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Egg masses treatment with micronutrient fertilizers has a suppressive effect on newly-emerged nymphs of the brown marmorated stink bug *Halyomorpha halys*

Elena Gonella, Bianca Orrù, Alberto Alma*

Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), Università degli Studi di Torino, Grugliasco, Italy

*Corresponding author: alberto.alma@unito.it, Phone +39 011 6708534, Fax +39 011 6708535

Abstract

The brown marmorated stink bug *Halyomorpha halys* is an invasive Asiatic pentatomid recently introduced in Europe. It is regarded as a major pest of many crops due to its marked polyphagy, high reproduction potential and high mobility. Among European countries where *H. halys* established in the last years, most of economic losses have been reported in Italy. A promising control approach against *H. halys* is based on the suppression of its gut primary symbiont '*Candidatus* Pantoea carbekii' (*P. carbekii*), vertically transmitted through maternal secretions containing symbiotic bacteria smeared during ovoposition, which are ingested by neonates. Symbiont elimination is regarded as a promising pest control strategy based on the application of antimicrobial substances.

Here an anti-symbiont activity is shown in response to the application of micronutrient fertilizers showing antimicrobial activity, resulting in *H. halys* nymphal mortality in laboratory conditions. Exposure to four commercial products, available for organic farming, was tested on isolated stink bug egg masses, by measuring survival to II nymphal instar of neonates emerging from treated eggs. Zinc, copper and citric acid biocomplexes showed the most effective impact on *H. halys* survival, causing more than 90% nymph mortality. Molecular diagnosis for *P. carbekii* confirmed that observed effects were attributable to missed symbiont acquisition.

Taken together, our results provide indication for the potential field use of micronutrient fertilizers as controls tool against *H. halys*. Future work will clarify operating details to design a new, eco-friendly approach for the control of this pest threatening Italian and European agriculture.

Key words: *Pantoea carbekii*, Pentatomidae, symbiont disruption, micronutrient biocomplexes, integrated pest management

Introduction

The brown marmorated stink bug *Halyomorpha halys* (Stål) is an invasive pentatomid species native of Asia, which has been accidentally introduced in North America in the 1990s and subsequently in Europe (Leskey & Nielsen 2018). More than 300 species of wild and cultivated plants can be attacked by this pest, whose feeding activity induces symptoms such as seed abortion, fruit deformation and discolorations, necrosis and other tissue alterations (Rice et al. 2014; Bariselli et al. 2016; Bosco et al. 2018). Moreover, its widely aggregative behaviour observed in overwintering adults makes this insect an important household nuisance pest as well (Inkley 2012). Even though in its native area *H. halys* is considered only as an occasional pest of few crops (Lee et al. 2013), its high invasive potential in areas where bioclimatic condition are favourable to its development makes this stink bug a very destructive pest in countries of new introduction. In Europe, *H. halys* was first detected in 2004 in Switzerland, where it is rarely harmful to vegetables and crops (Haye et al. 2014). Afterwards it was found in many countries of central and southern Europe; particularly, most of economic losses have been recorded in Italy (Bariselli et al. 2016). Indeed, in Italy *H. halys* has two generations per year, high reproductive rates, and high mobility. Furthermore it is widely present in areas where commercial exchanges favour massive movement of goods and materials; all these traits highly enhance its pest status (Costi et al. 2017).

Due to reduced effectiveness and high impact of chemical control of *H. halys*, alternative environmentally friendly tools are under investaigation (Haye et al. 2015; Gariepy et al. 2018). A promising approach for sustainable integrated control of economically relevant stink bugs pests could be the exploitation of gut primary symbioses typically occurring in these insects. Indeed, similarly to other Hemiptera, pentatomids rely on obligate bacterial symbionts complementing their nutritionally unbalanced diets (Moran et al. 2008). In stink bugs, these primary symbionts are hosted in caeca in the posterior midgut region. Transmission to the

progeny is achieved through a distinctive strategy, diverging from transovarial transmission commonly reported for other Hemiptera. Maternal secretions containing symbiotic bacteria are smeared on or laid close to egg masses during oviposition; nymphs immediately acquire symbionts by consuming this secretion (Prado et al. 2006). Aposymbiotic (i.e. deprived of their primary symbionts) individuals most commonly display reduced survival or fitness (Otero-Bravo & Sabree 2015). During the transmission process, symbionts live outside the insect gut for several days before being acquired by the next generation, being protected only by secretions.

