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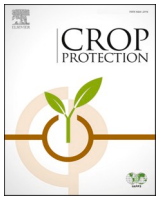

Field evaluation of symbiont-targeted control of *Halyomorpha halys* in hazelnut crop

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ABSTRACT

Halyomorpha halys has emerged as one of the most damaging pests for hazelnut production. Due to the continuous reduction of available options for chemical control, alternative strategies are required. A promising and sustainable alternative is the use of symbiont-targeted control. Treatment of the egg surface with a biocomplex containing copper, zinc, and citric acid has been shown to effectively prevent the acquisition of the endosymbiont '*Candidatus* Pantoea carbekii' by *H. halys* nymphs, resulting in high nymph mortality under laboratory conditions. The aim of this work was to assess the field efficacy of treatments targeting the *H. halys* symbiont in hazelnut orchards to optimize spray applications. Treatments with the anti-symbiont biocomplex were performed in an orchard infested with *H. halys* during 2022 and 2023. To assess product efficacy, *H. halys* egg masses were glued on cardboard tags and hung on different positions of plants canopy before the treatments. The wetting percentage of egg masses according to the position of the egg on the plant canopy was measured; eggs were then reared to evaluate nymphal mortality after hatching. Symbiont acquisition was measured by quantitative PCR. A preliminary test to set the spraying conditions revealed that a higher fan speed induced higher mortality of the newborns from treated egg masses, although it did not affect the wetting rate of cardboards. In addition, cardboard tags hung in the central part of the canopy and those closer to the sprayer showed the highest egg coverage, whereas those in the peripheral row were less covered. However, nymphal mortality was not correlated with the percentage of cardboard tags coverage, although it was correlated with symbiont acquisition. A significant reduction in the percentage of damaged hazelnuts was observed in the treated plot compared to an untreated control, confirming the efficacy of symbiont-targeted control in hazelnut orchards.

1. Introduction

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) is an invasive stink bug pest, well known for its broad host spectrum (Lee et al., 2013; Leskey and Nielsen, 2018; Morrison III et al., 2018) and native to eastern China, Korea and Japan (Xu et al., 2014). First reported outside its native range in Pennsylvania (Hoebeke and Carter, 2003), this insect is now widely distributed in North America (Abram et al., 2017; Leskey and Nielsen, 2018) and Europe (Cesari et al., 2018; Gariepy et al., 2021). In the recent years, the distribution has continued to expand, with populations detected for the first time in South America (Chile) (Faúndez and Rider, 2017), North Africa (Morocco) (Nouere et al., 2020) and in Central Asia (Uzbekistan, Kazakhstan) (Temreshev et al., 2018; Gandjaeva et al., 2022), showing a high degree of adaptation to local environmental conditions (Reznik et al., 2023).

Crop damage can be a direct consequence of the piercing-sucking feeding behaviour of *H. halys* (Bariselli et al., 2016; Vetek and Koranyi, 2017; Bosco et al., 2018; Daher et al., 2023; Ozdemir et al., 2023; Di Serio et al., 2024), or indirect due to the development of pathogens after feeding (Mitchell, 2004; Rice et al., 2014; Opoku et al., 2019; Scaccini et al., 2024a). In native regions, occasional outbreaks are reported with losses in apple, pear and peach production among the other crops (Lee et al., 2013). In invaded areas, high economic losses have been reported in several crops over the years. In 2010, apple and peach production was severely affected in the Mid-Atlantic states of the USA (Leskey et al., 2012). In Italy, mainly pears, but also persimmons, tomatoes, peaches and plums, were severely damaged (Bariselli et al., 2016). Damos et al. (2019) recorded up to 87% of damaged olives in Greece in 2018. Significant damage was also reported on kiwifruit, dry beans and green hot peppers in Europe (Bernardinelli et al., 2017; Vetek and Koranyi, 2017; Andreadis et al., 2018). Moreover, among the feeding hosts, hazelnuts are one of the most threatened in highly

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productive regions such as Italy, Turkey or Georgia (Bosco et al., 2018; Ak et al., 2019; de Benedetta et al., 2023). Damage varies according to the phenological stage of hazelnut development. Feeding before kernel expansion results in higher abortion rate, while feeding soon after kernel expansion results in shrivelled kernels. Piercing activity in late stages of kernel development results in corking damage (Hedstrom et al., 2014).

