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Impact of triflumuron on *Halyomorpha halys* (Hemiptera: Pentatomidae): laboratory and field studies

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Abstract

Halyomorpha halys, (the brown marmorated stink bug, BMSB), is a high-concern invasive species causing severe damage to orchards in many countries outside its native Asian range. Control options matching both effectiveness and sustainability are currently lacking. Inhibitors of chitin biosynthesis might be exploited for integrated management programs because of the overall better ecotoxicological profile in comparison with most neurotoxic insecticides used so far against BMSB. In this study, the activity of triflumuron, a benzoylphenyl urea hampering chitin biosynthesis, was tested on BMSB in laboratory and field conditions. In laboratory bioassays, the insecticide was sprayed on potted peach plants (30 cm high) and residues were aged in a glasshouse for 0, 7, 14 and 21 days. Then 3rd instar bugs were placed on the plants and continuously exposed to residues. Mortality was scored after 7, 14 and 21 day exposure. Triflumuron caused significantly higher mortality on BMSB nymphs in comparison with water controls at all aging periods. Moreover, aging of residues up to 21 days did not cause any significant reduction of activity. Field experiments were also carried out in 2019 in eight pear orchards. Injuries to fruits at harvest were compared between plots where triflumuron was added to insecticide sprays against BMSB and control plots managed exactly in the same way but without any triflumuron treatment. An overall mean of 9.99 ± 1.98% stink bug injured fruits was detected in plots managed with the strategy including triflumuron, whereas 19.45 ± 3.55% of fruits were injured in plots assigned to controls.

Keywords: Brown marmorated stink bug; Invasive species; Inhibitors of chitin biosynthesis; Integrated pest management; Reduced-risk insecticides.

Introduction

The brown marmorated stink bug (BSMB) - *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) - is a serious agricultural pest in most countries where it has been accidentally introduced (Bosco et al. 2017, Leskey and Nielsen 2018). Current management strategies rely mostly on neurotoxic broad-spectrum insecticides that are quite effective in preventing BMSB damages but have a short residual activity (Kuhar and Kamminga 2017). This forces farmers to increase the frequency of insecticide sprays (Leskey et al. 2012) causing the disruption of previously established integrated pest management (IPM) programs and the resurgence of secondary pests (Leskey and Nielsen 2018). The identification of more selective insecticides targeting BMSB would significantly reduce the environmental issues associated to its control and could contribute to IPM principles. Besides efficacy on BMSB and less severe side effects, residual activity of insecticides could play a key role in restoring sustainable management programs.

Benzoylphenyl ureas (BPUs) are classified by Insecticide Resistance Action Committee (IRAC, https://www.irac-online.org/modes-of-action) into group 15 (Inhibitors of chitin biosynthesis, type 0). These compounds interfere with chitin biosynthesis, thus hindering insect molts. Detrimental effects on fecundity and egg viability are reported as well (Pener and Dhadialla 2012). Although BPUs have been used as pesticides since the 1970s (Retnakaran and Wright 1987, Spomer and Sheets 2019), the exact mode of action is still quite obscure (Zhu et al. 2016). However, Douris et al. (2016) shed some light on the molecular targets of BPUs by demonstrating a direct interaction with the enzyme chitin synthase 1. These insecticides are persistent and do not show cross-resistance with neurotoxic active ingredients (Doucet and Retnakaran 2012, Arruda et al. 2020). Moreover, BPUs are considered less harmful to beneficial insects and non-target organisms than most nerve poisons (Sun et al. 2015). For these reasons, BPUs are desirable for inclusion in IPM programs in several cropping systems (Dhadialla et al. 2005).

Several studies focused on the effects of a number of insecticides on BMSB, but the activity of

BPUs has not been investigated extensively. Kamminga et al. (2012) reported promising effects of diflubenzuron and novaluron on BMSB nymphs in laboratory assays carried out in Petri dishes, no more investigations were undertaken. The first objective of this study was to evaluate the activity of triflumuron (the only BPU currently allowed on orchards in EU) on BMSB nymphs by means of laboratory assays. The effects of aged residues were also investigated because triflumuron is known to be persistent on vegetation (Marx 1977, Aplada-Sarlis et al. 1999) and this could be a crucial factor to reduce the frequency of insecticide sprays (Kuhar and Kamminga 2017). Potted peach plants were used in the experiments because this is more representative of real conditions for residues aging. Moreover, the use of living plants allowed a prolonged exposure of insects, which is needed because of the mode action of BPUs (Dhadialla et al. 2005). A second aim of the study was to test, by mean of a field experiment set up in 2019 on pear orchards, if the addition of triflumuron to IPM strategies against BMSB could lead to a decrease in injuries to fruit.

Materials and methods

Insects

BMSB nymphs were obtained from a laboratory colony established in 2015 at the Department of Agricultural and Food Sciences (University of Bologna). Bugs were reared in a walk-in climatic chamber at 25° C ± 2° C, 50%–70% RH, with a 14 L:10 D photoperiod. Adults and nymphs were fed twice per week with green beans, carrots and soybean seeds; fresh fruits (pears, apples or kiwi fruits) were provided weekly to adults only.

Insecticide treatments

The insecticide Alsystin 48SC (triflumuron 39.34% = 480 g L^{-1}) was provided by Bayer Crop Sciences (Milan, Italy) and applied at 25 mL hL^{-1}, the maximum field recommended concentration for pome and stone fruits. Potted peach plants (GF-677 rootstock, approximately 30 cm high) were sprayed using a hand sprayer up to dripping and allowed to dry completely before trials. Control plants were sprayed in the same way with tap water.

Laboratory bioassays

From July 2017 to November 2018, four aging periods (0, 7, 14 and 21 days) of insecticide residues were investigated, each in a separate experiment. Twelve experimental units, each consisting of a potted peach plant, were arranged for each aging period. Six plants were treated with triflumuron and the others, which were assigned to control, with tap water. Overall, 48 experimental units were set up and 480 BMSB nymphs were tested.

Once sprayed outdoor, plants and were transferred into a glasshouse box where residues of the treatments aged. Here the plants were maintained for 7, 14 or 21 days at the following conditions: T_{max} 30° C, T_{min} 15° C, 16 L:8 D photoperiod, minimum artificial lighting in photophase 70,000 lx. In the experiment of 0-day aging of residues, the peach plants were used immediately after treatment droplets dried.