The gut primary symbionts of *H. halys*, named '*Candidatus* Pantoea carbekii' (hereafter P. *carbekii*) (Bansal et al. 2014), inhabits the posterior midgut caeca of the host and the extrachorion secretions on the egg surface, and supplies the host with nutrients limited in its diet (Kenyon et al. 2015). Moreover, preventing vertical transmission of *P. carbekii* heavily affects the fitness of first generation nymphs of *H. halys* and their progeny (Taylor et al. 2014). The application of substances with antimicrobial activity has been tested on *H. halys* egg masses, in some cases showing high mortality (Mathews &Barry 2014; Taylor et al 2017). Hence, their use was proposed for symbiont-targeted control strategies against *H. halys*.

Even though stink bug primary symbionts are regarded as a promising target for the control of *H. halys* (Mathews &Barry 2014; Taylor et al 2017), at present specific control methods based on this strategy are still unavailable in Europe. Hence, research on European populations is required to implement integrated crop management solutions targeting the containment of this pest. In this study, the application was assessed of active substances currently in use in European agriculture and showing direct or indirect protective effects from pathogenic microorganisms on *H. halys* egg masses in laboratory conditions. Their effect on nymphal survival was tested along with the interruption of *P. carbekii* acquisition. An Italian population was selected, as in Europe most of economic damage is produced in this area.

Material & methods

Insect material

During spring and summer of 2018, adults of the brown marmorated stink bug were collected from different wild and cultivated host plants in the Piedmont region, Italy. Field-collected adults were reared at the DISAFA laboratories, in climatic chambers at 25 ± 1 °C, with an L:D of 16:8 photoperiod, in net cages (930 × 475 × 475 mm) containing seedlings of broad bean, apples, and shelled hazelnuts. *H. halys* egg masses were collected daily from the mass rearing to obtain two distinct groups, corresponding to 24 hour-old and 5 day-old egg masses, respectively.

Egg masses treatment

For this study three commercially available micronutrient EC fertilizers, suitable for organic farming, were selected: (1) a zinc, copper and citric acid biocomplex (Dentamet®, Diagro Srl, Italy); (2) a zinc, manganese and citric acid biocomplex (Bio-D®, Diagro); (3) a copper hydroxide 50% wettable powder (Keos®, Green Ravenna Srl, Italy). Moreover, the experimental product Dentamet A3 (Diagro) containing citric acid, zinc sulphate, and copper sulphate, was tested as well (4). All products were used on 24 hour-old egg masses at a final concentration of 1% in combination with 0.5% a Poly-1-p-menthene-based pesticide additive (NU-FILM-P®, CBC, Italy), to increase active ingredients penetration of maternal secretions covering *P. carbekii* cells (Kenyon et al. 2015). Finally, an untreated control (5) and a water + 0.5% additive control (6) were included. The two products showing the higher mortality rates on 24 hour-old egg masses were used to perform a second experiment on 5 day-old egg masses, along with controls.

A total of 120 egg masses were collected and randomly allocated to treatments, once the number of eggs per mass was recorded. Product applications were conducted with 24 hour-old and 5 day-old egg masses for each treatment and water + additive control (N=10); 20 replicates for the untreated control were collected as well. The treatment solutions were applied to the egg masses, individually placed into Petri dishes covered with filter paper, by employing a hand sprayer under a fume hood.

Nymphal rearing

After the treatment, egg masses were individually reared in climatic chamber (25 °C, RH 70%) in a clear plastic Petri dishes provided with a green bean as a food source, with a wider lid to provide ventilation; hatching percentages were checked daily. Newly hatched nymphs were fed with green beans until reaching second nymph instar. Mortality rates were calculated; dead nymphs were collected each day and stored at -80°C in RNA later® (Sigma-Aldrich, MO, USA). As live nymphs moulted to the second instar, they were collected as well and stored as described above.