Chemical control of *H. halys* relies mainly on broad-spectrum insecticides, mostly belonging to the pyrethroid, neonicotinoid, carbamate and organophosphate classes (Lee et al., 2013; Kuhar and Kamminga, 2017). However, the better performing insecticides are not compatible with IPM strategies, and most of them are currently not allowed in some of the invaded areas (e.g., Europe). Furthermore, the tendency of *H. halys* adults to fly away after disturbance makes open field treatments harmful for non-target organisms and potentially less effective (Lee et al., 2013; Kuhar and Kamminga, 2017). Several authors have suggested improvements to reduce the environmental impact of chemical control, such as using pheromones or attractive plants for attract-and-kill strategy or treating only the perimeter plants of orchards (Lee et al., 2013; Blaauw et al., 2015; Bosco et al., 2018; Kuhar and Kamminga, 2017). Sustainable alternatives to chemical control include physical exclusion (Chouinard et al., 2016; Candian et al., 2018, 2020, 2021; Fornasiero et al., 2023; Vergnani et al., 2023; Marshall and Beers, 2024), application of repellents such as sulphur (Scaccini et al., 2024b), treatments with plant extracts (Bulgarini et al., 2021), and biological control with egg parasitoids introduced from the native region of *H. halys* (Haye et al., 2020; Konopka et al., 2017; Scaccini et al., 2020; Falagiarda et al., 2023; Tortorici et al., 2023). Another low-impact control strategy that is constantly gaining interest is symbiotic control by means of symbiont-targeted methods. This strategy is based on preventing the acquisition of the primary endosymbiont by newborn nymphs, resulting in their rapid death (Gonella and Alma, 2023). Indeed, the primary endosymbiont of *H. halys*, '*Candidatus* Pantoea carbekii' (hereafter *P. carbekii*), is hosted in crypts of the midgut caeca and it is released a on the egg surface during ovipositions as part of a maternal secretion. Newly hatched nymphs acquire the endosymbiont by feeding on the eggs (Bansal et al., 2014; Kenyon et al., 2015). Preventing *P. carbekii* acquisition by *H. halys* nymphs results in high mortality rates in the first instars, while the few surviving insects suffer severe developmental impairment, reduced fertility and high mortality in the sibling generation (Taylor et al., 2014; Kenyon et al., 2015; Gonella et al., 2019). Several compounds have been tested for symbiont-targeted control of *H. halys* (Mathews and Barry, 2014; Taylor et al., 2017; Gonella et al., 2019, 2022), among which a micronutrient biocomplex containing copper, zinc and citric acid showed promising potential, as it completely prevented symbiont acquisition by newly hatched nymphs and resulted in high nymph mortality under laboratory conditions (Gonella et al., 2019, 2022).

Given the high potential of symbiont-targeted interventions, and since scaling up from the laboratory to the field can result in different treatment outcomes (Gonella et al., 2020), the aim of this work is to evaluate the anti-symbiont efficacy of field applications using the micronutrient biocomplex that has previously shown high symbiont-targeted containment of *H. halys*. The efficacy of spraying was evaluated on *H. halys* egg masses glued on cardboard tags with water-sensitive paper, placed on different positions, both in the field and on the plant. Our evaluation took into account: i) the percentage of egg mass coverage, ii) the effect on nymphal mortality, iii) the effect on *P. carbekii* acquisition rates, and iv) the resulting percentage of damaged hazelnuts.

2. Materials and methods

2.1. Insect material, egg mass collection, and cardboard tags preparations

Laboratory rearings of *H. halys* were established in 2022 and 2023 from field-collected adults obtained via plant beating starting from late April to four days before the last treatment. Insects were sampled from *Prunus avium* L. and *Morus alba* L. until the second half of July, and from *Corylus avellana* L. and *Fraxinus excelsior* L. from the second half of July onward. Insects were reared in 93.0 x 47.5 × 47.5 cm net cages (BugDorm®, NHBS Ldt, Devon, UK) in climatic chambers at 25 ± 1 °C, 16:8 h L:D photoperiod. Adults were fed with *Vicia faba* L. and *Glycine* max (L.) Merr. plants, as well as apples, green beans, and hazelnuts. Egg mass production was checked daily and egg masses were collected; only egg masses less than 4 days old were used for field treatments.

Each egg mass collected from the rearing was glued in the centre of a 5.0 × 10.0 cm red cardboard tag either directly or with a portion of the leaf on which the egg mass was laid. The egg mass was surrounded by 4 pieces of 0.2 × 0.9 cm water-sensitive paper, which were glued together to form a frame. On the diagonal of each cardboard tag, another 2 pieces of 1.1 × 0.9 cm water-sensitive paper were glued. The top of the tag was reinforced with duct tape and a hole was made to insert a string of iron wire to attach it to the hazelnut plant (Fig. S1b).