To set up experimental units, potted plants were placed in plexiglas cylinders (diameter 8 cm, height 30 cm), sealed with a fine net on the top and with a hole (diameter 8 cm) closed with a fine net on the base to allow air circulation and avoid mold growth. The soil surface on the pot was covered with nonwoven fabric, which was tightly wrapped to plant stem. Ten BMSB early 3rd instar nymphs were placed in each cylinder. Given that BMSB nymphs must feed on fruit or vegetables to complete development, carrots and green beans were provided ad libitum and changed twice per week. The green beans were hung on the plants to force the nymphs to climb on canopy in order to feed (Supplementary data Fig. SD1).

Experimental units were held at the same conditions reported for BMSB rearing and mortality was checked after 7, 14 and 21 days of continuous exposure of nymphs to residues. Besides the number of dead nymphs also their instars, and therefore the number of molts they successfully completed, was recorded. Moribund nymphs (i.e. unable to upright themself when flipped on their back) found at the last exposure interval were reared individually until they died or reached the adult stage (recorded as a recovery). Nymphs were held in ventilated 200-mL plastic jars at the same conditions and with the same food provisions as previously reported.

Field trials

From 21 June to 5 September 2019, field experiments were carried out in eight pear orchards located in Northern Italy, which suffered severe damages by BMSB in the previous years. Each orchard was considered as an experimental block and two contiguous plots (> 0.5 ha) were delimited. One plot was managed according to IPM strategies (here after referred as "farmer's strategy") recommended in Northern Italy. The other plot of each orchard underwent the same management practices as the former, but a treatment with Alsystin 48 SC at 25 mL hL^{-1} was also added (hereafter reported as triflumuron strategy). This treatment was carried out between 21 and 30 June targeting the first-generation nymphs of the BMSB. Of course, no triflumuron treatment was allowed in plots managed with farmer's strategy. The sprays of broad-spectrum insecticides, which included chlorpyrifos-methyl, acetamiprid and pyrethroids (deltamethrin, lambda-cyhalothrin and tau-fluvalinate), varied slightly among orchards, as farmers were allowed to make minor changes in order to adjust for pest pressure and phenology. These sources of variation were accounted in the block factor.

Fruit sampling was carried out in a transect of 3 rows x 15 m. The transects were established in the middle of each plot at least 10 m away from plot edges to avoid border effect from the perimeter of the orchards and to accommodate for drift of triflumuron spray. External injuries were checked at harvesting on a sample of 400 fruits per plot. Two hundred fruits were sampled at eye-level and 200 were sampled above 2.5-m height using a fruit picking ladder, because most severe injuries by BMSB are usually recorded on fruit growing in the upper parts of the trees (Bariselli et al. 2016).

Pears were examined directly on the trees and scored as injured if at least one distinct depression or discoloration on fruit surface compatible with BMSB punctures could be detected (Acebes-Doria et al. 2016). Although the occasional occurrence of Heteropteran bugs other than BMSB could not be ruled out, the overwhelming majority of fruit injuries were likely due to feeding activity by BMSBs. Damages to fruit by Heteropteran bugs have been rarely reported in orchards of Northern Italy and the symptoms used to score injuries to pears are quite specific to BMSB.

Statistical analysis

To assess the activity of triflumuron on BMSB nymphs in laboratory experiments, insecticide-treated plants and relative water controls were compared within each aging period of residues. Data were analyzed by general linear mixed models (GLMM) with first-order autoregressive covariance structure, binomial distribution, probit link function and Kenward–Roger method for estimating degrees of freedom. The number of dead insects out of the total nymphs tested was considered as dependent variable. The treatments (triflumuron and water control) were used as factors, the exposure intervals (7, 14 and 21 days) were included as repeated measures and their interaction (treatment x exposure interval) were tested as well.

To study the possible decrease in the activity of triflumuron residues (i.e. to compare mortality only in insecticide-treated plants among different aging period), nymphs mortality recorded in triflumuron experimental units was corrected by Schneider-Orelli formula (Schneider-Orelli 1947) considering as natural the mean mortality recorded in water controls aged for the same period. Corrected percentages of mortality matched the assumptions of parametric tests and were analyzed by a mixed design ANOVA without any transformation. Aging periods (0, 7, 14 and 21 days) were considered as between-subject factor and exposure intervals (7, 14 and 21) as within-subject factor. The interaction aging period x exposure interval was tested as well.

Pearson $\chi^2$was used to test the association between the ages of residues and the number of molts that dead nymphs exposed to triflumuron were able to successfully complete before dying.

A GLMM was carried out to analyze data from the field experiments. Binomial error distribution and log link function were selected, the number of injured fruits out of the total number of fruits checked was considered as dependent variable, treatments (farmer's strategy vs triflumuron strategy) and location of fruits (>2.5 m vs eye level) were considered as fixed factors, and orchards were included as random block factor.

All the analyses were performed using IBM SPSS Statistics (ver. 26).

Results

Laboratory bioassays

GLMMs detected significant effects on mortality by both main factors (i.e., treatment and exposure interval) for all aging periods of residues. Whereas the interaction treatment * exposure interval was significant only for 7- and 14 -day aged residues (Table 1). In other word, triflumuron residues caused overall higher mortality than water controls and longer exposure interval increased mortality (Fig. 1). Because of significant interactions, treatments were compared within each of the exposure intervals for 7-and 14-day old residues. For both aging periods, GLMMs did not detect significant difference between triflumuron and control at 7-day exposure but indicated higher mortality in triflumuron than in water at 14- and 21-day exposures (Table 1).

Mortality increased at longer exposure intervals also in the controls with a certain degree of variation among aging periods. Overall, the lowest number of dead nymphs was counted in the controls of residues tested immediately after drying. Whereas for other aging periods the natural mortality ranged from 16.06 ± 3.99% to 28.60 ± 9.80%, 23.55 ± 4.99% to 38.14 ± 7.30% and 30.07 ± 4.77% to 47.79 ± 6.61% for 7-, 14- and 21-day exposure, respectively.

The comparison of corrected percentages of mortality did not show any significant effect of aging periods on the activity of triflumuron residues ($F_{(3;\,20)} = 1.34$; $p = 0.29$). The interaction aging period * exposure interval was not significant too ($F_{(6; 40)} = 1.32$; $p = 0.27$). Therefore, the aging of residues up to 21 days did not significantly decrease the activity of the insecticide.

The number of molts that nymphs exposed to triflumuron were able to complete before dying did not differ among aging periods ($\chi^2 = 6.34$; df = 6; $p = 0.39$). Therefore, the ages of residues did not significantly affect the insecticide speed of action. Pooling all aging periods, 29.70% of BMSB nymphs died without completing any molt, 57.43% of individuals successfully molted from 3rd to 4th instar but died before or during the next molt, 12.87% reached the 5th instar but failed to cast the exuviae and died during the molt to adult stage (Supplementary data Fig. SD2).

