

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1720033> since 2020-02-19T14:07:22Z

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

**This is the pre-print version of the contribution published as:**

GONELLA E., ORRÙ B., ALMA A. – Egg masses treatment with micronutrient fertilizers has a suppressive effect on newly-emerged nymphs of the brown marmorated stink bug *Halyomorpha halys*. *Entomologia Generalis*, 3-4, 2019, 231-238.

**The publisher's version is available at:**

[https://www.schweizerbart.de/papers/entomologia/detail/39/91951/Egg\\_masses\\_treatment\\_with\\_micronutrient\\_fertilizers\\_has\\_a\\_suppressive\\_effect\\_on\\_newly\\_emerged\\_nymphs\\_of\\_the\\_brown\\_marmorated\\_stink\\_bug\\_Halyomorpha\\_halys](https://www.schweizerbart.de/papers/entomologia/detail/39/91951/Egg_masses_treatment_with_micronutrient_fertilizers_has_a_suppressive_effect_on_newly_emerged_nymphs_of_the_brown_marmorated_stink_bug_Halyomorpha_halys)

**When citing, please refer to the published version.**

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

3

4 Elena Gonella, Bianca Orrù, Alberto Alma\*

5

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

8

9 \*Corresponding author: [alberto.alma@unito.it](mailto:alberto.alma@unito.it), Phone +39 011 6708534, Fax +39 011 6708535

10

## 11 **Abstract**

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

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

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

32

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

## 35 **Introduction**

36 The brown marmorated stink bug *Halyomorpha halys* (Stål) is an invasive pentatomid species  
37 native of Asia, which has been accidentally introduced in North America in the 1990s and  
38 subsequently in Europe (Leskey & Nielsen 2018). More than 300 species of wild and cultivated  
39 plants can be attacked by this pest, whose feeding activity induces symptoms such as seed  
40 abortion, fruit deformation and discolorations, necrosis and other tissue alterations (Rice et al.  
41 2014; Bariselli et al. 2016; Bosco et al. 2018). Moreover, its widely aggregative behaviour  
42 observed in overwintering adults makes this insect an important household nuisance pest as  
43 well (Inkley 2012). Even though in its native area *H. halys* is considered only as an occasional  
44 pest of few crops (Lee et al. 2013), its high invasive potential in areas where bioclimatic  
45 condition are favourable to its development makes this stink bug a very destructive pest in  
46 countries of new introduction. In Europe, *H. halys* was first detected in 2004 in Switzerland,  
47 where it is rarely harmful to vegetables and crops (Haye et al. 2014). Afterwards it was found  
48 in many countries of central and southern Europe; particularly, most of economic losses have  
49 been recorded in Italy (Bariselli et al. 2016). Indeed, in Italy *H. halys* has two generations per  
50 year, high reproductive rates, and high mobility. Furthermore it is widely present in areas where  
51 commercial exchanges favour massive movement of goods and materials; all these traits highly  
52 enhance its pest status (Costi et al. 2017).

53 Due to reduced effectiveness and high impact of chemical control of *H. halys*, alternative  
54 environmentally friendly tools are under investigation (Haye et al. 2015; Garipey et al. 2018).

55 A promising approach for sustainable integrated control of economically relevant stink bugs  
56 pests could be the exploitation of gut primary symbioses typically occurring in these insects.  
57 Indeed, similarly to other Hemiptera, pentatomids rely on obligate bacterial symbionts  
58 complementing their nutritionally unbalanced diets (Moran et al. 2008). In stink bugs, these  
59 primary symbionts are hosted in caeca in the posterior midgut region. Transmission to the