2.2. Symbiont-targeted field treatments

Field treatments were performed in the summers of 2022 and 2023 in a commercial hazelnut orchard, using a commercial biocomplex (Dentamet®, Diachem SpA, Italy) whose efficacy against the *H. halys* symbiont was previously demonstrated (Gonella et al., 2019). The hazelnut orchard was located in Guarene (Cuneo Province, Italy. 44°44′03.9″N, 8°02′19.9″E). The site was selected based on monitoring data obtained the year before the start of trials, which showed high pest pressure (>2 *H. halys* nymphs per plant; >40% kernel damage). The planting plan was 5 × 5 m, with a single-trunk training system. The orchard was divided into two equal plots of 2400 m^2 each, one with symbiont-targeted treatments and the other untreated as control. The initial occurrence of *H. halys* in the orchard was monitored from the beginning of June in both years by plant beating technique and the presence of egg masses was checked by visual inspection of the plant canopy. Soon after observing the onset of *H. halys* oviposition in the orchard, the anti-symbiont treatments were started in the treated plot. The first application was made on June 27th, 2022 and June 8th, 2023, from which treatments were performed approximatively every 10 days in 2022 (four treatments) and every 15 days in 2023 (three treatments), until late July. Each treatment was applied with an Athos trailed round sprayer with a tank capacity of 1500 L (Dragone, Italy). Each application was made with 1000 L/ha of 0.4% v/v Dentament®; driving speed during spraying was 4.7 km/h. A preliminary trial was conducted within the first two applications in 2022 to determine the most suitable operating conditions for the treatments. Specifically, two airflow velocities were used by setting the fan speed to 1890 rpm and 2700 rpm, respectively. All subsequent treatments were conducted at 2700 rpm. No additional insecticide treatments were applied in the orchard.

Immediately before each treatment, cardboard tags were attached to seven plants; six plants were in two adjacent rows within the plot where the anti-symbiont treatment was applied, whereas the last one was in a separate row in the control plot. The first plant in the treated plot with the cardboard tags was 15 m away from the untreated plot; the same distance was maintained between the untreated plant with cardboard tags and the treated plot. Three out of the six plants in the treated plot were selected in the first row of the orchard, with one side of the canopy facing the orchard border, and the remaining three were in the second row, completely embedded in the field. Each plant had 10 cardboards on; cardboard tags were placed in different positions: along the row, facing the inter-row spacing, and in the centre of the plant (Fig. S1a). Each position was covered by two cardboard tags at different heights, one at about 1.5 m and the other at about 3.0 m. Thus, 70 tags were used for each experiment, 60 of which were treated and 10 were untreated controls. Cardboard tags were recollected 2 h after treatment (Fig. S1c) and returned to the laboratory.

2.3. Wetting percentage assessment

Egg masses collected in the field after spray were cut from the cardboard tags to assess the mortality induced in newborn nymphs. Cardboard tags without egg masses were scanned in groups of six. The scan resolution was 600 × 600 DPI, with maximum sharpness. Each cardboard tag was then separated from the others and the background was removed using the R package magick version 2.8.3, to keep only the water-sensitive paper image. The resulting images were manually adjusted using the GIMP software to remove any residual background. Mask thresholds to identify yellow pixels in images with different average brightness were defined using ImageJ software (Schneider et al., 2012). Once the thresholds were defined, the wetting percentage of each cardboard tag was automatically calculated using the Python packages NumPy (Harris et al., 2020) and OpenCV (Bradski, 2000). Wetting percentage was calculated as: (1-Ny/Nt)*100, where Ny is the number of yellow pixels and Nt is the total number of pixels. Results were manually checked on randomly selected cardboard tags by re-running the analysis with the same mask threshold in ImageJ software and comparing the results before proceeding with further analyses.

2.4. Nymphal rearing and mortality assessment

After removal from the cardboard tags, the egg masses were reared into 9 mm diameter Petri dishes with filter paper at the bottom. Rearings were performed in a climatic chamber at 25 ± 1 °C, 16:8 h L:D photoperiod and green beans were provided as food. Egg hatching and nymphal mortality were checked daily. Egg masses were maintained for up to three days after the last nymph molted to the second instar. All nymphs were collected and stored at −80 °C in RNAlater® for further RNA extraction, separating those that were dead from those that were alive at the end of the experiment.

