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SHORT COMMUNICATION

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Laboratory approach to the use of sulphur and kaolin as preventive control against *Drosophila suzukii*

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Abstract

Drosophila suzukii (Matsumura, 1931) is an invasive pest from South East Asia that was detected for the first time in Southern Europe in 2008. This species can damage a wide range of soft-skinned fruits crops affecting ripening fruits and causing important economic losses. Since the exclusive use of chemical insecticides for controlling *D. suzukii* may prompt the appearance of resistance and environmental pollution, alternative methods compatible with sustainable management are required. In this study, commercial formulations of powdered sulphur and kaolin were tested as a preventive method applied to blueberry fruits under laboratory conditions. In no-choice assay, powdered sulphur had a significant effect on oviposition and adult emergency with reductions of 76% and 77%, respectively. In addition, sulphur displayed a significant toxicity on males and lethal effect with over 40% adult mortality seven days after exposure. The choice assay confirmed and improved the powdered sulphur effects, with reductions of 98% and 96% in oviposition and adult emergence, respectively. In contrast, kaolin produced no significant reduction in infestation and adult mortality during no-choice and choice assays. These outcomes suggest that preventive use of powdered sulphur could be considered for sustainable control of *D. suzukii* in some berry crops.

Additional key words: spotted wing *Drosophila*; invasive pests; blueberry; berry crops; deterrent effects; fruit coating.

Abbreviations used: GLM (generalized linear model); MRL (maximum residue limit); SWD (spotted wing *Drosophila*).

Authors' contributions: Conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper: SPG and JMM.

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Introduction

The spotted wing *Drosophila* (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), is a highly polyphagous invasive pest from South East Asia (Walsh *et al.,* 2011). SWD was detected for the first time in Southern Europe (Spain and Italy) in 2008 (Cini *et al.,* 2012). Since then, this species has spread rapidly over Europe affecting a wide range of host plants including some economically important crops, especially berries (EPPO, 2014). In contrast to other *Drosophila* species, SWD infests healthy, ripening fruits laying eggs thanks to its serrated ovipositor (Walsh *et al.,* 2011). Larvae feed and develop within the fruits, which become unmarketable, resulting in a dramatic reduction of fruit production. SWD cause significant economic losses in berry production in North America (Goodhue *et al.,* 2011) and Europe (Lee *et al.,* 2011). In recent years, some authors have analyzed the effectiveness of chemical and others conventional insecticides against SWD (Bruck *et al.,* 2011). In general, organophosphates, pyrethroids and spynosins have shown strong activity against SWD adults. However, the exclusive use of these insecticides may prompt the appearance of resistance (Bruck *et al.,* 2011), environmental pollution, negative effects on beneficial arthropods and incompatibility with organic management and MRL's (maximum residue limit) zero tolerance policy of berry markets. In this context, alternative control techniques seem to be necessary, and some authors have carried out field and laboratory tests to analyze the effects of some organic products against SWD (Gargani *et al.,* 2013).

Sulphur has been widely used as fungicide in many crops over the last century. The insecticidal activity of this product has also been known, and there has been an increasing interest to include this chemical in Integrate Pest Management programs due to its low ecological impact and compatibility with organic management (Williams & Cooper, 2004). Kaolin particle films have originally been used to protect fruits from solar injury. Furthermore, negative effects of kaolin on insect activity (egg laying, movement or feeding) have been proved (Bürgel *et al.,* 2005). The present study analyses the role of powdered sulphur and kaolin as a barrier against SWD oviposition under laboratory conditions, with a view to ascertaining its potential preventive use as an alternative to traditional synthesis insecticides.

Material and methods

The SWD adults used in bioassays performed in this work came from a colony established in the Laboratory of Entomology of IFAPA "Las Torres-Tomejil" (Alcalá del Río, Seville; Spain) from larvae collected from field-infested raspberries in Huelva (southern Spain) during February 2014. A SWD population was reared on berry fruits, mainly blueberries (*Vaccinium* × *corymbosum* L. Ericaceae) and raspberries (*Rubus* × *idaeus* L. Rosaceae) In order to avoid endogamy, and ensure genotypic diversity as representative of the field populations, wild SWD from natural infested fruits were introduced into the colony several times during 2014 and 2015. Insects were kept at 22 ± 1 Cº, 65% RH, and 16:8 h (L:D) photocycle. Female and male SWD used in the bioassays were between 5 days old and 12 days old, and had no contact with fruits until they were used in the assays. The tested substances were powdered sulphur (90% DP formulation; Bago d'Ouro®; Sapec Agro, Valencia, Spain), and kaolin (95% WP formulation; Surround®; Basf Agro España, Barcelona, Spain). Since SWD eggs are difficult to see on structured fruits such as blackberry, raspberry and strawberry, making egg counts unreliable (Lee *et al.,* 2011), commercial blueberries (FresDoñarosa; Superexport Cia. Agraria S.L.; Huelva, Spain) were used in bioassays to ensure reliability of the counts. Given that the availability of commercial blueberry cultivars varied during assays, blueberries cv. 'Biloxy' were used in the no-choice assay, and for fruit firmness analyses and cv. 'Duke' were used in the choice assay.

