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Short note

Larvicidal activity of different entomopathogenic fungi on *Halyomorpha halys* (Heteroptera: Pentatomidae) under laboratory conditions

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Abstract
Halyomorpha halys, represents a significant threat to a diverse array of agricultural crops. The most prevalent method of control employed against *H. halys* in integrated fruit production is the use of chemical insecticides. Because of environmental concerns that arise from pesticide use, there is a pressing demand to identify alternative and more sustainable control measures that could serve as a potential substitute or supplement to conventional insecticides. Against this background, entomopathogenic fungi can be seen as a potential management tool worthy of further investigation. In the present study, the effects of egg spray treatments with two *Beauveria bassiana*-based mycopesticides and conidial suspensions of *B. bassiana*, *B. pseudobassiana*, *Metarhizium brunneum* and *M. robertsii* isolates were investigated under constant laboratory conditions (25 °C, 65% RH, 16L:8D). Egg hatching rate and nymphal mortality were recorded. Treatment of eggs with the selected fungi resulted in reduced nymph survival compared to a water-treated control. The fungal treatments did not significantly affect hatching success but increased mortality of first instar nymphs, with average mortality rates between 70% and 100% during development from egg to imago. The efficacy of the investigated fungi was demonstrated to be high under specific laboratory conditions, indicating a potential suitability of these fungi as biocontrol agents.

Keywords
Biocontrol, brown marmorated stink bug, bioassay, mycopesticides, *Beauveria*, *Metarhizium*

Introduction

The invasive brown marmorated stinkbug, *Halyomorpha halys* Stål (Heteroptera: Pentatomidae) is considered a prominent pest affecting different agricultural crops (Nielsen and Hamilton, 2009; Leskey et al., 2013; Swathy et al., 2024). The sucking activity of *H. halys* on fruit can cause considerable damage and losses especially in pome and stone fruit cultivation (Wermelinger et al., 2008; Bariselli et al., 2016; Maistrello et al., 2017). Effective management of *H. halys* can be a

decisive factor for successful fruit production. In Italian apple orchards, the current integrated pest management (IPM) strategy against *H. halys* is largely based on a combination of field monitoring and the use of broad-spectrum insecticides, alongside a classical biocontrol approach targeting area-wide management (Serratore et al., 2018; Fischnaller et al., 2022; Falagiarda et al., 2023). The utilization of conventional pesticides is a controversial issue due to pesticide drift and the potential impact on the environment, biodiversity and beneficial insects (Lamichhane et al., 2016; Tudi et al., 2021; Serrão et al., 2022). One of the current objectives for European agriculture is to reduce reliance on synthetic insecticides by promoting biological crop protection, in order to prevent insecticide-related adverse effects (Lamichhane et al., 2016; Jacquet et al., 2022). The use of microbial biocontrol agents, especially mycopesticides, is a promising method of improving the sustainability of agriculture (Bamisile et al., 2021; Vivekanandhan et al., 2022, 2024). The search for new and effective biocontrol agents is vital in this context. The aim of the present study was to evaluate a selection of entomopathogenic fungi for their control efficacy and potential suitability as a sustainable alternative or supplement to conventional insecticides in the control of *H. halys*. Previous studies reported on the efficiency of different entomopathogenic fungi (EPF) in controlling nymphal stages of *H. halys* (Parker et al., 2015; Tozlu et al., 2019; Mantzoukas et al., 2024). Most of these studies investigated the effect of direct spray treatments on nymphs. The present laboratory study investigated the effect of egg spray treatments with conidial suspensions of different EPF and commercially available mycopesticides.

Material and methods

Fungal spores were applied to egg masses (n = 6) with a hand operated thin-layer chromatography reagent sprayer (Supplementary Fig. S1d). Each of the experimental trials included a water-treated control (average spray deposition [H_2O] = 4.6 mg ± 0.95 cm^{-2}). Egg masses were collected from rearing cages (continuous laboratory rearing) by cutting them from nylon nets (oviposition site). Insects were reared in a climate-controlled room at 25 °C, 65% RH and 16:8 h light:dark (L:D). Adults were fed with green beans, carrots, sunflower seeds, kiwi fruit and *Peperomia* sp. (Piperaceae). The age of the eggs varied, ranging from 0–72 h at the time of treatment. After treatment, the eggs were air-dried for 5 minutes and then placed in glass petri dishes (⌀ 10 cm) lined with filter paper (Whatman No. 1). Nymphs of the second instar were transferred and kept in transparent netted polypropylene beakers. Every beaker contained a wetted cotton plug for hydration. Nymphs were fed with green beans and carrot slices. The food and cotton plugs were changed twice a week. Incubation of egg masses and nymph cohorts occurred in a climatic test chamber (FDM, Italy) at 25° C, 65% RH and 16:8 h L:D. Egg hatching success and mortality of nymphal stages were determined for each treatment. The ovo-larval mortality was calculated as follows:

$$\text{Ovo-larval mortality} = 100 - \frac{N_{adults}}{N_{eggs}} \times 100\ [\%]$$

N_{adults} = number of individuals per egg mass that develop into adults. N_{eggs} = number of eggs per egg mass. The ovo-larval mortality per egg mass was compared between experimental and control groups by one-sided t-test if data was normally distributed or by Mann-Whitney-U for non-normally

distributed data (Shapiro-Wilk: $p < 0.05$) and in case of unequal variance (Brown-Forsythe: $p < 0.05$) (SigmaPlot 13.0, Systat Software, Inc.). The experiments were replicated twice.

Tested fungi

Two *Beauveria bassiana*-based mycopesticides and five lab-grown fungal strains were tested in this study. *Metarhizium brunneum* BIPESCO 5 (alias Ma43, formerly *M. anisopliae var. anisopliae* BIPESCO 5/F52 [Eilenberg et al., 2008]), *M. brunneum* GT7, *M. robertsii* GT10, *Beauveria bassiana* XG/2B and *B. pseudobassiana* AWLB were applied in a concentration of 5 x 10^{10} conidia L^{-1}. *Metarhizium* strains were provided by Dr. Hermann Strasser - Institute of Microbiology, University of Innsbruck (Innsbruck, Austria). *Beauveria bassiana* XG/2B and *B. pseudobassiana* AWLB were isolated at Laimburg Research Centre (Vadena, Italy) from mycotised bark beetle, *Xylosandrus germanus* and codling moth, *Cydia pomonella,* respectively. Conidia were produced by solid state fermentation on pearl barley. Liquid pre-culture was used to inoculate sterilised barley (biphasic production process). Spore suspension was prepared by washing off conidia from barley kernels colonised by the fungus with sterile Tween 80 solution [0.1% (w/v)]. The suspension was filtered through a 120 µm mesh size nylon membrane. Conidial concentration was determined by haemocytometer counts. The viability (germination rate) of the wet harvested conidia was determined according to the method described by Laengle et al. (2005).

For examination of the germination rate an aliquot of spore suspension was diluted to 5 × 10^6 conidia mL^{-1}. One hundred microliters of diluted spore suspension were spread on agar plates (n = 6). Sabouraud 2% dextrose agar and Sabouraud 4% dextrose agar were used for *Beauveria* spp. and *Metarhizium* spp., respectively. Plates were incubated in the dark at 25 °C. 3 × 100 conidia per plate were checked microscopically for germ tube formation. Conidia were interpreted as germinated if the germ tube length exceeded the diameter of the conidia. Germination rate after 30 h exceeded 95% in all trials.

BOTANIGARD® OD (CERTIS BELCHIM B.V., Utrecht, Netherlands) was applied at a concentration of 2 mL L^{-1}. The concentration of VELIFER® (BASF Española S.L., Barcelona, Spain) was 1 mL L^{-1}.

Results and discussion

All fungal treatments caused increased ovo-larval mortality compared to corresponding controls (Table 1, Fig. 1). The spray treatment with *Metarhizium brunneum* GT7 conidia suspension even led to 100% mortality in three independent experiments. The average ovo-larval survival rate (egg to adult) after exposure to *Metarhizium* spp. conidia suspension was less than 5% in all experiments. BOTANIGARD® OD and VELIFER® treatments resulted in mean ovo-larval mortality rates of ≥ 87% and ≥ 95%, respectively. The *B. pseudobassiana* strain AWLB showed the lowest efficiency among the tested strains by causing average cumulative mortality rates of 70%, 86% and 92% in different trials. Apart from two exceptions, the main mortality always occurred during the first nymphal stage (Fig. 1).