2.5. Symbiont quantification in nymphs

The acquisition of living *P. carbekii* cells by dead and live nymphs coming from treated and control egg masses was quantified. Absolute quantification was performed by RNA extraction, retrotranscription and quantitative Real-time PCR (qPCR) as described by Gonella et al. (2019), using six serial dilutions of an external standard. Briefly, total RNA was extracted from dead and live nymphs using the SV Total RNA Isolation System (Promega, WI, USA) and retrotranscription was performed using the Reverse Transcription System (Promega, WI, USA) according to the manufacturer's instructions. qPCR reactions were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA). The mix consisted of 12.5 μL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 2.5 μL of 3 mM PcarQF and PcarQR primers, 6.5 μL of sterile water, and 1 μL of cDNA. The thermal protocol consisted of an initial denaturation at 94 °C for 3 min, followed by 50 cycles of 94 °C for 15 s and 53 °C for 30 s, and final melting curve generation from 53 to 95 °C measuring fluorescence every 0.5 s. To check for the presence of false negative samples due to inefficient cDNA preparation, a second Real-Time PCR was performed using a portion of the housekeeping insect's 18S rRNA gene as a target, as described by Marzachí and Bosco (2005). Briefly, the primer pair was MqFw and MqRv, the mix was prepared as described above, and the thermal protocol consisted of an initial denaturation at 94 °C for 3 min, then 36 cycles of 94 °C for 45 s and 65 °C for 1 min, followed by melting curve generation from 53 to 95 °C measuring fluorescence every 0.5 s. Only samples showing amplification with the reference gene were used for symbiont quantification. The concentration of *P. carbekii* cells was calculated as the number of expressed copies of 16S rRNA gene per pg of insect 18S rRNA gene.

2.6. Evaluation of stink bug-related damage on hazelnuts

To estimate the impact of symbiont-targeted control on production damage, 300 harvested hazelnuts were randomly selected each year from both treated and untreated plots to measure the incidence of stink bug-related damage. Hazelnuts were collected at least after the third plant from the border between treated and untreated plots, with a minimum distance of 30 m between the closer collection sites. Hazelnuts were shelled in the laboratory and inspected for the presence of evident kernel alterations due to stink bug feeding, defined as "cimiciato", resulting in darker color, tissue depression, and necrosis (Memoli et al., 2017). Apparently undamaged kernels were cut into four parts to check for internal symptoms. Finally, kernels were divided into two groups: those showing external or internal symptoms (cimiciato-damaged) and those showing no symptoms (healthy).

2.7. Statistical analysis

Statistical analyses were performed using R (version 4.2.3). Preliminary comparison between cardboard tags wetting percentage and nymphal mortality as a function of applied sprayer fan speed was performed using Kruskal-Wallis test followed by post hoc Bonferroni test (R package agricolae). The effect of the position of cardboard tags in the field or on the canopy on the wetting percentage was evaluated using the generalized linear model (glm) function with quasibinomial distribution and logit link function, the means were then separated by Bonferroni test (R package emmeans). Mortality of nymphs hatched from treated and untreated egg masses was compared using Kruskal-Wallis test followed by post hoc Bonferroni test. Regression analysis to correlate egg mass wetting percentage and nymphal mortality was performed using the generalized linear model (glm) function with quasibinomial distribution and logit link function. The correlation between nymphal mortality at different wetting percentages and according to the position of the cardboard tag was analysed using glm with quasibinomial distribution and logit link function, means were then separated by Bonferroni test (R package emmeans). Differences in *P. carbekii* acquisition rate between dead and live nymphs in treated and untreated samples were evaluated by Kruskal-Wallis test followed by post hoc Bonferroni test. Evaluation of damaged hazelnuts was performed as described by Bosco et al. (2018) via glm with binomial distribution and logit link function, with means separated by Bonferroni post hoc test.

3. Results

3.1. Effect of spraying conditions on treatment efficiency

A preliminary observation was made to assess the influence of airflow speed during spraying on the efficacy of the anti-symbiont treatment, i.e. on the wetting percentage of cardboard tags as well as on the mortality induced on newborn nymphs from treated egg masses. Increasing the fan speed of the sprayer did not result in a statistical difference in the total wetting percentage of the cardboard tags ($P = 0.91$, $df = 1$) or in the wetting percentage of the water-sensitive paper around the egg masses ($P = 0.28$, $df = 1$). In contrast, a significant difference was observed in the mortality of *H. halys* nymphs from egg masses sprayed at different fan speeds. A significantly higher mortality percentage was observed among nymphs from the egg masses exposed to the treatment with higher fan speed compared to the lower fan speed treatment ($P < 0.05$, $df = 1$) (Fig. S2). Based on these results, the higher sprayer fan speed was selected for the subsequent treatments.

