Entomopathogenic nematodes and fungi to control *Hyalesthes obsoletus* (Hemiptera: Auchenorrhyncha: Cixiidae)

Running title: Entomopathogens acting against *Hyalesthes obsoletus*

Abstract

Hyalesthes obsoletus Signoret (Hemiptera: Auchenorrhyncha: Cixiidae) is a univoltine, polyphagous planthopper that completes its life cycle, including the subterranean nymph cryptic stage, on herbaceous weeds. In vineyards, it can transmit '*Candidatus* Phytoplasma solani', an obligate parasitic bacterium associated with Bois noir (BN) disease of grapevine, from its host plants to grapevine when occasionally feeding on the latter. The main disease management strategies are based on vector(s) control. Insecticide treatments on grapevine canopy are completely inefficient on *H. obsoletus*, due to its life cycle; consequently, control of this planthopper focuses on the nymphs living on the roots of their host plants. Such practices, based on herbicide application and/or weed management, can reduce vector density in the vineyard but can impact the environment or may not be applicable, highlighting the necessity for alternative strategies. In this study, the efficacy of entomopathogenic nematodes (EPNs; *Steinernema carpocapsae*, *S. feltiae*, *Heterorhabditis bacteriophora*) and fungi (EPFs; *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea*, *Lecanicillium muscarium*) against *H. obsoletus* nymphs (EPNs) and adults (EPNs and EPFs) was assessed under laboratory and greenhouse conditions. The majority of examined EPNs and EPFs were able to kill *H. obsoletus* exhibiting a range of effectiveness. *S. carpocapsae* (among EPNs) and *I. fumosorosea* (among EPFs) were found to be the most effective biocontrol agents in all trials carried out. Advantages and limitations of such promising biocontrol agents were discussed. Ecological competency and conditions that can impede or enhance the EPNs and EPFs performance should be investigated to optimize their performance under field conditions.

Keywords: planthoppers; grapevine; Bois noir; sustainability**,** entomopathogens

Introduction

Hyalesthes obsoletus Signoret (Hemiptera: Auchenorrhyncha: Cixiidae) is a polyphagous planthopper able to transmit '*Candidatus* Phytoplasma solani' (CaPsol) to plants (Quaglino et al. 2013), including *Vitis vinifera* L. (Maixner 1994). CaPsol, a plant obligate parasitic bacterium, is associated with Bois noir (BN), a disease belonging to the complex of grapevine yellows which had high economic impact on viticulture in Europe in the last decades (Angelini et al. 2018). Although alternative insect vectors of CaPsol to grapevine were recently reported (Cvrkovic et al. 2014; Quaglino et al. 2019), BN epidemiology is principally determined by the life cycle of its main vector *H. obsoletus* (Mori et al. 2013).

H. obsoletus is a palaearctic, univoltine species that, in Europe, completes its life cycle mainly on bindweed (*Convolvulus arvensis* L.) and nettle (*Urtica dioica* L.) (Langer and Maixner 2004) but also on other host plants (Kosovac et al. 2019; Moussa et al. 2019). In summertime, females produce eggs on the root collar of host plants. After egg hatching, the nymphs migrate into the soil to the roots from which they can acquire CaPsol. After a latency period, *H. obsoletus* becomes able to transmit CaPsol to plants for the duration of its life. Overwintering occurs as second-third instar nymphs in the soil. In early spring, fourth and fifth instar nymphs migrate to the soil surface. Adults emerge from end of May till end of June and they fly from beginning of July to end of August, based on climate, host plant, and region (Cargnus et al. 2012; Maixner and Johannesen 2014; Alma et al. 2015).During their flights, *H. obsoletus* adults can occasionally feed on grapevine and, if infected, transmit CaPsol. However, due to their limited feeding activity on grapevine and the short lifespan of the adult stage, they cannot transmit CaPsol from vine to vine. Grapevine is therefore a dead-end host for the pathogen (Bressan et al. 2007).

No effective control measures directly targeting phytoplasmas are available. The main strategies to manage the spreading of phytoplasma-associated diseases are based on preventive measures, including the control of vectors before their emergence from the ground (Bianco et al.

2019). Due to its cryptic life cycle and polyphagous feeding habit, insecticide treatments on grapevine canopy are completely inefficient against *H. obsoletus*. Thus, strategies for its control focus on depriving the nymphs of their feeding substrate, the host plant roots. Before adult emergence, bindweed and nettle can be suppressed by planting of ground covering rosette plants, repeated mowing or weeding (Maixner and Mori 2013; Mori et al. 2014a). Since *H. obsoletus* presence depends on the distribution of its natural plant hosts both within and outside the vineyards, such strategies are limited by restrictions on the use of herbicides in uncultivated areas, as well as mechanical weeding on ditches and embankments because of soil landslide. In Israel, *H. obsoletus* populations within vineyards are successfully limited by a push and pull strategy using chaste tree (*Vitex agnus-castus* L.) (Sharon et al. 2015), but such a strategy cannot be employed in Europe where this plant hosts both *H. obsoletus* and CaPsol (Moussa et al. 2019).

Considering these limitations, a promising approach to control the vector populations could be based on the utilization of biocontrol agents such as entomopathogenic nematodes (EPNs) and fungi (EPFs). In particular, several *Steinernema* and *Heterorhabditis* EPNs have been reported as effective biocontrol agents against a broad range of insects with a cryptic life cycle like *H. obsoletus* (Grewal et al. 2005; Lacey and Georgis 2012; Guerrero and Pardey 2019). EPNs efficacy depends on their survival for a long time without their host targets in the soil, and their ability to find the hosts by ambush (i.e., *Steinernema carpocapsae*) or cruising (i.e., *Heterorhabditis bacteriophora*) strategy (Kaya et al. 1993; Grewal et al. 1994). Concerning EPFs, they are reported as important antagonists of soil-dwelling insect pests adapted to live in agricultural soils, such as the grapevine phylloxera in vineyards (Kirchmair et al. 2004). Interestingly, the EPF *Metharizium anisopliae* showed a great efficacy against *H. obsoletus* adults under laboratory conditions (Langer et al., 2005).