Field trials

The addition of triflumuron to insecticide treatments allowed in IPM strategy for BMSB led to a significant decrease in the percentage of injured fruits ($F_{(1; 28)} = 106.93$; $p < 0.001$). Fruit location had also a significant effect ($F_{(1; 28)} = 137.92$; $p < 0.001$), and the percentage of injured fruits was higher above 2.5 m than at eye level. The interaction strategy * fruit location was not significant ($F_{(1; 28)} = 0.81$; $p = 0.78$). A mean of 9.99 ± 1.98% injured fruits was detected in plots managed with triflumuron strategy, whereas 19.45 ± 3.55% of fruits were injured in plots assigned to farmer's strategy. Overall, 20.19 ± 3.42% of pears showed symptoms of feeding activity by BMSB above 2.5 m. This percentage dropped to 9.25 ±1.95 at eye level (Fig. 2).

Discussion

Triflumuron residues on potted peach plants showed a significant activity on BMSB nymphs that died increasingly between progressive nymphal instars. These results agreed with laboratory assays by Kamminga et al. (2012) who reported 90% and 65% mortality of BMSB nymphs fed for 7 days with fresh beans dipped in solutions of novaluron and diflubenzuron, respectively. Significant reduction in the abundance of BMSB and other stink bugs was also described for diflubenzuron applications to soybean in open field conditions (Herbert et al. 2013). On the other hand, Gradish et al. (2019) found neither contact nor residual toxicity of novaluron on BMSB nymphs. However, in this study, mortality was checked only in the short term, whereas prolonged exposure to BPU residues is necessary to detect activity of this group of insecticide (Dhadialla et al. 2005).

The activity of triflumuron on BMSB nymphs persisted up to 21 days of residue aging in glasshouse. Overall, the mortality of bugs exposed to the insecticide was higher than in controls for all ages of residues. Moreover, no significant decrease in mortality could be detected on insecticide-treated plants at increasing periods of residues aging. The environmental persistence of BPUs has been well documented and the slow decrease in residue concentration of triflumuron over time (Bayer Crop Sciences, unpublished data) is in line with the steady insecticidal activity of this active ingredient up to the longest aging period. Given that treated plants were maintained in a glasshouse, the only relevant insecticide degradation factor was photolysis. Rainfall, which was not considered in this study, seems indeed a minor issue as BPUs are known to adhere to plant waxes and to undergo to slight wash off (Retnakaran and Wright 1987, Doucet and Retnakaran 2012).

A relatively high mortality in the controls of some aging periods was related to the long-term exposure used in the bioassays. Fluctuations in nymphal survival has been often observed in BMSB laboratory colonies. Our findings were in line with Medal et al. (2013) who reported a juvenile mortality from 23% to 50% in BMSB rearing and with Fisher et al. (2020) who recorded overall low nymphal survival when testing the influence of temperature and humidity on BMSB.

Progressive poisoning through tarsal adsorption seemed the main way of insecticide contamination because most of BMSB nymphs did not die at the first molt, but many insects completed one or even two molts before succumbing. Ingestion has been usually considered as the major way of uptake for BPUs, but slow penetration through cuticle was also described (Hammann and Sirrenberg 1980, Retnakaran and Wright 1987, Sun et al. 2015). This second way of triflumuron uptake was likely prevalent in our experiments, given that untreated food was used and the bug probing on treated peach plants was negligible.

Given that intoxicated nymphs could survive for a couple of weeks, the damage they may cause before succumbing needs to be taken into account. In this context, sub lethal effects may be worth of consideration as secondary impacts on pest population (Cira et al. 2017, Depalo et al. 2017). In particular, BPUs could reduce hatch rate, impair feeding activity and increase susceptibility to pathogens on intoxicated BMSB (Catchot et al. 2021).

Early BMSB instars are more susceptible to insecticides than adults and several insecticides have demonstrated a significant activity on nymphs (Kuhar and Kamminga 2017, Gradish et al. 2019). Mostly broad-spectrum neurotoxic active ingredients have been so far investigated and, in comparison with these insecticides, BPUs are more compatible with IPM (Dhadialla et al. 2005, Doucet and Retnakaran 2012). Nevertheless, BPUs do not lack any impact on beneficials. Lethal effects have been found on nymphs of predatory bugs (Soares et al. 2019) and on larvae of ladybeetles (Cabrera et al 2017). Some sub-lethal effects have been reported on adult parasitoids either after direct contact with residues (Matioli et al. 2019) or when parasitized eggs of the hosts were treated (Goulart et al. 2012). Therefore, the possible detrimental effects of BPUs on natural enemies of BMSB seem worth of investigations to draw a comprehensive picture of the possible role of these insecticides in IPM scenario.

Besides the environmental side effects and the disruption of IPM practices, the overuse of a few neurotoxic insecticides to control BMSB often impaired the rotation of insecticide chemistries upon which insecticide resistance management strategy should rely (Hurley and Mitchell 2014). The potential for insecticide-resistance development in BMSB populations has not been established yet, but it would be wise to avoid over-reliance on neurotoxic active ingredients (Alford et al. 2020). From this standpoint, the inclusion of BPUs in IPM practices could help to restore active ingredient rotation as a strategy to delay the insurgence of resistance (Spomer and Sheets 2019).

The long-lasting activity of triflumuron, and possibly other BPUs, could be exploited to reduce damages by BMSB nymphs without the need of frequent treatments. The short residual activity, which lasts less than three days for most insecticides used against BMSB (Leskey et al. 2014), is widely recognized as a major problem (Kuhar and Kamminga 2017). This pest is polyphagous and very mobile; both nymphs and adults have big dispersal abilities and move across the landscape following phenology of crops and uncropped plants (Martinson et al. 2015, Hamilton et al. 2018). Repeated and unpredictable infestations of orchards are common and frequent sprays of short-lasting insecticides are needed.

The field experiments, which showed a significant decrease in the percentages of injured pears where triflumuron was added, reinforced the findings of laboratory assays. Field trials pointed out also that in 2019 strategies mainly relying on neurotoxic active ingredients did not achieve a satisfactory control of BMSB. The mean percentage of injured fruit recorded in plots managed by farmer's strategy (19.45 ± 3.55) far exceeded the economic threshold. Although the percentage of injured fruits dropped to 9.99 ± 1.98 in the plots where triflumuron was added, this was still not completely acceptable from an economic standpoint. A refinement of management strategies for BMSB seems therefore urgently needed and the development of treatment thresholds for this stink bug would be a relevant step forward (Short et al. 2017).