3.2. Effect of cardboard tags position on wetting percentage

The mean wetting percentage for cardboards placed in the treated rows was 57.02%. The position of the cardboard tags significantly affected their wetting percentage, both considering the position of the

plant in the orchard (border or inner row) and the position of the tag in the canopy (centre of the plant canopy, along the row, facing the inter-row spacing) (Fig. 1a). Since the lower nozzle of the sprayer sprayed under the first branches, the treatment easily reached the central part of the plants, both in the border and in the inner rows. For this reason, all the data generated from the cardboard tags in the central part of the plants were not divided into outer and inner rows. All of the cardboard tags placed on the untreated control plant for all the treatments showed no change in the water-sensitive paper colour, confirming that the wetting percentages recorded were due to the treatments and not to environmental moisture.

Overall, the highest cardboard coverage was obtained for tags placed in the central part of the hazelnut canopy, followed by those facing the inter-row spacing in the inner row, with no significant difference between them ($P = 0.36$, $Z = 1.83$, $df = 359$). Cardboards facing the inter-row spacing in the orchard border were sprayed with a similar coverage as those in the inner row, both facing the inter-row spacing ($P = 0.19$, $Z = -2.16$, $df = 359$) or being along the row ($P = 0.97$, $Z = 0.62$, $df = 359$), but they were significantly less wet than those in the central part of the plants ($P < 0.05$, $Z = 3.40$, $df = 359$). The least covered cardboards were those in the border along the row, significantly less wet than those in the central part of the plant ($P < 0.05$, $Z = 5.56$, $df = 359$) and those in the inner side facing the inter-row spacing ($P < 0.05$, $Z = -4.56$, $df = 359$) (Fig. 1b). However, the wetting percentage records showed high variability among cardboards, especially those along the row, where the wetting percentage ranged from a minimum of 1.12% to a maximum of 99.99%.

3.3. Effect of cardboard wetting percentage on nymphal mortality

Overall, the mean mortality of nymphs from treated egg masses (42.83 ± 2.00%) was significantly higher than that of nymphs hatched from control egg masses (25.24 ± 5.06%), regardless of the position of the cardboard (Kruskal-Wallis test, $P < 0.05$, $df = 1$). However, although a slightly higher mortality rate was observed in nymphs hatched from egg masses with more than 30% wetness (Table 1), the regression