The first experiment sought to determine the potential effects of sulphur and kaolin applications on adults' survival, as well as on oviposition and adult emergency. Experimental chambers were constructed in accordance with Emiljanowicz *et al.* (2014): 50 mL Falcon centrifuge tubes were modified making perforations for ventilation in the ends and by inserting a cut pipette tip in one of the sides, in which a small ball of cotton saturated with 10% w/v Honey-dH_2O solution was used as the adults' food. Two treatments plus a control were performed separately: (1) powdered sulphur treated blueberries, (2) kaolin treated blueberries, and (3) dH_2O-treated blueberries. Fruits were placed in a nylon mesh and powdered sulphur was applied directly (0.1 g per fruit); blueberries were gently rolled over the mesh to ensure that the entire surface of each fruit was covered. The mesh was carefully shaken in order to remove the surplus product, making it fall through the mesh. Dose per fruit was estimated by weighing 10 groups of 10 fruits beforehand, being 2.6 ± 0.3 mg/fruit after treatment. The field-recommended dose for kaolin was used for the assay (5%; 5 kg/100 L). Kaolin was applied using a hand sprayer with the blueberries placed in a nylon mesh. Once again, fruits were gently rolled over the mesh to ensure that the entire surface of each fruit was covered and the surplus product fell through the mesh. The same process was used to apply distilled water. All treated fruits were allowed to dry for 2 h before exposure to SWD. Each blueberry fruit was placed in experimental chambers with one female and one male SWD, and kept at 22 ± 1 Cº, 60 ± 5% RH, and a 16:8 h (L:D) photocycle. After 48 h, fruits were removed from the chambers and the number of eggs laid by SWD was counted under a stereomicroscope (×20). Each fruit was separately placed in a Petri dish and kept under laboratory conditions during 21 days recording number of emerged adults. Direct mortality produced by each treatment was recorded after 48 h. SWD couples were kept in the experimental chambers and under laboratory conditions, recording residual mortality seven days later. A total of 20 replicates per each treatment were established. Sibling samples of fruits of the same origin were treated in the same way and kept under the same conditions in order to analyze the effects of each treatment on fruit firmness. Fruit firmness was measured in grams force (gf) using a manual penetrometer (Effegi® tr FDP 500 g; Italy) fitted with a 1.5 mm Ø blunted needle. For each blueberry, two measures at the middle of the berry were recorded and averaged. A total of 93 berries (31 per treatment) were measured for firmness 48 h after treatment.

Choice assay was carried out to determine comparative deterrent effects on SWD oviposition. Blueberries were treated as in the no-choice assay. Fruits were exposed to SWD in 29 × 29 × 29 cm plastic cages (BugDorm® 1; Bio-Quip Products Inc., Rancho Rodríguez, CA; USA). Two blueberries (standardized at

5 g of fruit) per treatment (sulphur and kaolin) and a control (dH_2O) were placed in random order around the food source (25 mm plastic cup containing a small ball of cotton saturated with 10% w/v honey-dH_2O) placed in the center of the cage. Blueberries were spaced 4 cm from each other, and 5 cm from food source. A total of 3 females and 3 males per cage were exposed to 6 blueberries (2 per treatment and control) during 24 h. After 24 h, the blueberries were removed from the cage and the number of eggs laid by SWD females was counted under a stereomicroscope (×20). As in the no choice test, each fruit was separately placed in a Petri dish and kept under laboratory conditions during 21 days recording number of emerged adults. A total of 15 replicates were established for this experiment.

