ENTOMOPATHOGENIC POTENTIAL OF *BEAUVERIA BASSIANA* IN THE CONTROL OF ADULT APPLE BLOSSOM BEETLE (*TROPINOTA HIRTA*)

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(Received 3rd Oct 2025; accepted 23rd Dec 2025)

Abstract. Problems in phytomedicine associated with the extensive use of chemical pesticides have increased the need for alternative pest control strategies. The entomopathogenic fungus *Beauveria bassiana* is widely recognized for its potential in suppressing a broad range of insect pests in agriculture. The apple blossom beetle (*Tropinota hirta*) causes significant damage to fruit trees, ornamental plants and field crops by feeding on flowers, thereby preventing fruit set. The entomopathogenic activity of an autochthonous isolate of *B. bassiana* was evaluated under semi-controlled conditions in a black chokeberry (*Aronia melanocarpa*) plantation using two inoculation treatments applied to adult beetles: the dip method and the contaminated inflorescence method. By day 14 of the experiment, cumulative mortality of *T. hirta* reached 23% in the dip treatment and 13% in the contaminated inflorescence treatment. The delayed and relatively low mortality observed under semi-controlled field conditions may be influenced by environmental factors (e.g. temperature, relative humidity and UV radiation), characteristics of the fungal isolate, and the behaviour of adult beetles, including migration into the soil and feeding habits. Given the number of interacting factors potentially affecting infection efficiency, further studies using reference isolates of *B. bassiana* under controlled and semi-field conditions are required to more fully evaluate the entomopathogenic potential of the tested autochthonous isolate.
Keywords: *entomopathogenic fungi, inoculation methods, semi-field experiment, adult beetle mortality, integrated pest management*

Introduction

Problems in the field of agriculture and phytomedicine have always existed. With the increase in the human population, the demand for crop cultivation and the production of larger quantities of food and raw materials has also increased. As a result, these challenges have become more pronounced, and it is increasingly difficult to identify optimal and sustainable solutions. Although multiple factors contribute to these problems, climate change represents one of the most significant drivers, as it is causally linked to numerous contemporary issues in phytomedicine (Quesada Moraga et al., 2022).

Since their emergence, insect pests have had a destructive impact on agriculture by directly and indirectly damaging crops, orchards and vineyards, thereby reducing both yield and quality (Khan et al., 2023). Insects are responsible for approximately 10–16% of global agricultural production losses, and the damage they cause is increasing each year due to ecosystem disruptions associated with climate change and globalization (Savary et al., 2019). The effects of climate change are manifested through an increased number of pest generations per year, higher overwintering survival rates, expansion of

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suitable geographical ranges, and disruption of interactions between pests, host plants and natural enemies (Skendžić et al., 2021).

In addition to climate-related challenges, excessive and uncontrolled use of chemical plant protection products has contributed significantly to contemporary problems in phytomedicine (Gangwar et al., 2014). Frequent pesticide application leads to residues in food of animal and plant origin, environmental pollution, mortality of pollinators when treatments coincide with flowering, and the development of resistance in target pest populations. Consequently, there is an increasing need to promote more efficient and sustainable use of natural resources, including a reduction in chemical pesticide inputs (Gangwar et al., 2014; Hashimi, 2020; Faria and Wraight, 2020).

The apple blossom beetle (*Tropinota hirta* Poda) is a polyphagous pest that causes significant damage to a wide range of economically important crops, primarily fruit species, including black chokeberry (*Aronia melanocarpa* L.) (Yaşar and Uysal, 2013). Damage is caused by adult feeding on flowers and flower buds, resulting in sterility of attacked plants and the absence of fruit set, which ultimately reduces yield (Vuts et al., 2009). In some studies, damage levels of up to 70% of flowers have been reported for certain agricultural crops, indicating the need for effective control measures (Kutinkova and Andreev, 2004). Adult beetles are active during daylight hours and migrate towards the soil at dusk, burrowing to depths of three to five centimetres, while some individuals remain hidden among leaves and flowers until morning to maintain favourable moisture conditions (Çakmak and Şahin, 2018; Muradova, 2022).