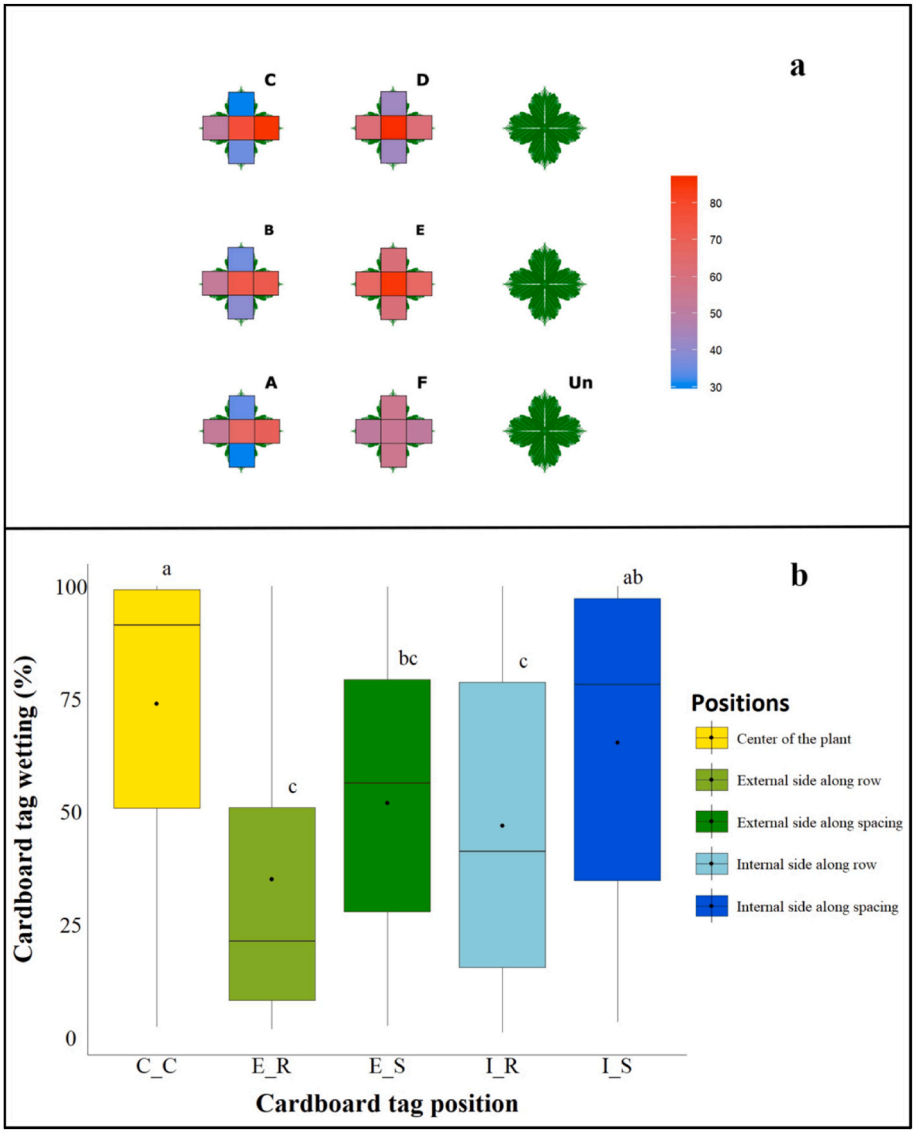

Fig. 1. (a) Heatmap of average wetting percentage of cardboard tags on treated plants. Mean values were calculated between all the treatments performed in 2022 and 2023 with the sprayer fan speed set at 2700 rpm. A, B, C represent plants in the perimetral row, whereas D, E, F represent plants in an inner row. "Un" corresponds to the selected plant within an untreated row, where no change in water sensitive paper color has been recorded. (b) Cardboard tags wetting percentage according to the position in field and on the canopy. Black dots represent the mean value. Internal lines represent the median value. Different letters indicate significantly different values according to glm with quasibinomial distribution and logit link function and means separation by Bonferroni test ($P < 0.05$).

Table 1
Mean mortality of nymphs from treated egg masses, according to the wetting percentage recorded in the corresponding cardboard. Nymphs are grouped according to carboard tag wetting percentage rates.

Wetting percentage	Percentage Mortality (mean ± SE)	No. egg masses
x ≤ 10	38.95 ± 5.40	36
10<x ≤ 20	44.32 ± 5.58	25
20<x ≤ 30	31.38 ± 6.91	17
30<x ≤ 40	46.48 ± 11.45	11
40<x ≤ 50	43.51 ± 10.88	11
50<x ≤ 60	39.28 ± 11.37	11
60<x ≤ 70	60.34 ± 12.37	10
70<x ≤ 80	41.04 ± 9.00	13
80<x ≤ 90	33.76 ± 7.58	12
90<x ≤ 100	44.60 ± 3.01	139

analysis showed no significant correlation between percentage wetness of the cardboard and nymphal mortality ($P = 0.16$, $R^2 = 0.01$). In confirmation, there was no correlation between nymphal mortality and the position of the cardboard tags ($P > 0.05$ for each combination, $df = 284$), although a moderate increase in nymphal mortality was obtained for egg masses placed facing the inter-row spacing compared to those placed along the row (42.57 ± 7.20 vs 35.45 ± 5.77% in the orchard border and 48.34 ± 3.83 vs 40.27 ± 3.37% in the inner row). Egg masses in the central part of the plants, despite being the most widely covered by the treatments, showed an intermediate percentage of nymphal mortality (42.41 ± 4.65). Interestingly, about half of the egg masses (regardless of their position) showed more than the 90% coverage (Table 1).

3.4. Acquisition of 'Candidatus Pantoea carbekii' by newborn nymphs

A total of 230 nymphs were used to quantify the infection with live *P. carbekii* cells; specifically, 110 nymphs from treated egg masses and 120 nymphs from untreated egg masses were randomly selected. A general reduction in *P. carbekii* acquisition was observed for dead nymphs from treated egg masses (median = 3.36×10^3 expressed copies of 16S rRNA gene/insect, IQR = 1.87×10^4) compared to live nymphs (median = 5.46×10^4 expressed copies of 16S rRNA gene/insect, IQR = 1.11×10^5) ($P < 0.05$, $df = 1$) (Fig. 2). However, such a difference was not observed for control nymphs, whose *P. carbekii* acquisition level did not show a significant difference between dead (median = 1.55×10^4, IQR = 1.19×10^5) and alive specimens (median = 2.50×10^4 expressed copies of 16S rRNA gene/insect, IQR = 1.89×10^5) ($P = 0.30$, $df = 1$). Complete prevention of *P. carbekii* acquisition was achieved for some tested dead (10 out of 53) and live nymphs (3 out of 57) from treated egg masses. All nymphs from control egg masses showed successful *P. carbekii* acquisition.

