Research Article

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# Toxicity to and egg-laying avoidance of *Drosophila suzukii* (Diptera: Drosophilidae) caused by an old alternative inorganic insecticide preparation

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## Abstract

**BACKGROUND: The application of synthetic insecticides remains the most used tool for the management of spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). However, management of this pest in the organic production of soft-skinned fruits is a complex task due to the restricted number of registered products. Here, we assess the toxicity of lime sulfur and evaluate whether lime sulfur-treated strawberry plants affected the oviposition and development of *D. suzukii*.**

**RESULTS: Lime sulfur exhibited adequate toxicity to *D. suzukii* ($LC_{50} = 26.6$ mL $L^{-1}$) without phytotoxicity to strawberry plants. When *D. suzukii* females were exposed to lime sulfur-treated plants in no-choice bioassays, oviposition was significantly (*t*-test, $P < 0.05$) reduced compared with that on untreated plants. In free-choice bioassays, *D. suzukii* females laid significantly (paired *t*-test, $P < 0.05$) more eggs on untreated plants. Furthermore, in the free-choice bioassays, immature development was slower for adults that originated from eggs laid on lime sulfur-treated plants than from those laid on untreated plants.**

**CONCLUSIONS: Lime sulfur showed adequate control and, therefore, has potential for use as a management tool against *D. suzukii* infestations in organic production systems. This old, alternative insecticide preparation not only caused adult fly mortality, but also reduced the number of eggs laid on lime sulfur-treated plants.**


**Keywords:** spotted-wing drosophila; lime sulfur; alternative pest control; organic production systems

## 1 INTRODUCTION

Successful organic cultivation of soft-skinned fruits (e.g., strawberries) requires effective and economical control of insect pests. However, because synthetic insecticides are not permitted in organic fruit production systems[1] and because of the continued requirement for more environmentally benign pest control methods (coupled with regulatory actions to reduce the risks associated with these methods), the return to old inorganic insecticide preparations has recently gained popularity in agriculture, particularly in organic production.[2–5]

The application of inorganic preparations rich in calcium polysulfides [under the general term 'lime sulfur' and easily prepared by combining water ($H_2O$) with elemental sulfur (S) and hydrated lime ($CaO{\cdot}H_2O$)][6,7] has been recommended since the 19th century, representing one of the oldest effective pest (including insects) control methods.[5,8] Degradation of lime sulfur upon application on the plant surface releases both hydrogen sulfide ($H_2S$) and elemental sulfur, which act as decouplers of the electron transport chain.[2] The $H_2S$ inhibits the cytochrome oxidize, whereas the elemental sulfur oxidizes the cytochrome B to C, releasing more $H_2S$ inside the cell.[9,10] Furthermore, because the ecological impacts of lime sulfur preparations are apparently low and are highly compatible with organic management practices,[3,4,11] their use (through spray applications on leaves or fruits) continues to be commonly recommended in integrated pest management (IPM) programs. Additionally, lime sulfur preparations are one of most common recommendations to control fungi and mite infestations in organic production systems.[11–13]

In the global production of strawberries, as observed for fields of other soft-skinned fruits,[14–17] recent invasions of the spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), have elevated these flies to one of the most prevalent strawberry pests.[18–22] However, despite recent efforts aimed at developing other management strategies, e.g., potential parasitoids,[23–26] mass trapping[27] and physical exclusion (netting),[28] the application of synthetic insecticides remains the most used tool for the management of *D. suzukii*.[19,29,30] As a result,

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the management of organic production becomes an even more complex task due to the restricted number of registered products.

Recent investigations evaluated the potential of using alternative insecticides,[31,32] including sulfur-based preparations,[30,33] to manage infestations of *D. suzukii*. However, although some sulfur-based commercial preparations did not provide adequate toxicity[30] against *D. suzukii* adults, application of sulfur powder reduces the oviposition of *D. suzukii* under laboratory conditions.[33] Thus, the current investigation aimed to evaluate the toxicity of inorganic lime sulfur preparations to *D. suzukii* and determine whether lime sulfur-treated strawberry plants would affect the oviposition and development of *D. suzukii*.