RNA extraction and Real Time PCR

Real Time PCR was used to determine the presence or absence of *P. carbekii* to assess the rate of effective acquisition of bacteria from the egg mass surface. A RNA-based approach was designed in order to avoid possible amplification of the DNA related to dead *P. carbekii* cells, eliminating the risk of false positive detection. A subset of stored nymphs was used, consisting of 10 individuals from the two treatments emerging as the most effective within the experiment on 24 day-old egg masses, as well as from the controls. RNA extraction was performed with the "SV Total RNA Isolation System" (Promega, WI, USA), accordingly to the supplier's suggestions. After extractions, RNA quality and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop, DE, USA). First strand cDNA was synthesized by using the

"Reverse Transcription System" (Promega) and Random Primers, following the manufacturer's instructions. cDNA was used as a template for Real Time PCR analysis with the newly designed *P. carbekii*-specific primers PcarQF (5'-ACAGACTAGAGTCTCGTAGA-3') and PcarQR (5'-TCACATCTTAAAGACACAAC-3'), amplifying a 207 bp fragment of the symbiont 16SrRNA gene. The following thermal conditions were applied: an initial denaturation at 94°C for 3 min was followed by 50 cycles consisting of denaturation at 94°C for 15 sec and annealing at 53°C for 30 sec. A final step for melting curve analysis from 70 to 95°C, measuring fluorescence every 0.5°C, was added. Moreover, to verify whether negative nymphs were truly deprived of *P. carbekii*, rather than missing due to sample quality, Real Time PCR targeting the insect's 18S rRNA gene (MqFw / MqRv) was used (Marzachì & Bosco 2005), under the conditions described by Gonella et al. (2015).

Statistical analysis

To compare hatching and mortality data obtained in this work, the percentages of dead specimens were derived with respect to the total number of emerged nymphs for each egg mass. Normalized mortality rates were calculated according to the Abbott's formula (Abbott 1925); moreover, absolute mortality rates were analysed with SPSS Statistics 25 (IBM Corp. Released 2017, Armonk, NY, USA), using a generalized linear model (GLM) with a binomial probability distribution and logit link function. Means were separated by a Bonferroni post hoc test ($P < 0.05$).

Results

To test the effect of applying micronutrient-based active substances on *H. halys* nymph survival, egg masses obtained from our laboratory colony were used; these egg masses counted an average of 24.21 eggs per mass. Binomial GLM analysis on 24 hour-old egg masses revealed that the mean egg hatching rates obtained after treatment with product (1) and (4) were

156 significantly lower than products (2) and (3); similarly significant differences were recorded
157 between untreated control and water + additive (Tab. 1). Furthermore, significantly different
158 percentages were found of nymphs dying before reaching II instar (df = 5; χ^2 = 443.600; P
159 <0.05) (Tab.1). The highest percentage of dead nymphs was found for the zinc, copper and
160 citric acid-based products (1) and (4). The application of substance (1) induced significantly
161 higher mortality than use of products (2) and (3), containing zinc, manganese and citric acid,
162 and copper hydroxide, respectively. However, all of tested commercial products caused
163 significantly increased mortality than both controls (untreated and water + additive).
164 Experiment on 5 day-old egg masses, performed using only products (1) and (4) and the
165 controls, showed similar results, as significant differences were recorded according to binomial
166 GLM on nymphal mortality rates (df = 3; χ^2 = 245.335; P <0.05) (Fig.1). Although slightly
167 lower percentages of dead nymphs were detected for both products, mortality rates were
168 significantly more abundant than untreated and water + additive controls in either cases. As in
169 experiments on 24 hour-old egg masses, the highest mortality was observed for product (1). On
170 the other hand, a significantly lower number of eggs hatched from treatment with product (4)
171 (Tab. 1).
172 To verify whether mortality results were indeed referable to missed *P. carbekii* acquisition, for
173 treatments (1) and (4), which caused the highest mortality rates, 10 dead I instar nymphs as well
174 as 10 II instar nymphs found live at the end of our experiments were used for RNA extraction
175 followed by *P. carbekii*-specific Real time PCR on cDNA. The results of this molecular analysis
176 revealed that all of dead I instar nymphs treated with either products, regardless of the applied
177 active substance, were deprived of the bacterial symbiont (Fig.2). Likewise, no *P. carbekii*-
178 positive samples were detected among live II instar nymphs obtained from egg masses exposed
179 to products (1) and (4). Real time PCR targeting 18SrRNA of nymph cDNA testing negative
180 for *P. carbekii* indicated effective amplication for all individuals, confirming the success of

sample processing. On the other hand, 95% of nymphs from the controls (either dead or live) carried the symbiont, even though a lower percentage of positive samples were observed after egg masses exposure to water + additive (Fig.2). Strikingly, about 10% of nymphs from the untreated control, found live at the end of the trials, tested negative for *P. carbekii*.

