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The screening of suitable insecticides is one of the key factors for successful applications of trunk injection technology on ornamental plants. In the present study, ~~we selected~~ six chemical pesticides and applied them inside the trunks of *Osmamthus fragrans* using a no-pressure injection system for the control of *Latoia lepida*. The leading quantity of chemicals, mortality and frass of *L. lepida* larvae, as well as the leaf loss, were evaluated after 77 days and 429 d ays. The results showed that 4% Imidacloprid + Carbosulfan and 21% Abamectin + Imidacloprid + Omethoate had the fastest conductivity and were completely ~~up taken~~ into the trunks within 14 days; however, the insecticide efficiency was extremely low. Additionally, the conductivity of 10% Emamectin Benzoate + Clothianidin and 2.5% Emamectin Benzoate was appropriate within 30 days, and they also had a longer duration of insecticide efficiency (>80% mortality) in the upper or lower leaves. These insecticides also showed a significantly lower on leaf loss and frass amount. We conclude that Emamectin Benzoate and Emamectin Benzoate + Clothianidin, have a rapid uptake into *O. fragrans*, an appropriate efficiency and longer duration. Hence, they may be ~~the most~~ suitable control option for *L. lepida* in *O. fragrans* plants.

Evaluation of insecticide trunk injections for the management of *Latoia lepida* (Cramer) in sweet olive trees, *Osmamthus fragrans*

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ABSTRACT

The screening of suitable insecticides is one of the key factors for successful applications of trunk injection technology on ornamental plants. In the present study, we selected six chemical pesticides and applied them inside the trunks of *Osmamthus fragrans* using a no-pressure injection system for the control of *Latoia lepida*. The leading quantity of chemicals, mortality and frass of *L. lepida* larvae, as well as the leaf loss, were evaluated after 77 days and 429 days. The results showed that 4% Imidacloprid + Carbosulfan and 21% Abamectin + Imidacloprid + Omethoate had the fastest conductivity and were completely up taken into the trunks within 14 days; however, the insecticide efficiency was extremely low. Additionally, the conductivity of 10% Emamectin Benzoate + Clothianidin and 2.5% Emamectin Benzoate was appropriate within 30 days, and they also had a longer duration of insecticide efficiency (>80% mortality) in the upper or lower leaves. These insecticides also showed a significantly lower on leaf loss and frass amount. We conclude that Emamectin Benzoate and Emamectin Benzoate + Clothianidin, have a rapid uptake into *O. fragrans*, an appropriate efficiency and longer duration. Hence, they may be the most suitable control option for *L. lepida* in *O. fragrans* plants.

INTRODUCTION

The sweet olive, *Osmamthus fragrans* (Thunb.) Lour., is a popular garden evergreen shrub or small tree that belongs to the family of Oleaceae. It has both ornamental and practical uses in vegetation, landscaping, and incensing (*Liu & Xiang, 2003*; *Lee et al., 2007*), and is widely planted in the Huaihe River basin and southern areas of China (*Wang et al.,* 2006). The nettle caterpillar or blue-striped nettle grub, *Latoia lepida* (Cramer; Lepidoptera: Limacodidae) is distributed throughout Southeast Asia (*Azharul Islam et al.,* 2009), especially in China, Japan, India, Sri Lanka, Indonesia and Vietnam (*Hirashima, 1898*). *L. lepida* larvae mainly feed on leaves of *O. fragrans*, which result in the leaves and twigs died back or fell, and growth restriction (*Wakamura et al., 2007*). Thus, this pest reduces the ornamental and practical uses of the plants (*Ju et al., 2007*). In addition, the exposure to the stinging spines on the dorsal surface of *L. lepida* could lead to skin redness, swelling, heating, pain and other clinical manifestations, such as fever, joint pain, and death in the human allergic population (*Qin et al., 1998*). Therefore, combating *L. Lepida* infestations has an economic value and a great significance for the protection of human health.

Currently, spraying chemicals on tree crowns is the main practice for *L. Lepida* control in China. However, this method can result in the release of pesticides into the atmosphere, water and non-target animals, which causes adverse consequences such as the death of a large number of natural enemies, poisoning of livestock and environmental pollution (*Wakamura et al., 2007*). This phenomenon is more commonly found in the green area of the city or the suburbs. In contrast, the trunk injection technology is an environment-compatible pesticide technology because of its high efficiency with liquid drugs, its broad insecticidal spectrum and its pollution-free, safe, simple operation, and is barely affected by the weather (*Navarro, 1992*; *Montecchio, 2013*). This technology is based on the injection of pesticides into the tree trunks, which transport the liquids from the conducting tissues to the site of action (*Mendel, 1998*; *Harrell, 2006*; *Mota-Sanchez et al., 2009*; *Doccola et al., 2011*); thus, it plays an important role in disease and insect pest control (*Mota-Sanchez et al., 2009*; *Takai et al., 2001*; *James et al., 2006*; *Darrieutort & Lecomte, 2007*). For example, by using trunk injection of emamectin benzoate, the treated ash trees exhibited less canopy decline relative to non-treated control trees (died for heavily *Agrilus planipennis* impacted) over a four-year period (*Flower et al., 2015*), and also exhibited nearly 99% mortality of *A. planipennis* feeding on treated tissue (*Smitley et al., 2010*; *McCullough et al., 2011*; *Herms et al., 2014*).