3.5. Damage evaluation

Similar percentages of damaged hazelnuts were recorded in 2022 and 2023 in the same plot (either treated or control). Cimiciato damage was significantly lower in hazelnuts from the treated plot compared to the untreated one in both years (P value < 0.05, df = 1) (Table 2).

4. Discussion

This study provides, for the first time, a comprehensive evaluation of the field efficacy of symbiont-targeted control of *H. halys*. As the efficacy of any active substance, including those targeting symbionts, can vary widely when scaled up from the laboratory to the field (Taylor et al., 2017), the aim of this work was to understand how the spraying conditions affect the treatment outcome in terms of ability to reach egg masses in different positions, and consequently to induce nymphal mortality. The most obvious result of this work was the lack of a clear correlation between the wetting percentage of the egg masses and

Table 2
Cimiciato damage comparison between the untreated control and the plot submitted to anti-symbiont treatment along two consecutive years. Different letters indicate statistically different values in the same year (glm with binomial distribution and logit link function, Bonferroni post hoc correction, $P < 0.05$).

Year	Plot	Cimiciato (% ± SE)
2022	Control	22.4 ± 2.4 a
	Treated	6.6 ± 1.4 b
2023	Control	24.1 ± 2.4 a
	Treated	7.4 ± 1.4 b

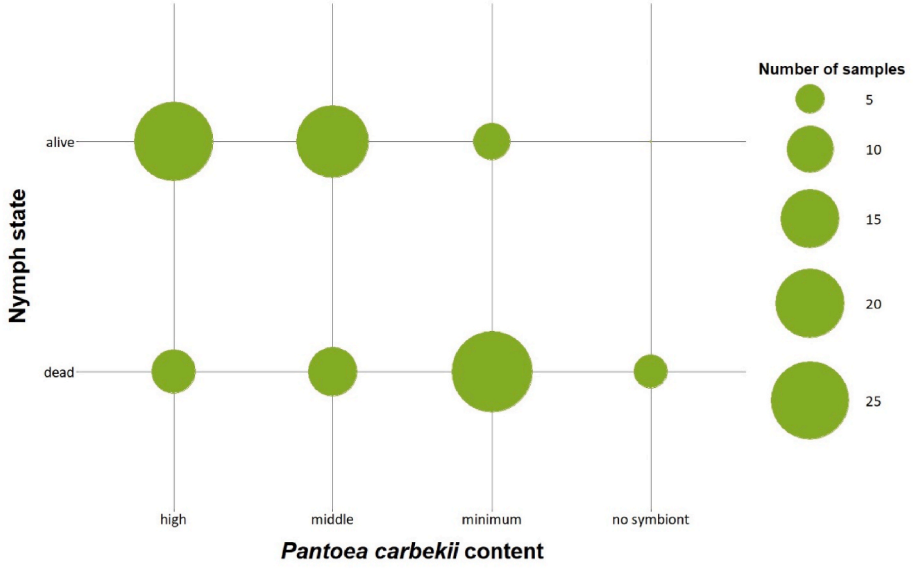

Fig. 2. Concentration of live *P. carbekii* cells in dead and live *H. halys* nymphs hatched from treated egg masses, measured as number of expressed copies of *P. carbekii* 16S rRNA gene per pg of insect DNA. "Minimum" *P. carbekii* content: symbiont concentration within the 33rd percentile. "Middle" *P. carbekii* content: symbiont concentration between 33rd and 66th percentile. "High" *P. carbekii* content: symbiont concentration above 66th percentile. The size of dots indicates the number of samples showing a given concentration.

nymphal mortality after egg hatching. This was noted already within the preliminary evaluation conducted to determine how sprayer fan speed might affect the egg mass coverage and the consequent nymphal mortality. There was no significant difference in the wetting percentage of cardboard tags when the fan speed was increased from 1890 rpm to 2700 rpm. This result may be due to the only partial reliability of results of the software analysis results on the scanned water-sensitive paper due to the irregular distribution and size of wet drops (Cerruto et al., 2019). However, changing the fan speed was not irrelevant to the outcome of symbiont-targeted control: increasing the fan speed resulted in augmented mortality of insects within the second instar. A possible explanation could be that the increased pressure made it easier for the anti-symbiont biocomplex to dilute or penetrate deeper into the secretion containing *P. carbekii* on the egg surface. Indeed, not only the amount of a substance reaching the egg mass is important for a proper control strategy, but also the ability of this substance to reach *P. carbekii* cells embedded in the maternal secretion can affect the outcome (Kenyon et al., 2015; Taylor et al., 2017).