## 2 MATERIALS AND METHODS

### 2.1 Insects and alternative insecticide preparations

The *D. suzukii* adult flies used in the bioassays were obtained from a stock colony reared under controlled conditions (i.e., temperature: $24 \pm 2$ °C; relative humidity: $55 \pm 10\%$; photoperiod: 12 h). The fly stock colony was initially established from ∼ 800 individuals obtained from a colony maintained at Embrapa Clima Temperado (Pelotas, RS, Brazil). All developmental stages of the fly stock colony were reared following previously described methods.[34,35]

Three alternative insecticide preparations containing ingredients that are used to manage insect pests in organic systems[13,36–38] were used in toxicological bioassays with *D. suzukii* adults. Lime sulfur preparations were prepared in accordance with methodology described elsewhere.[12,39–41] Briefly, a stock solution with a final density of 32° Baumé (i.e., 59° Brix) was prepared from which further dilutions were performed before application. The other two preparations were prepared according to a handout of 'natural' recipes empirically used by organic farmers in southeast Brazil. A potassium permanganate (0.15%) + lime (1.0%) mixture was prepared from permanganate potassium powder (Ref.: 02690; Neon Comercial Ltda, São Paulo, Brazil), lime (bought at a local market) and distilled water in the proportions 1: 6.7: 667, respectively. The third preparation (i.e., 0.15% sulfur +1.5% salt +1.0% detergent) was a solution composed of sulfur powder (1.5 g), NaCl (15 g) and detergent (10 mL) mixed in 1 L of distilled water.

### 2.2 Concentration–mortality bioassays

Lethal concentrations (LC) of the alternative insecticide preparations were estimated for *D. suzukii* using concentration–mortality bioassays. These toxicological bioassays were conducted using a contact/ingestion exposition protocol adapted from the IRAC Susceptibility Test Method No. 026.[42] Briefly, a dental wick (Cremer S.A., Blumenau, Brazil) was impregnated with 1.9 mL of the insecticide solution and placed in a 200-mL glass vial. The vials were sealed at the top by a foam plug to prevent fly escape. After placement of the insecticide-impregnated dental wicks, groups of 25 mated flies were released into the glass vials for 24 h, after which mortality levels were assessed. Complete absence of movement after mechanical stimulation by a fine-tipped brush indicated a dead fly. For the bioassays with each alternative insecticide preparation, preliminary bioassays were conducted across a broad range (i.e., 1 to 1000 mL $L^{-1}$) to select the highest concentration with no mortality and the lowest concentration that did kill all *D. suzukii*. For each insecticide concentration, four replicates (i.e., each replicate a vial containing 25 flies and the insecticide-impregnated dental wick) were used to determine mortality.

### 2.3 Effect of lime sulfur on egg-laying avoidance (with and without choice) under semi-field conditions

To assess the activity of the lime sulfur on female oviposition behavior, flies were provided with choice and no-choice oviposition bioassays under semi-field conditions. Strawberry plants were cultivated in plastic pots (4 L) in the greenhouse to avoid previous *D. suzukii* infestation. When each had three ripe fruits, the plants were sprayed with the lime sulfur preparation at 30 mL $L^{-1}$ (approximately the $LC_{50}$ estimated for *D. suzukii* in the current investigation), a commonly recommended concentration for mite pest control.[12,43,44] Untreated (i.e., control) plants were sprayed with distilled and deionized water. To spray the plants, a hand sprayer (0.5 L) was pressed 15 times around the plant, which resulted in a deposition of ∼ 10 mL of solution per plant. Pots with lime sulfur-treated and untreated plants were then transferred to the field and partially buried, placing the plants close to ground level.