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

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

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

85

## 86 **Material & methods**

### 87 **Insect material**

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

### 95 **Egg masses treatment**

96 For this study three commercially available micronutrient EC fertilizers, suitable for organic  
97 farming, were selected: (1) a zinc, copper and citric acid biocomplex (Dentamet®, Diagro Srl,  
98 Italy); (2) a zinc, manganese and citric acid biocomplex (Bio-D®, Diagro); (3) a copper  
99 hydroxide 50% wettable powder (Keos®, Green Ravenna Srl, Italy). Moreover, the  
100 experimental product Dentamet A3 (Diagro) containing citric acid, zinc sulphate, and copper  
101 sulphate, was tested as well (4). All products were used on 24 hour-old egg masses at a final  
102 concentration of 1% in combination with 0.5% a Poly-1-p-menthene-based pesticide additive  
103 (NU-FILM-P®, CBC, Italy), to increase active ingredients penetration of maternal secretions  
104 covering *P. carbekii* cells (Kenyon et al. 2015). Finally, an untreated control (5) and a water +  
105 0.5% additive control (6) were included. The two products showing the higher mortality rates  
106 on 24 hour-old egg masses were used to perform a second experiment on 5 day-old egg masses,  
107 along with controls.

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

#### 114 **Nymphal rearing**

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

#### 122 **RNA extraction and Real Time PCR**

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



132 “Reverse Transcription System” (Promega) and Random Primers, following the manufacturer’s  
133 instructions. cDNA was used as a template for Real Time PCR analysis with the newly designed  
134 *P. carbekii*-specific primers PcarQF (5’-ACAGACTAGAGTCTCGTAGA-3’) and PcarQR  
135 (5’-TCACATCTTAAAGACACAAC-3’), amplifying a 207 bp fragment of the symbiont  
136 16SrRNA gene. The following thermal conditions were applied: an initial denaturation at 94°C  
137 for 3 min was followed by 50 cycles consisting of denaturation at 94°C for 15 sec and annealing  
138 at 53°C for 30 sec. A final step for melting curve analysis from 70 to 95°C, measuring  
139 fluorescence every 0.5°C, was added. Moreover, to verify whether negative nymphs were truly  
140 deprived of *P. carbekii*, rather than missing due to sample quality, Real Time PCR targeting  
141 the insect’s 18S rRNA gene (MqFw / MqRv) was used (Marzachi & Bosco 2005), under the  
142 conditions described by Gonella et al. (2015).

### 143 **Statistical analysis**

144 To compare hatching and mortality data obtained in this work, the percentages of dead  
145 specimens were derived with respect to the total number of emerged nymphs for each egg mass.  
146 Normalized mortality rates were calculated according to the Abbott’s formula (Abbott 1925);  
147 moreover, absolute mortality rates were analysed with SPSS Statistics 25 (IBM Corp. Released  
148 2017, Armonk, NY, USA), using a generalized linear model (GLM) with a binomial probability  
149 distribution and logit link function. Means were separated by a Bonferroni post hoc test ( $P <$   
150 0.05).

### 151 **Results**

152 To test the effect of applying micronutrient-based active substances on *H. halys* nymph  
153 survival, egg masses obtained from our laboratory colony were used; these egg masses counted  
154 an average of 24.21 eggs per mass. Binomial GLM analysis on 24 hour-old egg masses revealed  
155 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 ( $df = 5$ ;  $\chi^2 = 443.600$ ;  $P$   
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 ( $df = 3$ ;  $\chi^2 = 245.335$ ;  $P <0.05$ ) (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