In this study, the efficacy of different EPNs and EPFs against *H. obsoletus* nymphs and adults under laboratory and greenhouse conditions were assessed to develop effective and innovative approaches to control the main vector of CaPsol.

Materials and methods

Hyalesthes obsoletus collection

Nymphs of *H. obsoletus* were collected on two dates immediately before the different experiments. The collection for lab bioassay was done in the middle of May 2019; nymphs were obtained from pots of stinging nettle plants at the rearing facility in Julius Kühn-Institute in Siebeldingen, Germany; collection for the greenhouse trials was performed in late May 2019 from roots of nettle plants growing at the borders of a highly BN-affected vineyard in Mosel area (49.9198°N, 7.0627°E), Germany. Nymphs were placed in falcon tubes filled with the same soil in which they were collected and sent to the lab for trials. Nymphs identity was confirmed based on the taxonomic key by Stöckmann et al. (2013).

Adults of *H. obsoletus* were collected from bindweed and stinging nettle plants using a sweep net and mouth aspirator from mid-June till the end of July 2019, based on the trial requirements, in the vicinity of highly BN-affected vineyards in Mosel area (49.9198°N, 7.0627°E, and 49.1928°N, 8.0830°E), Germany. Collected adults were placed in collapsible insect mesh cages with shoots of nettle plants as food source, transferred to the lab, and identified by taxonomic key (Bertin et al. 2010). *H. obsoletus* adults were subject to immediate use in bioassays and greenhouse efficacy trials.

Entomopathogenic nematodes

Three EPNs were applied in both bioassays and greenhouse trials against *H. obsoletus* nymphs and adults. In detail, the utilized EPNs (*Steinernema carpocapsae, S. feltiae, Heterorhabditis bacteriophora*, and a combination (1:1) of *S. feltiae* and *H. bacteriophora*) were purchased from E-nema® Company (Schwentinental, Germany) (Table 1) and maintained at 4 °C. Immediately before using, each EPN (supplied in powder) was suspended in tap water and tested

for its viability by counting the infective juveniles (IJs) under stereomicroscope. IJs without a response to stimulators were considered dead (Lacey 1997). All EPNs showed a viability higher than 95%. Before the application, each EPN was serially diluted in tap water to reach the required concentration for bioassay (200 IJs ml^{-1}) and greenhouse trials (400 IJs ml^{-1}) (Guerrero and Pardey 2019).

Entomopathogenic fungi

Three distinct EPF isolates of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosoroseus* (provided by Dr. Dietrich Stephan - Institute for Biological Control, Julius Kühn-Institut, Darmstadt, Germany), were applied in preliminary screening against *H. obsoletus* adults. In addition to these isolates, one commercial isolate of *M. anisopliae* and one of *Lecanicillium muscarium* were purchased from Koppert Biological Systems Company (Verona, Italy) and applied in greenhouse trials against *H. obsoletus* adults (Table 1). EPFs were cultivated in Petri dishes on malt extract peptone agar (30 g of malt extract, 3 g of peptone, 15 g of agar in 1 l of distilled water) previously autoclaved at 121 °C for 10 min. After inoculation, EPFs were grown for 5 days at room temperature (22 °C) and for 6 days at 4 °C. For each EPF isolate, fungal conidia were recovered from the mycelium, suspended in distilled water and counted (three times per isolate) at 400X magnification through a haemocytometer. For each EPF isolate, stock solutions of conidial suspension were prepared at the concentrations of 10^6, 10^8 and 10^{10} conidia ml^{-1} and stored at 4 °C until use.

Entomopathogenic nematodes: laboratory bioassays

Bioassay to evaluate the efficacy of EPNs against *H. obsoletus* nymphs was conducted in 12-well cell culture plates with lids. According to the protocols of Kaya and Stock (1997) and Lewis (2000), in each well, filled with 1 g of autoclaved sand, 1 ml of EPN suspension (200 IJs ml^{-1}) was applied and one nymph was placed; control plates were treated with distilled water before placing

139 the nymphs. Bioassay to evaluate the efficacy of EPNs against *H. obsoletus* adults was conducted in
140 plastic Petri dishes (9 cm diameter) padded with filter paper. Following Lewis (2000) procedures, in
141 each dish, fresh shoots of stinging nettle (5 cm) were placed as a food source together with 12
142 adults of *H. obsoletus*, and 1 ml of EPN suspension (200 IJs ml^{-1}) was applied using a hand-held
143 sprayer. Control dishes were sprayed with distilled water before placing the adults. The edges of
144 Petri dishes were dried with tissue paper to prevent the adults sticking to water droplets. Three
145 replicates of each plate (nymphs)/Petri dish (adults) were made per EPN as well as the control. All
146 plates/dishes were placed in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6 days.
147 Mortality readings were taken daily for 6 consecutive days. To confirm that insect mortality was
148 caused by the activity of the EPNs, the presence of EPN was evaluated by dissecting nymphs and
149 adult insect bodies under a 40X magnifying stereomicroscope. This was done after rinsing single
150 dead nymphs and adults in a conical flask filled with 20 ml of distilled water, for removing
151 nematodes from their surface. Nymphs and adults were then placed on a moist filter paper padded
152 plastic Petri dish and maintained at 25 °C for 3 days according to Glazer and Lewis (2000).
153
154 Entomopathogenic nematodes: greenhouse trials
155 To evaluate the efficacy of EPNs against *H. obsoletus* nymphs and adults, greenhouse trials
156 were conducted on potted stinging nettle (*Urtica dioca* L.) and faba bean (*Vicia faba* L.) plants,
157 respectively. The nettle plants (diameter about 0.1 m and height approximately 0.4 m) were grown
158 in 3.0 l pots, while the bean plants (diameter about 0.1 m and height approximately 0.25 m) in 1.0 l
159 pots and placed in transparent plastic ventilated cages; all plants were in good vegetative condition
160 and did not show symptoms of biotic and abiotic stresses. In each pot with nettle, 5 ml of EPN
161 suspension (400 IJs ml^{-1}) was applied to the soil using a hand-held sprayer, and a total of 20
162 nymphs were placed after treatment. The control potted plants were treated with 5 ml of distilled
163 water before placing the nymphs. On each caged bean plant, 10 ml of EPN suspension (400 IJs ml^{-1},
164 with 0.02% Tween 80) was applied using a hand-held sprayer, and 15 *H. obsoletus* adults were