Since the normality and linearity of residuals were not matched for the no-choice and choice assays, generalized linear models (GLMs) were run to test the effects of treatment on the number of laid eggs and emerged adults using the R v.3.1.3 software package. GLMs were carried out including treatment as a factor, and the number of laid eggs and emerged adults (per fruit and per 5 g of fruit in no-choice and choice assays, respectively) as dependent variables fitted to a Poisson distribution with a log link function. Where differences were detected by GLM multiple comparisons, post-hoc Tukey HSD tests ($p<0.05$) were performed using "glht" function in the "multcomp" package. GLM procedures use the Wald statistic ("z") value and Pr([|z|]) to analyze the effects of each factor on the response variable, testing the hypothesis that the corresponding parameter (regression coefficient) is zero (Crawley, 2005). Firmness data matched the normality and linearity of residuals; therefore, one-way ANOVA was performed to assess the effects of treatments on fruit firmness. Adult mortality among treatments was compared using a χ^2 test run with Statistix v.9.1. Abbott-corrected mortalities (Abbott, 1925) were used to plot adult mortality.

Results and discussion

After 48 h, control percentage mortality in the no-choice assay was 2.5. Abbot-corrected mortality caused by sulphur was 23.1% being significantly higher than control ($\chi^2 = 8.5$; $p<0.01$). In addition, Abbot-corrected mortality caused by sulphur on males was 40.0% being significantly higher than control (0.0%) after 48 h ($\chi^2 = 10.0$; $p<0.01$). In contrast, no significant differences were found between sulphur (5.3%) and control (5.0%) mortality rates on females after 48h. Kaolin displayed no significant toxic effect ($\chi^2 = 2.9$; $p>0.05$) with an Abbot-corrected mortality of 5.1% (Fig. 1). The same results were obtained with this product when males (5.0 and 0.0% for kaolin and control, respectively) and females (5.3 and 5% for kaolin and control, respectively) were analyzed separately ($\chi^2 = 1.1$ and 0.4 for males and females respectively; $p>0.05$ in the two cases). Seven days after application, control percentage mortality in the no-choice assay was 7.5%. Mortality displayed by sulphur was significantly higher than that obtained for the control group ($\chi^2 = 16.1$; $p<0.001$), with a total Abbot-corrected mortality of 43.2%. This mortality caused by sulphur was due to the significant effects on males (73.6 and 5% for sulphur and control, respectively; $\chi^2 = 20.4$; $p<0.001$) with no significant differences between sulphur (11.1%) and control (10%) mortality rates on females after seven days ($\chi^2 = 0.8$; $p>0.05$; Fig. 1). Mortality caused by kaolin was not significantly different from control mortality ($\chi^2 = 2.6$; $p>0.05$) with a total Abbot-corrected mortality of 13.5% (Fig. 1) and no significant effects on males (21.1 and 5.6% for kaolin and control, respectively; $\chi^2 = 3.14$; $p>0.05$) and females (5.6 and 10% for kaolin and control, respectively; $\chi^2 = 0.2$; $p>0.05$). The no-choice assay revealed significant differences in the mean number of SWD eggs per fruit treatments (z = -4.5; $p<0.001$). Powdered sulphur treatment displayed the lowest mean (0.6 ± 0.2 eggs/fruit) with significant differences with respect to the control treatment (2.5 ± 0.7 eggs/fruit; z = -4.4; $p<0.001$) and kaolin treatment (1.8 ± 0.3 eggs/fruit; z = -3.3; $p<0.01$). There were no significant differences between the mean number of eggs per fruit in kaolin and control treatments (z = -1.5; $p>0.05$; Fig. 2a). Results also showed significant differences in the mean number of emerged adults per fruit among treatments (z = -3.3; $p<0.001$). Once again, powdered sulphur treatment had the lowest mean adults/fruit) with significant differences with the control treatment (1.3 ± 0.5 adults/fruit; z = -3.2; $p<0.01$) and kaolin treatment (0.9 ± 0.2 adults/fruit;

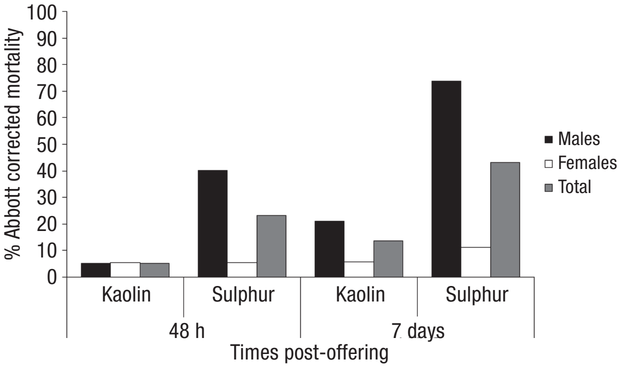

Figure 1. Abbott-corrected mortalities caused by treatments (sulphur and kaolin) in *Drosophila suzukii* adults 48 hours and 7 days after treatment.