Discussion

This work provided experimental evidences of extensive suppressive effect caused to *H. halys* nymphal survival after exposure to micronutrient fertilizers, as a consequence of interrupted acquisition of *P. carbekii*. Indeed, the chemical composition of these products entails anti-microbial activity as a side effect of fertilizer application. Products (1), (2), and (4), displaying the most severe effect on nymphal survival, contained zinc and citric acid. Zinc is widely used as a pesticide active ingredient to control different plant pathogens, exhibiting lethal effects on many Gram negative bacteria (Fones et al. 2010; Navarrete et al. 2015; Aggarwal et al. 2018). Similarly, citric acid, as well as other organic acids, has been shown to display broad range bactericidal activity majorly related to pH reduction and disruption of cell transmembrane transport (Finten et al. 2017). Product (1), whose application resulted in the highest mortality rates, was previously shown to inhibit growth of *Xylella fastidiosa*, reducing the severity of symptoms related to this pathogen in olive trees (Scortichini et al. 2018). Copper was present in products (1), (3), and (4). The involvement of this element in plant pathogen control is widely recognized (Scheck & Pscheidt 1998; Narciso et al. 2012), and our results confirmed a lethal effect on *P. carbekii* as well. Moreover, higher mortality, as a result of bactericidal effect, was exhibited when copper was used in combination with zinc and citric acid, while the application of copper hydroxide alone was less effective in reducing nymphal survival. Similarly, the use of manganese in place of copper in product (2) limited the lethal effect on nymphs. Therefore, a crucial involvement in *P. carbekii* suppression can be assumed for Zn- and Cu-hydracid

206 complexes, which are generated in products (1) and (4). Additionally, application of these
207 products - especially product (4) - caused a partial ovicidal effect, resulting in even higher total
208 nymph mortality. Indeed, considering both unhatched eggs and dead nymphs, overall mortality
209 was in average 95% for treatments on 24 hour-old egg masses and 90% on 5 day-old egg
210 masses.
211 Mortality rates detected in this work were generally more abundant than values reported by
212 Mathews and Barry (2014) and Taylor et al. (2017); however, the products tested by these
213 authors widely diverged with micronutrient fertilizers in their composition. Mathews and Barry
214 (2014) examined the use of compost tea, whose activity is due to a combined effect of biotic
215 and abiotic agents (Palmer et al. 2010). The products tested by Taylor et al. (2017) included
216 insecticides, antibiotics and other antimicrobials. Interestingly, the product showing the highest
217 mortality according to these authors was a surfactant mixture (Naiad). This substance was
218 suggested to hamper symbiont acquisition due to a combination of antimicrobial activity and
219 ability to penetrate the egg secretion coating (Taylor et al. 2017). A similar combined effect
220 may be assumed to be exerted after administering the four products tested in our work, as the
221 pesticide additive added prior to product application on egg masses is used as a wetting agent
222 similar to Naiad. Although the mortality caused by spraying water + additive alone was not
223 significantly divergent from untreated control, this treatment was related to a higher number of
224 dead nymphs, suggesting partial removal of *P. carbekii* cells, as indicated also by Real Time
225 PCR data. Likewise, application of water + additive resulted in a lower number of hatched eggs
226 than untreated control, suggesting an egg toxic effect at least in our experimental conditions.
227 Moreover, the significant efficiency in penetrating egg coating was coupled with high
228 persistence potential of the anti-*P. carbekii* activity, as similar results were obtained using
229 newly laid as well as mature egg masses.

Real Time PCR screening of nymph cDNA provided confirmation for the unsuccessful acquisition of *P. carbekii* by nymphs treated with the most effective products. Most of nymphs deprived of their symbiont dead; strikingly a 10% of tested untreated populations was able to survive in the absence of *P. carbekii*. Live *P. carbeckii*-free *H. halys* individuals were observed both from treated egg masses and in the controls. Since this was found for nymphs from the same egg mass, limited genetic variability can be presumed, on the other hand the introduction of a different symbiotic organism capable to replace *P. carbekii* in nutrient provisioning cannot be ruled out. A potential substitute symbiont should not be affected by antimicrobial administration on the egg surface; therefore it should either: i) be unsensitive to the application of tested products, or ii) undergo vertical transmission through a different route (e.g. transovarial transmission). Despite the terminal gut portion of *H. halys* was previously reported to be widely dominated by *P. carbekii* in American populations (Kenyon et al. 2015), further work deeply examining the microbiome composition of in Italian population of the brown marmorated stink bug is required, to identify candidate species possibly involved in symbiont replacement.