165 released. The control caged plants were treated with 10 ml of distilled water with 0.02% Tween 80
166 before releasing insect adults. Three replicates, arranged in randomized blocks, were made per EPN
167 as well as the control. Potted plants were kept in a controlled chamber (25 °C, 72% RH, 16:8
168 photoperiod). Mortality was recorded 6 days after treatment. As described above for the bioassay,
169 mortality due to nematodes infection was confirmed by dissecting the dead nymph and adult insect
170 bodies under a 40X magnifying stereomicroscope.
171
172 Entomopathogenic fungi: laboratory bioassays
173 Initially, the nine EPF isolates provided by the Institute for Biological Control - Julius
174 Kühn-Institute (JKI) (Table 1) were screened for their entomopathogenic activity against *H.*
175 *obsoletus* adults in plastic Petri dishes (8.5 cm diameter), filled to a depth of 5 mm with a mixture
176 of plaster of Paris and charcoal (10:1) and moistened with distilled water (Green 1964; Maixner
177 2005). In each Petri dish, fresh bindweed (*Convolvulus arvensis* L.) shoots (5 cm) were placed as a
178 food source, 1 ml of EPF conidia suspension (10^8 conidia ml^{-1}) was applied using a hand-held
179 sprayer, and 12 adults were released. The control dishes were treated with distilled water before the
180 release of insects. Three replicates were conducted per EPF isolate as well as the control. Petri
181 dishes were kept in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod). Mortality readings
182 were taken after 6 days. Single dead insects from each EPF isolate were placed in malt extract
183 peptone agar plates kept for 3 days at room temperature (22 °C) and for 5 days at 4 °C. Mortality
184 due to EPF infection was confirmed through the observation of EPF mycelium growth on the insect
185 body. Subsequently, EPF isolates, found active against *H. obsoletus* in the initial screening test,
186 were employed in a bioassay applying conidial suspension at different concentrations (10^6, 10^8, 10^{10}
187 conidia ml^{-1}). The bioassays were conducted in plastic Petri dishes as described above for the initial
188 screening test. Three replicates were conducted per concentration per EPF isolate as well as the
189 control. Petri dishes were kept in controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6
190 days. Mortality readings were taken daily for 6 consecutive days. Single dead insects from each

bioassay were placed in malt extract peptone agar plates and checked to confirm EPF-related mortality as described above.

Entomopathogenic fungi: greenhouse trials

Greenhouse trials were conducted on potted stinging nettle plants, placed singly in transparent plastic ventilated cages, to evaluate the efficacy against *H. obsoletus* adults of the previously selected JKI EPF isolates along with two commercial EPFs (*M. anisopliae* and *L. muscarium*). On each caged plant, 1 ml of EPF suspension (10^8 conidia ml^{-1}) was applied on the potted plants using a hand-held sprayer, and 15 *H. obsoletus* adults were released. The control caged plants were treated with 1 ml of distilled water before the release of insects. Three replicates were made per EPF as well as the control. Caged potted plants were kept in a controlled chamber (25 °C, 72% RH, 16:8 photoperiod) for 6 days. Mortality readings were taken after 6 days. EPF-related mortality was confirmed by observing the EPFs mycelium growth directly on insect bodies on stinging nettle or placing single dead insects in malt extract peptone agar plates as described above.

Statistical analyses

In the bioassays, the median lethal time (LT_{50}) for EPNs against nymphs and adults was calculated from daily mortality data. The median lethal concentration (LC_{50}) of EPFs against adults was log_{10} transformed and calculated from the data obtained on the 4th day post-inoculation (dpi). LT_{50} and LC_{50} results with their fiducial confidence limits were obtained by R statistical package "ecotox" (Hlina 2020) based on the probit analysis. In the screening (EPFs) and greenhouse trials (EPNs and EPFs), mortality data of nymphs and adults were tested for normality and equality of variance with the Shapiro-Wilk test followed by Levene's test of homogeneity of variance. Greenhouse trials data were subjected to ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons. Graphical representations of the statistical

analysis results were produced using R statistical package "ggplot2" (Wickham et al. 2020). All the analyses were done using R (version 3.6.2) (R Core Team 2019).

Results

Biocontrol efficacy of entomopathogenic nematodes against *H. obsoletus* nymphs and adults

In the bioassay, *S. carpocapsae* was the most effective with a LT_{50} value of 3.24 and 3.69 days post-inoculation (dpi) for *H. obsoletus* nymphs and adults, respectively (Fig. 1a, b). *S. feltiae* was the second most effective with a LT_{50} of 3.49 and 4.05 dpi for nymphs and adults, respectively (Fig. 1c, d). The mixture of *H. bacteriophora* and *S. feltiae* showed a LT_{50} of 3.65 and 4.61 dpi for nymphs and adults, respectively (Fig. 1e, f). *H. bacteriophora* (Hf) was the least effective with a LT_{50} of 4.34 and 5.06 dpi for nymphs for adults (Fig. 1g, h) (Supplementary Material Table S1). In the greenhouse trials, results of ANOVA [nymphs ($F_{4,10} = 82.55$; $p < 0.001$); adults ($F_{4,10} = 87.96$; $p < 0.001$)], followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* nymphs and adults treated with the different EPNs were significantly higher than the non-treated control. Among EPNs, *S. carpocapsae* showed the highest efficacy against *H. obsoletus* nymphs (average mortality rate 86.67%) and adults (81.67%), while *H. bacteriophora* the lowest (56.67% vs nymphs; 55.00% vs adults) (Fig. 2a, b; Supplementary Material Table S2).

