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Laboratory trials to investigate potential repellent/oviposition deterrent effects of selected substances on *Drosophila suzukii* adults

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Abstract

Drosophila suzukii (Matsumura) (Diptera Drosophilidae), commonly named spotted wing drosophila, is a highly polyphagous invasive pest from Asia, causing large economic losses in soft and stone fruit production. To prevent *D. suzukii* from ovipositing into host fruits four essential oils (neem oil, patchouli oil, celery oil and catnip oil) as well as single components of two other essential oils (p-menthane-3,8-diol and 3-butylidenephthalide) were tested at two different concentrations for their repellent and/or oviposition deterrent effects on *D. suzukii* in laboratory trials on raspberries. A no-choice test assay in modified Hesler-plates employing seven 8-10 days old *D. suzukii* per treatment was carried out for each treatment. Flies were allowed to oviposit for 24 hours on 3 raspberries/test unit and were examined 72 hours later for the presence of larval instars. Only the treatments with neem oil at 1% and p-menthane-3,8-diol at 1% and 10% significantly reduced the mean number of *D. suzukii* larvae/fruit compared to the control.

Key words: no-choice test, spotted wing drosophila, essential oils, p-menthane-3,8-diol, 3-butylidenephthalide.

Introduction

Drosophila suzukii (Matsumura) (Diptera Drosophilidae), commonly named spotted wing drosophila, is an invasive species from Asia spreading worldwide since 2008 and causing tremendous economic losses in soft- and stone-fruit production (Mazzi *et al*., 2017). *D. suzukii* is a highly polyphagous pest able to survive at low temperatures during winter in the northern hemisphere (Cini *et al*., 2012). Unlike other Drosophilidae, *D. suzukii* prefers late ripening or ripe thin-skinned fruits as oviposition sites (Cini *et al*., 2012), which makes pest management with insecticides close to harvest difficult, due to the required minimum waiting period between the insecticide treatment and the harvest. Only few alternative methods and tools are available for the efficient control of *D. suzukii*. The most promising method currently is the use of physical barriers such as exclusion netting of whole plants and orchards or in combination with high plastic tunnels to protect the host fruits from *D. suzukii* (Walsh *et al*., 2011; Haye *et al*., 2016; Leach *et al*., 2016; Ioriatti *et al*., 2017; Boehnke *et al*., 2019, Ebbenga *et al*., 2019). Extensive sanitation measures (i.e. removal of dropped fruits from the ground) can be effective at low infestation rates and in cultures with narrow picking intervals (i.e. berries) (Walsh *et al*., 2011; Haye *et al*., 2016). However, at high infestation rates fruits become unmarketable and sanitation measures like picking are unviable. The use of antagonists such as entomopathogens (nematodes and fungi), predators and parasitoids are under investigation (Walsh *et al*., 2011; Haye *et al*., 2016; Ibouh *et al*., 2019). Several trials under laboratory and field conditions showed that the pupal parasitoids *Pachycrepoideus vindemiae* Rondani (Hymenoptera Pteromalidae) and *Trichopria drosophilae* Perkins (Hymenoptera Diapriidae) are currently the most promising natural enemies for *D. suzukii* in Europe (Chabert *et al*., 2012; Rossi-Stacconi *et al*., 2015; 2017; 2019; Daane *et al*., 2016; Mazzetto *et al*., 2016; Wang *et al*., 2018; Ibouh *et al*., 2019). However, biological agents can currently only facilitate the reduction of the *D. suzukii* population growth, but not prevent effectively damage to the fruits (Rossi-Stacconi *et al*., 2017; 2019; Gonzalez-Cabrera *et al*., 2019).