As a conclusion, the experimental evidences provided by this work in laboratory conditions suggest that foliar application of micronutrient fertilizers on *H. halys*-infected crops has the potential to induce high nymphal mortality. Specifically, the use of zinc, copper and citric acid biocomplexes could results in the most effective containment of *H. halys* populations. However, in order to develop standard operating procedures for the control of the brown marmorated stink bug, some issues are still to be clarified. In particular, field efficiency and persistence of product application should be evaluated, to establish treatment number, timing and dose. Moreover, the interaction of these substances with non-target organisms, including natural enemies, which have a direct role in the control of *H. halys* (Leskey et al. 2018).

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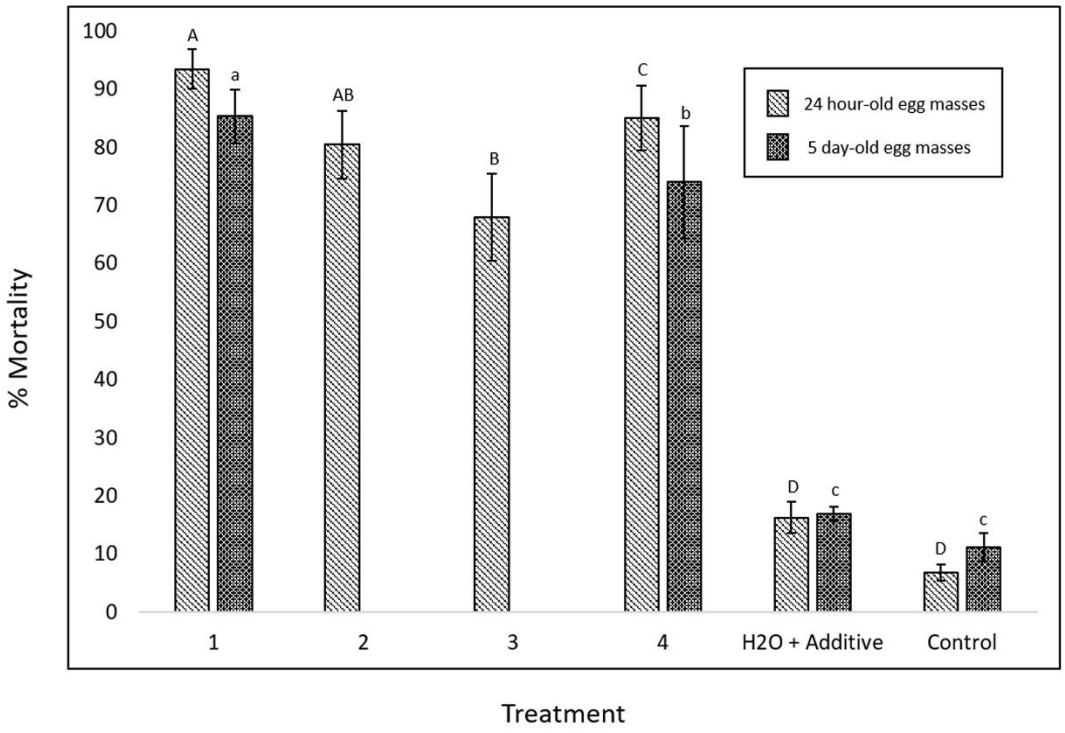
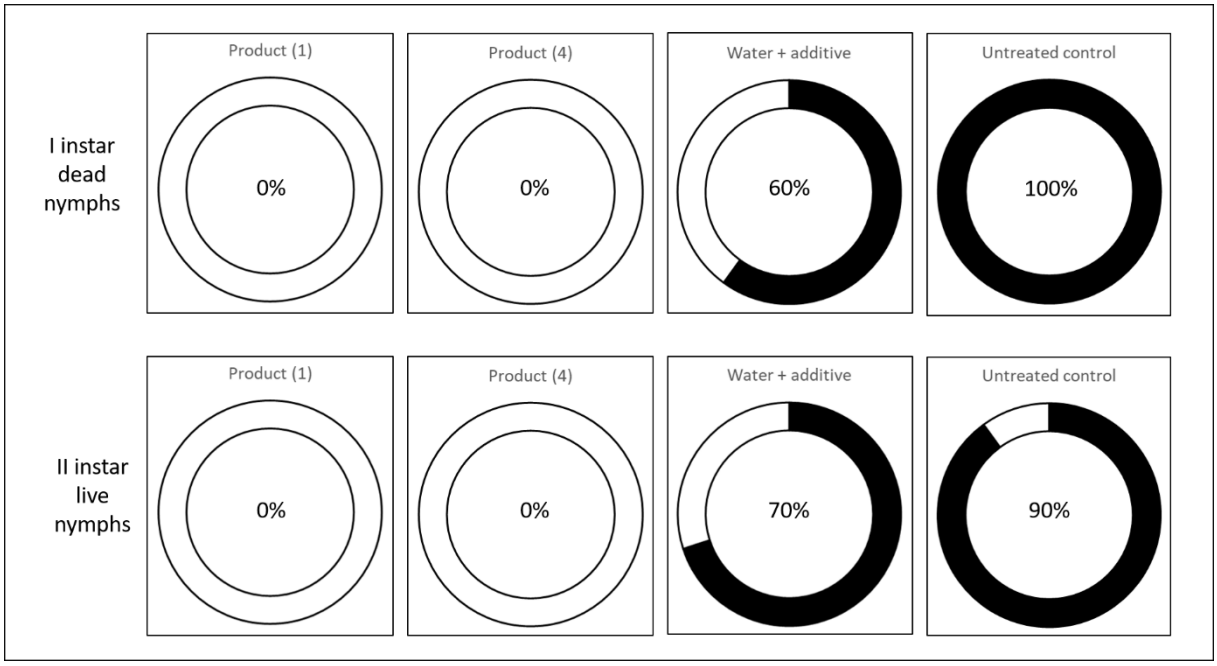
Table 1. Data recorded during laboratory experimental application of micronutrient EC fertilizers to 24 hour-old and 5 day-old *H. halys* egg masses. Results are expressed as average values ± SE. For egg hatching rates, different letters indicate significantly different values according to binomial GLM analysis + Bonferroni's test. Separate statistical tests were conducted for 24 hour-old egg masses (df = 5; $\chi^2 = 41.376$; $P < 0.05$) and 5 day-old egg masses (df = 3; $\chi^2 = 29.332$; $P < 0.05$). Normalized mortality rates were obtained with respect to untreated control according to the Abbott's Formula.