z = -2.3; $p<0.05$). There was no significant difference in the mean number of adults emerged from the control and kaolin treatments (z = -1.2; $p>0.05$). Furthermore, powdered sulphur and kaolin coating treatments did not affect fruit firmness and no significant differences were found with respect to the control treatment ($F_{2,90} = 1.56$; $p>0.05$). The outcomes obtained in the no-choice assay also showed that the differences among treatments for emerged adults were related to the number of eggs laid (approximately 50% in all the three cases) but not to an increase of mortality of the eggs (Fig. 2a). Results from the choice assay confirmed and were consistent with those obtained in the no-choice assay (Fig. 2b). Significant differences in eggs per fruit were found among treatments (z = -3.8; $p<0.001$). Powdered sulphur treated fruits were almost not attacked by females, with 0.07 ± 0.07 eggs/5g fruit (only one egg laid over the total number of replicates). Control (2.9 ± 0.4 eggs/5g fruit) and kaolin (2.6 ± 0.6 eggs/5g fruit) treatments displayed significantly higher means than sulphur treatment (z = -3.7, and -3.6 respectively; $p<0.001$ in both cases). In addition, no significant difference was observed in oviposition between the control and kaolin treatments (z = -0.4; $p>0.05$). Emerged adults followed a similar pattern with significant differences among treatments (z = -3.8; $p<0.001$; Fig. 2b). Powdered sulphur treatment showed a significantly lower mean (0.07 ± 0.07 adults/5 g fruit) than the control (1.7 ± 0.4 adults/5 g fruit) and kaolin (2.0 ± 0.5 adults/5 g fruit) treatments (z = -3.1, and -3.3 respectively; $p < 0.01$ in both cases). There was no significant difference in the mean number of adults emerged from the control and kaolin treatments (z = 0.7; $p>0.05$).

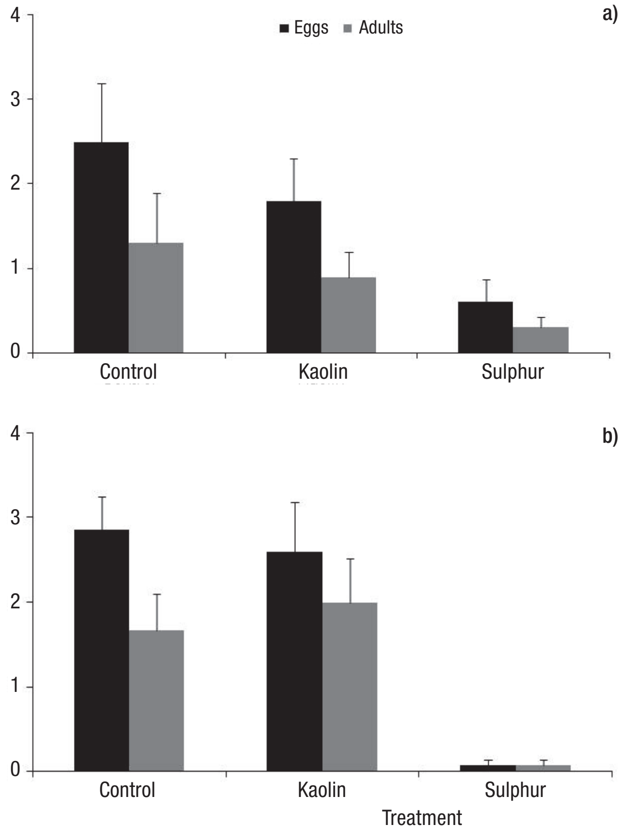

Figure 2. Mean number of laid eggs and emerged adults per fruit for treatments (sulphur and kaolin) and control groups in the no-choice assay after 48 hours of exposure (a). Mean number of laid eggs and emerged adults per 5 g of fruit for treatments and control groups in the choice assay after 24 hours of exposure (b). Vertical lines show standard errors.