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

185

## 186 **Discussion**

187 This work provided experimental evidences of extensive suppressive effect caused to *H. halys*  
188 nymphal survival after exposure to micronutrient fertilizers, as a consequence of interrupted  
189 acquisition of *P. carbekii*. Indeed, the chemical composition of these products entails anti-  
190 microbial activity as a side effect of fertilizer application. Products (1), (2), and (4), displaying  
191 the most severe effect on nymphal survival, contained zinc and citric acid. Zinc is widely used  
192 as a pesticide active ingredient to control different plant pathogens, exhibiting lethal effects on  
193 many Gram negative bacteria (Fones et al. 2010; Navarrete et al. 2015; Aggarwal et al. 2018).  
194 Similarly, citric acid, as well as other organic acids, has been shown to display broad range  
195 bactericidal activity majorly related to pH reduction and disruption of cell transmembrane  
196 transport (Finten et al. 2017). Product (1), whose application resulted in the highest mortality  
197 rates, was previously shown to inhibit growth of *Xylella fastidiosa*, reducing the severity of  
198 symptoms related to this pathogen in olive trees (Scortichini et al. 2018). Copper was present  
199 in products (1), (3), and (4). The involvement of this element in plant pathogen control is widely  
200 recognized (Scheck & Pscheidt 1998; Narciso et al. 2012), and our results confirmed a lethal  
201 effect on *P. carbekii* as well. Moreover, higher mortality, as a result of bactericidal effect, was  
202 exhibited when copper was used in combination with zinc and citric acid, while the application  
203 of copper hydroxide alone was less effective in reducing nymphal survival. Similarly, the use  
204 of manganese in place of copper in product (2) limited the lethal effect on nymphs. Therefore,  
205 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.

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

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

254

## 255 **Acknowledgements**

256 The authors are grateful to Ester Ferrari, Silvia Moraglio and Sara Visentin for their essential  
257 help in insect rearing and laboratory activities. Financial support was provided by Cassa di  
258 Risparmio di Cuneo (Italy), within the project “Eco-friendly control strategies against  
259 *Halyomorpha halys* (HALY-End)”, and by Diagro, through the research contract “Symbiont-  
260 targeted control of *Halyomorpha halys* and other stink bugs by using integrated fertilizers and  
261 resistance inducers”.

262

## 263 **References**

264 Abbott WS (1925): A method of computing the effectiveness of an insecticide. J Econ Entomol  
265 18: 265-267.

266 Aggarwal M, Hussaini MY, De La Fuente L, Navarrete F, Cogan NG (2018): A framework for  
267 model analysis across multiple experiment regimes: investigating effects of zinc on *Xylella*  
268 *fastidiosa* as a case study. J Theor Biol 457: 88-100.

269 Bansal R, Michel AP, Sabree ZL (2014): The crypt-dwelling primary bacterial symbiont of the  
270 polyphagous pentatomid pest *Halyomorpha halys* (Hemiptera: Pentatomidae). Environ  
271 Entomol 43: 617-625.

272 Bariselli M, Bugiani R, Maistrello L, (2016): Distribution and damage caused by *Halyomorpha*  
273 *halys* in Italy. EPPO Bull 46: 332-334.

274 Bosco L, Moraglio S, Tavella L (2018): *Halyomorpha halys*, a serious threat for hazelnut in  
275 newly invaded areas. J Pest Sci 91: 661–670.

276 Costi E, Haye T, Maistrello L (2017): Biological parameters of the invasive brown marmorated  
277 stink bug, *Halyomorpha halys*, in southern Europe. J Pest Sci 90: 1059-1067.

278 Finten G, Agüero MV, Jagus RJ, (2017) Citric acid as alternative to sodium hypochlorite for  
279 washing and disinfection of experimentally-infected spinach leaves, LWT-Food Sci Technol  
280 82: 318-325.

281 Fones HN, Davis CAR, Rico A, Fang F, Smith JAC, Preston GM (2010): Metal  
282 hyperaccumulation armors plants against disease. PLoS Pathog 6: e1001093.

283 Garipey TD, Bruin A, Konopka J, Scott-Dupree C, Fraser H, Bon M-C, Talamas E (2018): A  
284 modified DNA barcode approach to define trophic interactions between native and exotic  
285 pentatomids and their parasitoids. Mol Ecol doi: 10.1111/mec.14868.

286 Gonella E, Pajoro M, Marzorati M, Crotti E, Mandrioli M, Pontini M, et al. (2015): Plant-  
287 mediated interspecific horizontal transmission of an intracellular symbiont in insects. Sci. Rep.  
288 5: 15811.

289 Haye T, Abdallah S, Garipey T, Wyniger D (2014): Phenology, life table analysis and  
290 temperature requirements of the invasive brown marmorated stink bug, *Halyomorpha halys*, in  
291 Europe. J Pest Sci 87: 407–418.