For the no-choice oviposition bioassay, groups of four untreated or four lime sulfur-treated plants were transferred to the field, and a cage comprising a plastic pipe (2.5 cm in diameter) covered with organza ($1.2 \times 1.2 \times 0.8$ m) was immediately placed over the plants. The four plants were transplanted in different corners of the cage. On the top of each cage, a zipper allowed access to the inside through which 30 mated *D. suzukii* females (3–4 days old) were released. Flies were released 1 h after the plants were caged. In the free-choice oviposition bioassay, two plants were lime sulfur-treated and two were untreated. Of note, during the semi-field experiments (August 2016), no rain fell and day length remained between 11.2 and 11.4 h. The mean temperature ranged from 19.7 to 20.9 °C, and the relative humidity range was 69–73%. Cage locations were under direct sunlight for ∼ 70% of the day length, with cages in an open, well-ventilated environment. In both oviposition bioassay types, 24 h after the release of flies, the strawberry fruits were harvested, and the oviposited eggs were counted using a stereoscopic microscope. In each bioassay type, five replicates (i.e., a group of four plants) were used.

### 2.4 Pre-imaginal development and emergence bioassays

After the egg counts, the strawberry fruits were kept in plastic containers (0.5 L) on a 1 cm layer of vermiculite (Ref. 23071; Caldesul Co., Porto Alegre, Brazil) for egg hatch and immature development. The plastic containers were closed at the top with a sleeve-like voile fabric. From day 8 to day 16 after oviposition, all emerged adults were collected and sexed daily.

### 2.5 Statistical analyses

Concentration–mortality curves were estimated with probit analyses using the PROBIT procedure in the SAS statistical software package.[45] The number of eggs laid, adult emergence, sex ratio, and egg-to-adult viability from each cage in the no-choice oviposition bioassay were analyzed by a Student's *t*-test or a Mann–Whitney rank sum test when assumptions of normality and homoscedasticity were not satisfied. All comparisons were performed using SigmaPlot v. 12.5 (Systat Software, San Jose, CA, USA). For the free-choice oviposition bioassay, the same parameters were analyzed by a paired Student's *t*-test on SigmaPlot v. 12.5. Additionally, the daily number of emerged adults and the daily emergence percentage in each treatment were submitted to nonlinear regression analysis using the curve fitting procedure of SigmaPlot v. 12.5. Regression analyses were conducted to detect trends in the pre-imaginal development period that resulted from the lime sulfur treatment or the choice of female oviposition.

  

**Table 1.** Toxicity of lime-sulfur to *Drosophila suzukii* adults ($n = 502$, $\chi^2 = 2.3$, $df = 4$; $P = 0.51$)

| Slope ± SE | $LC_{20}$ (95% CI) | $LC_{50}$ (95% CI) | $LC_{80}$ (95% CI) |
|---|---|---|---|
| 3.4 ± 0.30 | 15.1 (13.4–16.6) | 26.6 (24.3–29.6) | 47.0 (40.6–57.1) |

Lethal concentration (LC) values were estimated based on concentration–mortality bioassays using probit analyses. CI, confidence interval. All concentrations are expressed in mL of lime-sulfur preparation per L of water.

Regression models for each treatment were considered significantly different when the confidence limits of their parameters did not overlap.

## 3 RESULTS

### 3.1 Concentration–mortality bioassays

Of the three tested alternative insecticide preparations, only the lime sulfur solution increased mortality in response to increasing concentrations. As shown in Table 1, the lime sulfur $LC_{50}$ value was 26.6 (24.3–29.6) mL $L^{-1}$, which is approximately the recommended concentration (30 mL $L^{-1}$) for controlling mite pests in neotropical organic production systems. However, with the highest concentration (i.e., 1000 mg $mL^{-1}$) of both potassium permanganate + lime and sulfur + salt + detergent preparations, mortality did not exceed 11.3 ± 2.3% and 6.5 ± 3.3% of the tested flies, respectively.

### 3.2 Semi-field oviposition bioassays (with and without choice)

In the no-choice bioassay, the groups of 30 females released into each cage with lime sulfur-treated plants laid 18.5 ± 4.4 eggs per plant, which was significantly fewer eggs ($t = 3.04$, df = 8, $P = 0.016$) than the 35.3 ± 3.4 eggs laid per untreated plant (Fig. 1a). Similarly, in the choice bioassay, the groups of 30 females released into each cage laid 20.0 ± 4.8 eggs per lime sulfur-treated plant, which was 48.6 ± 9% fewer ($t = 4.44$, df = 4, $P = 0.011$) than the 38.0 ± 3.7 eggs laid on each untreated plant (Fig. 1b).