Biocontrol efficacy of entomopathogenic fungi against *H. obsoletus* adults

In the initial screening, results of ANOVA ($F_{9,20} = 20.39$; $p < 0.001$), followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* adults were significantly higher than the non-treated control in all EPFs treatments, except *B. bassiana* strain 1124 and *M. anisopliae* 1428 (Fig. 2c). In the bioassay, the seven effective EPFs were employed to define their proper concentration leading to 50% of mortality of *H. obsoletus* adults. *I. fumosorosea* strains 1497 and 1499 were found to be the most virulent against *H. obsoletus* adults with a LC_{50} ($\log_{10}$

concentration) of 6.07 and 6.46 conidia ml^{-1}, respectively. *M. anisopliae* strains 1429 and 1430 showed a LC_{50} of 8.21 and 8.51 conidia ml^{-1}, respectively. *B. bassiana* strains1125 and 1126 were the least virulent with a LC_{50} of 8.89 and 9.23 conidia ml^{-1}, respectively (Fig. 3; Supplementary Material Table S3). In the greenhouse trials, results of ANOVA ($F_{9,20} = 23.87$, $p < 0.001$), followed by Tukey's HSD test, revealed that the average mortality rate of *H. obsoletus* adults treated with the different EPFs were significantly higher than the non-treated control. Among EPFs, *I. fumosorosea* strain 1497 showed the highest efficacy against *H. obsoletus* adults (average mortality rate 91.1%). This percentage was not significantly different in comparison to that obtained by *I. fumosorosea* strain 1499 (84.45%), *M. anisopliae* strain 1111 (75.56%), and *L. muscarium* strain 2222 (80.00%). On the contrary, it was significantly different in comparison to that obtained by *M. anisopliae* strains 1429 (68.89%) and 1430 (66.67%), and *B. bassiana* strains 1125 (68.89%). *B. bassiana* strain 1126 showed the lowest efficacy (60.00%) (Fig. 2d; Supplementary Material Table S4).

Discussion

Due to the complex life cycle of *Hyalesthes obsoletus*, most strategies to control its populations in the vineyard agro-ecosystem are not effective or can impact the environment (Maixner and Mori 2013). In the last years, biocontrol has been proposed and frequently utilized as sustainable strategy to control plant pathogen insect vectors (Kumar 2016; Abdel-Razek et al. 2017; Abd El-Ghany et al. 2018). Entomopathogenic nematodes (EPNs) and fungi (EPFs) have been largely employed as effective biocontrol agents against insects with a cryptic life cycle, including phytoplasma vectors (Grewal et al.2005; Lacey and Georgis 2012; Guerrero and Pardey 2019), making this approach promising also for the main vector of '*Candidatus* Phytoplasma solani' to grapevine, *H. obsoletus*.

The results obtained in this study demonstrated that all the examined EPNs are able to kill *H. obsoletus* nymphs and adults and that the EPFs, except *Beauveria bassiana* strain 1124 and *Metharizium anisoploae* strain 1428, are able to control the adults in both laboratory bioassays and

268 greenhouse trials, exhibiting a range of effectiveness related to their virulence against the target
269 insect. In all conducted trials, *Steinernema carpocapsae* and *Isaria fumosorosea* were found to be
270 the most effective biocontrol agents of *H. obsoletus* among the examined EPNs and EPFs,
271 respectively.

272 Concerning *Steinernema* spp., our findings are in agreement with Le Vieux et al. (2013)
273 showing that, in laboratory bioassay performed against the vine mealy bug *Planococcus ficus*, the
274 EPN *Steinernema yirgalemense* moved 15 cm vertically downward, and infected its insect target
275 inducing a mortality of 95%. Another study demonstrated that the combination of *S. yirgalemense*
276 with specific adjuvants increased its biocontrol activity against the vine mealy bug on grapevine
277 leaves in both laboratory and semi-field conditions (Platt et al. 2019). Such ev idence fortifies the
278 possibility of applying *Steinernema* spp. in the open field against both subterranean forms and
279 adults of *H. obsoletus*. Among tested EPNs, also *Heterorhabditis bacteriophora* showed a high
280 efficacy in *H. obsoletus* biocontrol. Interestingly, this EPN was reported to be effective against the
281 nymphs of *Haplaxius crudus*, the insect vector of '*Candidatus* Phytoplasma palmae' associated
282 with Palm Lethal Yellowing disease in Florida (Guerrero and Pardey 2019), and of *Aeneolamia*
283 spp., a putative vector of genetically distinct phytoplasmas (Pérez et al. 2018). Moreover, *H.*
284 *bacteriophora* strongly reduced the survival of the root-form of grapevine phylloxera (English-Loeb
285 et al. 1999). This evidence highlighted that, in vineyard agroecosystems, treatments based on the
286 application of *H. bacteriophora* could be effective against multiple insect pests. Based on all these
287 evidences, it would be interesting to apply a combination of *S. carpocapsae*, found here as more
288 effective against *H. obsoletus* nymphs and adults, and *H. bacteriophora*, reported in previous
289 studies as the most effective EPN against phytoplasma insect vectors and grapevine insect pests
290 with a cryptic life stage.

291 Concerning *Isaria fumosorosea*, found here as the most effective EPF against *H. obsoletus*,
292 previous studies showed its biocontrol activity against various nymphal stages of the green
293 leafhopper *Empoasca decipiens* Paoli under laboratory and greenhouse conditions (Tonou et al.