The negative consequences associated with chemical pesticide use further complicate the control of the apple blossom beetle. Because *T. hirta* is present during flowering, optimal timing for chemical control coincides with peak pollinator activity, making chemical treatments environmentally unacceptable as a long-term solution (Vuts et al., 2009). Therefore, there is a clear need to develop alternative control strategies that are effective under the restrictive environmental and regulatory conditions imposed during flowering.

Alternative control approaches, including cultural, mechanical and biotechnical methods, offer the advantage of being applicable throughout the flowering period. Soil cultivation aimed at reducing egg survival, mechanical removal of adults and the use of visual or chemical attractants can contribute to population reduction; however, these methods alone are often insufficient to achieve satisfactory control (Schmera et al., 2004; Subchev et al., 2011; Aydin and Yaşar, 2019). Consequently, the development of more effective and environmentally friendly control strategies for *T. hirta* remains necessary.

Biological control using entomopathogenic fungi represents one such environmentally sustainable approach for managing not only the apple blossom beetle, but also numerous other agricultural pests. Many insect species are susceptible to entomopathogenic fungi, and more than 500 fungal species are currently recognized as potential biological control agents (Ivezić, 2008). The advantages of fungal-based biological control include high selectivity towards target pests, minimal risk to mammals and the environment, reduced reliance on chemical pesticides and mitigation of resistance development, as well as potential persistence in the environment that can support long-term pest suppression. However, disadvantages include slower host mortality compared to chemical insecticides, high requirements for relative humidity, limited effectiveness at low pest densities, restrictions on fungicide use during application periods, and higher production and storage costs (Wang, 2003; Mascarin et al., 2020).

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Among entomopathogenic fungi, *Beauveria bassiana* (Bals. - Criv.) Vuill. (Ascomycota: Hypocreales) is one of the most widely studied and commercially applied species due to its broad host range and multiple modes of action (Rondot and Reineke, 2018; Barra-Bucarei et al., 2020). The importance of the genus *Beauveria* is reflected in the global market, where approximately 40% of registered fungal biocontrol products are based on this genus, with *B. bassiana* accounting for the majority of these formulations (Kovač, 2021). Although the number of registered *B. bassiana*-based products fluctuates due to regulatory changes, their use remains widespread (Mascarin and Jaronski, 2016). Furthermore, *B. bassiana* is generally considered safe for pollinators, although its potential sublethal effects on pollinator behaviour and cognition remain insufficiently studied (Carlesso et al., 2020). Despite its promise, the development, registration and implementation of fungal biopesticides remain challenging due to regulatory complexity, high costs and limited standardization (Kovač, 2021; Inglis et al., 2021).

The aim of this study was to evaluate the entomopathogenic potential of an autochthonous isolate of *B. bassiana* for the control of adult *T. hirta* under semi-controlled field conditions using two inoculation methods: the dip method and the contaminated inflorescence method. The working hypothesis was that *B. bassiana* would induce significant mortality of adult beetles under semi-controlled field conditions when applied using either inoculation approach.

Materials and methods

Origin and cultivation of the Beauveria bassiana isolate

The isolate of *B. bassiana* was isolated from the mycotic potato beetle (*Leptinotarsa decemlineata* Say), after which it was identified by the conventional PCR method (according to Gebremariam et al., 2021) to the species level, using the DNA extraction method according to Elias et al. (2004).

A pure culture of the isolate is grown in a climate chamber on potato dextrose agar media (PDA) (Sigma-Aldrich), at 23 °C, in the dark, and kept in the collection of the Department of Plant Pathology, University of Zagreb Faculty of Agriculture.

Preparation of spore suspension

Petri dishes with PDA were inoculated with *B. bassiana* spores and incubated at 23 °C for 21 days in the dark. After incubation, 10 ml of sterile water with 0.01% surfactant (Tween-80, Sigma – Aldrich) was poured into each Petri dish containing the developed pure culture of the fungus. The spores were separated from the culture with a sterile silicone brush to obtain spore suspension. The spore suspension was then filtered to remove mycelial fragments, and the spore concentration was adjusted to 3.3×10^8 spores/ml with a Neubauer hemocytometer. The spore suspension (3.3×10^8 spores/ml) was left for 12 hours before conducting the experiment at a temperature of 23 ± 1 °C, in the dark to accelerate and synchronize spore germination.

