



Dose-response and sublethal effects from insecticide and adjuvant exposure on key behaviors of *Trissolcus japonicus*

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With 5 figures

Abstract: In its native range, *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) is a main egg parasitoid of *Halyomorpha halys* (Stål) (Hemiptera; Pentatomidae). Adventive populations of this parasitoid have been spreading in Europe and North America, allowing active distribution efforts in these areas to utilize it against *H. halys* in agriculture. We tested exposure of adults and parasitized egg masses to residues of a range of insecticides and an adjuvant that the wasp could encounter in perennial horticultural crops, and how exposure might affect key behaviors. The insecticides included *Burkholderia rinojensis*, Methoxyfenozide, Sulfoxaflor, and Spinosad, and the adjuvant NuFilm®P. The lethal effects were investigated after exposure for 1, 6, and 24 hours to residues applied to Munger cells using a Potter spray tower to determine mortality levels. Sulfoxaflor and Spinosad resulted in 73% and 100% mortality after 24 hours at field rates, while Methoxyfenozide caused 19% mortality at the same rate. Little mortality occurred with *Burkholderia rinojensis* and NuFilm®P at recommended rates after 24 hours. Regarding sublethal effects, Spinosad, Sulfoxaflor, and *Burkholderia rinojensis* negatively impacted reproduction. Sulfoxaflor caused a lower angular velocity in parasitoids exposed to *H. halys* traces. Spinosad, Methoxyfenozide, and NuFilm®P induced a significantly lower overall circadian activity. Our results showed that precision timing of insecticide sprays to pests is necessary, especially for biocontrol programs that involve the release of parasitoids such as *T. japonicus*. Our results also illustrate how sensor data acquisition and behavioral analysis software can be utilized to detect important sublethal effects such as circadian disruption in minute parasitoids.

Keywords: *Halyomorpha halys*; biological control; IPM; circadian rhythm; egg parasitoid

1 Introduction

Biological invasions have caused substantial economic losses globally, amounting to an estimate of 1.288 trillion US \$, and have also contributed significantly to the decline in biodiversity (Diagne et al. 2021). One of the biological invaders causing significant economic losses to crops in Europe and the United States is the Asian insect species, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) (Leskey & Nielsen 2018). For instance, hazelnut crops can be greatly affected by feeding damage of *H. halys* (Hedstrom et al. 2014). Moreover, severe damage was found in mid-Atlantic apple orchards due to high density of *H. halys* populations (Leskey & Nielsen 2018). In Europe, notable *H. halys* damage to tree

fruits occurred in Northern Italy on a range of crops such as pear, apple, and kiwifruit (Bariselli et al. 2016; Francati et al. 2021). This pest is able to spread rapidly and become invasive owing to its high dispersal capabilities and hitchhiking behavior (Leskey & Nielsen 2018), whereby it takes advantage of global trade and transportation for passive movement (Haye et al. 2015). Short-term management of *H. halys* could be performed by means of chemical control (Leskey & Nielsen 2018). However, the most effective insecticides against *H. halys* are non-selective, including pyrethroids, carbamates, and organophosphates. Dependence on these materials often lead to secondary pest outbreaks (Leskey & Nielsen 2018), substantial ecological impacts, and unwanted collateral effects on non-targets (Desneux et al. 2007) making

this approach unsustainable. Biological control is an attractive alternative for managing *H. halys* as well as avoiding the disruption of existing IPM approaches targeting primary and secondary pests. Thus, *H. halys* biological control programs should be integrated with careful use of insecticides targeting other key pests in the crop system, or at minimum, costs and benefits of different insecticidal treatments should be understood.

Trissolcus japonicus (Ashmead) (Hymenoptera: Scelionidae) is the principal egg parasitoid of *H. halys* in its native range, with parasitism rates up to 70% (Talamas et al. 2013). Since the discovery of adventive *T. japonicus* populations in the Pacific Northwest (Hedstrom et al. 2017), distribution efforts have been made within the same area. Classical biological control (CBC) is a well-known practice that can significantly reduce dependence on toxic insecticides and thereby improve environmental quality (Borowiec & Sforza 2022). This practice has been extensively employed worldwide since the first known introduction of an insect biological control agent (BCA) (i.e., *Rodolia cardinalis*) in 1889 (Caltagirone & Doult 1989). However, the success of a biocontrol strategy can be severely affected by pesticides (Desneux et al. 2007). For example, in hazelnut orchards in Oregon's Willamette Valley, a complex of pest species poses significant threats to the crop, among which *H. halys* is a key pest (Hedstrom et al. 2014; Miller et al. 2019). As a result, a wide range of pesticides have been employed for their control. Therefore, in regions where large-scale agricultural systems exist (e.g., the United States), the effectiveness of *T. japonicus* releases could be affected owing to the chemical control program of this wide array of pests. Thus, it is crucial to comprehensively assess the risks of residue exposure to commonly-used insecticides in these programs on *T. japonicus*. This assessment is necessary to establish sustainable and long-term control against *H. halys*.