Certainly, a variety of insecticide options is a key link for the successful applications of trunk injection technology. *Byrne et al* (2012) found that the uptake of 10% dinotefuran was more rapid than the uptake of 5% imidacloprid in California avocado groves; both chemicals showed a good control of avocado thrips, *Scirtothrips perseae*, and no residues were detected within the fruits. In contrast, although 10% acephate showed a rapid uptake and provided a good control of

thrips in bioassays, acephate residues and its insecticidal metabolite, methamidophos, were detected in the fruits for up to 4 weeks after the injection; the uptake of 5% avermectin was considerably slow, and it was ineffective against avocado thrips (Byrne *et al.,* 2012). Another study found that the trunk injection of imidacloprid, thiamethoxam and clothianidin for the control of the citrus greening disease, *Diaphorina citri*, on grown king mandarin trees resulted in approximately 50% mortality of the psyllid within 45 days; in general, imidacloprid had a better control effect than others (*Ichinose et al., 2010*). Therefore, the evaluation of pest control using trunk injections of different chemicals allows for a quick and effective assessment of the optimal trunk injection agent. However, little has been reported on the success of insecticide treatments by using trunk injection technique to control *L. lepida* on *O. fragrans* trees.

In this study, we selected six chemical pesticides to be injected, without pressure, into the trunks of *O. fragrans* for the control of *L. lepida*. First, the leading quantity of chemicals was estimated at different times within a month. In addition, the mortality of *L. lepida* larvae and the leaf loss were evaluated in bioassays at 77 (nearly the span period of the two generation larvae) and 429 days (duration of inter annual) after the treatment, respectively. Finally, we also investigated the amount of frass of *L. lepida* larvae at above time points. Our goal was to assess which type of chemicals had a superior performance with regards to the uptake rate, effect against target pest and its duration.

MATERIAL AND METHODS

Plants and insects

The study was conducted in a garden located in the Zhejiang Academy of Agricultural Sciences (30°18′75″ N; 120°28′60″ E), Hangzhou, China. Sweet olive trees, *O. fragrans* var. thunbergii, were 10-15 years old, and planted in a total of three rows with a tree spacing of approximately 30 cm, and had a well-structured crown and a uniform growth trend. We randomly selected 21 individual trees and measured the height of the tree, the width of the canopy, and the diameter at breast height. The means (± sem) of these measures were 4.53 ± 0.20 m, 2.39 ± 0.10 m, and 0.12 ± 0.01 m, respectively. These trees were managed with common watering and fertilization techniques; however, they were not subjected to chemical pesticides. Fifth instar larvae of *L. lepida* with similar weight were collected from sweet olive trees planted in the Hangzhou Blue Ocean Ecology Park (30°08′71″ N; 120°31′49″ E) and were used for the bioassay. None of the study species are protected in China, so no specific permits were required for collections or field activities.

Insecticides

The insecticide (TC) included 95% imidacloprid and 70% emamectin benzoate (Guangdong Dafeng Plant Protection Technology Co., Ltd.), 95% abamectin (Hebei Weiyuan Group Co., Ltd.), 95% clothianidin emulsifiable concentrate (Nanjing Lebang Chemical Products Co., Ltd.), 98% omethoate (Lianyungang Dongjin Chemical Co., Ltd.), and 92% carbosulfan (Jiangsu Xingnong Co., Ltd.). Then, they were diluted and formulated (or mixed) following the six trunk injection chemicals described in Table 1.

Insecticide application by trunk injection

On 28 April 2014, 21 brown, plastic bottles, 6 cm high (from the bottom to the bottle neck) and 4 cm in diameter were prepared in the laboratory. Each bottle was supplied with 30 mL trunk injection insecticide (n = 3 for each treatment) and three with distilled water (no insecticides) as the controls. A hole of approximately 30 mm in depth and 4 mm in diameter was drilled downward at the angle of 45° using a rechargeable drill (Model TSR/1080-LI, Bosch Power Tools Co., Ltd., Shanghai, China) at approximately 30 cm above the ground in the main trunk of each tree. The bottle tip was cut open using a razor and inserted into the hole to completely inject the formulated agent into the trunk. The quantity of residual agent in the bottles was visually observed and recorded at 9, 14, 23, and 30 days after the injection. During the assays, the temperature was 23.40 ± 0.71°C, and atmospheric humidity was 67.75 ± 2.10 %; in addition, 5 rainy (showers) days occurred.

Laboratory bioassay

The mortality of *L. lepida* larvae was measured after feeding on isolated leaves from the treated plants at 77 days and 429 days. Eight branches in the four directions from the bottom and top of the canopy were randomly collected from each tree. Each branch was 25-30 cm length and had approximately 16 leaves. Debris and insects were removed from the branches and leaves before the test. First, the leaf area was measured using a Laser Area Meter (Model CI-203, CID Inc., USA). Each branch was place vertically in a glass bottle (6 cm in diameter, 9 cm in height) and fixed at the bottle neck with polystyrene foam. The glass bottle was filled with distilled water and was placed in the middle of a plastic funnel (upper diameter, 40 cm; lower diameter, 5 cm; height, 20 cm). Lastly, the entire funnel was placed on the mouth of another glass bottle. The funnel neck (ca. 4 cm in length) was inserted into the glass bottle and fixed. Meanwhile, the frass of larvae was collected in the bottom bottle. The inner wall of the funnel was coated with Teflon cream Fluon® to avoid the escape of falling larvae.