294 2003; Kodjo et al. 2011). Similar efficacy was found by treatments with *Metarhizium anisopliae*
295 (strain Ma43) and *Beauveria bassiana* (strain Bba113) (Tonou et al. 2003; Kodjo et al. 2011). For
296 all these EPFs, percentage of mortality and LC_{50} values reported against *E. decipiens* were
297 comparable to those observed in this study against *H. obsoletus* adults. Moreover, promising results
298 obtained in the present study with two strains of *M. anisopliae* confirmed its entomopathogenic
299 activity against *H. obsoletus* adults under laboratory conditions (Langer et al. 2005). Interestingly,
300 *Beauveria bassiana*, two strains of which showed a great biocontrol activity against *H. obsoletus*
301 adults in the present work, was found naturally infecting and causing visual symptoms on *H.*
302 *obsoletus* adults in Georgia (Caucasus region) (Chkhaidze et al. 2017). Moreover, *B. bassiana*
303 showed an efficacy in biocontrol of young stages and adults of *Scaphoideus titanus* Ball, the insect
304 vector of Flavescence dorée phytoplasma, in semi-field and field trials (Mori et al. 2014b). All these
305 evidences underlined that *B. bassiana* can control both the insect vectors of phytoplasmas
306 associated with the main grapevine yellows diseases; thus, *B. bassiana* strains represent really
307 promising EPFs for application in vineyards.

308 Effectiveness of EPNs and EPFs, as well as other living organisms used as biocontrol
309 agents, depends on a range of climatic and environmental parameters allowing their liveliness and
310 entomopathogenic activity. In particular, it is crucial that the target insect stage is present when
311 climatic parameters are optimal for EPNs and/or EPFs (Lacey and Georgis 2012; Wang and Wang
312 2017). In the case of *H. obsoletus*, it is known that the duration of the cryptic (subterranean) phase
313 of its life cycle, involving the nymph stages, is dependent on the degree day units that can be
314 estimated based on forecasting models measuring the accumulated heat units (Maixner and Mori
315 2013). Such models allow understanding of the life cycle of the insect as well as narrowing the
316 spraying window of products for plant protection, including EPNs and EPFs. In particular, the
317 spraying window should prioritize two important aspects: (i) the ecological competency of EPNs as
318 well as EPFs; (ii) the proper timing for application against the different stages and instars of *H.*
319 *obsoletus*. In Europe and the Mediterranean area, considering the life cycle of *H. obsoletus* and the

environmental conditions suitable for EPNs and EPFs utilized in the present study, it should be recommended to apply EPNs and EPFs on *H. obsoletus* host plants in the open field from mid of September to October and/or in early spring to optimize the activity of each biocontrol agent and avoid resistance in the insect target populations. Moreover, given their ability to colonize the soil after their inoculation (Meyling and Eilenberg 2006; Denno et al. 2008), EPNs and EPFs could reduce the *H. obsoletus* population density for a long time. According to our result EPFs could be applied also with foliar application from end of May till end of June against newly hatched adults before grapevine infestation. Optimized application of entomopathogenic nematodes (on the soil) and fungi (on the plants) can increase the control of *H. obsoletus* nymphs and adults, respectively.

In conclusion, the majority of EPNs and EPFs utilized in the present study showed a considerable biocontrol activity against *H. obsoletus* nymphs and adults in laboratory bioassays and greenhouse trials. The ecological competency of both EPNs and EPFs, the conditions that can impede or enhance their performance, the barriers that can block infection from taking place on the target host, and the possible actions on non-target species should be carefully investigated for a better understanding of their potential performance under field conditions.

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Acknowledgments

Compliance with ethical standard

Conflict of interest

Authors declare no conflict of interest.

Ethical approval

This research did not involve participation of animals or humans.

463 **Figure captions**

464 **Fig. 1**. Time-to-death curve of different EPNs against nymphs and adults of *H. obsoletus*. Black
465 dots represent the observations. Black curves were computed using the glm smoothing method
466 within ecotox. Shaded grey areas represent the 95% confidence intervals (not visible due to small
467 size). *S. carpocapsae* (Sc) vs *H. obsoletus* nymphs (**a**) and adults (**b**); *S. feltiae* (Sf) vs nymphs (**c**)
468 and adults (**d**); *H. bacteriophora* + *S. feltiae* (Hb + Sf) vs nymphs (**e**) and adults (**f**); *H.*
469 *bacteriophora* (Hb) vs nymphs (**g**) and adults (**h**).

470 **Fig. 2.** Average mortality rate of *Hyalesthes obsoletus* nymphs and adults treated with EPNs and
471 EPFs. EPNs greenhouse trials vs *H. obsoletus* nymphs (**a**) and adults (**b**); EPFs initial screening test
472 vs *H. obsoletus* adults (**c**); EPFs greenhouse trials vs *H. obsoletus* adults (**d**). On each bar: letters (a-
473 d) indicate significant differences ($p < 0.05$) based on ANOVA followed by Tukey's HSD test; bars
474 indicate the standard errors (SE). Acronyms in (**a**) and (**b**): C (non-treated control); Hb + Sf (*H.*
475 *bacteriophora* + *S. feltiae*); Hb (*H. bacteriophora*); Sc (*S. carpocapsae*); Sf (*S. feltiae*). Acronyms
476 in (**c**) and (**d**): 1124, 1125, 1126 (*B. bassiana* strains JKI-BI-1124, JKI-BI-1125, JKI-BI-1126);
477 1428, 1429, 1430 (*M. anisopliae* strains JKI-BI-1428, JKI-BI-1429, JKI-BI-1430); 1497, 1499,
478 1500 (*I. fumosorosea* strains JKI-BI-1497, JKI-BI-1499, JKI-BI-1500); 1111 (*M. anisopliae* strain
479 1111); 2222 (*L. muscarium* strain 2222); C (non-treated control).