The present study aimed to assess the lethal and sublethal effects of residues of four insecticides and an adjuvant commonly used in hazelnut and other tree crops on female *T. japonicus* adults. These insecticides have a range of modes of action (MA) with reported specificity (i.e., MA: 4C, 5, 18, and UNB), as well as the spreader-sticker adjuvant NuFilm®P (hereinafter "Nufilm"). A range of concentrations including the recommended field rate was tested to assess the lethal effects on *T. japonicus*. Although previous studies have provided some understanding of the lethal and sublethal effects of insecticides on *T. japonicus* (Lowenstein et al. 2019; Ludwick et al. 2020; Ribeiro et al. 2021), the selection of insecticides was limited to those utilized for the control of *H. halys* or lacked a comprehensive reporting of sublethal effects. Sublethal effects are a critical parameter to understand the risk of exposure to insecticides on beneficial insects, especially when there are many forms of adverse effects including changes in physiology, behaviors, and reproductive success (Desneux et al. 2007).

In this study, we addressed these gaps by introducing novel parameters for sublethal assessments of insecticides used in hazelnut crops in Oregon. We also estimated lethal concentration values based on the lethal assessment of the studied treatments. The findings of this study will aid in the development of appropriate consideration of spatiotemporal contexts which regard the compatibility of *T. japonicus* releases with ongoing integrated pest management (IPM) measures.

2 Materials and methods

2.1 Insect rearing

Halyomorpha halys were collected from Salem, OR from June to September 2020, and supplemented with field-collected adults as needed from June to September of 2021 and 2022. The adults and nymphs were kept in a greenhouse with supplemental lighting (16L:8D full spectrum LED lights, 30 °C high 21 °C low) in collapsible fine mesh cages (60 cm × 60 cm × 90 cm, Bioquip 1466CB). All life-stages of *H. halys* were reared on a diet of bean plants (wax bush beans, royal purple bush beans, green beans), fresh green beans (French beans), snap peas, carrots, unsalted raw sunflower seeds, raw almonds, and sweet bell peppers. Fresh bean plants were provided as oviposition substrate. The egg masses were removed every Monday, Wednesday, Friday, and Saturday from leaf material, or mesh cages using a fine-tipped paint brush, and light pressure. The eggs were stored in a small petri dish and sealed with Parafilm in a residential refrigerator (2–4 °C) for up to 30 days until use. We opted for the use of refrigerated rather than frozen egg masses due to their significantly higher fitness, especially when stored for less than two months, as reported by Wong et al. (2021) and Bittau et al. (2021).

Trissolcus japonicus rearing was performed using freshly collected and stockpiled refrigerated egg masses in Percival Growth Chambers (Percival Scientific, Perry, Iowa, USA) (16L:8D, 26 °C high, 24 °C low). The eggs were placed in 473 mL wax lined paper soup containers with a single drop of honey streaked onto the side of the cup using a #3 insect pin. Female *T. japonicus* (7 days old) were introduced to the fresh *H. halys* eggs at a 10:1 host to parasitoid ratio and were given 1 week to parasitize eggs before being removed. The parasitized eggs were observed daily for emergence.

2.2 Lethal effect assessment

Female parasitoids of *T. japonicus* of 4–7 days old were exposed to a range of insecticides and an adjuvant at different concentrations (Table S1), including field application rates. The tested insecticides included the proteobacterial insecticide *Burkholderia rinojensis* (MA: UNB, i.e., bacterial agent of uncertain mode of action), Spinosad (MA: 5), Sulfoxaflor (MA: 4C), Methoxyfenozide (MA: 18), and the

adjuvant Nufilm. For all insecticides, the tested concentrations (Table S1) were prepared by serial ten-fold dilutions with deionized water starting from the field concentration rate. When needed, higher concentrations were sprayed to improve the fit of the dose-response models. The opposite was true for the adjuvant Nufilm, as the tested concentrations were prepared by serial two-fold increases starting from the recommended field rate, given its presumably inert nature. Overall, 12 concentrations of Spinosad were tested compared to ten for *Burkholderia rinojensis*, and seven for Sulfoxaflor, Nufilm, and Methoxyfenozide (Table S1).

Spraying was performed using Potter's spray tower (Burkard Manufacturing Co. Ltd., UK) on two square glass plates for each concentration (i.e., 0.5 mL per plate). The plates were left to dry for 30 minutes under a fume hood and then assembled into Munger cells (Hassan 1992) which were connected to a vacuum source at a 1L.h⁻¹ rate. Each concentration was repeated three times, and each Munger cell included five to seven parasitoids for a total of 15 to 21 tested female parasitoids per concentration. The control treatment consisted of the adjuvant Nufilm. The mortality of *T. japonicus* was recorded at 1 hour (h), 6 h, and 24 h to assess the insecticide lethal effects on the parasitoids and estimate the lethal concentrations (i.e., LC50 and LC90).