### 3.3 Pre-imaginal development and emergence bioassays

In the no-choice oviposition bioassay, the egg-to-adult viability (Mann–Whitney rank sum test; $P = 0.31$) and the offspring female sex ratios (Mann–Whitney rank sum test; $P = 0.69$) were not significantly different between the eggs laid on lime sulfur-treated plants and those on untreated plants. Additionally, no significant differences (egg-to-adult viability: Mann–Whitney rank sum test, $P = 0.55$; offspring sex ratio: paired *t*-test, $P = 0.69$) were recorded for these parameters for the eggs laid on both plant types in the choice oviposition bioassay. The overall egg-to-adult viability was 94.4 ± 2.3%, and the female sex ratio was 49.6 ± 2.0%.

The cumulative number of flies developed from eggs laid on lime sulfur-treated plants was significantly ($t = 2.48$, df = 8, $P = 0.038$) lower than the number that developed from eggs laid on untreated plants in the no-choice oviposition bioassay (Fig. 2a). As shown in Fig. 2(b), in the choice oviposition bioassay, the cumulative number of flies developed from eggs laid on lime sulfur-treated plants was also significantly ($t = 5.48$, df = 4, $P = 0.005$) fewer than the number that developed from eggs laid on untreated plants.

Although the peak occurrence for daily emergence both in numbers and in percentages was not significantly different between

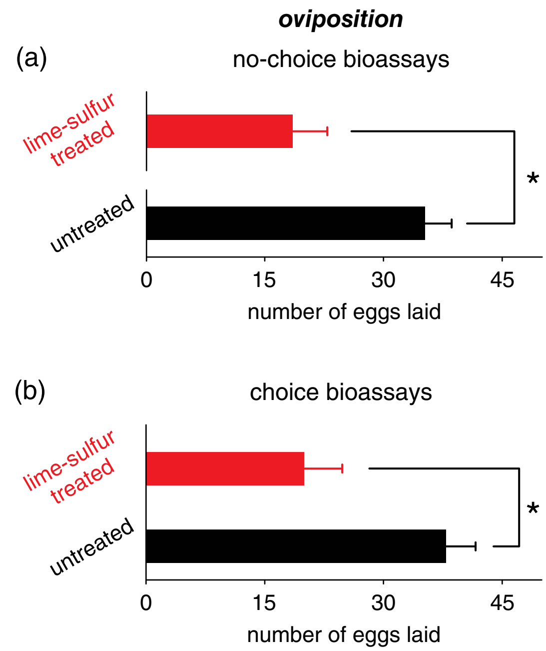


**Figure 1.** Total number of eggs laid per plant by *Drosophila suzukii* females on lime-sulfur-treated or untreated strawberry plants under semi-field conditions. (a) Sexually mature *D. suzukii* females were confined in cages containing four lime-sulfur-treated or four untreated strawberry plants in a no-choice bioassay. (b) The female flies were subjected to a choice bioassay under semi-field conditions and were confined in cages containing four strawberry plants, with two lime-sulfur-treated and two untreated plants. Asterisks indicate significant differences between treatments according to Student's *t*-test (a) or paired Student's *t*-test (b) ($P < 0.05$).

lime sulfur-treated and untreated plants in the no-choice oviposition bioassay (Table 2, Fig. 3a,b), these peak occurrences were delayed for eggs laid on lime sulfur-treated plants in the choice oviposition bioassay (Table 2, Fig. 3c,d).

## 4 DISCUSSION

The application of sulfur-based insecticide preparations is an old tool commonly used to manage fungi, mite and insect infestations in organic production systems.[11,13,37,43,46] Here, we demonstrated that a lime sulfur insecticide preparation was toxic to *D. suzukii* adults and caused egg-laying avoidance in *D. suzukii* females without resulting in phytotoxic effects on strawberry plants.

