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Evaluation of *Pimpinella anisum* essential oils on egg-laying behaviour of *Drosophila suzukii*: A Multiple-choice test approach

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Abstract

Drosophila suzukii, spotted wing drosophila, is recognized as an economically significant new invasive pest of stone and, especially, berry fruits. This pest has been registered on the EPPO-A2 quarantine list. Due to the limited presence of natural predators on the European continent, this pest quickly proliferates, posing a significant threat to the agricultural economy by causing substantial losses in fruit production. As *D. suzukii* infestations commonly occur during the ripening stage, the main obstacle with chemical control methods is the risk of high residue in fruits. To face this issue, it is crucial to explore alternative control strategies. One of the possible effective methods involves the use of plant-derived bioactive compounds. Currently, there is ongoing research to identify essential oils with potential insecticidal properties for combating this challenge. The objective of this study was to assess the insecticidal and behavioural impacts of anise (*Pimpinella anisum* L.) essential oils on *D. suzukii* adults in a multiple-choice test using blueberries treated with three concentrations of the anise essential oil. Additionally, the study monitored the emergence of adults from the treated berries. Using GC-MC chromatography, the chemical composition of anise essential oil was determined, in order to understand the possible mode of action. The essential oil of anise showed high efficacy in the multiple choice test, since a lower number of laid eggs was recorded at all three application concentrations (1%, 5% and 10%) compared to the control (water and acetone). Our findings suggest that anise essential oil can serve as an alternative to chemical insecticides, which would enable the use of bioinsecticides based on this oil, in conventional, integrated, and organic crop production.

Key words*: Drosophila suzukii*, essential oil, oviposition, bioinsecticide, eco-friendly.

Introduction

Drosophila suzukii Matsumura, or spotted wing drosophila, (Diptera: Drosophilidae) is an economically significant pest of soft-skinned stone and berry fruit. The vast majority of members of the *Drosophila* genus are not considered significant agricultural pests because they lay their eggs in overripe, decaying, and damaged fruits, unlike *Drosophila suzukii* which lays eggs in intact and ripe fruits (Cini et al., 2012; Asplen et al., 2015). Spotted wing drosophila is native to Asia but in the past decades, it has invaded America and Europe and become widely distributed in temperate climate regions which gave it the status of a cosmopolitan species over time (Rota-Stabelli et al., 2013). The dispersal of this pest may occur through either passive or active means. The passive diffusion through the conveyance of infested fruits is likely the main reason for its rapid spread (Cini et al., 2012). In the region, it was registered for the first time in Croatia (Milek et al., 2011), followed by Bosnia and Herzegovina (Ostojić et al., 2014) and Serbia (Toševski et al., 2014). This pest has a high reproductive rate and short generation time with numerous generations per year, which serves as a favourable base for their fast invasion and high levels of damage to crops (Kanzawa, 1939; Kirschbaum et al., 2020; Walsh et al., 2011).

Males have dark spots on the wings, while females possess a large serrated ovipositor that enables them to cut through the skin of fruit in order to lay eggs. Larval feeding in the fruit causes tissue collapse, rendering it unmarketable even if it is not visibly damaged, while oviposition scars made by females expose fruits to secondary pathogens infestation (Kanzawa, 1939; Walsh et al., 2011). These pests cause the greatest damage to raspberries, blackberries, and cherries, especially to their late varieties. The control of spotted-wing drosophila mostly involves insecticide use, leading to resistance development and posing risks to the environment and beneficial organisms. Current control methods pose a significant risk of residues in fruits, because of many chemical treatments conducted during the ripening stage. The limited efficacy of chemical insecticides against *D. suzukii* larvae inside the fruit, requires treatments targeted at adults (Cini et al., 2012).