292 Haye T, Fischer S, Zhang J, Garipey T (2015): Can native egg parasitoids adopt the invasive  
293 brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), in Europe? J  
294 Pest Sci 88: 693-705.

295 Kenyon LJ, Meulia T and Sabree ZL (2015): Habitat visualization and genomic analysis of  
296 ‘*Candidatus Pantoea carbekii*,’ the primary symbiont of the brown marmorated stink bug.  
297 Genome Biol Evol 7: 620–635.

298 Inkley DB (2012): Characteristics of home invasion by the brown marmorated stink bug  
299 (Hemiptera: Pentatomidae). J Entomol Sci 47: 125-130.

300 Lee DH, Short BD, Joseph SV, Bergh JC, Leskey TC (2013): Review of the biology, ecology,  
301 and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the  
302 Republic of Korea. *Environ Entomol* 42: 627–641.

303 Leskey TC, Nielsen AL (2018): Impact of the invasive brown marmorated stink bug in North  
304 America and Europe: History, Biology, Ecology, and Management. *Annu Rev Entomol* 63:  
305 599–618.

306 Marzachì C, Bosco D (2005): Relative quantification of chrysanthemum yellows (16Sr I)  
307 phytoplasma in its plant and insect host using real-time polymerase chain reaction. *Mol.*  
308 *Biotechnol.* 30: 117-127.

309 Mathews CR, Barry S (2014): Compost tea reduces egg hatch and early-stage nymphal  
310 development of *Halyomorpha halys* (Hemiptera: Pentatomidae). *Fla Entomol* 97: 1726-1732.

311 Moran NA, McCutcheon JP, Nakabachi A (2008): Genomics and evolution of heritable  
312 bacterial symbionts. *Annu Rev Genet* 42: 165-190.

313 Narciso JA, Ference CM, Ritenour MA, Widmer WW (2012): Effect of copper hydroxide  
314 sprays for citrus canker control on wild-type *Escherichia coli*. *Lett Appl Microbiol* 54: 108-  
315 111.

316 Navarrete F, De La Fuente L (2015): Response of *Xylella fastidiosa* to zinc: decreased  
317 culturability, increased exopolysaccharide production, and formation of resilient biofilms under  
318 flow conditions. *Appl Environ Microbiol* 80: 1097–1107.

319 Otero-Bravo A, Sabree ZL (2015): Inside or out? Possible genomic consequences of  
320 extracellular transmission of crypt-dwelling stinkbug mutualists. *Front Ecol Evol* 3: 64.



321 Palmer AK, Evans KJ, Metcalf DA (2010): Characters of aerated compost tea from immature  
322 compost that limit colonization of bean leaflets by *Botrytis cinerea*. J Appl Microbiol 109: 1619  
323 1631.

324 Prado SS, Rubinoff D, Almeida RPP (2006): Vertical transmission of a pentatomid caeca-  
325 associated symbiont. Ann Entomol Soc Am 99: 577–585.

326 Rice KB, Bergh CJ, Bergmann EJ, Biddinger DJ, Dieckhoff C, Dively G, *et al.* (2014): Biology,  
327 ecology, and management of brown marmorated stink bug (Hemiptera: Pentatomidae) J Integr  
328 Pest Manag 5: 1–13.

329 Scheck HJ, Pscheidt JW (1998): Effect of copper bactericides on copper-resistant and -sensitive  
330 strains of *Pseudomonas syringae* pv. *syringae*. Plant Dis 82: 397-406.

331 Scortichini M, Chen J, De Caroli M, Dalessandro G, Pucci N, Modesti V, *et al.* (2018): A zinc,  
332 copper and citric acid biocomplex shows promise for control of *Xylella fastidiosa* subsp. *pauca*  
333 in olive trees in Apulia region (southern Italy). Phytopathol Mediterr 57: 48–72.