The natural degradation of lime sulfur preparations is known to result in both $H_2S$ and elemental sulfur, which are toxicologically active against pests.[47] Whereas hydrogen sulfides inhibit cytochrome oxidase, elemental sulfur oxidizes cytochrome b to cytochrome c with the release of more $H_2S$.[2,9,10] Although not addressed in detail in this study, a reasonable assumption is that hydrogen sulfides exerted the primary toxicity against *D. suzukii*, because in previous investigations,[30] other sulfur-based preparations did not provide adequate control of *D. suzukii*. Furthermore, the other sulfur-based preparation (sulfur + NaCl + detergent) tested in this investigation also resulted in very low mortality to *D. suzukii*, even when applied pure (without further dilution). Notably, although the potassium permanganate + lime preparation is recognized as an alternative preparation with potent fungicide, bactericide and molluscicide activities,[36] the application of pure (i.e., without further dilution) preparation resulted in very low mortality to *D. suzukii*. Thus, the potential use of these two

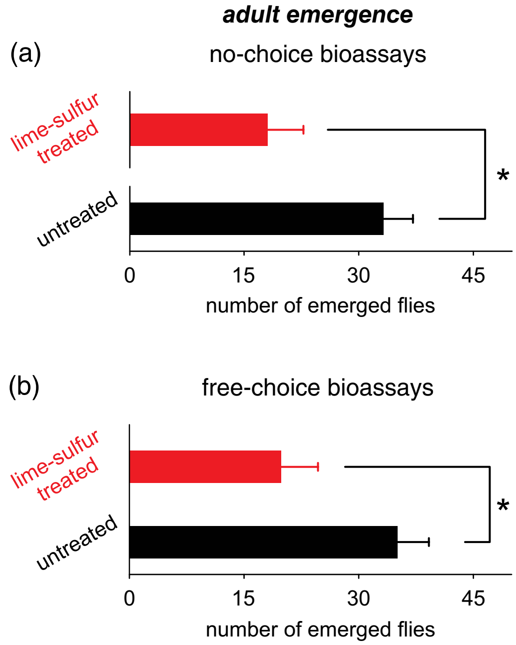


**Figure 2.** Number of emerged *Drosophila suzukii* adults originated from eggs laid on lime-sulfur-treated or untreated strawberry plants. (a) Adults emerged from eggs laid on lime-sulfur-treated or untreated strawberry plants in a no-choice bioassay. (b) Adults emerged from eggs laid on lime-sulfur-treated or untreated strawberry plants in a free-choice bioassay. Asterisks indicate significant differences between treatments according to Student's *t*-test (a) or paired Student's *t*-test (b) ($P < 0.05$).

preparations in the management of *D. suzukii* infestations in the organic production of soft-skinned fruits is completely excluded.

Although pesticide products with a limited spectrum of activity may contribute to the safety of the environment and non-target organisms, such traits increase the complexity for alternative pesticides to enter niche markets, and for the development and commercialization of these products.[48–50] Therefore, to encourage the widespread use of alternative pesticides, products with activity against multiple pests are required. Application of the lime sulfur preparation represents a good example of an alternative product capable of controlling multiple insect pests in the organic production of strawberries. In strawberry production systems worldwide, phytophagous mites (particularly the two-spotted spider mite, *Tetranychus urticae* Koch)[51–53] and more recently, *D. suzukii* are considered the most prevalent pests,[18–20,54] and both pest systems can be adequately controlled by lime sulfur preparations. For example, the lime sulfur $LC_{80}$ for *D. suzukii* is in the concentration range known to provide adequate control of *T. urticae*.[37]

Similar to the effect recorded for sulfur powder applications,[33] application of the lime sulfur preparation to strawberry plants resulted in egg-laying avoidance in *D. suzukii* females in the no-choice oviposition bioassay. Because drosophilids have relatively short generation times and high reproductive outputs (with several overlapping generations during each crop season),[34,55,56] reductions in the number of eggs laid will not fully prevent damage to strawberry fruits; however, such reduction certainly has negative effects on population growth, and therefore, the lime sulfur preparation could maintain the size of the fly population below the economic threshold.