2.3 Sublethal effect assessment

To assess the sublethal effects of each treatment at the recommended field rate, parasitized egg masses (PEM) that were on the verge of emergence (i.e., 10 days old counting from parasitism) were sprayed in a similar manner to that of the lethal assessment. Spinosad caused complete mortality of the emerging parasitoids in preliminary trials at the field rate. Thus, the highest dose that caused no mortality within the lethal assessment bioassays was used instead of the recommended field concentration (i.e., 5.1 mL.ha⁻¹) (Table S1). Distilled water was used as control.

Three sub-experiments were conducted to assess the sublethal effects (Fig. S1):

The first consisted of a no-choice bioassay to assess the foraging behavior of female parasitoids ($n = 120$, 20 replicates per treatment) on baited filter papers with traces of gravid female *H. halys*, using a slightly modified experimental design compared to that of previous studies (Malek et al. 2021; Scala et al. 2022). Only naïve parasitoids were tested (i.e., no previous experience with host footprints/chemicals except for the egg mass from which they emerged). Emerged parasitoids (3–5 days old, see Scala et al. 2022) from the sprayed PEM were recorded on circular baited filters (24 mm diameter) for five minutes in small Petri dishes (Falcon®, 50 mm diameter) using a digital camera (Canon T5i) with an 18–55 mm lens (Canon EF-S 18–55mm f/3.5–5.6 IS STM). The recordings were taken between 08:00 and 14:00 under natural light conditions. The recorded footage was analyzed using Ethovision® XT9 (Noldus Information

Technology BV, the Netherlands) to examine mean angular velocity (deg/s), total residence time on the baited filter paper (s), and mean velocity (cm/s).

In the second sub-experiment, we monitored the circadian locomotor activity of female parasitoids ($n = 180$, 30 replicates per treatment) that emerged from the sprayed PEM. The locomotor activity was assessed with three insect locomotor activity monitors (LAM25H, Trikinetics, Waltham, MA, USA) using 0–1 days old female parasitoids following the protocol of Paul et al. (2022). The monitoring activity took place for a total of nine days, from which the first and last days were excluded, resulting in seven complete days of activity. Two honey droplets were added before the beginning of monitoring, similarly to Paul et al. (2022).

In the third and final sub-experiment, no-choice tests ($n = 60$; 10 replicates per treatment) were carried out to investigate the parasitism capacity of the emerged female parasitoids from the sprayed PEM. The tested females had no prior contact with a stink bug egg mass except for the one from which they emerged, and were of seven to 10 days old to guarantee that they had reached their maximum egg load (see Paul et al. 2022). The parasitoid wasps were provided with refrigerated (4 °C, up to 1 month) egg masses of *H. halys* for 24 h. The egg masses were then stored in a Percival climatic chamber (16L:8D, 26 °C high, 24 °C low) and were controlled for progeny emergence after 12 days, and up to 30 additional days from that date. The tested parasitoids were maintained on honey droplets as food source until they reached the required age. Honey was not procured during the bioassay (i.e., when they were provided with the egg mass for 24 h).

2.4 Statistical analyses

All analyses were carried out on R version 4.1.1 (R Core Team 2021, Vienna, Austria). The lethal effects of insecticides were analyzed by fitting log-logistic regression functions following the AIC criterion for model fitness within the “drc” package (Ritz et al. 2015). The effective lethal concentrations were calculated using the “ED()” function of the same package. The models were also evaluated by Neill's lack of fit test by determining their *F*- and *P*- values. For the first sub-experiment within the assessment of the sublethal effects (i.e., foraging behavior), a Wilcoxon signed-rank test was used to compare between the treatments and control in pairwise comparisons. For the second sub-experiment, the data collected from the activity monitors were analyzed using the rethomics packages developed by Geissmann et al. (2019). The total daily activity and intensity of activity were compared between treatments by fitting linear models, which were verified for normality assumptions and homoscedasticity. For the third sub-experiment, total parasitoid emergence was compared between treatments using a Kruskal-Wallis rank test followed by Dunn's test. The plots were created using the “Tidyverse” packages (Wickham et al. 2019).

3 Results

3.1 Lethal effect assessment

The probability of mortality for *T. japonicus* after 1 h, 6 h, and 24 h of exposure to Sulfoxaflor, Spinosad, *Burkholderia rinojensis*, Methoxyfenozide, and Nufilm was plotted using nonlinear log-logistic regression models (Fig. 1). Sulfoxaflor caused a high average mortality level of parasitoids, which was almost 7% after 1 h of exposure and 73% after 24 h of exposure at the recommended field rate (Fig. 1). Spinosad caused 83% mortality after 6 h of exposure and 100% mortality after 24 h at the recommended rate (Fig. 1). *Burkholderia rinojensis* exposure did not result in mortality after 6 h of exposure at rates higher than the recommended field dose value (up to 34 fold) (Fig. 1). Moreover, it caused just 5% mortality after 24 h of exposure at the recommended field rate (Fig. 1). The adjuvant Nufilm caused high mortality among parasitoids exposed at 63 fold of field rates, but no mortality was recorded at the recommended field rate (Fig. 1). Methoxyfenozide caused 4, 12.5 and 19% mortality after 1, 6, and 24 h (Fig. 1) at the recommended field rate, respectively.