Larvae that were starved for 24 h were transferred to the leaves using a brush and stabilized after 12 h of observation before the test. Two larvae were introduced into each branch. The mortality of the larvae fed on the tested leaves during the last 5 days was recorded after 77 days and 249 days. In addition, the number of leaves eaten or damaged by the larvae was recorded, and the residual leaf area was measured with a Laser Area Meter. The larva frass was weighted using an electronic scale (model EX223, Ohaus Inc., USA).

Statistical Analysis

The data were analyzed using the Shapiro-Wilk test to determine if the data had a normal distribution and homogeneity of variances. If the data were normally distributed and exhibited similar variances, they were further analyzed using a one-way ANOVA to compare the differences in the ratio of agents injected in different periods. The corrected mortality of larvae on the upper or lower branches treated with different agents, the ratio of damaged leaves, the area of residual leaves and the amount of frass were analyzed and compared using a two-way ANOVA and Duncan multiple range tests after 77 days and 249 days after the injection. The correlation between the mean amount of frass and the mean leaf area consumed by *L. lepida* was assessed using simple linear-regression analysis, then via an F-test. All the statistical analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

198
199 ## RESULTS
200 **Leading quantity of chemical agents**
201 Within 30 days of the injection, 4 agents were completely ~~injected into~~ the trunk, namely A+I+O,
202 EB, I+Ca and EB+A. Among them, I+Ca had the fastest injection speed and was completely
203 injected within 14 days, followed by A+I+O and EB+A (23 days; Fig. 1). However, only 77.5%
204 of EB+CL and 56.7% of I were injected within 30 days. In addition, the injection speed of
205 EB+CL showed no significant differences in all the measured time points. Within 9 days, the
206 quantity of A+I+O and I+Ca injected was the largest, over 80% of the total.
207 **Larvae mortality**
208 The mortality of larvae fed on the isolated upper leaves with EB+CL and EB after 77 days of the
209 injection treatment was significantly different than that of larvae feeding on trees with other
210 treatments. The corrected mortality with EB+CL was 100%, whereas the data for A+I+O, I+Ca, I
211 and EB+A were not significantly different from that of the control, especially for A+I+O and
212 I+Ca (mortality = 0; Fig. 2).
213 The mortality of larvae fed on the lower leaves with EB+CL, EB, I and EB+A was
214 significantly different than that of larvae feeding on trees with other treatments. The mortality
215 with EB+CL was 100%, while the data for A+I+O and I+Ca was not significantly different from
216 that of the control, especially for I+Ca (mortality = 0). After 429 days, the mortality of larvae fed
217 on the upper or lower leaves after the EB+CL and EB treatment was significantly different than
218 that for larvae feeding on trees with other treatments (Fig. 2). These results indicate that I+Ca
219 had a good injection speed; however it had no effects on the survival of larvae.
220 **Leaf loss**
221 After 77 days, the ratio of upper damaged leaves, in total, was approximately 20% or less for EB
222 and EB+CL and significantly less than that of other treatments (Fig. 3); in addition, the same
223 situation occurred in the area of damaged leaves (Fig. 4). However, in comparison with the upper
224 leaves, the data of lower damaged leaves for all the agents were significantly different than that
225 of the control. The ratio of lower damaged leaves, in total, was less than 12% for EB+CL,
226 A+I+O and EB (Fig. 3).
227 After 429 days, the ratio of upper damaged leaves, in total, for EB+CL, EB, A+I+O and I was
228 not significantly different from that of the control; however, it was less than that of other agents.
229 Instead, the areas of upper damaged leaves for A+I+O and I were not significantly different from
230 that of the control (Fig. 4). The ratio of lower damaged leaves for EB+CL, EB and A+I+O was
231 less than that of I and the control, whereas the data for I, I+Ca, and EB+A were not significantly
232 different from that for the control. In addition, the area of lower damaged leaves for I+Ca was
233 significantly different from that of the control (Fig. 4).
234 **Larva frass**
235 After 77 days, the amount of frass from larvae feeding on upper or lower leaves during the last 5
236 consecutive days for all the treatments, except for I+Ca, was less than that of the control. The
237 data for EB+CL and EB were more obvious.
238 After 429 days, the frass ~~of~~ EB and EB+CL were ~~less than that of others~~. The data from larva

feeding on upper leaves with I+Ca were significantly different from that of the control, but it was not significantly different from that of the control for larvae feeding on lower leaves. Moreover, the correlation analysis showed that the amount of frass had a significantly positive correlation with the area of damaged leaves (R=0.7583, F=35.1894, n=28, P<0.001) (Fig. 6).

Finally, the leading quantity of chemicals, the mortality and frass of *L. lepida* larvae, as well as the leaf loss, were evaluated ~~intuitively,~~ and the results are shown in Table 2. The performance of the EB treatment was the best, followed by the EB+CL treatment. However, the injection of the latter was slightly less effective.