Powdered sulphur is traditionally and widely used as a fungicide in several crops including berries (*e.g.* strawberry). Although the insecticide effects of this product are well known (Bloem *et al.,* 2005), to our knowledge no previous studies have tested its effects on SWD. The results obtained here show a significant 48 h and seven-day lethal effect of sulphur on adults after 48 h of exposure (Fig. 1) with a significant toxicity on males when this product was applied on the fruit as coating before infestation. Previously, Bruck *et al.,* (2011) have been also obtained higher mortality rates for males compared with females testing different insecticides. Although these authors have not reported further discussion about this result, tentative explanation may be advanced. Differences in body size (in general, females are larger than males) and activity rate (males actively move in search of females) could explain a higher susceptibility of males to toxic effects. It is important to stress that since the exposure of SWD to insecticides may vary greatly under field conditions and laboratory experiments do not simulate field conditions accurately, the results obtained here could be considered a worst-case situation (direct exposure in small experimental chambers during 48 h) but can be useful in order to firstly detect the toxicity of sulphur against SWD. Field observations suggest that the lower impact of SWD in strawberry crops in southern Spain may be due to the widespread use of powdered sulphur during the fruit season to prevent other pest and plant diseases (Molina, 2015). Powdered sulphur clearly reduced the number of egg laid, and consequently the number of emerged adults per fruit (Figs. 2a and 2b). In the no-choice assay after 48 h of exposure, reduction reached over 76% and 77% in both response variables with respect to the control treatment. Reduction was even greater in the choice assay, reaching (after 24 h) over 98%, and 96% with respect to the control treatment in eggs and adults per 5g of fruit, respectively. These results clearly indicate a repellent effect of powdered sulphur on SWD oviposition. Furthermore, the toxicity of powdered sulphur in males shown in the choice assay could account for the reduction in fruit infestation by affecting the population levels of males and consequently the oviposition capacity of females. In this sense, combination of lethal effects on males and repelent effects on oviposition could be complementary in preventive treatments against *D. suzukii.* Further research is required in order to elucidate the contribution of toxicity in the effects of sulphur coating.

Although some authors have reported mortality caused by some kaolin formulations (Leskey *et al.,* 2010), kaolin had no lethal effects on adults (Fig. 1). Under our experimental conditions, kaolin had no deterrent effect on SWD oviposition and there was no reduction in the number of laid eggs and emerged adults in choice and no choice assays (Figs. 2a and 2b). This product appears to be effective against other dipteran pests (Mazor & Erez, 2004; Leskey *et al.,* 2010; Pascual *et al.,* 2010), and several authors have reported that kaolin forms a barrier when applied on fruits, hindering visual and tactile recognition and thus preventing oviposition (Glenn *et al.,* 1999; Bürgel *et al.,* 2005). The results obtained here suggest that this is not the case with SWD and the coating formed by kaolin on blueberries did not affect the oviposition process and thus did not prevent egg laying. However, González-Núñez & Sánchez-Ramos (2015) reported deterrent effects on SWD oviposition when cherries were coated with kaolin. Although coating with kaolin was performed to ensure that the entire surface of each fruit was covered, influence of natural wax and other blueberry traits on the kaolin efficacy cannot be excluded. Obviously, further research with different host fruits and in field conditions is necessary in order to definitively rule out kaolin as an alternative for the management of *D. suzukii.*

The treatments tested in this study did not affect fruit firmness. Some authors have reported that SWD lay fewer eggs in firmer substrates and prefer ripe fruits rather than green or ripening fruits for oviposition (Lee *et al.,* 2011). However, Swoboda-Bhattarai & Burrack (2014) found no relationship between modification of firmness caused by the coating tested and the level of infestation. Therefore, since the products tested in this study did not change fruit firmness, we can surmise that the effects on oviposition caused by powdered sulphur were directly linked to its deterrent and toxic effects. It should be stressed that a full evaluation of coating tested in this study requires assessment of how affects the residue of the dust on pre-harvest and marketing of the treated fruits in order to define compatible strategies with management of the different crops attacked by *D. suzukii* (*e.g.* powered sulphur is currently used in strawberry as fungicide, therefore this product could be easily integrated as pesticide in this crop).

In summary, laboratory assays revealed that powdered sulphur applied on blueberries displayed significant mortality on adults reaching over 40% seven days after exposure, as well as deterrent effects with a reduction in oviposition and adult emergence reaching over 76% and 96% in no-choice and choice assays, respectively. In contrast, kaolin produced no significant mortality and reduction in infestation. The treatments had no effect on fruit firmness. Finally, although the information presented here is of particular value for assessing the preventive use of powdered sulphur for the management of SWD, different host fruits must be tested and further field research is needed to support the findings reported in this study. This could be an alternative tool to chemical control compatible with crop organic management and residuals reduction strategies.

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