to one-way analysis of variance (ANOVA). Means were compared using Tukey's HSD test at $P < 0.05$ significance level (Institute SPSS 2018). Student's $t$ test was performed for comparison of both fungi and two methods.

## Results

The results showed that color of *B. bassiana* colony on media was white, while it was green-brownish in *M. anisopliae*. The estimated lethal concentrations ($LC_{50}$ and $LC_{90}$) for *B. bassiana* and *M. anisopliae* treated on the ELB larvae are presented in Table 1. Based on the findings, $LC_{50}$ value of *B. bassiana* on 1st instar larvae of ELB by immersion and spraying methods were $3.88 \times 10^2$ and $7.52 \times 10^2$ conidia/ml, respectively. As well as, estimated $LC_{50}$ of *M. anisopliae* on 1st instar larvae of ELB was $7.05 \times 10^2$ and $11.85 \times 10^2$ conidia/ml, respectively (Table 1). Likewise, the $LC_{90}$ due to the 2 pathogens in immersion method was more than the spraying one.

The mortality means of 1st, 2nd, and 3rd larval instars of ELB treated with *B. bassiana* and *M. anisopliae* using immersion and spraying methods are presented in Tables 2 and 3. Moreover, the findings revealed that the mortality rates of both pathogens were significantly different ($t = 13.12$, $P < 0.001$). Moreover, there was a statistical significant difference between both methods in case of each pathogen ($t = 9.93$, $P < 0.001$ for *B. bassiana* and $t = 8.13$, $P < 0.001$ for *M. anisopliae*). In addition, the mortality rates due to *B. bassiana* at the immersion method on 1st, 2nd, and 3rd larval instars of ELB were significantly different ($df = 3$, $F = 12.61$, $P < 0.001$).

Mortality due to *B. bassiana*, the mortality of *M. anisopliae* on each of the 3 larval instars in immersion method was higher than in the spraying one (Table 3). In the immersion method of *M. anisopliae,* there were differences among different concentrations than the control ($df = 4$, $F = 7.55$, $P = 0.001$). Also, in the spraying method, significant differences ($df = 4$, $F = 8.97$, $P < 0.001$) were recorded among different concentrations.

**Table 2** Mortality percent of *Xanthogaleruca luteola* larvae by *Beauveria bassiana* using immersion and spraying methods

| Concentration conidia/ml | Immersion | | | Spraying | | |
|---|---|---|---|---|---|---|
| | L1 | L2 | L3 | L1 | L2 | L3 |
| $10^8$ | 64.00 $^{a}$ | 54.67 $^{a}$ | 44.00 $^{a}$ | 48.00 $^{a}$ | 37.67 $^{a}$ | 27.34 $^{a}$ |
| $10^7$ | 53.33 $^{ab}$ | 42.66 $^{ab}$ | 40.00 $^{a}$ | 41.33 $^{ab}$ | 30.33 $^{a}$ | 25.33 $^{a}$ |
| $10^6$ | 42.66 $^{ab}$ | 38.66 $^{ab}$ | 31.66 $^{a}$ | 31.98 $^{ab}$ | 27.26 $^{a}$ | 25.04 $^{a}$ |
| $10^5$ | 36.07 $^{b}$ | 26.86 $^{bc}$ | 27.06 $^{a}$ | 26.66 $^{bc}$ | 21.34 $^{ab}$ | 20.18 $^{a}$ |
| Control | 5.34 $^{c}$ | 6.67 $^{c}$ | 1.67 $^{b}$ | 6.68 $^{c}$ | 8.33 $^{b}$ | 5.34 $^{b}$ |

*L1* the 1st instar larvae, *L2* the 2nd instar larvae, *L3* the 3rd instar larvae. The same letters in each column had no statistically significant differences.