Site selection for egg-laying is a central behavioral decision for insects that is strongly affected by genetic and environmental factors, with the decision influencing lifetime reproductive fitness by favoring (or not) offspring survival.[57–61] In this study, we recorded not only a reduction in the number of eggs laid, but also a significant delay in the emergence peak for the individuals from eggs laid on lime sulfur-treated plants. These responses to lime sulfur treatment might be a consequence of the decisions of *D. suzukii* females, as proposed in the theory of optimal oviposition behavior,[62,63] and reflect the abilities of drosophilid females to actively probe the environment and evaluate site quality before depositing each egg.[57–61]

Thus, in this investigation, the potential of using an old, alternative insecticide preparation (lime sulfur) was demonstrated as a *D. suzukii* management tool in organic production of soft-skinned

**Table 2.** Summary of the model parameters obtained from the Gaussian peak nonlinear regression curves shown in Fig. 3

| | | No-choice bioassay | | | Choice bioassay | | |
|---|---|---|---|---|---|---|---|
| Parameter[a] | Treatment | Value (95% CL)[b] | *t*-value | *P*-value | Value (95% CL)[b] | *t*-value | *P*-value |
| | | Daily emergence in number of emerged flies | | | | | |
| a | Untreated | 18.78 (17.10–20.46)a | 35.5 | < 0.0001 | 20.53 (18.76–22.30)a | 36.9 | < 0.0001 |
| | Lime-sulfur treated | 8.27 (6.52–10.01)b | 15.1 | 0.0006 | 9.67 (8.64–10.71)b | 29.6 | < 0.0001 |
| b | Untreated | 0.70 (0.63–0.77)a | 32.4 | < 0.0001 | 0.68 (0.61–0.74)a | 32.9 | < 0.0001 |
| | Lime-sulfur treated | 0.88 (0.67–1.09)a | 13.3 | 0.0009 | 0.84 (0.74–0.94)a | 26.1 | 0.0001 |
| $X_0$ | Untreated | 11.87 (11.79–11.96)a | 456.3 | < 0.0001 | 11.83 (11.75–11.91)a | 492.7 | < 0.0001 |
| | Lime-sulfur treated | 11.89 (11.67–12.11)a | 172.5 | < 0.0001 | 12.16 (12.06–12.27)b | 363.2 | < 0.0001 |
| | | Daily emergence in percentage of emerged flies | | | | | |
| *a* | Untreated | 53.97 (46.69–61.24)a | 23.6 | 0.0002 | 58.49 (53.46–63.52)a | 37.0 | < 0.0001 |
| | Lime-sulfur treated | 45.34 (35.99–54.70)a | 15.4 | 0.0006 | 48.71 (43.48–53.94)a | 29.6 | < 0.0001 |
| *b* | Untreated | 0.74 (0.62–0.85)a | 20.7 | 0.0002 | 0.68 (0.61–0.74)a | 32.9 | < 0.0001 |
| | Lime-sulfur treated | 0.89 (0.68–1.10)a | 13.6 | 0.0009 | 0.84 (0.74–0.94)a | 26.1 | 0.0001 |
| $X_0$ | Untreated | 11.79 (11.67–11.90)a | 313.3 | < 0.0001 | 11.83 (11.75–11.91)a | 492.7 | < 0.0001 |
| | Lime-sulfur treated | 11.92 (11.71–12.14)a | 175.6 | < 0.0001 | 12.16 (12.05–12.27)b | 363.2 | < 0.0001 |

[a] Coefficients from a three-parameter Gaussian peak nonlinear regression model: $Y = a * \exp(-0.5 * ((X - X_0)/b)^2)$; *a* = maximum peak value at y-axis (number of emerged flies); *b* = rate of change; $X_0$ = location of the peak on the x-axis (time).
[b] Different letters in a column within each parameter indicate significant differences due to non-overlap of the 95% confidence limits (CL).