480 **Fig. 3**. Dose-response curve of EPFs against *H. obsoletus* adults. Black dots represent the
481 observations. Black curves were computed using the glm smoothing method within ecotox. Shaded
482 grey areas represent the 95% confidence intervals. *B. bassiana* strains JKI-BI-1125 (1125) (**a**), JKI-
483 BI-1126 (1126) (**b**); *M. anisopliae* strains JKI-BI-1429 (1429) (**c**), JKI-BI-1430 (1430) (**d**); *I.*
484 *fumosorosea* strains JKI-BI-1497 (1497) (**e**), JKI-BI-1499 (1499) (**f**), JKI-BI-1500 (1500) (**g**).

485 **Table 1**. Entomopathogenic nematodes (EPNs) and fungi (EPFs) used against *Hyalesthes obsoletus*

486 adults and nymphs

Agent	Scientific name	Strain acronym	Source	*H. obsoletus* target stage
EPNs	*Steinernema carpocapsae*	Sc	E-nema®	nymphs and adults
	Heterorhabditis bacteriophora	Hb		
	Steinernema feltiae	Sf		
	S. feltiae & *H. bacteriophora*	Hb+Sc		
EPFs	*Beauveria bassiana*	JKI-BI-1124	Julius Kühn-Institut	adults
		JKI-BI-1125		
		JKI-BI-1126		
	Metarhizium anisopliae	JKI-BI-1428		
		JKI-BI-1429		
		JKI-BI-1430		
	Isaria fumosorosea	JKI-BI-1497		
		JKI-BI-1499		
		JKI-BI-1500		
	Metarhizium anisopliae	1111	Koppert Biological Systems	
	Lecanicillium muscarium	2222	Company	