Considering all the stated problems, biological control of *D. suzukii* is essential due to the significant economic impact this pest has on fruit crops. Implementing biological control strategies, such as the introduction of natural enemies, the use of microbial agents, or applying bioinsecticides based on essential oils offers a sustainable and environmentally friendly approach to managing *D. suzukii* populations and, therefore, reducing crop damage (Bošković et al., 2023). This way of control can help mitigate losses and preserve fruit quality for growers in conventional and organic agriculture farming systems. Botanical insecticides are beneficial for use in crop protection considering their natural origin, reduced environmental impact, and leaving low or no residues in food compared to synthetic pesticides (Isman, 2004; Vuković & Šunjka, 2021). These types of bioinsecticides are derived from plants and often contain bioactive compounds that target specific pests while posing minimal risk to non-target organisms like beneficial insects and pollinators. Additionally, botanical insecticides typically have shorter residual effects in the environment, reducing the risk of persistence and their potential harm to ecosystems (Bale et al., 2007; Šunjka, & Mechora, 2022). Their mode of action can also help in managing pest resistance, as pests may be less likely to develop resistance to botanical compounds compared to synthetic chemicals due to the complex mode of action of these natural products, which often target multiple physiological pathways in the pest (Isman, 2000; Campos et al., 2019). Overall, the use of botanical insecticides is a sustainable and eco-friendly way of crop protection, ensuring long-term agricultural sustainability.

This study aimed to evaluate the insecticidal and behavioural effects of anise essential oils on *D. suzukii* adults in a multiple-choice test with blueberries treated with different concentrations of essential oil. The emergence of adults from treated berries was also monitored.

Material and Methods

Rearing of *D. suzukii* colony

The adults of *D. suzukii* used in these bioassays were obtained from a laboratory colony maintained in the Insectarium facility of CIHEAM Bari, Italy, where the experimental part was also conducted. The insects were maintained in Plexiglas cages (30 × 30 × 30 cm) in a climatic chamber (temperature 22–24°C, with relative humidity (RH) 65±5%, and photoperiod of 12L:12D (light:dark)) and were constantly provided with a water wick and artificial diet (Dalton et al., 2011). Male and female adults used in this bioassay were between 4 and 7 days old. Organic, healthy, and undamaged blueberry fruits were used for the bioassay.

Essential oil solution

The essential oil tested in the multiple-choice test was anise oil (*Pimpinella anisum* L.), obtained from Nature in Bottle, New York, USA (Avena Lab – Farmadria ©). It was dissolved in pure acetone at three concentrations (1%, 5%, and 10%). Water (Control 1) and acetone (Control 2) were used as control treatments.

Multiple-choice bioassay

The experiment involved conducting a multiple-choice test in a plastic arena with ventilation holes to prevent gas saturation. Organic blueberries were prepared by submerging them for two seconds in three essential oil solutions or control treatments prior to setting the experiment. After drying, the berries were separately placed in Petri dishes in the arena. The dishes with wet wipes and diet medium were also placed in the arena as a water and food source. Flies were introduced into the arena (5 males and 5 females). The rest of the treated blueberries, which were to be used in the bioassay for the following three days, were put in a covered plastic container and kept under the same conditions as the arenas. Treated berries, from the container, were placed in arenas for four days in total, in the same way as described for the first day. The aim of the bioassay was to treat all blueberries only on the first day to assess the anise essential oil effect after 24, 48, 72, and 96 h, to see whether the deterrent effect on *D. suzukii* females diminishes after the time of treatment passes. The bioassay was kept in a climate chamber room under control conditions (22°C ± 2°C, 65±5% mRH, 12h:12h light: dark cycle). After 24 hours, egg counting was performed and recorded on the berries using a stereomicroscope. The berries were then transferred to individual cups for adult emergence observation (Bošković et al., 2023).

GC–MS Analysis of the anise EO composition

For the chemical characterization of anise essential oils, gas chromatography with mass spectrometry (GC–MS Shimadzu QP2010) was used with the following conditions: a fused silica HP-5 column; carrier gas He (1.0 mL/min); temperature programmed from 55°C to 240°C with a temperature increase of 3°C/min; injection port temperature 250°C; and detector temperature 280°C. The constituents were identified by comparing their retention indices and MS spectra with those in the databases available in the licensed MassFinder 4 software (EssentialOil 4a and Adams2205 databases).