The application of repellent or oviposition deterring substances to prevent *D. suzukii* adults from egg-laying into the fruits with the objective to establish a push-pull system in combination with an attractant is another option to control the pest. Currently, there is a range of attractants for *D. suzukii* available (Landolt *et al*., 2012; Cha *et al*., 2012; 2015; Kleiber *et al*., 2014; Tait *et al*., 2018, Spies and Liburd, 2019). Several studies demonstrated the repellent potential activity of various alcohols, such as 1-octen-3-ol and geosmin, and esters of anthranillic acids (i.e. butyl anthranilate) against *D. suzukii* in laboratory (Krause Pham and Ray, 2015; Wallingford *et al*., 2015; 2016) and field trials (Wallingford *et al*., 2016; 2017; 2018). Additionally, several other substances and natural products were able to deter oviposition by *D. suzukii* like varieties of hop (*Humulus lupulus* L.) on grapes (Reher *et al*., 2019), ethanolic seed extracts of *Annona* species on strawberries (Bernardi *et al*., 2017) and powder coating formulations such as sulphur on blueberries (Pérez-Guerrero and Molina, 2016). In this context essential oils have been described to contain compounds with insecticidal, repellent and oviposition deterrent properties like monoterpenoids, sesquiterpenes and specific alcohols (Regnault-Roger *et al*., 2012; Sathantriphop *et al*., 2015; Wallingford *et al*., 2015; Lee, 2018). Laboratory trials with selected essential oils (citronella, eucalyptus, thyme, geranium, peppermint) and their single major components (thymol, citronellol, menthol, geraniol) against *D. suzukii* showed promising repellent potential (Renkema *et al*., 2016; 2017).

For the present study a systematic extensive literature search according to the European Food Safety Authority guidance document (EFSA, 2010) with the online database OVID (including AGRICOLA, CAB Abstracts, OVID Medline and Books@Ovid) as literature source was carried out. Based on the results of the systematic extensive literature search, test substances were selected for the subsequent laboratory trials if a repellent and/or oviposition deterrent effect on members of the order Diptera had been reported, if they were yet untested against *D. suzukii* and if they had potential for practical use. This search revealed that especially neem oil (*Azadirachta indica* Juss.), celery oil (*Apium graveolens* L.), catnip oil (*Nepeta cataria* L.) and patchouli oil (*Pogostemon cablin* [Blanco] Benth.) (commercial standard mixtures), as well as p-menthane-3,8-diol (single component of the essential of *Corymbia citriodora* (Hook.) K.D. Hill et L.A.S. Johnson) and 3-butylidenephthalide as one of the major active components of the essential oil of *Angelica sinensis* (Oliv.) Diels had shown deterrent effects on species of the order Diptera (Chen *et al*., 1996; Barasa *et al*., 2002; Schultz *et al*., 2004; Bernier *et al*., 2005; Tuetun *et al*., 2005; Trongtokit *et al*., 2005; Benelli *et al*., 2014; Zhu *et al*., 2012; Champakaew *et al*., 2015). The objective of this study was to evaluate these essential oils and single components of essential oils for their repellent and/or oviposition deterrent potential against *D. suzukii* in no-choice laboratory trials on raspberries (*Rubus idaeus* L. 'Autumn bliss').

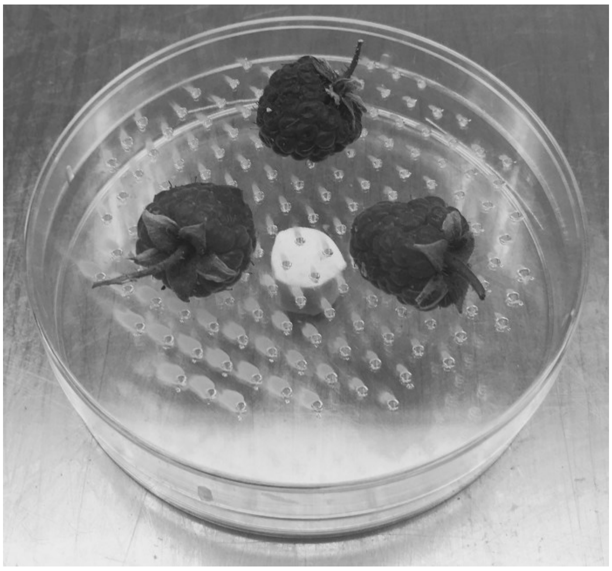

Figure 1. Test unit (Hesler-Plate) with test fruits (raspberries) for laboratory experiments.