BPUs lack any lethal effect on adult insects and have no chance to decrease the pressure exerted by highly damaging adult bugs that migrate into crops from surrounding areas (Acebes-Doria et al. 2016). Therefore, triflumuron and other BPUs might be considered for field applications only as a tool in a wider management strategy. Control methods aimed at preventing the invasion of crops by adult bugs such as exclusion nets (Candian et al. 2018), insecticide netting screens and sprays at orchard perimeter (Blaauw et al. 2015) might take major benefits from few and long-lasting BPU treatments aimed decreasing nymphal populations and hampering reproduction cycle that can occur within the orchards.

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Figure Caption

Fig. 1. Mortality of *Halyomorpha halys* nymphs exposed for 7, 14 and 21 days to residues of triflumuron (black line) or control (gray line) on potted peach plants. Each panel represent a different aging period of residues in glasshouse conditions. Vertical bars indicate standard errors of the means. Asterisks indicate significant differences between treatments, which were compared within exposure intervals, in the case of significant interaction of treatment * exposure interval detected by GLMM.

Fig. 2. Percentages of fruit with feeding injuries by *Halyomorpha halys* recorded in 2019 in pear orchards in Northern Italy. GLMM detected significant differences between strategies ($p<0.001$) and between fruit locations ($p<0.001$). Vertical bars indicate standard errors of the means.

Impact of triflumuron on *Halyomorpha halys* (Hemiptera: Pentatomidae): laboratory and field studies

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Abstract

Halyomorpha halys, (the brown marmorated stink bug, BMSB), is a high-concern invasive species causing severe damage to orchards in many countries outside its native Asian range. Control options matching both effectiveness and sustainability are currently lacking. Inhibitors of chitin biosynthesis might be exploited for integrated management programs because of the overall better ecotoxicological profile in comparison with most neurotoxic insecticides used so far against BMSB. In this study, the activity of triflumuron, a benzoylphenyl urea hampering chitin biosynthesis, was tested on BMSB in laboratory and field conditions. In laboratory bioassays, the insecticide was sprayed on potted peach plants (30 cm high) and residues were aged in a glasshouse for 0, 7, 14 and 21 days. Then 3rd instar bugs were placed on the plants and continuously exposed to residues. Mortality was scored after 7, 14 and 21 day exposure. Triflumuron caused significantly higher mortality on BMSB nymphs in comparison with water controls at all aging periods. Moreover, aging of residues up to 21 days did not cause any significant reduction of activity. Field experiments were also carried out in 2019 in eight pear orchards. Injuries to fruits at harvest were compared between plots where triflumuron was added to insecticide sprays against BMSB and control plots managed exactly in the same way but without any triflumuron treatment. An overall mean of 9.99 ± 1.98% stink bug injured fruits was detected in plots managed with the strategy including triflumuron, whereas 19.45 ± 3.55% of fruits were injured in plots assigned to controls.

Keywords: Brown marmorated stink bug; Invasive species; Inhibitors of chitin biosynthesis; Integrated pest management; Reduced-risk insecticides.

Introduction

The brown marmorated stink bug (BSMB) - *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) - is a serious agricultural pest in most countries where it has been accidentally introduced (Bosco et al. 2017, Leskey and Nielsen 2018). Current management strategies rely mostly on neurotoxic broad-spectrum insecticides that are quite effective in preventing BMSB damages but have a short residual activity (Kuhar and Kamminga 2017). This forces farmers to increase the frequency of insecticide sprays (Leskey et al. 2012) causing the disruption of previously established integrated pest management (IPM) programs and the resurgence of secondary pests (Leskey and Nielsen 2018). The identification of more selective insecticides targeting BMSB would significantly reduce the environmental issues associated to its control and could contribute to IPM principles. Besides efficacy on BMSB and less severe side effects, residual activity of insecticides could play a key role in restoring sustainable management programs.

Benzoylphenyl ureas (BPUs) are classified by Insecticide Resistance Action Committee (IRAC, https://www.irac-online.org/modes-of-action) into group 15 (Inhibitors of chitin biosynthesis, type 0). These compounds interfere with chitin biosynthesis, thus hindering insect molts. Detrimental effects on fecundity and egg viability are reported as well (Pener and Dhadialla 2012). Although BPUs have been used as pesticides since the 1970s (Retnakaran and Wright 1987, Spomer and Sheets 2019), the exact mode of action is still quite obscure (Zhu et al. 2016). However, Douris et al. (2016) shed some light on the molecular targets of BPUs by demonstrating a direct interaction with the enzyme chitin synthase 1. These insecticides are persistent and do not show cross-resistance with neurotoxic active ingredients (Doucet and Retnakaran 2012, Arruda et al. 2020). Moreover, BPUs are considered less harmful to beneficial insects and non-target organisms than most nerve poisons (Sun et al. 2015). For these reasons, BPUs are desirable for inclusion in IPM programs in several cropping systems (Dhadialla et al. 2005).

Several studies focused on the effects of a number of insecticides on BMSB, but the activity of

BPUs has not been investigated extensively. Kamminga et al. (2012) reported promising effects of diflubenzuron and novaluron on BMSB nymphs in laboratory assays carried out in Petri dishes, no more investigations were undertaken. The first objective of this study was to evaluate the activity of triflumuron (the only BPU currently allowed on orchards in EU) on BMSB nymphs by means of laboratory assays. The effects of aged residues were also investigated because triflumuron is known to be persistent on vegetation (Marx 1977, Aplada-Sarlis et al. 1999) and this could be a crucial factor to reduce the frequency of insecticide sprays (Kuhar and Kamminga 2017). Potted peach plants were used in the experiments because this is more representative of real conditions for residues aging. Moreover, the use of living plants allowed a prolonged exposure of insects, which is needed because of the mode action of BPUs (Dhadialla et al. 2005). A second aim of the study was to test, by mean of a field experiment set up in 2019 on pear orchards, if the addition of triflumuron to IPM strategies against BMSB could lead to a decrease in injuries to fruit.

Materials and methods

Insects

BMSB nymphs were obtained from a laboratory colony established in 2015 at the Department of Agricultural and Food Sciences (University of Bologna). Bugs were reared in a walk-in climatic chamber at 25° C ± 2° C, 50%–70% RH, with a 14 L:10 D photoperiod. Adults and nymphs were fed twice per week with green beans, carrots and soybean seeds; fresh fruits (pears, apples or kiwi fruits) were provided weekly to adults only.