DISCUSSION

The selection of appropriate trunk injection agents is key for the successful implementation of the trunk injection technology (*Dedek et al., 1986*; *Takai et al., 2004*). For a no-pressure injection system (the only pressure in the system is that of gravity), it is important for the liquid chemicals in the external or injection plastic bottles to move into the plant fas ystems such as this one seem to be less advantageous because their lack of pressure can make the uptake slow; however, this system is inexpensive and simple. In the present study, we found that four agents (i.e., A+I+O, EB, I+Ca and EB+A) completely moved into the trunks within 30 d; additionally, more than 80% of A+I+O and I+Ca were introduced into the trunks at 9 d. However, for 4% imidacloprid, only 56.7% of the agent was introduced within 30 d, and its effect on the mortality of *L. lepida* larvae was poor. In contrast, the conductivity and insecticide efficiency of imidacloprid on avocado groves (*Byrne et al., 2012*) and ash trees (*Mota-Sanchez et al., 2009*) were acceptable, although the authors did not mention whether the agents were completely introduced into the plants. The reason for this outcome may be that the chemical conductivity was affected by the injection time, procedure, tree size, growth, and even the type, concentration and formulation of the chemicals (*Harrell, 2006*; *McCullough et al., 2005*; *Cowles et al., 2006*; *Tanis et al., 2012*).

We also found that EB alone or mixed with other agents (i.e., EB+A and EB+CL) had a better conductivity and insecticide efficiency. Although only 77.5% of the amount of EB+CL was introduced, its insecticide efficiency maintained a level as high as that of EB (>80%). We suggest that the mixed EB and CL may have a synergistic effect. Interestingly, other chemicals showed a better conductivity but a low insecticide efficiency, such as A+I+O and I+Ca. Specifically, the lava mortality was zero in the I+Ca treatment group. The reasons that the I+Ca treatment group may explain this result are that 1) the concentrations of chemicals were too low to have insecticide efficiency, and/or 2) the effective components of the chemical were not compatible with the metabolites of plant because there are significant differences in the ability to metabolize exogenous compounds among different plant species [25].

Previous studies had shown that the concentrations of trunk-injected chemicals among plant tissue types were different in the plants as a whole, but the leaves had much greater concentrations (*Mota-Sanchez et al., 2009*; *Takai et al., 2004*; *Xu et al., 2004*). Therefore, for leaf-feeding insect pests, the leaf loss can indicate the insecticide efficiency of the residual concentration of chemicals. EB (emamectin benzoate) acts as an antagonist for gamma-

aminobutyric acid-gated chloride channels, which cause a disruption of nerve impulses and a rapid paralysis in a range of Lepidopteran species (*Kass et al., 1980*; *Ishaaya et al., 2002*). In addition, it has excellent control effects on nematodes (*Kazuya et al., 1999*; *Cheng et al., 2015*) and emerald ash borer (*Flower et al. 2015*) with both trunk or with soil injections. Similar to our study, we found that the conductivities of EB+CL and EB were acceptable, and they also had a longer duration of insecticide efficiency (429 days). However, another mixed agent, EB+A, only showed insecticide efficiency on the lower leaves and failed to persist through time. This outcome is possibly because different agent mixtures had different active ingredients in different concentrations. In the A+I+O treatment group, the leaf loss from the lower canopy was less than that from the upper canopy, which indicate that the concentration of the agent was mainly retained in the lower leaves.

The amount of frass excreted by the insect pests can be used as the main indicator of the insecticide efficiency of chemical pesticides (*Paguia et al., 1980*; *Yang et al., 2006*). In the present study, the frass of *L. lepida* larvae were investigated to further assess the insecticide efficiency of treated leaves. We found that the frass was affected in various degrees by all the treatments except for I+Ca; in addition, frass amount in the EB and EB+CL treatment groups was less than that in other treatments, which suggest that such agents might have a strong ~~virulence~~ on the larvae. A previous study demonstrated that a decrease in the food uptake was significantly correlated with the decrease in the frass amount of insect pests (*Yang et al., 2006*). This result is in agreement with our study. Interestingly, insects can reduce the toxicity of chemical agents through an excretion mechanism (*Bues et al., 2005*; *Liu et al., 2006*). Therefore, the detection and analysis of frass could be an important way to further determine the metabolic residues of injected chemical agents.

CONCLUSION

Overall, we conclude that the Emamectin Benzoate and Emamectin Benzoate + Clothianidin trunk-injected agents have a rapid uptake into *O. fragrans*, and they showed a significant insecticide efficiency and longer duration than other treatments. However, the safety of these injection agents in the flowers of *O. fragrans* must be further studied in future.

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Table 1(on next page)

Active ingredients and their formulation for the trunk injection.

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1 Table 1. Active ingredients and their formulation for the trunk injection.

Trunk injection chemicals (abbreviation or code)	Active ingredients	Formulation (%)
EB+CL	Emamectin benzoate + Clothianidin	10
A+I+O	Abamectin + Imidacloprid + Omethoate	21
EB	Emamectin Benzoate	2.5
I	Imidacloprid	4
I+Ca	Imidacloprid + Carbosulfan	4
EB+A	Emamectin benzoate+Abamectin	2.5

2

3

Table 2(on next page)

Overall performance of the pharmaceutical drugs with regards to injection rate, insecticidal effect, reduction of leaf loss and defecation of L. lepida.

Overall performance of the pharmaceutical drugs with regards to injection rate, insecticidal effect, reduction of leaf loss and defecation of L. lepida.