Nymphs from fungal treatment groups mainly died within 3–7 days of hatching and manifested a clearly visible mycosis (Supplementary Figs S1a,c). Unhatched, morphologically intact eggs did not show any visible signs of mycosis. This indicates that infection of nymphs probably occurred at hatching or during the subsequent aggregation on eggshells. First instar nymphs normally remain on

the egg chorion or in proximity until first moult (Hristozova and Harizanova, 2022). By remaining on the egg surface, nymphs inevitably encounter the deposited fungal propagules. The conidial load to which newly emerged nymphs were exposed by remaining on the eggshells was sufficient to induce fungal infection in most of the specimens. This led to increased nymph mortality. First-instar nymphs were susceptible to all tested *Beauveria* and *Metarhizium* isolates, including the commercial mycopesticide products BOTANIGARD® OD and VELIFER®. On average, 52% of water-treated (control) eggs successfully developed into adults (939 imagines from 1798 eggs [72 egg masses]). The survival rate of the control may seem low, but it is comparable to results from previous studies, ranging between 61% and 39% (Nielsen et al., 2008; Haye et al., 2014).

Table 1 – Mean ovo-larval mortality (cumulative mortality from egg to adult). *: t-test not performed due to non-normal data distribution (Shapiro-Wilk: $p < 0.05$) or because of unequal variance (Brown-Forsythe: $p < 0.05$). ns: not significant ($p > 0.05$). na: not available (Mann-Whitney-U test not performed). σ: Population standard deviation.

Isolate / product	Trial	Mean ovo-larval mortality ± σ [%]		One-sided t-test (p)	Mann-Whitney-U (p)
		Experimental treatment	Control		
BOTANIGARD® OD (*B. bassiana* GHA)	1	**87.4 ± 12.63**	(64.0 ± 22.70)	< 0.05	na
BOTANIGARD® OD (*B. bassiana* GHA)	2	**98.4 ± 3.55**	(69.3 ± 13.74)	*	< 0.01
BOTANIGARD® OD (*B. bassiana* GHA)	3	**97.6 ± 2.52**	(71.0 ± 18.54)	*	< 0.05
VELIFER® (*B. bassiana* PPRI 5339)	1	**100 ± 0.00**	(52.2 ± 18.45)	*	< 0.01
VELIFER® (*B. bassiana* PPRI 5339)	2	**95.5 ± 8.72**	(34.5 ± 15.67)	< 0.01	na
VELIFER® (*B. bassiana* PPRI 5339)	3	**96.0 ± 9.00**	(61.1 ± 16.20)	< 0.01	na
Beauveria bassiana XG/2B	1	**85.7 ± 19.33**	(52.3 ± 19.71)	< 0.05	na
Beauveria bassiana XG/2B	2	**91.9 ± 14.61**	(33.6 ± 14.88)	< 0.01	na
Beauveria bassiana XG/2B	3	**70.4 ± 17.77**	(34.5 ± 15.67)	< 0.01	na
Beauveria pseudobassiana AWLB	1	**100 ± 0.00**	(49.7 ± 35.70)	*	ns
Beauveria pseudobassiana AWLB	2	**97.6 ± 3.48**	(15.9 ± 12.30)	*	< 0.01
Beauveria pseudobassiana AWLB	3	**96.2 ± 5.44**	(26.5 ± 8.13)	< 0.01	na
Metarhizium brunneum BIPESCO 5	1	**99.2 ± 1.77**	(52.3 ± 19.71)	*	< 0.01
Metarhizium brunneum BIPESCO 5	2	**99.2 ± 1.77**	(33.6 ± 14.88)	*	< 0.01
Metarhizium brunneum BIPESCO 5	3	**96.1 ± 6.02**	(45.3 ± 14.58)	< 0.01	na
Metarhizium brunneum GT7	1	**100 ± 0.00**	(49.7 ± 35.70)	*	ns
Metarhizium brunneum GT7	2	**100 ± 0.00**	(52.3 ± 19.71)	*	< 0.01
Metarhizium brunneum GT7	3	**100 ± 0.00**	(33.6 ± 14.88)	*	< 0.01
Metarhizium robertsii GT10	1	**100 ± 0.00**	(49.7 ± 35.70)	*	ns
Metarhizium robertsii GT10	2	**99.2 ± 1.86**	(15.9 ± 12.30)	*	< 0.01
Metarhizium robertsii GT10	3	**97.6 ± 5.32**	(26.5 ± 8.13)	< 0.01	na