334 Taylor CM, Coffey PL, DeLay BD, Dively G (2014): The Importance of Gut Symbionts in the  
335 Development of the Brown Marmorated Stink Bug, *Halyomorpha halys* (Stål). PLOS ONE 9:  
336 e90312.

337 Taylor CM, Johnson V, Dively G (2017): Assessing the use of antimicrobials to sterilize brown  
338 marmorated stink bug egg masses and prevent symbiont acquisition. J Pest Sci 90: 1287–1294.

339

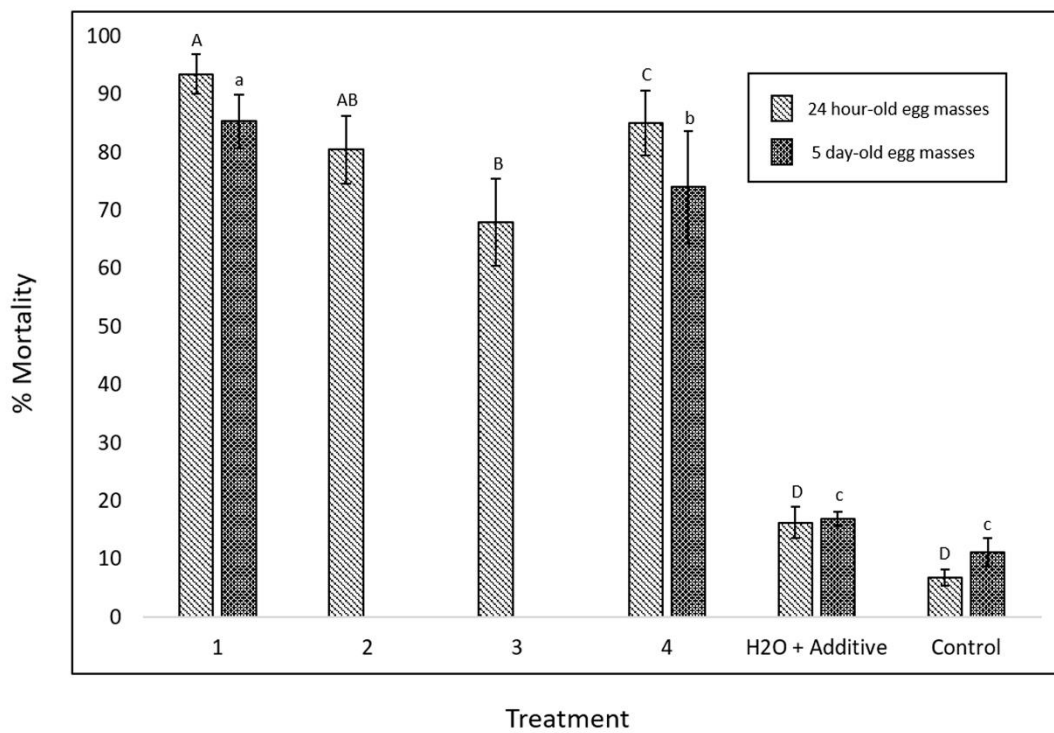
340 **Table 1.** Data recorded during laboratory experimental application of micronutrient EC  
 341 fertilizers to 24 hour-old and 5 day-old *H. halys* egg masses. Results are expressed as average  
 342 values  $\pm$  SE. For egg hatching rates, different letters indicate significantly different values  
 343 according to binomial GLM analysis + Bonferroni's test. Separate statistical tests were  
 344 conducted for 24 hour-old egg masses ( $df = 5$ ;  $\chi^2 = 41.376$ ;  $P < 0.05$ ) and 5 day-old egg masses  
 345 ( $df = 3$ ;  $\chi^2 = 29.332$ ;  $P < 0.05$ ). Normalized mortality rates were obtained with respect to  
 346 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 $\pm$ 1.12	68.60 $\pm$ 1.78 a	92.60 $\pm$ 0.29
	Product (2)	25.6 $\pm$ 0.95	81.64 $\pm$ 1.24 b	90.96 $\pm$ 0.86
	Product (3)	26 $\pm$ 1.03	82.30 $\pm$ 1.22 b	87.67 $\pm$ 1.44
	Product (4)	24.2 $\pm$ 1.71	66.94 $\pm$ 2.20 a	91.58 $\pm$ 0.68
	Water + additive	19.70 $\pm$ 2.04	71.06 $\pm$ 1.91 ab	64.36 $\pm$ 15.17
	Untreated control	24.75 $\pm$ 1.46	82.22 $\pm$ 1.68 b	0.00
5 days	Product (1)	26.40 $\pm$ 1.10	82.57 $\pm$ 2.26 c	87.84 $\pm$ 0.76
	Product (4)	21.00 $\pm$ 1.57	60.95 $\pm$ 1.96 a	82.57 $\pm$ 3.38
	Water + additive	21.06 $\pm$ 2.10	68.05 $\pm$ 1.77 ab	37.25 $\pm$ 4.71
	Untreated control	22.90 $\pm$ 1.93	75.10 $\pm$ 1.76 bc	0.00