Table 2. Overall performance of the pharmaceutical drugs with regards to injection rate, insecticidal effect, reduction of leaf loss and defecation of *L. lepida.*

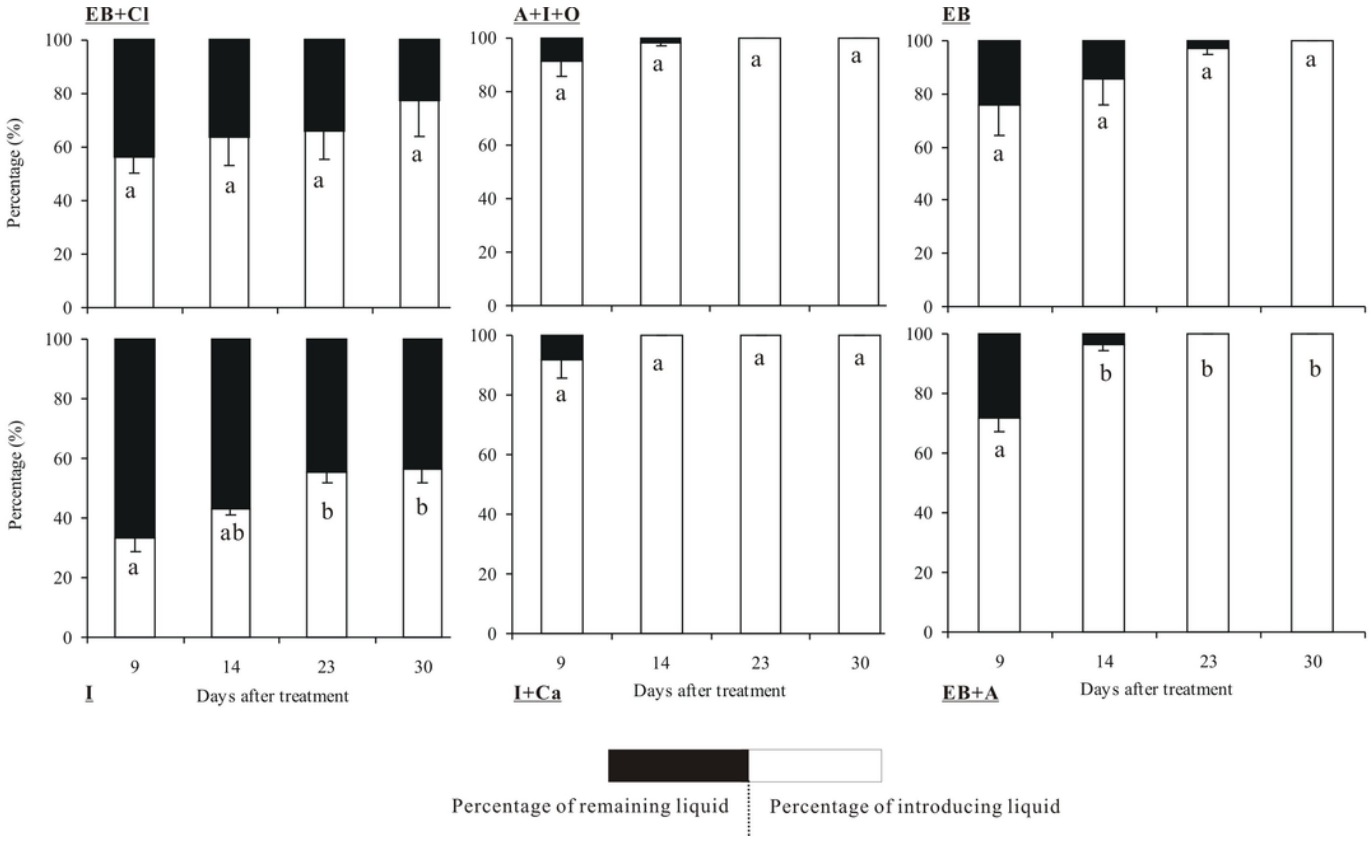
Drug treatment	Introducing effect	Insecticidal effect	Reduction of leaf loss	Reduction of defecation
EB+CL	√	√√	√√	√√
A+I+O	√√	-	√	√
EB	√√	√√	√√	√√
EB+A	√√	√	-	√
I	√	√	-	√
I+Ca	√√	×	√	-

Note: √√ indicates best performance, √ indicates regular performance, - indicates insufficient performance, × indicates no effect.

1

Percentage (%) of the six types of agents injected into the trunk of O. fragrans in different post-treatment periods