In order to check spore viability, 5 µl of spore suspension was applied to a microscope slide with PDA, and germination was determined after 24 hours of incubation. Spores were considered to have germinated if the spore germ tube was longer than the diameter of the spore. As a result, spore suspensions that germinated more than 90% were used in experiments (Zemek et al., 2021).

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DOI: http://dx.doi.org/10.15666/aeer/2402_19631975
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Testing the effect of inoculation treatments

The prepared spore suspension was used to quantify the entomopathogenic potential of *T. hirta* adults by conducting two experiments (*Figure 1*).

1) Inoculation treatment using the "dip" method (according to Polat et al., 2022) and

2) Inoculation treatment using the "contaminated inflorescence" method (according to Husberg and Hokkanen, 2001).

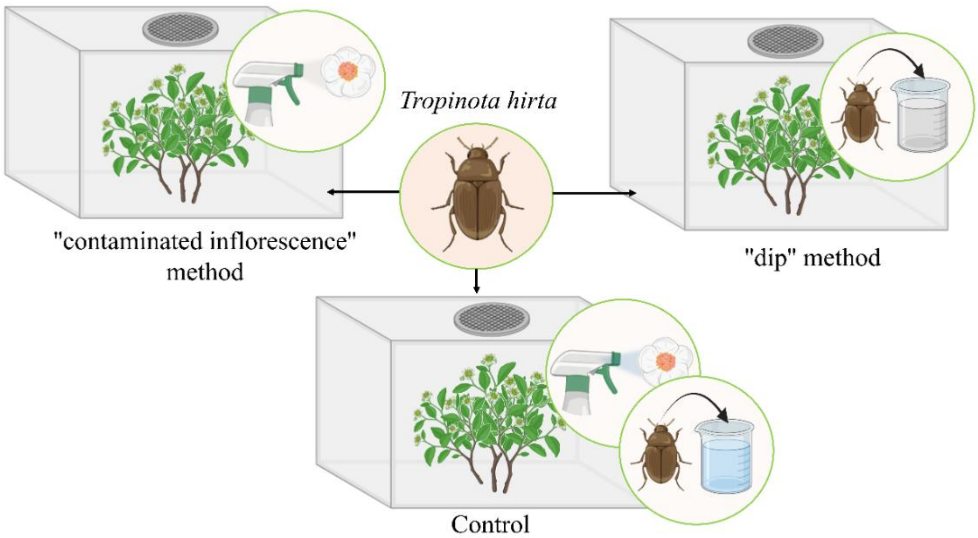

Figure 1. *Schematic representation of inoculation treatments of Tropinota hirta adults. Created with BioRender.com*

For the purpose of conducting the experiments, in April 2024, *T. hirta* adults were collected from black chokeberry plantations during flowering (Gornji Laduč, Croatia, plantation location 45°52'57.7"N 15°42'45.3"E) with inflorescences, for the purpose of feeding the beetles. A total of 90 adult individuals were collected during a sunny and windless day. Before the start of both experiments, *T. hirta* adults were placed in a refrigerator at 4 °C (anesthetic effect) for 10 minutes to make the beetles less mobile (according to Rayl and Wratten, 2016).

Two experiments were set up, each comprising two variants and three replicates (three containers per variant). The containers (35 × 35 × 35 cm) used in the experiment were designed so that adults could migrate to the soil. The sides of the containers were lined with mesh to prevent contact with other insects, except those facing the ground.

Inoculation treatment using the "dip" method

Three replicates were used in the experiment, each with 10 *T. hirta* adults. The test variant contained adults immersed in a suspension of *B. bassiana* spores (3.3×10^8 spores/ml) for 10 seconds, while the control variant included adults immersed in a solution of sterile distilled water and 0.01% surfactant (Tween-80).