Insecticide treatments

The insecticide Alsystin 48SC (triflumuron 39.34% = 480 g L^{-1}) was provided by Bayer Crop Sciences (Milan, Italy) and applied at 25 mL hL^{-1}, the maximum field recommended concentration for pome and stone fruits. Potted peach plants (GF-677 rootstock, approximately 30 cm high) were sprayed using a hand sprayer up to dripping and allowed to dry completely before trials. Control plants were sprayed in the same way with tap water.

Laboratory bioassays

From July 2017 to November 2018, four aging periods (0, 7, 14 and 21 days) of insecticide residues were investigated, each in a separate experiment. Twelve experimental units, each consisting of a potted peach plant, were arranged for each aging period. Six plants were treated with triflumuron and the others, which were assigned to control, with tap water. Overall, 48 experimental units were set up and 480 BMSB nymphs were tested.

Once sprayed outdoor, plants and were transferred into a glasshouse box where residues of the treatments aged. Here the plants were maintained for 7, 14 or 21 days at the following conditions: T_{max} 30° C, T_{min} 15° C, 16 L:8 D photoperiod, minimum artificial lighting in photophase 70,000 lx. In the experiment of 0-day aging of residues, the peach plants were used immediately after treatment droplets dried.

To set up experimental units, potted plants were placed in plexiglas cylinders (diameter 8 cm, height 30 cm), sealed with a fine net on the top and with a hole (diameter 8 cm) closed with a fine net on the base to allow air circulation and avoid mold growth. The soil surface on the pot was covered with nonwoven fabric, which was tightly wrapped to plant stem. Ten BMSB early 3rd instar nymphs were placed in each cylinder. Given that BMSB nymphs must feed on fruit or vegetables to complete development, carrots and green beans were provided ad libitum and changed twice per week. The green beans were hung on the plants to force the nymphs to climb on canopy in order to feed (Supplementary data Fig. SD1).

Experimental units were held at the same conditions reported for BMSB rearing and mortality was checked after 7, 14 and 21 days of continuous exposure of nymphs to residues. Besides the number of dead nymphs also their instars, and therefore the number of molts they successfully completed, was recorded. Moribund nymphs (i.e. unable to upright themself when flipped on their back) found at the last exposure interval were reared individually until they died or reached the adult stage (recorded as a recovery). Nymphs were held in ventilated 200-mL plastic jars at the same conditions and with the same food provisions as previously reported.

Field trials

From 21 June to 5 September 2019, field experiments were carried out in eight pear orchards located in Northern Italy, which suffered severe damages by BMSB in the previous years. Each orchard was considered as an experimental block and two contiguous plots (> 0.5 ha) were delimited. One plot was managed according to IPM strategies (here after referred as "farmer's strategy") recommended in Northern Italy. The other plot of each orchard underwent the same management practices as the former, but a treatment with Alsystin 48 SC at 25 mL hL^{-1} was also added (hereafter reported as triflumuron strategy). This treatment was carried out between 21 and 30 June targeting the first-generation nymphs of the BMSB. Of course, no triflumuron treatment was allowed in plots managed with farmer's strategy. The sprays of broad-spectrum insecticides, which included chlorpyrifos-methyl, acetamiprid and pyrethroids (deltamethrin, lambda-cyhalothrin and tau-fluvalinate), varied slightly among orchards, as farmers were allowed to make minor changes in order to adjust for pest pressure and phenology. These sources of variation were accounted in the block factor.

Fruit sampling was carried out in a transect of 3 rows x 15 m. The transects were established in the middle of each plot at least 10 m away from plot edges to avoid border effect from the perimeter of the orchards and to accommodate for drift of triflumuron spray. External injuries were checked at harvesting on a sample of 400 fruits per plot. Two hundred fruits were sampled at eye-level and 200 were sampled above 2.5-m height using a fruit picking ladder, because most severe injuries by BMSB are usually recorded on fruit growing in the upper parts of the trees (Bariselli et al. 2016).

Pears were examined directly on the trees and scored as injured if at least one distinct depression or discoloration on fruit surface compatible with BMSB punctures could be detected (Acebes-Doria et al. 2016). Although the occasional occurrence of Heteropteran bugs other than BMSB could not be ruled out, the overwhelming majority of fruit injuries were likely due to feeding activity by BMSBs. Damages to fruit by Heteropteran bugs have been rarely reported in orchards of Northern Italy and the symptoms used to score injuries to pears are quite specific to BMSB.

Statistical analysis

To assess the activity of triflumuron on BMSB nymphs in laboratory experiments, insecticide-treated plants and relative water controls were compared within each aging period of residues. Data were analyzed by general linear mixed models (GLMM) with first-order autoregressive covariance structure, binomial distribution, probit link function and Kenward–Roger method for estimating degrees of freedom. The number of dead insects out of the total nymphs tested was considered as dependent variable. The treatments (triflumuron and water control) were used as factors, the exposure intervals (7, 14 and 21 days) were included as repeated measures and their interaction (treatment x exposure interval) were tested as well.

To study the possible decrease in the activity of triflumuron residues (i.e. to compare mortality only in insecticide-treated plants among different aging period), nymphs mortality recorded in triflumuron experimental units was corrected by Schneider-Orelli formula (Schneider-Orelli 1947) considering as natural the mean mortality recorded in water controls aged for the same period. Corrected percentages of mortality matched the assumptions of parametric tests and were analyzed by a mixed design ANOVA without any transformation. Aging periods (0, 7, 14 and 21 days) were considered as between-subject factor and exposure intervals (7, 14 and 21) as within-subject factor. The interaction aging period x exposure interval was tested as well.

Pearson $\chi^2$was used to test the association between the ages of residues and the number of molts that dead nymphs exposed to triflumuron were able to successfully complete before dying.

A GLMM was carried out to analyze data from the field experiments. Binomial error distribution and log link function were selected, the number of injured fruits out of the total number of fruits checked was considered as dependent variable, treatments (farmer's strategy vs triflumuron strategy) and location of fruits (>2.5 m vs eye level) were considered as fixed factors, and orchards were included as random block factor.

All the analyses were performed using IBM SPSS Statistics (ver. 26).

Results

Laboratory bioassays

GLMMs detected significant effects on mortality by both main factors (i.e., treatment and exposure interval) for all aging periods of residues. Whereas the interaction treatment * exposure interval was significant only for 7- and 14 -day aged residues (Table 1). In other word, triflumuron residues caused overall higher mortality than water controls and longer exposure interval increased mortality (Fig. 1). Because of significant interactions, treatments were compared within each of the exposure intervals for 7-and 14-day old residues. For both aging periods, GLMMs did not detect significant difference between triflumuron and control at 7-day exposure but indicated higher mortality in triflumuron than in water at 14- and 21-day exposures (Table 1).