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Fig.1

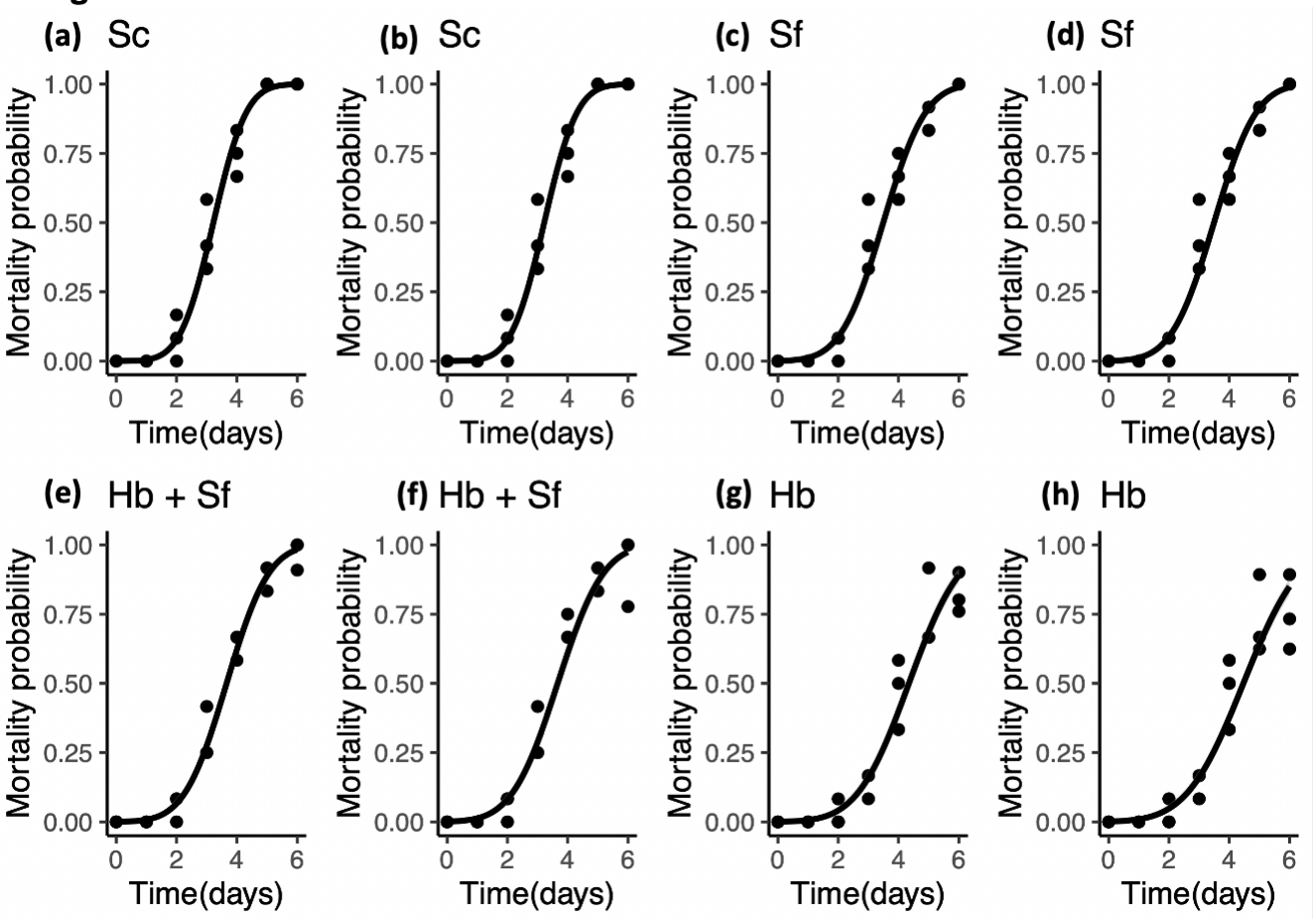

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Fig.2

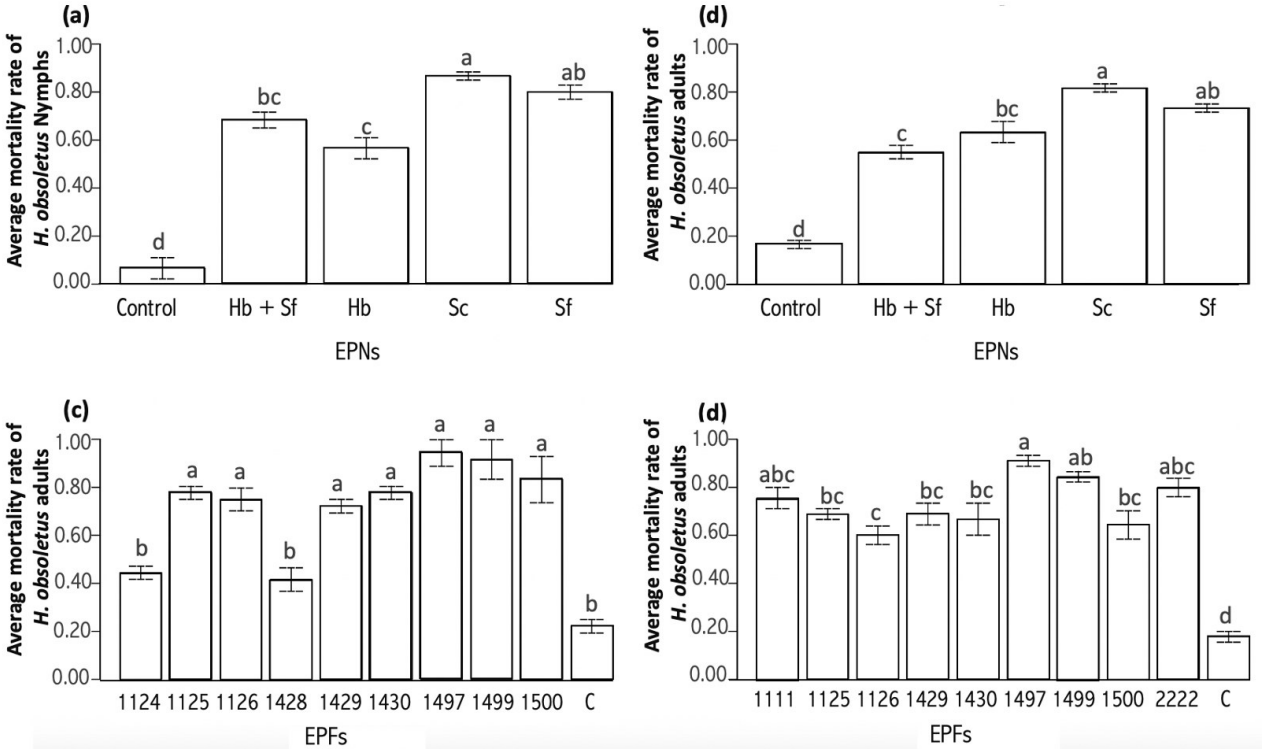

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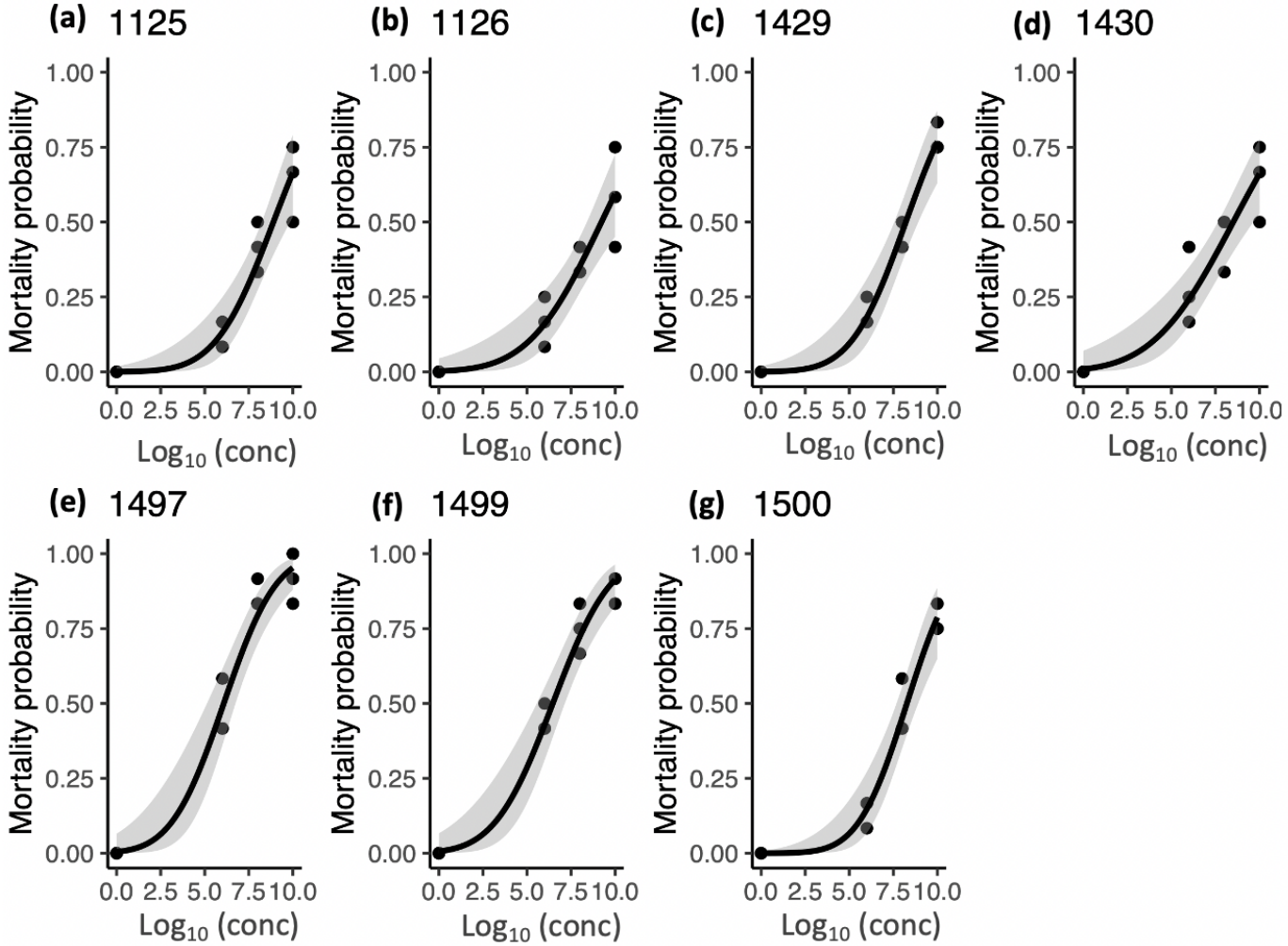
Fig.3