The effective lethal concentrations for each insecticide and Nufilm were estimated based on mortality after 6 h and 24 h with a 95% confidence interval (Table S2). LC50 for Spinosad after 6 h was 96.34 mL.ha⁻¹ and 90.09 mL.ha⁻¹ after 24 h of exposure. LC50 for Sulfoxaflor after 6 h was 0.11 Kg.ha⁻¹ and 1.1 Kg.ha⁻¹ after 24 h of exposure. As for Methoxyfenozide, the LC50 after 6 h was 15.89 mL.ha⁻¹ and 14.51 mL.ha⁻¹. *Burkholderia rinojensis* and Nufilm had the highest LC50 estimates after 6 h (2.6207e+95 and 14 019, respectively). The same was true after 24 h, as the estimates for LC50 were 3.7802e+11 mL.ha⁻¹ and 6 596.95 mL.ha⁻¹, in that order.

3.2 Sublethal effect assessment

No statistical differences were found between treatments in terms of residence time or walking velocity in the pairwise comparisons with the control (i.e., distilled water). Nonetheless, the lowest residence time on the baited filter was recorded for Nufilm, and the slowest velocity was recorded for the Spinosad and Distilled water treatments (Fig. 2). Regarding angular velocity, the average value recorded within the Sulfoxaflor treatment was significantly

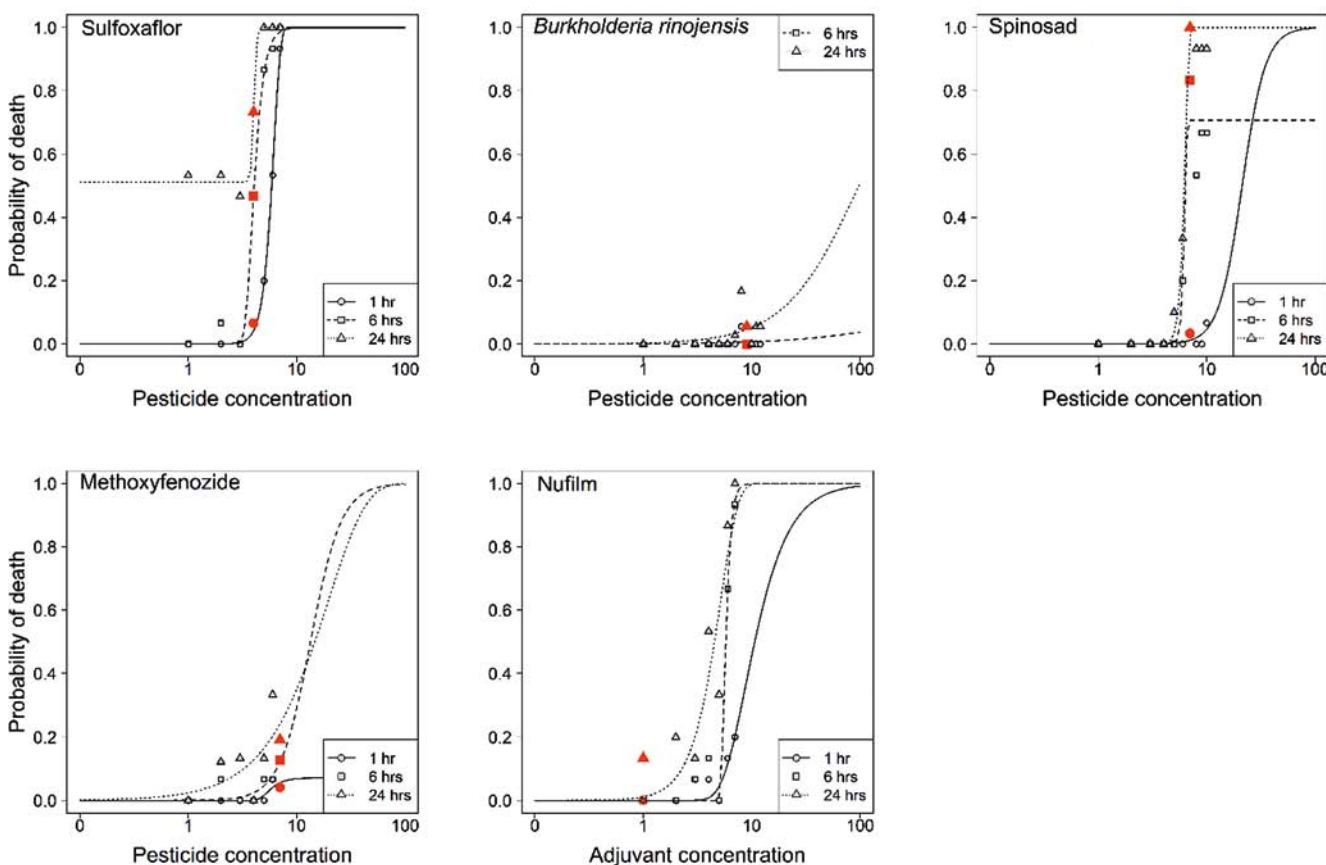


Fig. 1. Curves of log- logistic fitted probability of *Trissolcus japonicus* death at the various insecticide and adjuvant concentrations after 1,6, and 24 hours of exposure. Potter spray tower and Munger cells were used under controlled Laboratory conditions. No mortality was found for whatsoever for *Burkholderia rinojensis* after 1 hour, thus no curve is shown. The red data points represent the recommended field rates.