Mortality increased at longer exposure intervals also in the controls with a certain degree of variation among aging periods. Overall, the lowest number of dead nymphs was counted in the controls of residues tested immediately after drying. Whereas for other aging periods the natural mortality ranged from 16.06 ± 3.99% to 28.60 ± 9.80%, 23.55 ± 4.99% to 38.14 ± 7.30% and 30.07 ± 4.77% to 47.79 ± 6.61% for 7-, 14- and 21-day exposure, respectively.

The comparison of corrected percentages of mortality did not show any significant effect of aging periods on the activity of triflumuron residues ($F_{(3; 20)} = 1.34$; $p = 0.29$). The interaction aging period * exposure interval was not significant too ($F_{(6;\ 40)} = 1.32$; $p = 0.27$). Therefore, the aging of residues up to 21 days did not significantly decrease the activity of the insecticide.

The number of molts that nymphs exposed to triflumuron were able to complete before dying did not differ among aging periods ($\chi^2 = 6.34$; df = 6; $p = 0.39$). Therefore, the ages of residues did not significantly affect the insecticide speed of action. Pooling all aging periods, 29.70% of BMSB nymphs died without completing any molt, 57.43% of individuals successfully molted from 3rd to 4th instar but died before or during the next molt, 12.87% reached the 5th instar but failed to cast the exuviae and died during the molt to adult stage (Supplementary data Fig. SD2).

Field trials

The addition of triflumuron to insecticide treatments allowed in IPM strategy for BMSB led to a significant decrease in the percentage of injured fruits ($F_{(1;\ 28)} = 106.93$; $p < 0.001$). Fruit location had also a significant effect ($F_{(1;\ 28)} = 137.92$; $p < 0.001$), and the percentage of injured fruits was higher above 2.5 m than at eye level. The interaction strategy * fruit location was not significant ($F_{(1;\ 28)} = 0.81$; $p = 0.78$). A mean of 9.99 ± 1.98% injured fruits was detected in plots managed with triflumuron strategy, whereas 19.45 ± 3.55% of fruits were injured in plots assigned to farmer's strategy. Overall, 20.19 ± 3.42% of pears showed symptoms of feeding activity by BMSB above 2.5 m. This percentage dropped to 9.25 ±1.95 at eye level (Fig. 2).

Discussion

Triflumuron residues on potted peach plants showed a significant activity on BMSB nymphs that died increasingly between progressive nymphal instars. These results agreed with laboratory assays by Kamminga et al. (2012) who reported 90% and 65% mortality of BMSB nymphs fed for 7 days with fresh beans dipped in solutions of novaluron and diflubenzuron, respectively. Significant reduction in the abundance of BMSB and other stink bugs was also described for diflubenzuron applications to soybean in open field conditions (Herbert et al. 2013). On the other hand, Gradish et al. (2019) found neither contact nor residual toxicity of novaluron on BMSB nymphs. However, in this study, mortality was checked only in the short term, whereas prolonged exposure to BPU residues is necessary to detect activity of this group of insecticide (Dhadialla et al. 2005).

The activity of triflumuron on BMSB nymphs persisted up to 21 days of residue aging in glasshouse. Overall, the mortality of bugs exposed to the insecticide was higher than in controls for all ages of residues. Moreover, no significant decrease in mortality could be detected on insecticide-treated plants at increasing periods of residues aging. The environmental persistence of BPUs has been well documented and the slow decrease in residue concentration of triflumuron over time (Bayer Crop Sciences, unpublished data) is in line with the steady insecticidal activity of this active ingredient up to the longest aging period. Given that treated plants were maintained in a glasshouse, the only relevant insecticide degradation factor was photolysis. Rainfall, which was not considered in this study, seems indeed a minor issue as BPUs are known to adhere to plant waxes and to undergo to slight wash off (Retnakaran and Wright 1987, Doucet and Retnakaran 2012).

A relatively high mortality in the controls of some aging periods was related to the long-term exposure used in the bioassays. Fluctuations in nymphal survival has been often observed in BMSB laboratory colonies. Our findings were in line with Medal et al. (2013) who reported a juvenile mortality from 23% to 50% in BMSB rearing and with Fisher et al. (2020) who recorded overall low nymphal survival when testing the influence of temperature and humidity on BMSB.

Progressive poisoning through tarsal adsorption seemed the main way of insecticide contamination because most of BMSB nymphs did not die at the first molt, but many insects completed one or even two molts before succumbing. Ingestion has been usually considered as the major way of uptake for BPUs, but slow penetration through cuticle was also described (Hammann and Sirrenberg 1980, Retnakaran and Wright 1987, Sun et al. 2015). This second way of triflumuron uptake was likely prevalent in our experiments, given that untreated food was used and the bug probing on treated peach plants was negligible.

Given that intoxicated nymphs could survive for a couple of weeks, the damage they may cause before succumbing needs to be taken into account. In this context, sub lethal effects may be worth of consideration as secondary impacts on pest population (Cira et al. 2017, Depalo et al. 2017). In particular, BPUs could reduce hatch rate, impair feeding activity and increase susceptibility to pathogens on intoxicated BMSB (Catchot et al. 2021).

Early BMSB instars are more susceptible to insecticides than adults and several insecticides have demonstrated a significant activity on nymphs (Kuhar and Kamminga 2017, Gradish et al. 2019). Mostly broad-spectrum neurotoxic active ingredients have been so far investigated and, in comparison with these insecticides, BPUs are more compatible with IPM (Dhadialla et al. 2005, Doucet and Retnakaran 2012). Nevertheless, BPUs do not lack any impact on beneficials. Lethal effects have been found on nymphs of predatory bugs (Soares et al. 2019) and on larvae of ladybeetles (Cabrera et al 2017). Some sub-lethal effects have been reported on adult parasitoids either after direct contact with residues (Matioli et al. 2019) or when parasitized eggs of the hosts were treated (Goulart et al. 2012). Therefore, the possible detrimental effects of BPUs on natural enemies of BMSB seem worth of investigations to draw a comprehensive picture of the possible role of these insecticides in IPM scenario.

Besides the environmental side effects and the disruption of IPM practices, the overuse of a few neurotoxic insecticides to control BMSB often impaired the rotation of insecticide chemistries upon which insecticide resistance management strategy should rely (Hurley and Mitchell 2014). The potential for insecticide-resistance development in BMSB populations has not been established yet, but it would be wise to avoid over-reliance on neurotoxic active ingredients (Alford et al. 2020). From this standpoint, the inclusion of BPUs in IPM practices could help to restore active ingredient rotation as a strategy to delay the insurgence of resistance (Spomer and Sheets 2019).