Materials and methods

Test organisms

The laboratory trials were conducted with *D. suzukii* flies from an existing colony. For the rearing of the flies a modified *Drosophila* diet (Yoon, 1985) (6.70 g of mashed potato mix, 1.70 g of dried brewer's yeast, 0.17 g of methyl 4-hydroxybenzoate, 20 ml of canned pineapple juice and 20 ml of apple vinegar) was used. The diet was presented in polypropylene tubes (⌀ 50 mm; 100 mm height) and rearing was maintained in a climate-controlled chamber at 23 ± 1°C, 80 ± 5% RH and 16L:8D photoperiod. The light period started at midnight and ended at 4:00 pm. The diet served as nutrition and oviposition site and was changed every 3-4 days. After oviposition the polypropylene tubes (with diet and laid eggs) were closed with foam stoppers (⌀ 50 mm; 25 mm height), removed from the rearing box and kept under the same climate-controlled conditions in the climate-chamber for further development of the larvae.

Flies emerged in the polypropylene tubes 11-14 days after they were removed from the rearing boxes. For the experiments newly emerged flies were transferred to tubes with fresh media and were held there additional seven days for maturation (total age: 8-10 days) before they were used in bioassays.

Test fruits

Untreated raspberries (*Rubus idaeus*) were used as test fruits for the experiments. Ripe fruits (BBCH 89-897) (Schmid *et al*., 2001) were harvested from an organically managed raspberry orchard, which was free from *D. suzukii* infestation, as proved by checking a sample of 120 randomly collected raspberries for the presence of drosophilid eggs or larvae with the stereomicroscope. In total, approximately 4000 raspberries were picked randomly throughout the whole orchard. After harvesting the fruits were stored in plastic boxes, transferred immediately to laboratory, frozen at −34 °C and stored at this temperature until the trial start (4 months later).

Test units, chemicals and arenas

The selected test substances for the laboratory trials comprised celery oil, neem oil, catnip oil, patchouli oil, p-menthane-3,8-diol and 3-butylidenephthalide (table 1). All test odorants were diluted in hexane (v/v), except for celery oil (acetone), and applied in two concentrations (1%, 10%). Solvents were used as control. An amount of 500 µl was applied per treatment. For the no-choice test modified Hesler-Plates (Wallingford *et al*., 2017) (figure 1)

Table 1. Test substances and test design.

Test substances (sources)	Purity	Treatment (v/v)	n trial series	n replicates per trial series	Total n replicates
Celery oil (Sigma-Aldrich, Austria)	100%	Control, 1%, 10%	3	5	15
Catnip oil (Aromaland, Germany)	100%	Control, 1%, 10%	3	5	15
Patchouli oil (Sigma-Aldrich, Austria)	100%	Control, 1%, 10%	3	5	15
Neem oil (Naissance, UK)	100%	Control, 1%, 10%	3	5	15
p-menthane-3,8-diol (Chemos, Germany)	>92%	Control, 1%, 10%	3	5	15
3-butylidenephthalide (Sigma-Aldrich, Austria)	>96%	Control, 1%, 10%	3	5	15

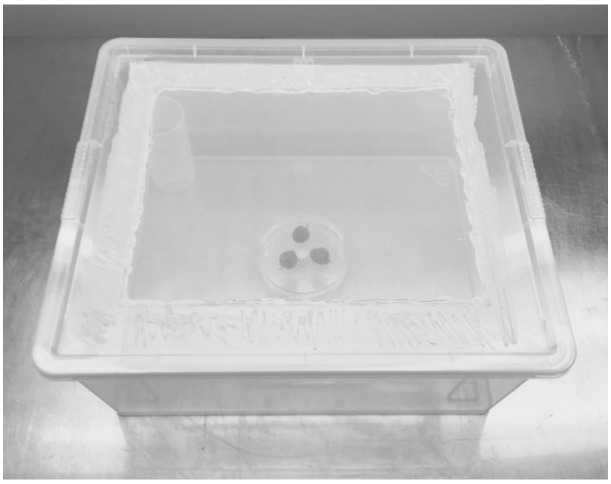

Figure 2. Test arena with test unit (Hesler-Plate) for laboratory experiments.

were used to release the test substance odours and to present the test fruits to the adults of *D. suzukii*. To provide a consistent emission of the volatiles of the test odorants, the lid of each Petri dish (Semadeni, Switzerland; polystyrene; ⌀ 90 mm; 25 mm height) was perforated with a laser (Dr. Bohrer Lasertec GmbH, Austria) resulting in 157 equally distributed holes (⌀ 0.8-1 mm). The Hesler-Plate was placed in the middle of the test arena (figure 2), which consisted of a polypropylene box (KIS C Box, Ormello, Italia; 40 × 34 × 17 cm) closable with a lid with a 30 × 22 cm rectangular window covered with curtain tissue (IKEA Curtain "Teresia", Austria; mesh size: 0.3 mm) for ventilation.