When the effect of egg mass position was tested, the result was opposite to the effect of spraying conditions, as the position affected wetting percentage but not mortality induction. The greatest egg mass coverage was obtained when the cardboards were placed in the central part of the plant. This was an expected result: given the single-trunk training system used in the orchard tested and the height of the first branch, the lower nozzles could easily spray the central part of the plants. Cardboard tags that were hung facing the inter-row spacing and thus closer to the sprayer were easily reached by the product, whereas the most difficult position to reach was the one along the row. Plants at the edge of the orchard were generally less efficiently wetted than those in the inner row; this result was expected since leaf density tended to be higher in the edge row, making it more likely that some leaves would prevent the treatment from reaching the cardboard tags.

Despite the differences observed in product coverage, no correlation was found between the percentage of wetting of cardboards (and consequently their position) and nymphal mortality. This result suggests that even a low coverage of the egg surface allows the antibacterial compounds contained in the applied biocomplex to reduce the amount of symbiotic cells available for nymphal acquisition. Considering that *H. halys* sampling usually indicates the highest insect abundance at the field borders (Bosco et al., 2018), where product spraying is more difficult, the possibility to achieve significant mortality induction even with low egg coverage supports the success of symbiont-targeted methods. However, to maximize the outcome of symbiont-targeted treatments, the use of appropriate management practices (e.g.: pruning to reduce the canopy vigor) can be suggested to increase the chance of effectively reaching egg masses.

It must be pointed out that the average nymphal mortality obtained under field conditions was lower than the mortality recorded in laboratory experiments with the same formulation (Gonella et al., 2019). This difference could be due to different product concentrations (0.4% in this study, 1.0% in Gonella et al. (2019)) and to differences between laboratory and field conditions. The lower mortality observed here is related to the incomplete prevention of *P. carbekii* acquisition that was achieved, at least for most of the samples; in contrast, laboratory trials showed the complete absence of live symbiotic cells in nymphs from egg masses treated with the same biocomplex (Gonella et al., 2019, 2022). Also Taylor et al. (2017) showed a lower efficacy in preventing *P. carbekii* acquisition in field trials compared to laboratory conditions using different formulations. Nevertheless, we have shown that the reduction in symbiont acquisition is sufficient to induce significant mortality even considering the unavoidable limitations of field conditions. The more than 3-fold reduction in damaged hazelnuts in the treated plots strengthens the hypothesis that, under field conditions, it is not strictly necessary to completely prevent symbiont acquisition to achieve an acceptable reduction in economic damage by applying thee to four anti-symbiont treatments during the summer season. An additional advantage of symbiont-targeted control is its compatibility with the propagative biological control programs underway in Europe using the egg parasitoid wasp *Trissolcus japonicus* (Ashmead) (Falagiarda et al., 2023). Field releases of *T. japonicus* are predicted to provide stable parasitoid establishment and successful containment of *H. halys* in Europe (Haye et al., 2020; Tortorici et al., 2023). The introduction of a second allochthonous egg parasitoid, *Trissolcus mitsukurii* (Ashmead), is expected to increase *H. halys* mortality, so the possibility of using the better performing species or biotypes depending on the area has been suggested (Gutierrez et al., 2023). However, parasitoid occurrence in cultivated areas may be severely hampered by the use of chemical insecticides against their host. The application of symbiont-targeted control, relying on low-impact antimicrobials rather than on insecticidal molecules, has been shown to be harmless to *T. japonicus*, and no alteration of parasitism by this wasp has been observed (Orrù et al., 2023).

5. Conclusion

This work represents, to the best of our knowledge, the most thorough investigation of the efficacy of symbiont-targeted control of *H. halys* under field conditions, and opens up future perspectives for further implementation of this strategy in hazelnut orchards. Future studies could focus on the application of anti-symbiont treatments on different host plants. Furthermore, the performance of low-impact strategies combining biological control and symbiont-targeted control deserves to be investigated under a realistic scenario in field conditions. However, our results support the effectiveness of the proposed approach in keeping *H. halys* populations low enough to reduce the associated economic damage.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

Matteo Dho: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Gonella:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Alberto Alma:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Matteo Dho reports financial support was provided by Ferrero Trading Lux S.A. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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