The long-lasting activity of triflumuron, and possibly other BPUs, could be exploited to reduce damages by BMSB nymphs without the need of frequent treatments. The short residual activity, which lasts less than three days for most insecticides used against BMSB (Leskey et al. 2014), is widely recognized as a major problem (Kuhar and Kamminga 2017). This pest is polyphagous and very mobile; both nymphs and adults have big dispersal abilities and move across the landscape following phenology of crops and uncropped plants (Martinson et al. 2015, Hamilton et al. 2018). Repeated and unpredictable infestations of orchards are common and frequent sprays of short-lasting insecticides are needed.

The field experiments, which showed a significant decrease in the percentages of injured pears where triflumuron was added, reinforced the findings of laboratory assays. Field trials pointed out also that in 2019 strategies mainly relying on neurotoxic active ingredients did not achieve a satisfactory control of BMSB. The mean percentage of injured fruit recorded in plots managed by farmer's strategy (19.45 ± 3.55) far exceeded the economic threshold. Although the percentage of injured fruits dropped to 9.99 ± 1.98 in the plots where triflumuron was added, this was still not completely acceptable from an economic standpoint. A refinement of management strategies for BMSB seems therefore urgently needed and the development of treatment thresholds for this stink bug would be a relevant step forward (Short et al. 2017).

BPUs lack any lethal effect on adult insects and have no chance to decrease the pressure exerted by highly damaging adult bugs that migrate into crops from surrounding areas (Acebes-Doria et al. 2016). Therefore, triflumuron and other BPUs might be considered for field applications only as a tool in a wider management strategy. Control methods aimed at preventing the invasion of crops by adult bugs such as exclusion nets (Candian et al. 2018), insecticide netting screens and sprays at orchard perimeter (Blaauw et al. 2015) might take major benefits from few and long-lasting BPU treatments aimed decreasing nymphal populations and hampering reproduction cycle that can occur within the orchards.

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Figure Caption

Fig. 1. Mortality of *Halyomorpha halys* nymphs exposed for 7, 14 and 21 days to residues of triflumuron (black line) or control (gray line) on potted peach plants. Each panel represent a different aging period of residues in glasshouse conditions. Vertical bars indicate standard errors of the means. Asterisks indicate significant differences between treatments, which were compared within exposure intervals, in the case of significant interaction of treatment * exposure interval detected by GLMM.

Fig. 2. Percentages of fruit with feeding injuries by *Halyomorpha halys* recorded in 2019 in pear orchards in Northern Italy. GLMM detected significant differences between strategies ($p<0.001$) and between fruit locations ($p<0.001$). Vertical bars indicate standard errors of the means.

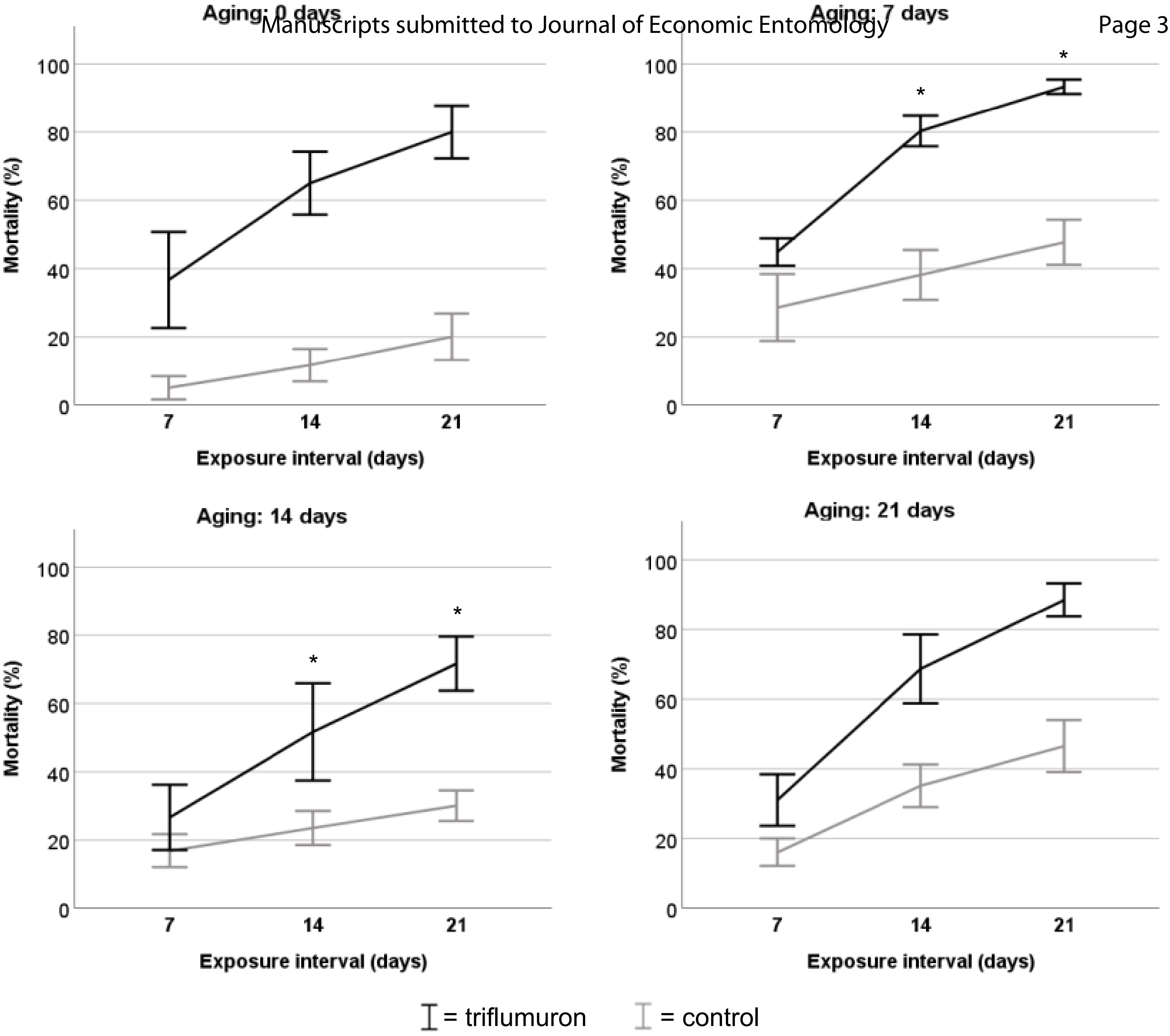
Table 1. Percentages of mortality (mean ± standard error) of *Halyomorpha halys* nymphs recorded for the different aging periods of residues at increasing exposure intervals. GLMMs were carried separately for each aging period and tests of fixed effects are reported. In the case of significant interaction of treatment * exposure interval, treatments were compared within each exposure interval and the *p* level is reported below mortality means.