Laboratory trials

The laboratory trials were carried out for each test substance separately. Bioassays were conducted in climate controlled walk-in chambers at 23 ± 1 °C, 80 ± 5 RH, and 16L:8D photoperiod. Preparations for the no-choice test started on the test day by unfreezing 50 frozen raspberries in a glass Petri dish for 2 hours at room temperature. Fifteen polypropylene tubes with seven adult *D. suzukii* flies (5 ♀ + 2 ♂, age: 8-10 days) each were prepared per test unit. Main trials started between 3:00 pm and 4:00 pm at the end of the light phase when oviposition was peaking (Lin *et al*., 2014). In the middle of every Hesler-Plate a cotton dental wick was placed and 500 µl of each specific treatment was applied. After five minutes of evaporation the perforated lid of the Petri dish was closed and 3 raspberries were put on it in the centre in a triangle shaped form. Afterwards the Hesler-Plates were placed in the middle of the test arenas. One prepared polypropylene tube with seven adult *D. suzukii* was positioned in the corner of each test arena. The foam stopper was removed and the lid of the test arena was closed and sealed with sticky tape to avoid the flies from escaping. To avoid olfactory interference, every treatment was conducted in a separate climate-controlled chamber. After 24 hours flies were removed from the test arena with an exhauster. Three days later raspberries were transferred individually to polypropylene cups (⌀ 35 × 38 mm) and frozen at −16 °C for further analysis. For counting of the larvae, raspberries were unfrozen and dissected under the stereomicroscope. All larval instars were considered for analysis.

Statistical analysis

The mean numbers of *D. suzukii* larvae per test fruit in the different treatments and trial series were compared for significant differences with an ONEWAY ANOVA (Bonferroni-Test) (SPSS Statistics, version 22).

Results

From the six tested substances only neem oil 1% and p-menthane-3,8-diol (1% and 10%) exhibited a statistically significant repellent and/or oviposition deterrent effect on *D. suzukii* adults compared to their control (figure 3). Neem oil 1% was able to reduce the mean number of larvae per test fruit from 38.8 for the control group to 27.9 for the 1% treatment (table 2). However, at higher concentrations of neem oil (10%) the number of larvae per test fruits increased and the deterrent effect of neem oil was no longer observed. Similar to neem oil, the application of p-menthane-3,8-diol resulted in a significant reduction of the mean number of larvae/test fruit in the 1% treatment and a slighter reduction at 10%.

Celery oil did not show a deterrent effect on *D. suzukii* flies, neither in the 1% nor in the 10% treatment. No deterring properties were also determined for catnip oil for which the opposite effect was noticed. Both treatment concentrations of catnip oil resulted in higher mean numbers of *D. suzukii* larvae/fruit than the control treatment.

Table 2. Influence of treatments on the mean number of *D. suzukii* larvae/test fruit compared to the control and reduction/increase (in percentage) compared with the control.

Test substance	Mean number larvae/fruit			Reduction/increase mean number of *D. suzukii* larvae/fruit (%)	
	Control	1% treatment	10% treatment	1% treatment	10% treatment
Celery oil	31.6	26.6	27.5	−16.0	−13.1
3-butylidenephthalide	41.7	40.5	36.6	−3.1	−12.3
Catnip oil	39.4	40.3	42.0	+7.4	+6.7
Patchouli oil	40.4	37.4	39.3	−7.3	−2.8
p-menthane-3,8-diol	43.6	31.5	34.1	−27.8	−22.0
Neem oil	38.8	27.9	35.8	−28.1	−7.7