After inoculation, adults were placed in containers with freshly collected inflorescences for feeding. The prepared test and control containers were placed in a randomized block design for 14 days in semi- controlled field conditions. Fresh chokeberry inflorescences (10 inflorescences/container) were collected every 24 hours and placed in the containers for feeding.

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Inoculation treatment using the "contaminated inflorescence" method

For the purpose of conducting the experiment, three replicates were used, each with 10 *T. hirta* adults. The test variant contained adults untreated with a suspension of fungal spores and 10 chokeberry inflorescences treated with a suspension of fungal spores at a concentration of 3.3×10^8 spores/ml in a volume sufficient to cover the entire surface of the inflorescence (until the excess suspension began to flow from the treated surface). The control containers contained untreated insects and 10 chokeberry blossoms treated in the same way as the test containers, with a solution of sterile distilled water and 0.01% surfactant.

Fresh chokeberry blossoms were collected every 24 hours and placed in the containers for the insects to feed on (untreated by spore suspension).

Meteorological data

Meteorological data for the 14-day experimental period (April 8–22, 2024) were obtained from the nearest official meteorological station of the Croatian Meteorological and Hydrological Service (DHMZ). For graphical presentation alongside mortality, mean daily air temperature and relative humidity were used (*Figure 2*).

The average air temperature during the experimental period was 15.7 °C, with an average maximum of 20.4 °C and a minimum of 6.8 °C. A pronounced temperature peak was recorded on April 14, followed by a marked decrease in air temperature from April 16 onward. Relative humidity showed notable fluctuations during the experimental period, with higher values coinciding with the period of decreasing temperatures.

In addition, an increase in precipitation was recorded during the second half of the experimental period, and a cold snap with frost occurred on April 21. The average maximum soil temperature at a depth of 10 cm was 24.2 °C, while the minimum was 7.4 °C during the experiment (DHMZ, 2024).

Data analysis

Mortality of *T. hirta* adults was recorded at 48-hour intervals. Cumulative mortality (%) was calculated for each replicate as the number of dead adults divided by the initial number of adults (10 per replicate). For statistical comparison, final mortality was evaluated at day 14 by constructing 2×2 contingency tables (dead vs. alive) for each pairwise comparison (dip vs. contaminated inflorescence; dip vs. control; contaminated inflorescence vs. control). Because of small expected frequencies, differences among treatments were tested using Fisher’s exact test (two-tailed). Mean mortality (%) and standard deviation (±SD) were calculated from three independent replicates per treatment. Statistical significance was set at $p < 0.05$.

Results

The entomopathogenic potential of the *B. bassiana* isolate against *T. hirta* adults was evaluated using two inoculation treatments (dip method and contaminated inflorescence method), alongside an untreated control. Adult mortality was monitored over a 14-day period and is presented as cumulative mortality (%), together with mean daily air temperature and relative humidity recorded during the experiment (*Figure 2*). Final mortality values calculated at day 14 are summarized in *Table 1*.

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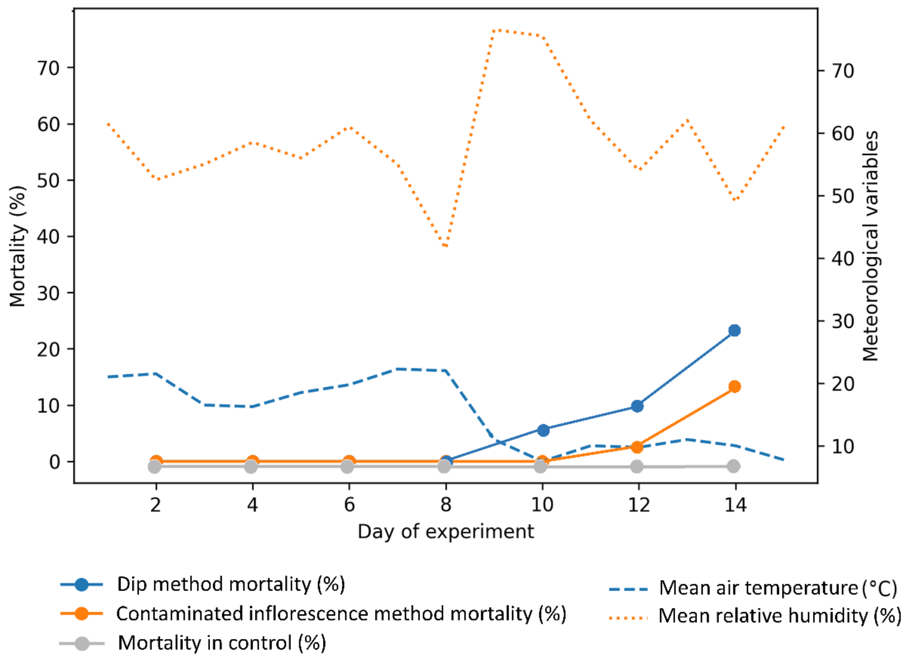