  

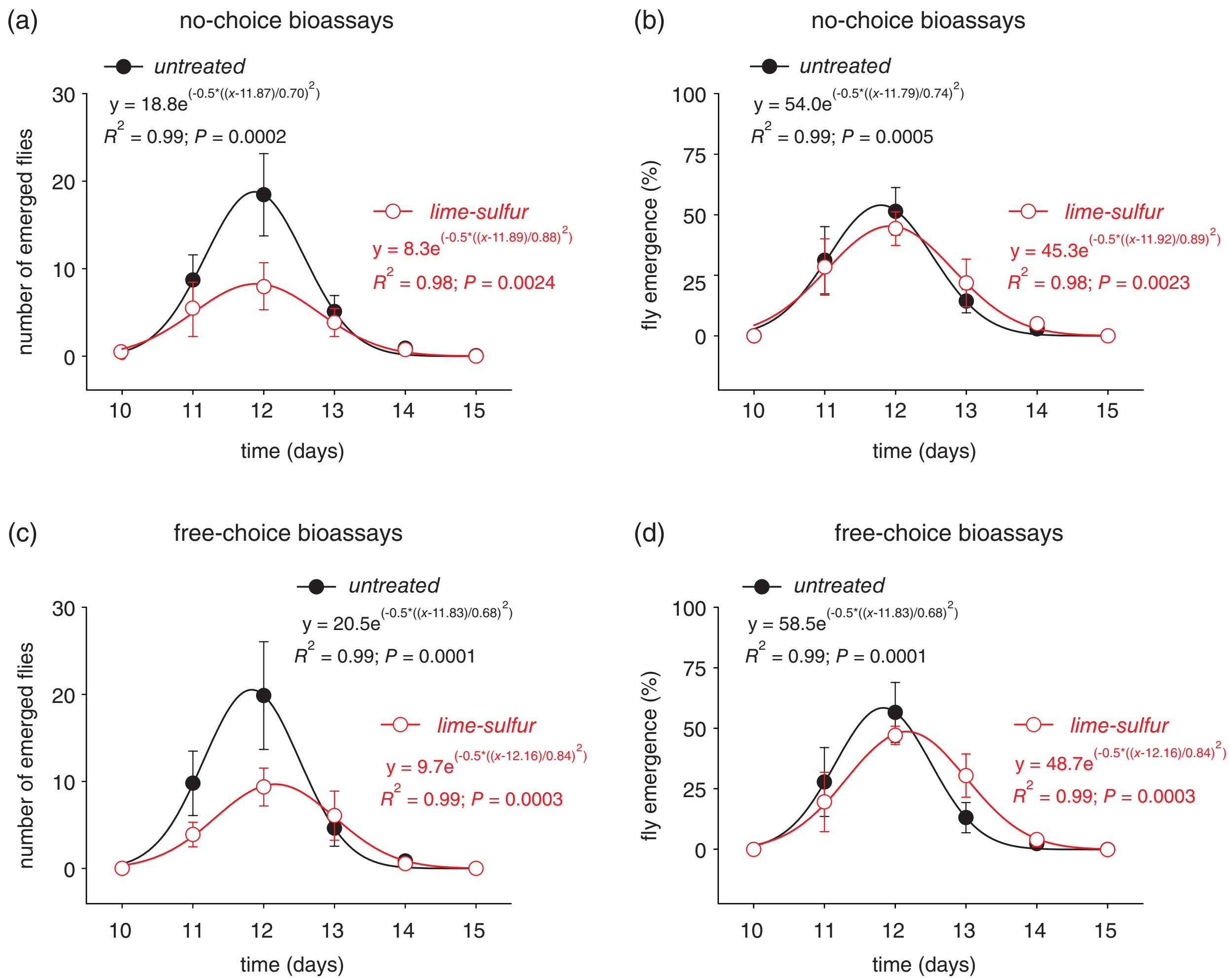


**Figure 3.** The daily emergency of *D. suzukii* adults in number (a,c) and in percentage of emerged flies (b,d) that were developed from eggs laid on lime-sulfur-treated or untreated plants in oviposition bioassays with and without free choice. Symbols represent means of the observed data and lines represent the fits to the data.

fruits (strawberries). Further investigations should evaluate the contributions of environmental (e.g., social cues coming from conspecifics or the presence of aversive tastants) and genetic factors (e.g., neural basis that governs simple decision-making processes) to the preference for oviposition sites, which will contribute to a better understanding of the sublethal effects induced by lime sulfur on *D. suzukii* females.

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