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Electronic Supplementary Material

Table S1. Median lethal time (LT_{50}) of different EPNs against nymphs and adults of *H. obsoletus*. Means represent the accumulated mortality per treatment. Hb (*H. bacteriophora*), Sf + Hb (*S. feltiae* + *H. bacteriophora*), Sc (*S. carpocapsae*), Sf (*S. feltiae*); dpi (days post inoculation).

Treatment	Nymphs		Adults	
	Accumulated % mortality mean ± SE	LT_{50} (dpi) [a]	Accumulated % mortality mean ± SE	LT_{50} (dpi) [a]
Hb	31.57 ± 7.62	4.34 (4.11 – 4.59)	23.48 ± 5.66	5.06 (4.77 – 5.42)
Sf + Hb	43.25 ± 8.84	3.65 (3.50 – 3.80)	24.14 ± 5.15	4.61 (4.25 – 5.03)
Sc	46.82 ± 9.46	3.24 (3.06 – 3.37)	40.08 ± 8.44	3.69 (3.46 – 3.90)
Sf	40.83 ± 8.78	3.49 (3.30 – 3.68)	34.92 ± 8.21	4.05 (3.82 – 4.29)

[a] 95% lower and upper fiducial limits are shown in parentheses

516 **Table S2**. Average mortality rate of *Hyalesthes obsoletus* nymphs and adults treated with EPNs.
517 Sc (*S. carpocapsae*), Sf (*S. feltiae*), Sf + Hb (*S. feltiae* + *H. bacteriophora*), Hb (*H.*
518 *bacteriophora*).

519

Treatment	Nymphs		Adults	
	Mean ± SE	Group	Mean ± SE	Group
Sc	86.67 ± 1.67	a	81.67 ± 1.66	a
Sf	80.00 ± 2.89	ab	73.33 ± 1.66	ab
Sf+Hb	68.33 ± 3.33	bc	63.00 ± 4.41	bc
Hb	56.67 ± 4.41	c	55.00 ± 2.89	c
Control	6.67 ± 4.41	d	16.67 ± 1.67	d

520
521 Means followed by similar letters in the group column are not significantly different according to
522 Tukey's HSD test ($p \geq 0.05$)

523 **Table S3**. LC_{50} of EPFs against *Hyalesthes obsoletus* adults calculated 4 days after treatment.

524 Means represent the mortality rate per cage.

525

EPFs	Strain	log_{10} conc	% mortality mean ± SE	log_{10} LC_{50} [a]
B. bassiana	JKI-BI-1125	6	11.11 ± 2.77	8.89 (8.43 - 9.46)
		8	41.67 ± 4.81	
		10	63.89 ± 7.84	
	JKI-BI-1126	6	16.66 ± 4.81	9.23 (8.59 – 10.15)
		8	36.11 ± 2.78	
		10	58.33 ± 9.68	
M. anisopliae	JKI-BI-1429	6	19.44 ± 2.77	8.21 (7.99 – 8.43)
		8	44.44 ± 2.77	
		10	77.78 ± 2.78	
	JKI-BI-1430	6	27.78 ± 7.35	8.51 (7.78 – 9.41)
		8	44.44 ± 5.56	
		10	63.89 ± 7.35	
I. fumosorosea	JKI-BI-1497	6	47.22 ± 5.55	6.37 (5.91 – 7.29)
		8	86.11 ± 2.78	
		10	91.67 ± 4.81	
	JKI-BI-1499	6	44.44 ± 2.78	6.46 (5.99 – 6.85)
		8	75.00 ± 4.80	
		10	88.89 ± 2.78	
	JKI-BI-1500	6	13.89 ± 2.78	8.27 (7.99 – 8.55)
		8	47.22 ± 5.56	
		10	77.78 ± 2.78	

526 [a] 95% lower and upper fiducial limits are shown in parentheses

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531 **Table S4**. Mean mortality rate of *Hyalesthes obsoletus* adults treated with EPFs in greenhouse.

532

EPFs	Strain	% mortality mean ± SE	Group
Control	-	17.78 ± 2.20	d
B. bassiana	JKI-BI-1125	68.89 ± 2.20	bc
	JKI-BI-1126	60.00 ± 3.85	c
M. anisopliae	JKI-BI-1429	68.89 ± 4.40	bc
	JKI-BI-1430	66.67 ± 6.67	bc
I. fumosorosea	JKI-BI-1497	91.10 ± 2.20	a
	JKI-BI-1499	84.45 ± 2.20	ab
	JKI-BI-1500	64.40 ± 5.88	bc
M. anisopliae	JKI-BI-1111	75.56 ± 4.40	abc
L. muscarium	JKI-BI-2222	80.00 ± 3.85	abc

533

534 Means followed by similar letters in the group column are not significantly different according to Tukey's HSD ($p \geq 0.05$)