Figure 2. *Mortality of Tropinota hirta adults treated with Beauveria bassiana using the dip method and contaminated inflorescence method in relation to mean daily air temperature and relative humidity during the 14-day experimental period. Mortality values are expressed as cumulative percentages of dead adults. Mortality values represent cumulative percentages based on 30 adults per treatment (three replicates of 10 individuals)*

Table 1. *Final mortality of Tropinota hirta adults at day 14 after inoculation with Beauveria bassiana*

Treatment	Dead adults (n/30)	Mean mortality (%) ± SD
Dip method	7 / 30	23.3 ± 5.8
Contaminated inflorescence method	4 / 30	13.3 ± 5.8
Control	0 / 30	0.0 ± 0.0

Values represent mean mortality (%) ± SD calculated from three independent replicates (10 adults per replicate)

No mortality was observed in either treatment or in the control group during the first eight days of the experiment, when mean daily air temperatures remained relatively stable and relative humidity showed moderate fluctuations. In the dip treatment, the first dead adults were recorded on day 10, after which cumulative mortality increased by day 14. At the end of the observation period used for statistical analysis (day 14), the dip treatment resulted in a total of 7 dead adults out of 30, corresponding to a mean mortality of 23.3 ± 5.8% (*Table 1*).

In contrast, mortality in the contaminated inflorescence treatment was lower and delayed. Dead adults were recorded only at later observation points, resulting in a total of 4 dead individuals out of 30 by day 14 and a mean mortality of 13.3 ± 5.8% (*Table 1*). No mortality was recorded in the control group throughout the experiment (0.0 ± 0.0%).

Statistical comparison of final mortality at day 14 showed that mortality in the dip treatment was higher than in the contaminated inflorescence treatment; however, this difference was not statistically significant (Fisher's exact test, $p > 0.05$). In contrast,

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 24(2):1963-1975.
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mortality in the dip treatment was significantly higher than in the control group ($p < 0.05$), while mortality in the contaminated inflorescence treatment did not differ significantly from the control.

Following incubation of dead adults in a humid chamber, white mycelium developed on all dead individuals (100%) from both treatments, whereas no mycelial growth was observed on beetles from the control group. This confirms that mortality in the treated groups resulted from infection with *B. bassiana* rather than from contamination with other microorganisms. Successful infection and proliferation of *B. bassiana* within the beetle's body were further demonstrated by the external development of mycelium on cadavers (*Figure 3*). Morphological examination confirmed that the fungal structures belonged to the genus *Beauveria*.