**Table 3** Mortality percent of *Xanthogaleruca luteola* larvae by *Metarhizium anisopliae* using immersion and spraying methods

| Concentration conidia/ml | Immersion | | | Spraying | | |
|---|---|---|---|---|---|---|
| | L1 | L2 | L3 | L1 | L2 | L3 |
| $10^8$ | 42.67$^{a}$ | 34.85$^{a}$ | 30.67$^{a}$ | 34.87$^{a}$ | 27.83$^{a}$ | 21.34$^{a}$ |
| $10^7$ | 36.00$^{a}$ | 32.01$^{a}$ | 31.07$^{a}$ | 26.67$^{a}$ | 23.27$^{a}$ | 19.70$^{a}$ |
| $10^6$ | 33.33$^{a}$ | 28.40$^{a}$ | 21.53$^{a}$ | 25.93$^{a}$ | 26.00$^{a}$ | 14.93$^{a}$ |
| $10^5$ | 26.47$^{a}$ | 24.97$^{a}$ | 21.53$^{a}$ | 21.34$^{a}$ | 22.70$^{a}$ | 16.13$^{a}$ |
| Control | 5.33$^{b}$ | 2.67$^{b}$ | 2.67$^{b}$ | 2.67$^{b}$ | 4.00$^{b}$ | 4.00$^{a}$ |

*L1* the 1st instar larvae, *L2* the 2nd instar larvae, *L3* the 3rd instar larvae. The same letters in each column had no statistically significant differences.

## Discussion

Because in the spray method, the probability of the spore collision level with the insect's body is higher, more mortality rates were observed in the immersion method. Therefore, both *B. bassiana* and *M. anisopliae* on 1st instar larvae of ELB were pathogenic. Both of the $LC_{50}$ and $LC_{90}$ values on ELB adults in immersion method were calculated more than the spraying method (Ebrahimifar et al. 2017).

Overall, the highest mortality rate in both methods was observed at 1st instar larvae that was statistically significantly different than the control. The findings revealed significant difference than the control in immersion method among different concentrations. In the spraying method, 3rd instar larvae showed no differences between different concentrations and the control. Accordingly, at the biggest larval instars, the mortality was decreased, which means the susceptibility of 1st instar larvae were more than 2nd and 3rd larval instars.

Thus, in both methods in the case of *M. anisopliae,* increasing of concentration and decreasing larval instars increased the mortality rates. Generally, the highest and lowest mortality in immersion (42.67 and 21.53%) and in spraying (34.87 and 16.13%) methods were observed at the concentrations of $10^8$ and $10^5$ conidia/ml, respectively.

The mortality recorded and increased over time in the case of both the pathogens. The mortality rates at different times on different instar larvae were varied (Figs. 1 and 2). These findings indicated that EPF required 48 to 72 h until they penetrate the insect cuticle and germinate. Eight days were reported as the lethal duration of 50% of *M. anisopliae* on elm bark beetle at the concentration of $10^7$ spores/ml, while the duration were 11.63 and 8.38 days for *B. bassiana* isolate IRAN 441C and *M. anisopliae* isolate DEMI 01 at $10^9$ spores/ml on *Oryctes*

*elegans* (Col., Curculionidae), respectively (Latifian and Rad 2012). The median lethal concentration, median lethal time, speed of replication in the host, production of toxins, and secondary metabolites are important factors to measure pathogenicity or virulence of a pathogen (Tanada and Harry 2012).

## Conclusion

The findings of the present study showed that *B. bassiana* on different instar larvae of ELB were more virulent than *M. anisopliae*. Also, the mortality rate due to the immersion method was greater than spraying one in both pathogens. Both fungi were pathogenic on the larvae of ELB; therefore, usage of this eco-friendly microbial agent with other non-chemical methods can be recommended. Of course, under natural conditions, their efficiency can presumably change; thus, more comprehensive studies are necessary.

### Abbreviations

ELB: Elm leaf beetle; LC: Lethal concentration; RH: Relative humidity; CL: Confidence limit; SE: Standard error

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### Authors' contributions

JE designed and conducted the experiments of research, and was a major contributor in writing the manuscript. AJ prepared the entomopathogenic fungi, analyzed, and interpreted the data regarding the research. The authors read and approved the final manuscript.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Competing interests

The authors declare that this work is not subject to any conflict of interest. The research project did not involve any human participants and/or animals (with the exception of insects and fungi).

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## Publisher's Note