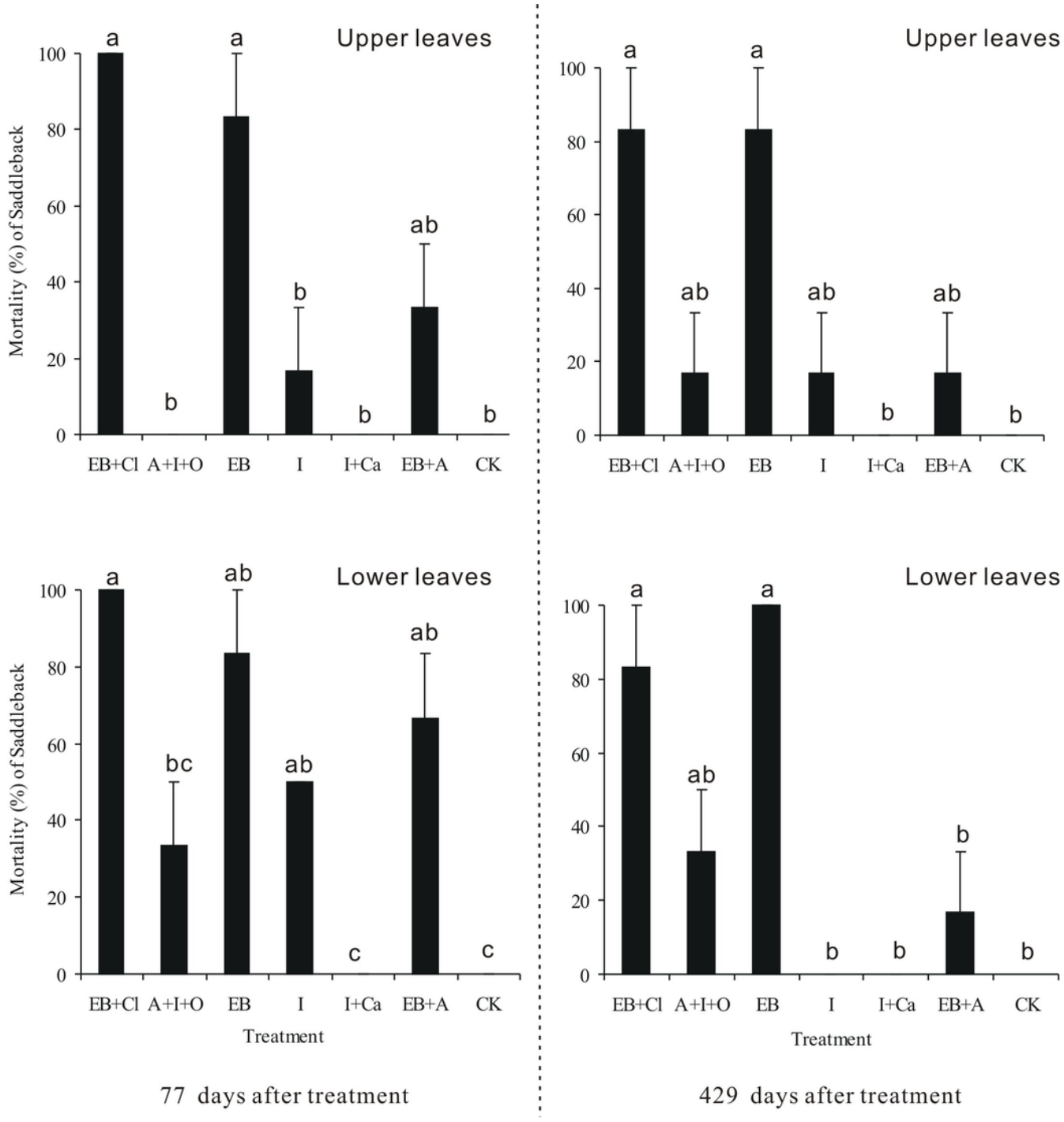
Percentage (%) of the six types of agents injected into the trunk of O. fragrans in different post-treatment periods.