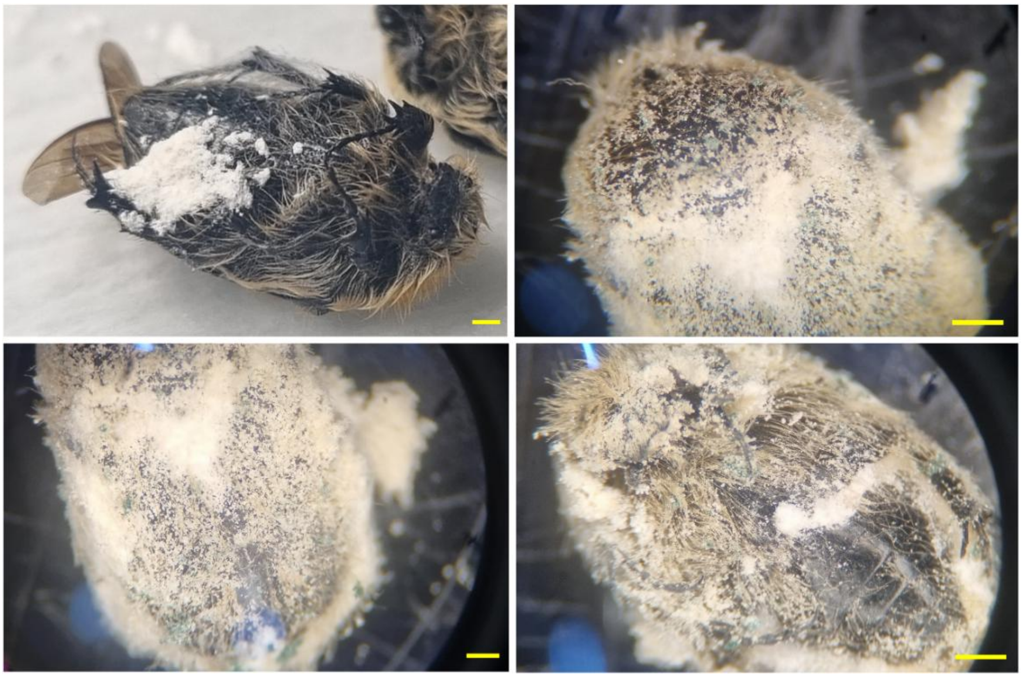

Figure 3. *Stereomicroscopic view of the appearance of Beauveria bassiana white mycelium and spores on dead Tropinota hirta adults in the treated variants after inoculation treatments. Scale bar 1 cm*

Discussion

The results of the present study were obtained by conducting two experiments that evaluated the effectiveness of *B. bassiana* spores in infecting adult apple blossom beetles (*T. hirta*) under semi-controlled field conditions. Overall mortality remained relatively low in both treatments, reaching 23.3% in the dip method and 13.3% in the contaminated inflorescence method by day 14 of the experiment, while no mortality was recorded in the control group.

The first mortality of beetles immersed in the spore suspension (dip method) was recorded on day 10 of the experiment, whereas mortality in the treatment using contaminated inflorescences was observed only at later observation points. Although mortality occurred earlier and reached higher numerical values in the dip treatment, statistical analysis showed that the difference between the two treatments at day 14 was

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not significant. Therefore, the observed differences between treatments should be interpreted as trends rather than as clear evidence of superior efficacy of one inoculation method over the other. The earlier onset and higher numerical mortality observed in the dip treatment may be associated with methodological differences between the inoculation approaches. Immersion of beetles in the spore suspension likely enabled more uniform adhesion of fungal spores to the entire insect surface, aided by the presence of a surfactant that reduced surface tension and facilitated contact between spores and the cuticle. In contrast, the contaminated inflorescence method relies on indirect contact and feeding behaviour, which may result in variable spore acquisition among individuals. Such variability could contribute to delayed infection and lower overall mortality in this treatment. However, given the absence of a statistically significant difference, these explanations should be regarded as possible contributing factors rather than definitive mechanisms.

Incubation of dead beetles under favourable conditions for fungal growth resulted in the development of characteristic white mycelium on all individuals from both treated variants, whereas no mycelial growth was observed on beetles from the control group. This confirms that *B. bassiana* was the direct cause of mortality in treated insects and that infection was successfully established in all individuals that died during the experiment. It should be emphasized that this research was conducted under semi-controlled field conditions, which differ substantially from laboratory conditions. Studies conducted under controlled laboratory settings generally report higher and more rapid mortality of *T. hirta* following treatment with *B. bassiana* (Atmaca et al., 2018; Uçar et al., 2022). Under laboratory conditions, the influence of individual environmental factors such as temperature, relative humidity and the absence of UV radiation can be isolated and precisely evaluated, allowing a clearer assessment of fungal virulence and host susceptibility. In contrast, under semi-controlled field conditions, exposure to fluctuating environmental factors often reduces or delays fungal infection and disease development. Similar discrepancies between laboratory and field performance of entomopathogenic fungi have been reported in recent studies, which highlight that environmental exposure frequently limits fungal efficacy outside controlled conditions (Mascarin et al., 2020; Inglis et al., 2021). Therefore, laboratory and semi-controlled field studies should be considered complementary, and direct comparisons between them should be made with caution.