Egg masses age	Treatment	Average number of eggs per mass	Average egg hatching rate	Normalized mortality rate to II nymphal instar (%)
24 hours	**Product (1)**	25.8 ± 1.12	68.60 ± 1.78 a	92.60 ± 0.29
	Product (2)	25.6 ± 0.95	81.64 ± 1.24 b	90.96 ± 0.86
	Product (3)	26 ± 1.03	82.30 ± 1.22 b	87.67 ± 1.44
	Product (4)	24.2 ± 1.71	66.94 ± 2.20 a	91.58 ± 0.68
	Water + additive	19.70 ± 2.04	71.06 ± 1.91 ab	64.36 ± 15.17
	Untreated control	24.75 ± 1.46	82.22 ± 1.68 b	0.00
5 days	**Product (1)**	26.40 ± 1.10	82.57 ± 2.26 c	87.84 ± 0.76
	Product (4)	21.00 ± 1.57	60.95 ± 1.96 a	82.57 ± 3.38
	Water + additive	21.06 ± 2.10	68.05 ± 1.77 ab	37.25 ± 4.71
	Untreated control	22.90 ± 1.93	75.10 ± 1.76 bc	0.00

Fig. 1. Mortality rates recorded for *H. halys* neonate nymphs after treatment with different micronutrient fertilizers. The percentage of dead nymphs before reaching II instar was calculated for 24 hour-old (light columns) and 5 day-old (dark columns) egg masses. Bars indicate standard errors. Different letters indicate significantly different values according to binomial GLM + Bonferroni's test ($P < 0.05$).



356 **Figure 2.** Percentage of nymphs carrying *P. carbekii* according to Real Time PCR on cDNA.



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