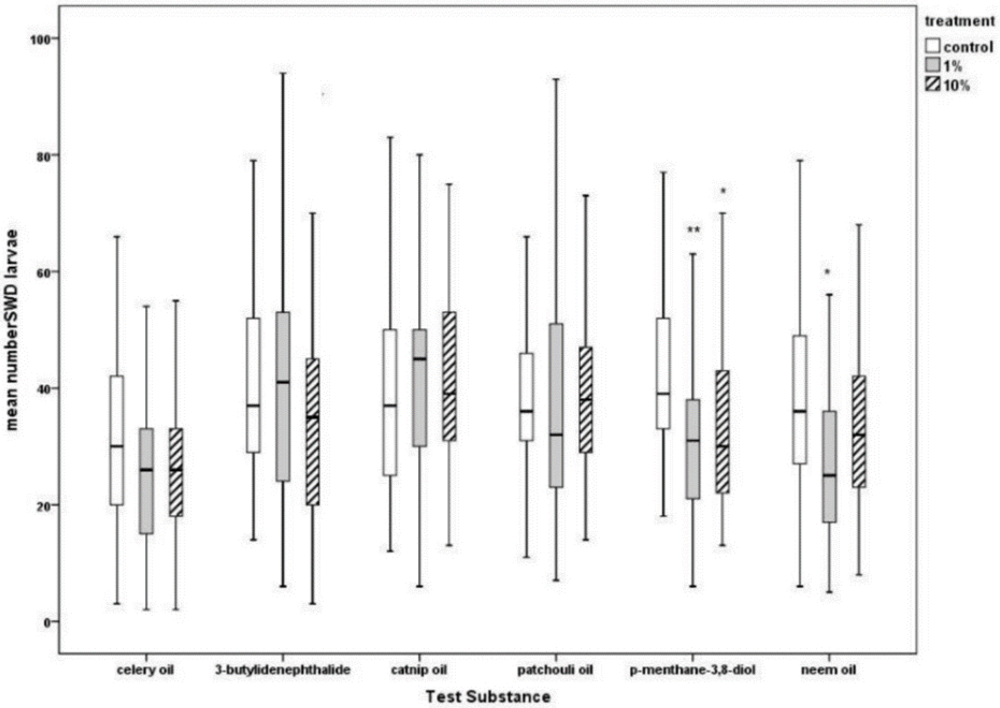

Figure 3. Mean number of *D. suzukii* larvae per fruit of test substances at applied concentrations (1%, 10%) compared to control treatment (acetone/hexane). Asterisks indicate significant differences (Bonferroni-Test): *(p < 0.05); **(p < 0.01).

Similar to catnip oil, patchouli oil and 3-butylidenephthalide also showed higher total average numbers of larvae per test fruit than celery oil but no oviposition deterrent effect on *D. suzukii* flies.

Discussion and conclusion

To evaluate oviposition deterrent effects of substances against Drosophilidae choice-test assays and/or no-choice test assays were used in laboratory and field trials (Krause-Pham and Ray, 2015; Wallingford *et al*., 2015; 2016; 2017; 2018; Pérez-Guerrero and Molina, 2016; Renkema *et al*., 2017; Bernardi *et al*., 2017). For the present study, it was decided to use a no-choice test design to avoid the risk of possible unintentional interactions of the test substances, like e.g. toxic fumigants effects, with the test flies. This effect was observed during pre-trials with a choice test assay with 1-octen-3-ol, a proven repellent of *D. suzukii* (Wallingford *et al*., 2015; 2016; 2017; 2018; Verschut *et al*., 2019), when disorientated test flies and a high mortality rate were noticed (Lethmayer *et al*., 2019). 1-octen-3-ol is a common volatile fungal semiochemical, which is linked to the Parkinson disease that can cause neurodegeneration and locomotor deficits in *Drosophila melanogaster* Meigen (Inamdar *et al*., 2013). To avoid such side-effects, control and test treatments were carried out in separated test arenas and climate chambers.

The attractiveness of the host fruit is also a factor for the repellent/deterrent potential of a chemical substance. Attractive host fruit species will trigger a more intense appealing response on the test flies than fruit species that are less suitable for oviposition (Lee *et al*., 2011; Bellamy *et al*., 2013). Hence, the repellent/deterrent effect of a substance can differ with the level of attractiveness of the host fruits. Most studies on repellent and oviposition deterrent effects on *D. suzukii* were conducted with blueberries (Erland *et al*., 2015; Krause-Pham and Ray, 2015; Pérez-Guerrero and Molina, 2016; Renkema *et al*., 2017; Sampson *et al*., 2017; 2019), although other fruit species like raspberries or strawberries are better hosts for oviposition and development (Lee *et al*., 2011; Bellamy *et al*., 2013). The goal of the present study was to find effective repellent/deterrent substances that are able to protect highly attractive host fruit species. Therefore, it was decided to use raspberries as test fruits.