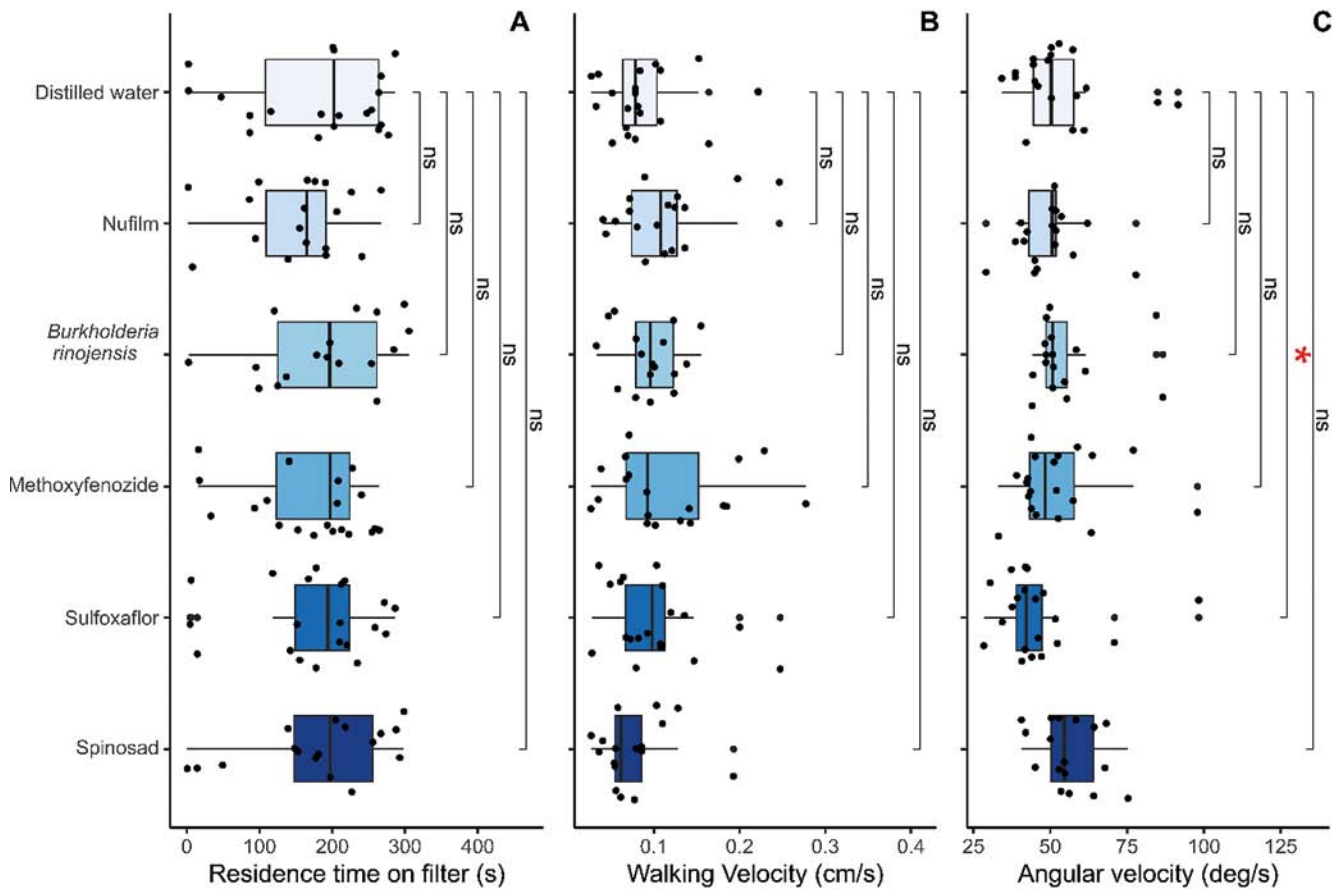


Fig. 2. Box plots with jitter dots showing the foraging behavior of 5–7 days old emerged *Trissolcus japonicus* from the treated parasitized egg masses at the recommended field rates (spinosad rate was 20-fold below the recommended rate because of the complete mortality caused in emerged parasitoids in preliminary trials), following the contact with a baited substrate with traces of female *Halyomorpha halys*. The parameters are (A) Residence time (s) on baited filter paper, (B) Walking velocity (cm/s), and (C) Angular velocity (deg/s). Pairwise comparisons indicated with an asterisk showed significant differences (Wilcoxon signed-rank test, $P < 0.05$); ns: non-significant. Boxplots represent the interquartile range with a vertical bar as the median. The whiskers represent 1.5× the interquartile range. The jitter dots represent individual data points.

lower compared to that of the control ($W = 120$, $P = 0.031$) (Fig. 2).