2

Mortality of L. lepida larvae

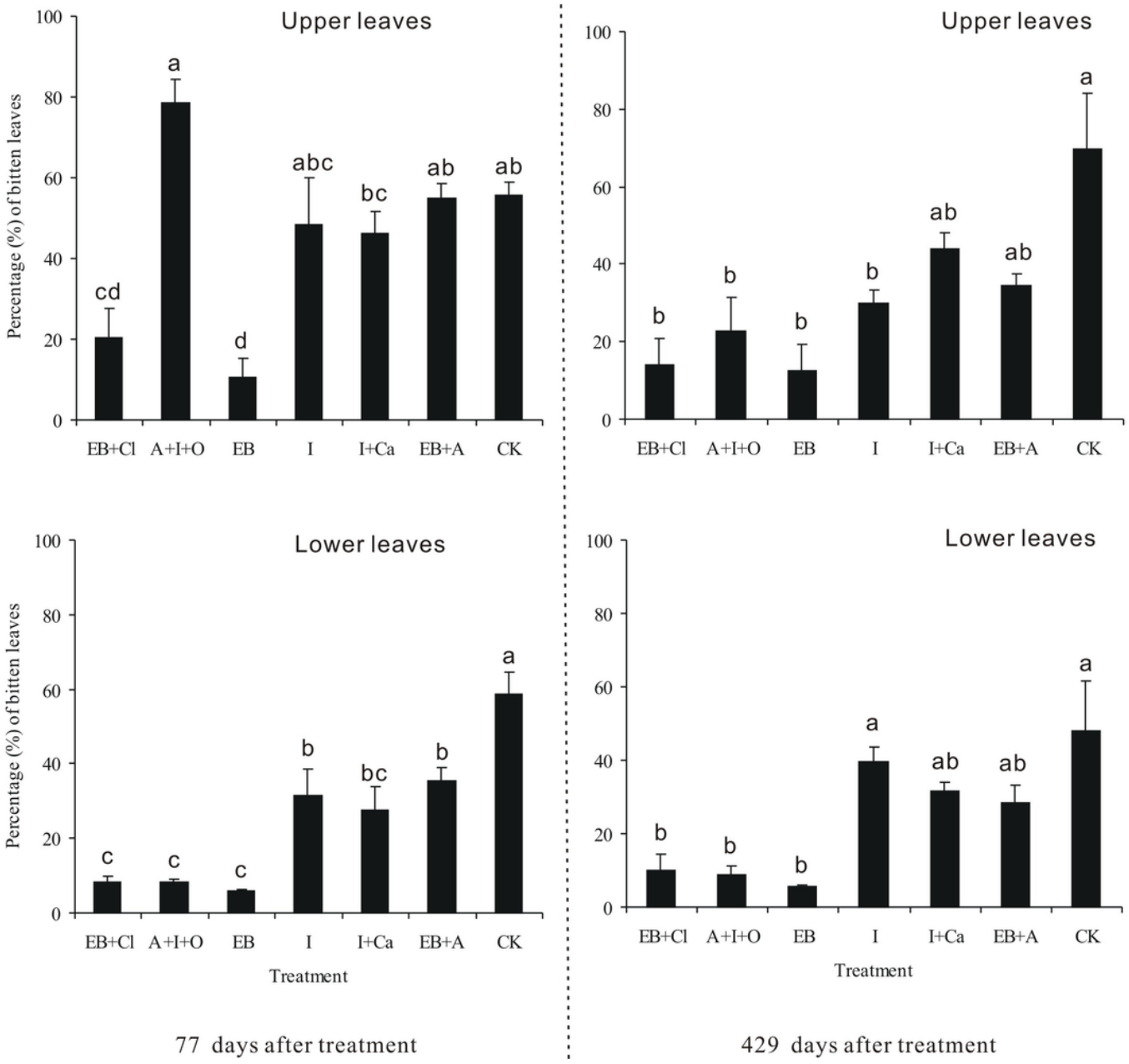
Mortality of L. lepida larvae fed on different parts of O. fragrans leaves in different treatment periods.



3

Percentage of different isolated leaves eaten by L. Lepida larvae

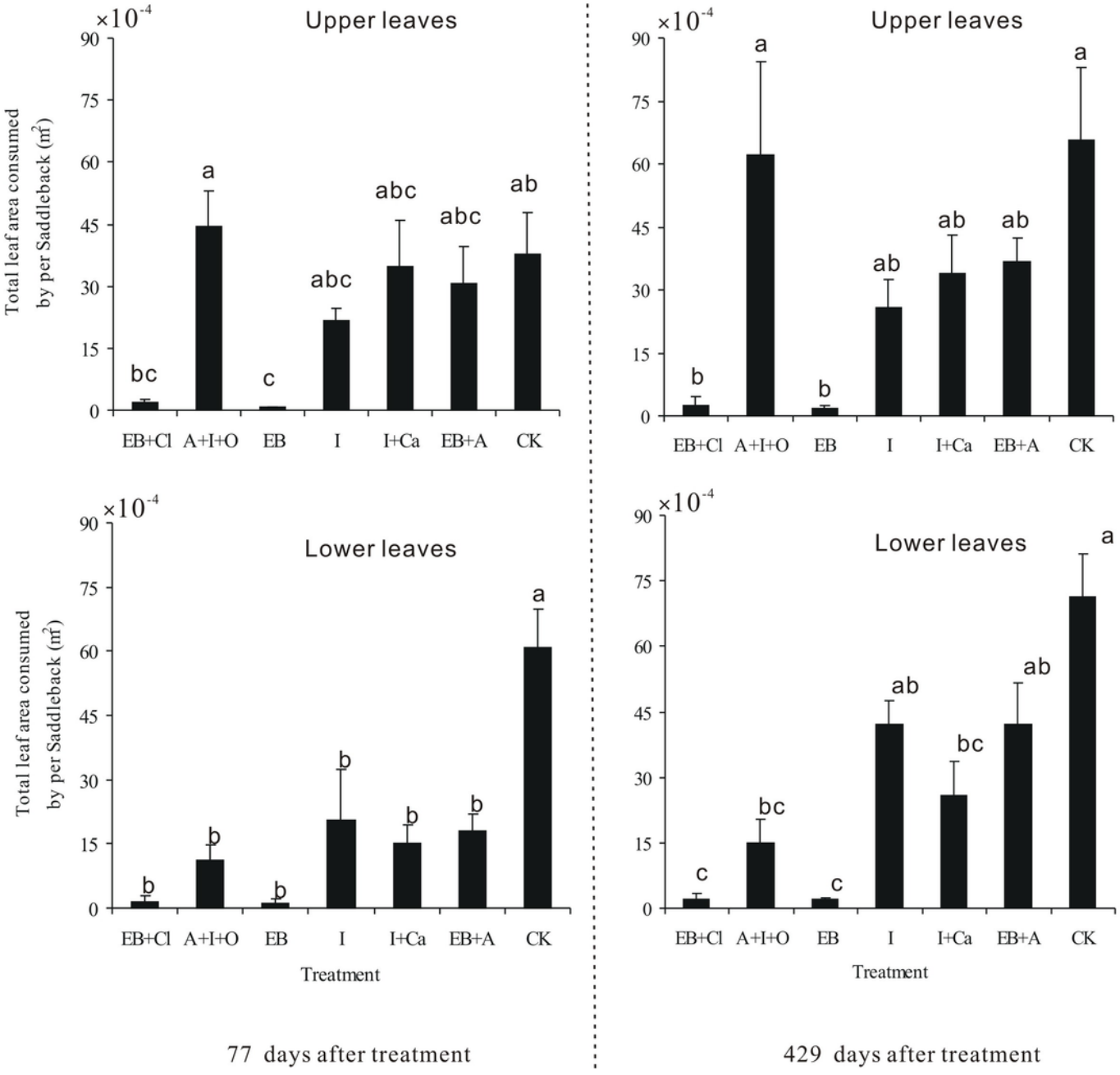
Figure 3-Percentage of different isolated leaves eaten by L. Lepida larvae in different treatment groups at different observation times