The goal of the present work was to identify essential oils and components of essential oils with a repellent and/or oviposition deterrent effect on *D. suzukii*. Celery oil, catnip oil, patchouli oil and 3-butylidenephthalide did not demonstrate a deterrent effect at the different test concentrations in the no-choice test assays. Previously reported repellent and/or oviposition deterrent effects of celery oil, catnip oil and patchouli oil against other members of the order Diptera like mosquitoes (Culicidae), house flies (Muscidae) and stable flies (Muscidae) (Schultz *et al*., 2004; Bernier *et al*., 2005; Tuetun *et al*., 2005; Trongtokit *et al*., 2005; Kumar *et al*., 2014; Zhu *et al*., 2012; 2015) could not be observed in the present trials with *D. suzukii*. Only neem oil (1%) and p-menthane-3,8-diol (1%, 10%) showed a significant reduction of larvae per test fruit.

For gravid females finding the optimum substrate is crucial for the survival of their offspring. To achieve this goal, they rely on chemical cues that guide them to an adequate oviposition site. The mechanisms that lead to a repellent or oviposition deterrent response are still poorly understood (Chin *et al*., 2018). If a chemical substance causes a repulsive effect can depend on numerous factors like the composition, concentration, ratio of components, residence time or quantity of an odour and its volatile organic compounds (Verschut *et al*., 2019). For example, the concentration of a volatile compound can influence the behaviour of flies in different ways. Thus, odours can be attractive at lower concentrations and deterrent at higher ones - and reverse. In bioassays with *D. melanogaster* ethyl hexanoate and butyl butyrate attracted at lower concentrations and repelled at higher concentrations (Stensmyr *et al*., 2003). Experiments in a caged greenhouse demonstrated that traps filled with a higher amount of ethyl acetate (55 and 550 µl) caught fewer *D. suzukii* flies than traps with less (5.5 µl) (Kleiber *et al*., 2014). It is assumed that the varying test results of neem oil at different concentrations are based on the same effect of varying behaviour at different concentrations of volatiles.

The composition of a mixture and the ratio of the components can also be crucial for their repulsive potential. Essential oils can contain up to a hundred different phytochemicals with varying ratios and concentrations (Regnault-Roger *et al*., 2012; Turek and Stintzing, 2013). The composition of the essential oil depends on many parameters like the plant species itself, its nutrition, climatic conditions, irrigation, plant management, life stage or harvest time of the plant (Regnault-Roger *et al*., 2012). Therefore, the repellent and deterring potential of essential oils can differ.

The test duration of 24 hours for the laboratory trials was based on a literature review of similar experiments with choice- and no-choice-tests on *D. suzukii* and *D. melanogaster* (Pérez-Guerrero *et al*., 2016; Krause-Pham *et al*., 2015; Bernardi *et al*., 2017; Renkema *et al*., 2017; Reher *et al*., 2019). However, although there was an oviposition deterrent effect of p-menthane-3,8-diol (1%, 10%) and neem oil (1%) on *D. suzukii*, the number of larvae per fruit was still high (table 2). A reason for that could be that the volatile organic compounds of the test substances completely evaporated before the end of the trial. In this case, females would only have delayed their oviposition, which would lead to a decreased egg-laying rate (Renkema *et al*., 2016). Therefore, for future trials additional shorter test durations are proposed to be able to measure the development of repellent/deterrent effects of the tested substances over the experimental period.

Due to the fact that only a few *D. suzukii* larvae are necessary to make a crop unmarketable, oviposition deterrent substances have to be highly efficient (Dam *et al*., 2019). Although neem oil (1%) and p-menthane-3,8-diol (1%, 10%) were able to reduce the mean number of *D. suzukii* larvae per test fruit, the deterring effect was not considered effective enough to continue with trials in the field. Additionally, for future trials it is proposed to work with individual components of essential oils instead with the whole mixture to determine the individual deterring potential of the components.

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