The daily locomotor activity of parasitoids differed among treatments. The total daily activity was significantly higher for parasitoids treated with Spinosad compared to those treated with distilled water ($F_{1,174} = 4.843$, $P < 0.001$) (Fig. 3). For Methoxyfenozide and Nufilm, activity was lower than those treated with distilled water (49 and 46 times respectively, $F_{1,171} = 5.626$, $P < 0.001$, Fig. 4).

Parasitism capacity was significantly different among treatments ($\chi^2 = 20.1086$, $df = 5$, $p < 0.01$). *Trissolcus japonicus* parasitoids emerging from PEM treated with distilled water were the most capable of parasitizing *H. halys* egg masses, with 91% emergence of new parasitoids. Nufilm and Methoxyfenozide followed with 70% and 63% emergence of new parasitoids compared to untreated control, respectively. The lowest parasitoid emergence rates compared to distilled water were recorded for Sulfoxaflor, Spinosad, and

Burkholderia rinojensis at 46.5%, 40%, and 32%, respectively (Fig. 5).

4 Discussion

In the present study, we examined the lethal and sublethal effects of insecticides belonging to a range of action groups with reported specificity, from very soft (i.e., *Burkholderia rinojensis*) to more conventional (i.e., sulfoxaflor). We also investigated the potential effects of the spreader-sticker adjuvant Nufilm. Regulations for registering adjuvants and co-formulants are less stringent compared to active ingredients because of their considered inert nature. However, active ingredients may exhibit different effects on non-targets when combined with co-formulants compared to when tested alone. Indeed, certain pesticide formulations could be multifold more toxic than the active ingredient itself to adult

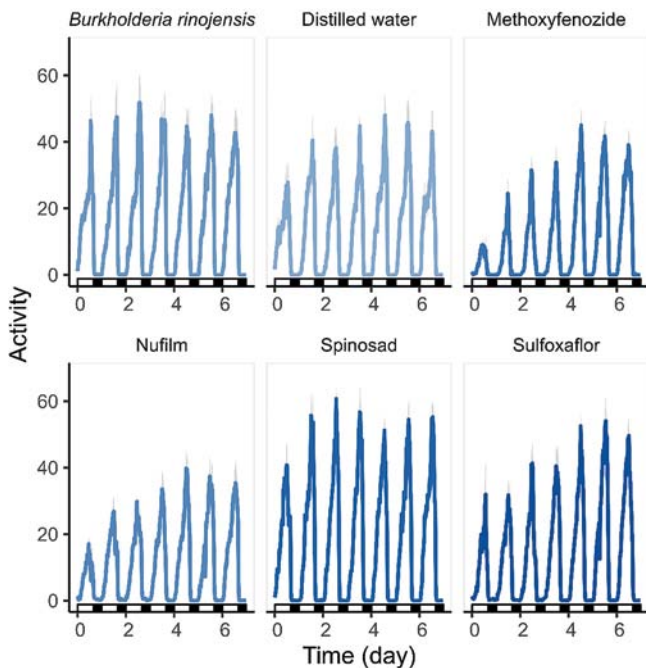


Fig. 3. Temporal locomotor activity of emerged *Trissolcus japonicus* from the treated parasitized egg masses at the recommended field rates (spinosad rate was 20-fold below the recommended rate because of the complete mortality caused in emerged parasitoids in preliminary trials). The plot shows the daily activity (i.e., the daily number of beam crosses per day) of the parasitoids over the course of the complete 7 days of locomotor activity monitoring. The parasitoids were 0–1 days old at the beginning of the monitoring.

honeybees and larvae (Straw et al. 2022). The same authors argued that adjuvants added to pesticide formulations via tank mixes are understudied due to the belief of their inert nature, leading to a lack of toxicity testing for beneficial insects (Straw et al. 2022). Among the few studies that tested adjuvants alone, some significant sublethal effects on bees were found, such as learning impairment and reduced colony fitness. Here, Nufilm was tested alone and had no lethal effects on parasitoids at the recommended field rate, whereas the same dose significantly reduced the total daily locomotor activity. This could have serious implications on the fitness of parasitoid wasps in field conditions, such as limited success in finding host egg masses and/or food sources. Our results further highlight the importance of additional studies examining the effects of adjuvants on beneficial insects. In addition, the results obtained from monitoring daily locomotor activity revealed that this parameter could detect important sublethal effects such as circadian disruption following exposure to substances deemed inert.