Environmental factors such as temperature, relative humidity, light intensity and UV radiation are known to strongly influence the viability, germination and infectivity of *B. bassiana* spores (Fernandes et al., 2007; Wraight et al., 2007; Jackson et al., 2010). Recent studies further highlight the importance of environmental competence of entomopathogenic fungi as a key determinant of their success in pest control under field conditions (Quesada-Moraga et al., 2022; Quesada-Moraga et al., 2023). In the present study, high air temperatures recorded during the initial phase of the experiment, followed by a decrease in temperature and an increase in relative humidity, may have influenced the timing and extent of infection. Elevated temperatures and potential UV exposure during daytime may reduce spore viability or delay germination, while cooler and more humid conditions are generally more favourable for fungal development. As UV radiation and microclimatic conditions were not directly measured, their effects can only be discussed as potential contributing factors.

The morphology and behaviour of *T. hirta* adults may also have limited infection efficiency. The dense hair coverage of the beetle body may act as a physical barrier

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between fungal spores and the insect cuticle, reducing the likelihood of successful penetration. In addition, adult beetles exhibit daily migration behaviour, burrowing into the soil during nighttime and returning to inflorescences during the day (Çakmak and Şahin, 2018; Muradova, 2022). Behavioural interactions between insects and entomopathogenic fungi have recently been emphasized as an important, yet often underestimated, factor influencing infection success under field conditions (Shrestha et al., 2022; Keyser and Meyling, 2023). Such behaviour may intermittently expose beetles to favourable conditions for fungal development while also limiting prolonged contact with viable spores.

The contaminated inflorescence method presents additional limitations, as it does not ensure uniform exposure of all individuals to fungal spores. Although beetles were observed feeding on treated inflorescences, the extent and duration of feeding could not be controlled, potentially resulting in inconsistent inoculation. Similar limitations of indirect exposure methods have been discussed in recent field-oriented studies on entomopathogenic fungi (Mascarin et al., 2020; Meyling and Eilenberg, 2021).

Although the mortality rates observed in this study were relatively low, the results demonstrate that environmental conditions, fungal isolate characteristics and insect behaviour collectively influence the success of biological control using entomopathogenic fungi under semi-controlled field conditions. Given the limited availability of field-based studies on the entomopathogenic activity of *B. bassiana* against *T. hirta*, further research is needed to clarify these interactions. Future studies should include reference isolates of *B. bassiana* with known virulence as positive controls and improved formulations, such as encapsulated spores, to reduce the inhibitory effects of environmental factors and enhance infection efficiency (Faria and Wraight, 2020; Felizatti et al., 2021).

Conclusion

Control of adult apple blossom beetle *Tropinota hirta* using an autochthonous isolate of *Beauveria bassiana* was evaluated using two inoculation treatments (dip method and contaminated inflorescence method) under semi-controlled field conditions. Neither treatment resulted in high mortality of beetles, with cumulative mortality reaching 23% in the dip treatment and 13% in the contaminated inflorescence treatment by day 14 of the experiment.

Given the number of interacting variables that may influence infection efficiency under semi-controlled field conditions, including environmental factors, fungal isolate characteristics and insect behaviour, further research is required to better assess the entomopathogenic potential of the tested isolate. Future studies should include a reference isolate of *B. bassiana* as a positive control to allow direct comparison of pathogenicity. In addition, the use of encapsulated formulations of *B. bassiana* spores and further optimisation of inoculation methods may help to reduce the inhibitory effects of environmental conditions and increase direct contact between fungal propagules and the insect host.

Acknowledgements. This work was supported by funding from the University of Zagreb Faculty of Agriculture.

Conflict of Interest. The authors declare no competing interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2402_19631975
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