347

348

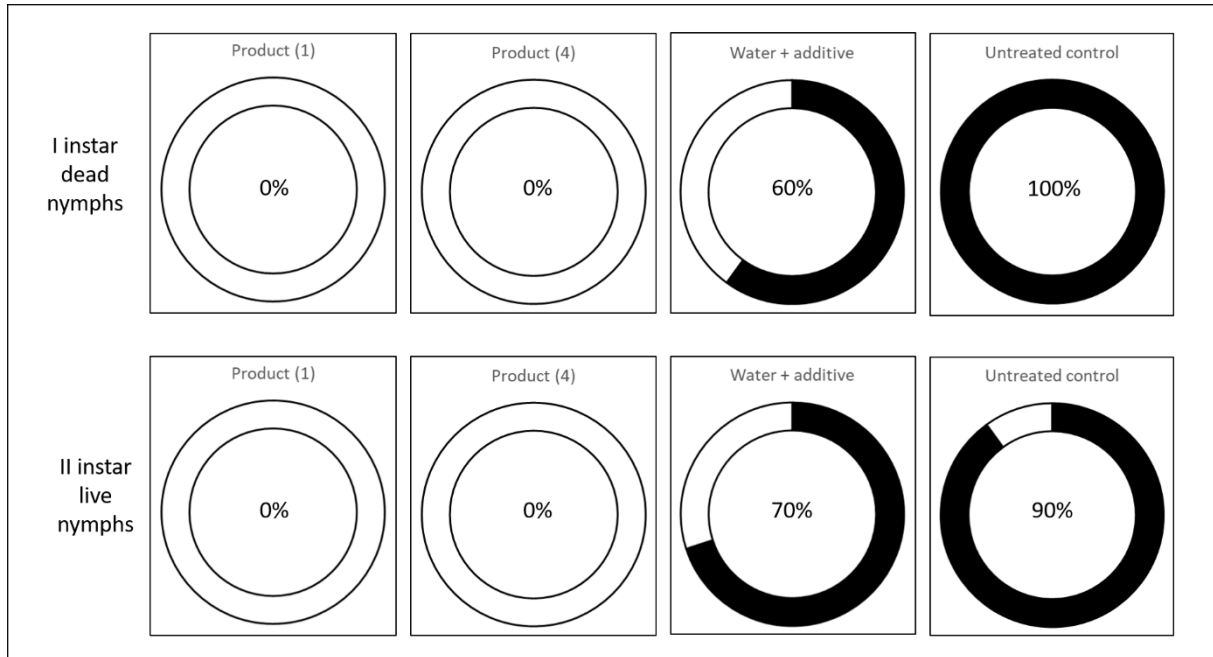
349 **Fig. 1.** Mortality rates recorded for *H. halys* neonate nymphs after treatment with different  
350 micronutrient fertilizers. The percentage of dead nymphs before reaching II instar was  
351 calculated for 24 hour-old (light columns) and 5 day-old (dark columns) egg masses. Bars  
352 indicate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



354

355

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



357