0-day aged residues		**Exposure intervals**	
Treatment	7 days	14 days	21 days
Triflumuron	36.67 ± 14.06	65.00 ± 9.22	80.00 ± 7.72
Control	5.00 ± 3.42	11.67 ± 4.77	20.00 ± 6.83
GLMM effects	**F**	**df**	***p***
Treatment	14.35	1; 9.89	0.004
Exposure interval	10.58	2; 21.12	0.001
Treatment * Exposure interval	0.42	2; 21.12	0.661

7-day aged residues		**Exposure intervals**	
Treatment	7 days	14 days	21 days
Triflumuron	44.85 ± 4.01	80.30 ± 4.48	93.33 ± 2.11
Control	28.60 ± 9.80	38.14 ± 7.30	47.69 ± 6.61
	$p = 0.064$	$p < 0.001$	$p < 0.001$
GLMM effects	**F**	**df**	***p***
Treatment	21.03	1; 10.33	0.001
Exposure interval	27.88	2; 20.84	<0.001
Treatment * Exposure interval	7.50	2; 20.84	0.004

14-day aged residues		**Exposure intervals**	
Treatment	7 days	14 days	21 days
Triflumuron	26.67 ± 9.55	51.67 ± 14.24	71.67 ± 7.92
Control	16.85 ± 4.90	23.55 ± 4.99	30.07 ± 4.77
	$p = 0.327$	$p = 0.023$	$p = 0.002$
GLMM effects	**F**	**df**	***p***
Treatment	6.05	1; 11.09	0.032
Exposure interval	18.05	2; 21.03	<0.001
Treatment * Exposure interval	3.82	2; 21.03	0.038

21-day aged residues		**Exposure intervals**	
Treatment	7 days	14 days	21 days
Triflumuron	31.06 ± 7.38	68.64 ± 9.88	88.48 ± 4.79
Control	16.06 ± 3.99	35.15 ± 6.11	46.52 ± 7.56
GLMM effects	**F**	**df**	***p***
Treatment	13.23	1; 11.31	0.004
Exposure interval	29.13	2; 20.92	<0.001
Treatment * Exposure interval	2.51	2; 20.92	0.105


Aging: 0 days
Aging: 7 days
Aging: 14 days
Aging: 21 days
Mortality (%)
0
20
40
60
80
100
Exposure interval (days)
7
14
21
*
= triflumuron
= control

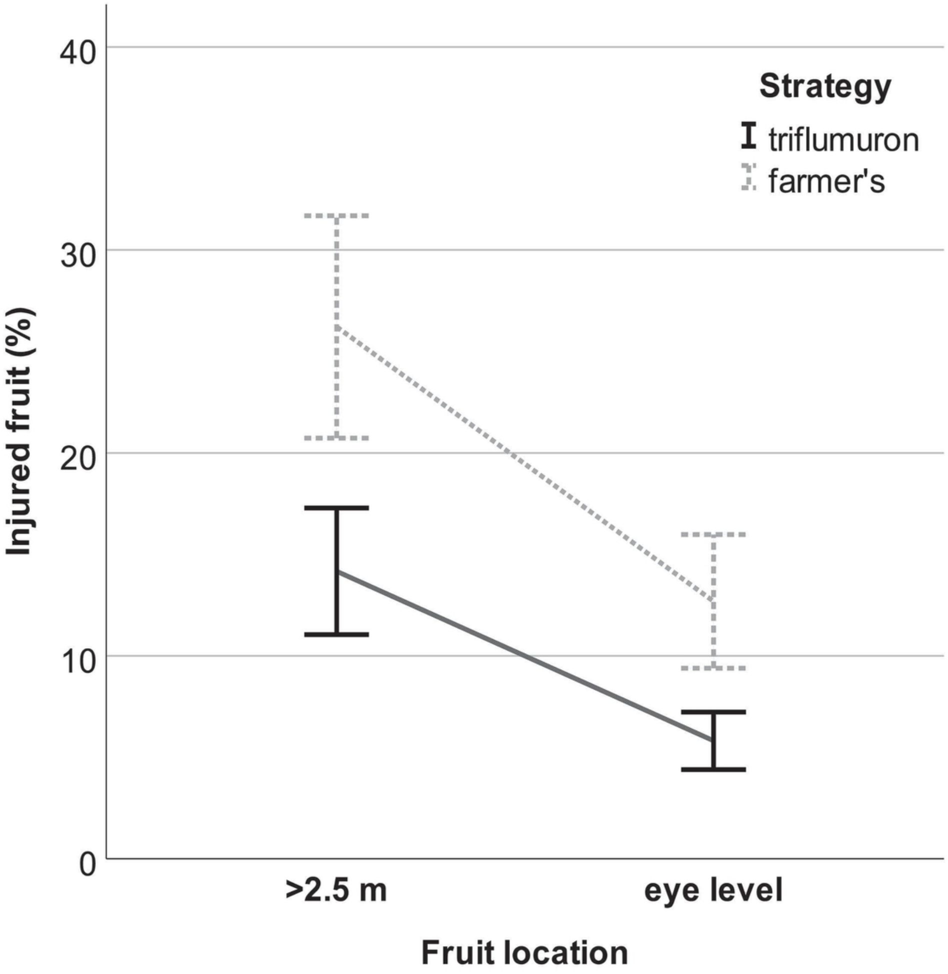


Fig. 2. Percentages of fruit with feeding injuries by Halyomorpha halys recorded in 2019 in pear orchards in Northern Italy. GLMM detected significant differences between strategies ($p<0.001$) and between fruit locations ($p<0.001$). Vertical bars indicate standard errors of the means.

99x99mm (300 x 300 DPI)

SUPPLEMENTARY DATA

Impact of triflumuron on *Halyomorpha halys* (Hemiptera: Pentatomidae): laboratory and field studies

Antonio Masetti, Laura Depalo and Edison Pasqualini

Fig. SD1. Experimental units with potted peach plants inserted in plexiglas cylinders.

N3 / N4

N4 / N5

N5 / adult

Fig. SD1. Individuals of *Halyomorpha halys* died at the ecdysis. Unsuccessful molting is a typical symptom of the activity of benzoylphenyl ureas.