4

Average areas of isolated leaves eaten by L. Lepida larvae

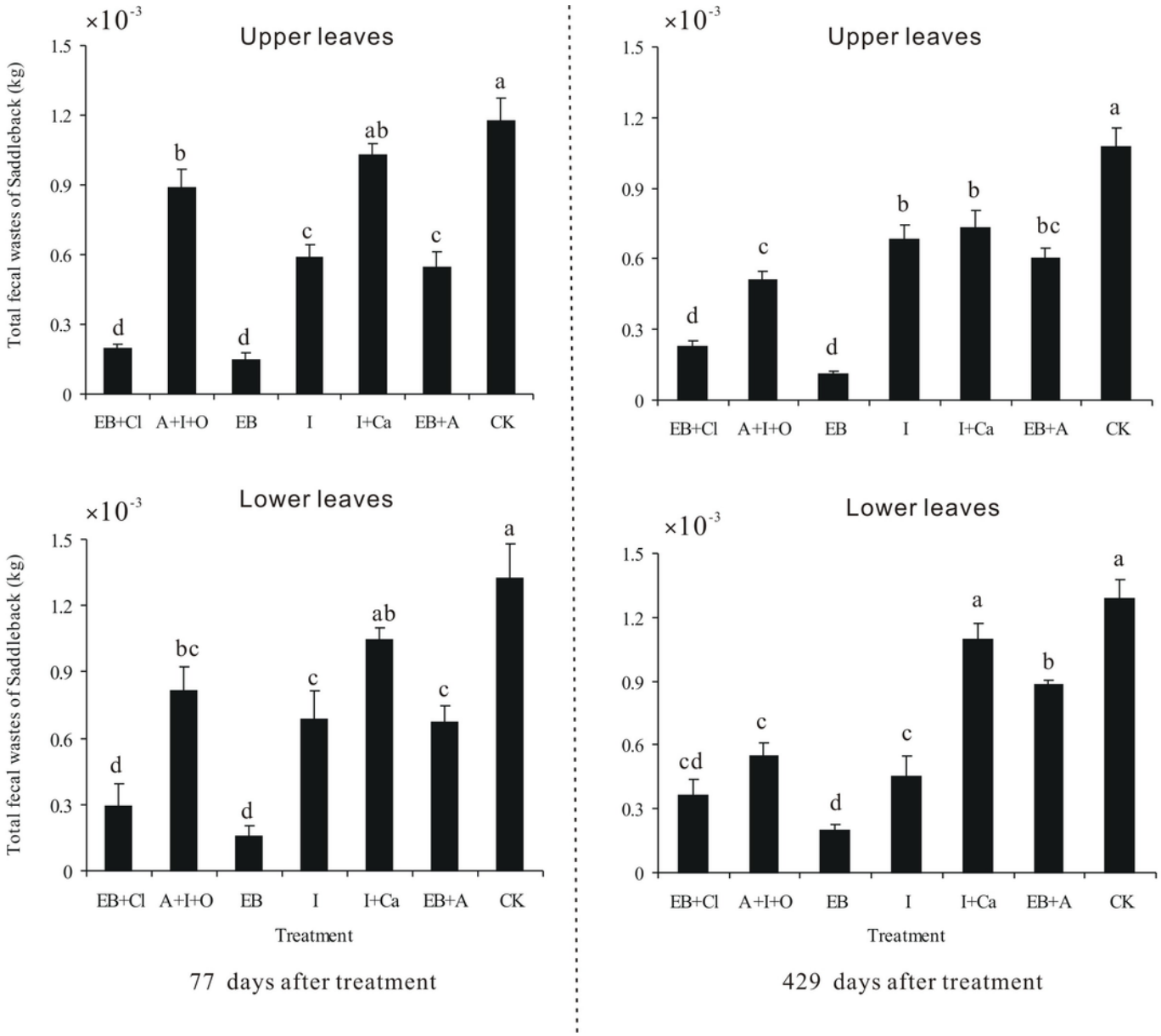
Average areas of isolated leaves eaten by L. Lepida larvae in different treatment groups at different observation times.



5

Frass a mounts of L. Lepida larvae

Frass a mounts of L. Lepida larvae fed on upper or lower leaves for 5 consecutive days in all the treatment groups.



6

Correlation between the mean amount of frass and mean leaf area

Correlation between the mean amount of frass and mean leaf area eaten by L. Lepida larvae (F =35.1894, n=28, P <0.001).