Burkholderia rinojensis, a bacterial strain isolated from the soil and used as a biological insecticide, showed notable discrepancies in its effects on *T. japonicus* female parasitoids. It caused little mortality in the lethal assessment bioassays, no significant effects on locomotor activity and

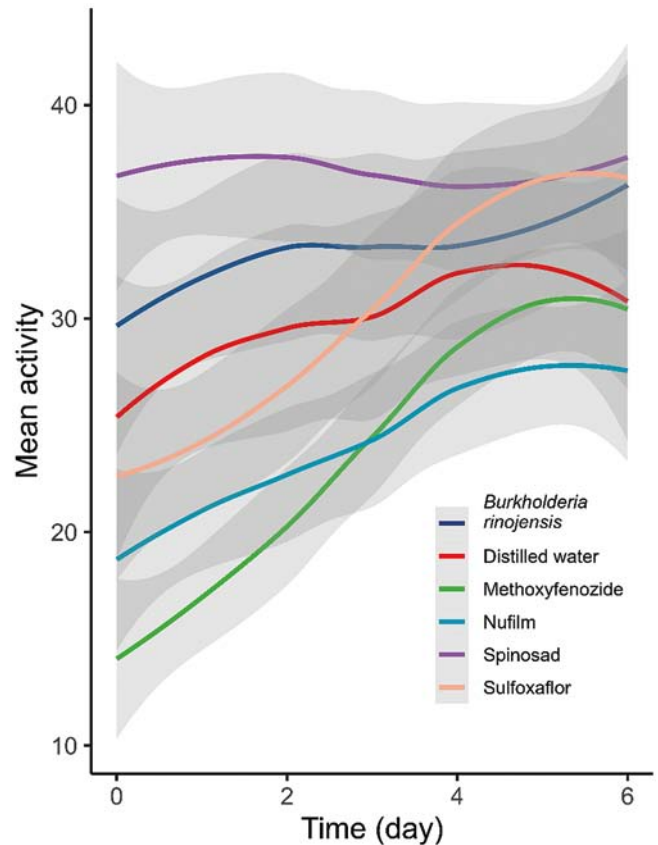


Fig. 4. The intensity of the temporal locomotor activity (i.e., the number of beam crosses per active interval) of emerged *Trissolcus japonicus* from the treated parasitized egg masses at the recommended field rates (spinosad rate was 20-fold below the recommended rate because of the complete mortality caused in emerged parasitoids in preliminary trials), over the course of the complete 7 days of activity monitoring. The parasitoids were 0–1 days old at the beginning of the monitoring.

attraction to the chemical cues of *H. halys*. However, our data showed a notable impact of this compound on reproductive capacity, as measured by emergence of next-generation parasitoids. Wasps emerging from PEM exposed to the residues of this insecticide were the least capable of parasitizing new *H. halys* egg masses. To the best of our knowledge, there are limited studies in the literature that have assessed the reproductive capacity of insects following treatments with this bacterial insecticide. In one study (Kil et al. 2014), an Enterobacteria strain of *Burkholderia* sp. was found to affect the oviposition and fecundity of the bean bug *Riptortus pedestris* (Fabricius) (Hemiptera: Alydidae). In another study, *Burkholderia* spp. strain A396 (subsequently defined as *Burkholderia rinojensis*, see Cordova-Kreylos et al. 2013), had a significant effect on the reproduction capacity of *Myzus persicae* (Hemiptera: Aphididae) (Shannag & Capinera 2018). Even though *Burkholderia rinojensis* is understudied, it is not unusual for biopesticides that are deemed safe for non-target ben-

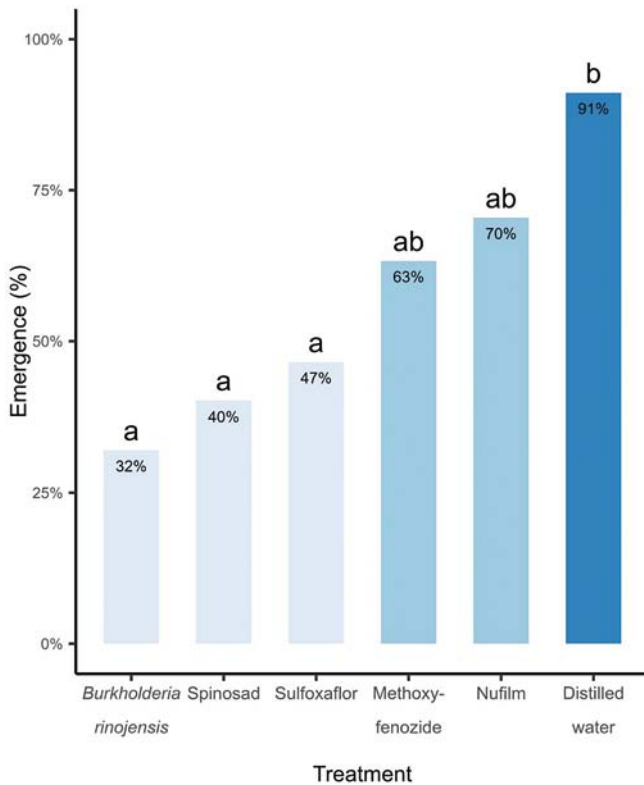


Fig. 5. The reproductive capacity of emerged *Trissolcus japonicus* from the treated parasitized egg masses at the recommended field rates (spinosad rate was 20-fold below the recommended rate because of the complete mortality caused in emerged parasitoids in preliminary trials). This capacity was measured based on the proportion of emergence from new refrigerated egg masses of *Halyomorpha halys* exposed to 7–10 days old female parasitoids. Different letters indicated significant differences (Kruskal-Wallis test, $P < 0.01$).