Egg treatments with the investigated EPF and mycopesticides proved to be an effective control measure under specific and constant laboratory conditions. The present laboratory results indicate a potential suitability of the tested *Beauveria* spp. and *Metarhizium* spp. strains, among them *M. brunneum* BIPESCO 5 (alias Ma43), a registered low-risk active substance (EU, 2022). Apart from our results, there have already been some reports on the efficacy of *Beauveria* isolates against nymphs. As reported by Parker et al. (2015), formulations of BOTANIGARD® (*B. bassiana* GHA) were significantly effective against second instar nymphs. Mantzoukas et al. (2024) reported a *B.*

varroae isolate as a promising biocontrol agent for *H. halys*. Further studies under semi-field and natural field conditions are required in order to substantiate the efficacy of the investigated fungi against *H. halys*. The persistence and efficiency of entomopathogenic fungi are influenced by a variety of factors. Physical and climatic conditions, as well as the use of agrochemicals may inhibit the performance of these fungi in the orchard (Khun et al., 2021; Quesada-Moraga et al., 2024). One aspect that may be potentially advantageous for EPF performance under natural conditions is that egg masses of *H. halys* are mainly deposited on the undersides of leaves, where the conidia are partially protected from harmful solar radiation.

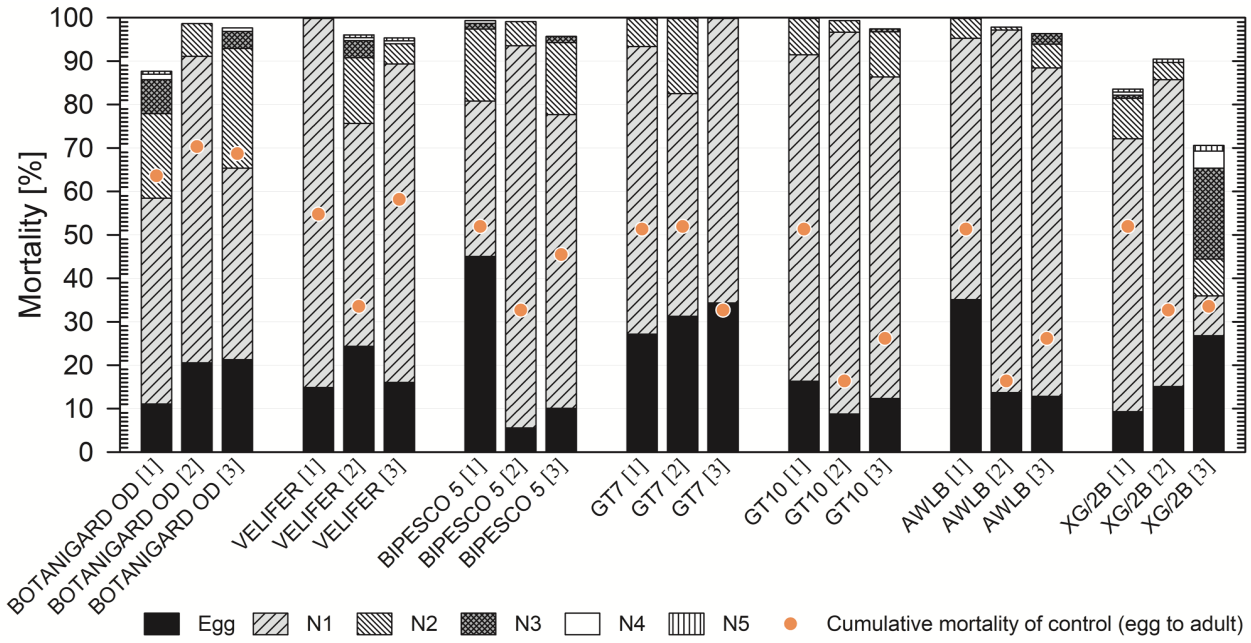

Fig. 1 – Total mortality per treatment. Bars: cumulative mortality of egg and nymphal stages after fungal exposure. Points: ovo-larval mortality after control treatments with water. Mortality data refers to total egg number per treatment (sum of six egg masses).

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