official arthropods to induce severe sublethal effects, such as reduced fecundity (Biondi et al. 2013).

Methoxyfenozide is a selective ecdysteroid agonist insecticide with relatively few adverse effects on non-target beneficial insects. It is used to control tortricid and other Lepidopteran pest species in different crops in Europe and North America. This insecticide's reported sublethal effects mainly involve males' reduced capacity to locate calling females in targeted insects (Reinke & Barrett 2007). Here, this insecticide caused lower acute toxicity in *T. japonicus* compared to Spinosad and Sulfoxaflor. However, similar to Nufilm, the sublethal effects caused by treatment with Methoxyfenozide were apparent in the assessment of locomotor activity. Interestingly, the overall daily activity and intensity of activity of exposed wasps were significantly reduced after the first week after emergence. This indicates that there is a high probability of reduced host searching and lower parasitism rates, as wasps are likely to be less active especially during the period in which they have reached

adequate maturity in terms of egg load (Paul et al. 2022). Consequently, given the synovigenic nature of *T. japonicus* and the fact that it is not a host-specific parasitoid, a higher attack on the eggs of non-target hosts might occur to secure *T. japonicus* progeny.

Sulfoxaflor and Spinosad, the insecticides belonging to action groups 4C and 5 respectively, caused the highest mortality in female *T. japonicus* at recommended field rates. These results are in accordance with the literature, as both active ingredients were found to be highly toxic to various Hymenoptera wasps and beneficial arthropods (Biondi et al. 2012). The harm was not only limited to acute toxicity, as significant sublethal effects were also observed. Sulfoxaflor residues notably affected the foraging behavior of the exposed parasitoids due to their significantly lower angular velocity. It should be noted that parasitoids interested in the chemical traces released by their hosts typically exhibit a higher angular velocity, slower walking velocity, and greater residence time within areas baited with such cues (Peri et al. 2006). Hence, this result suggests a lower interest in the footprint traces of the host following exposure to Sulfoxaflor residues. Moreover, Spinosad residues induced a significantly higher total daily activity among the treated female parasitoids. This is probably due to the mode of action of this active ingredient as spinosyns allosterically activate nicotinic acetylcholine receptors, causing hyperexcitation of the nervous system. Similarly to the *Burkholderia rinojensis* treatment, female *T. japonicus* that emerged from PEM treated with Spinosad and Sulfoxaflor were significantly less fertile than those of the control. Reduced reproductive activity of egg parasitoids due to Spinosad residue exposure is in accordance with previous reports in the literature (Biondi et al. 2012). Moreover, decreased reproductive success is among Sulfoxaflor's reported sublethal effects, which is extensively studied in pollinators like bumblebees (Siviter et al. 2020). Here, the significant decrease in reproduction is probably solely due to the direct effect of the active ingredients (i.e., Spinosad, Sulfoxaflor, and *Burkholderia rinojensis*). Indeed, despite potential alterations in the host's symbiont density following insecticide sprays, Orrù et al. (2022) found that *T. japonicus* could still successfully parasitize *H. halys* egg masses subject to symbiotic control.

Our data stress the importance of careful timing of pesticide sprays in order to minimize significant lethal and sublethal effects to these important biological control agents. The results presented in this study are due to residue exposure rather than direct application (e.g., topical application). Hence, when scheduling insecticide sprays and releasing *T. japonicus* as a BCA, it is crucial to consider both the LC50 values and the minimum required time for molecule breakdown to minimize their impact on the natural enemies. Moreover, the mere lack of lethal effects of a certain insecticide does not impede the occurrence of serious sublethal effects such as impaired locomotor activity, foraging behavior, and/or decreased reproduction. Nevertheless,

sensor data acquisition and behavioral analysis software should be utilized as a parameter to detect important sublethal effects such as disruption of circadian locomotor activity. Such parameter could reveal significant sublethal effects within non-targets caused by understudied substances that are assumed to be inert, such as adjuvants. Further studies are needed on the mechanisms by which the proteobacterial insecticide *Burkholderia rinojensis* affects reproduction, which could help avoid this phenomenon especially for non-targets. Nevertheless, not only the temporal but also the careful spatial distribution of *T. japonicus* is important; releasing parasitoids within hedgerows and field edges should minimize insecticide effects on the latter.

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Figure S1, S2; Table S1, S2