Statistical Analysis

To perform the statistical analysis of data, statistical software "Statistica" version 14.0.0.15 (Tibco Software Inc., Palo Alto, CA, USA, 2021) was used for the analysis of variance (one-way ANOVA). The Duncan’s multiple range test was used for comparing the difference between treatments. The chi-square test was used to determine the statistical significance between male and female emergence.

Results and Discussion

The results of the multiple-choice bioassay with anise essential oil are shown in Table 1. At a concentration of 1%, after the first day, the average number of eggs laid was 0.5. On the second and third day it was slightly lower (0.75). After the fourth day, the highest average number of eggs was recorded (2.5 ± 0.58). For a concentration of 5%, the mean value of 0.25 was recorded after the first and second day, 0.5 after the third, and the highest average number of eggs was recorded at the end of the fourth day and it was 1.5. In a concentration of 10%, after the first and second day no egg laying was recorded. After the third day, an average number of eggs of 0.25 was registered, and after the fourth day, 0.5. A statistically significant difference, regarding egg-laying activity, has been observed between both controls and all three treatments after the first, second, third, and fourth day for all three applied concentrations ($p<0.05$).

In all three applied concentrations of anise essential oil, there was no emergence of the adults after the first and second day. Their emergence was recorded after the third day (0.25) for all three concentrations. After the fourth day, there was a slight increase in the number of emerging adults. At a concentration of 1%, the average number was 1.5, while at a concentration of 5% that number was 0.5. In a concentration of 10%, the average number was the same as on the third day, 0.25. A statistically significant difference, using the Duncan's multiple range test, in the number of emerged adults, was recorded between both controls and all three treatments after the first, second, third, and fourth day for all three concentrations ($p<0.05$). The difference in the sex of the emerged adults in the treatment with anise essential oil was not statistically significant. The emergence rate of eggs laid on the treated berries with the essential oil was lower compared to the control treatments.

Tab 1. Multiple-choice bioassay results

Mean number of laid eggs - **Mean ± SD**								
Treatment	Day 1		Day 2		Day 3		Day 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1%	0.5	0.58	0.75	0.96	0.75	0.96	2.5	0.58
5%	0.25	0.5	0.25	0.5	0.5	1	1.5	1.29
10%	0	0	0	0	0.25	0.5	0.5	0.58
Control 1	6.5	2.89	9.25	2.22	7.5	1.91	7.5	2.89
Control 2	5.25	2.22	10	4.69	8.75	2.63	6.25	2.99
F-test	14.05		18.54		27.71		9.56	
p value	0.00		0.00		0.00		0.00	
Mean number of emerged adults - **Mean ± SD**								
Treatment	Day 1		Day 2		Day 3		Day 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1%	0	0	0	0	0.25	0.5	1.5	0.58
5%	0	0	0	0	0.25	0.5	0.5	0.58
10%	0	0	0	0	0.25	0.5	0.25	0.5
Control 1	5.0	2.16	8.0	1.83	6.25	1.5	6.0	2.94
Control 2	4.25	2.22	9.25	4.35	7.25	1.71	5.0	2.94
F-test	13.53		20.23		49.05		7.79	
p value	0.00		0.00		0.00		0.00	
Sum of emerged female and males **(SUM)**								
Treatment	Day 1		Day 2		Day 3		Day 4	
	F	M	F	M	F	M	F	M
1%	0	0	0	0	1	0	3	3
5%	0	0	0	0	0	1	2	0
10%	0	0	0	0	1	0	0	1
Control 1	10	10	15	17	11	14	9	15
Control 2	10	7	19	18	12	17	11	9
χ^2	182.85		164.29		85.55		148.18	
df	92		92		64		120	
p value	0.00		0.00		0.37		0.41	
Emergence Rate %								
Treatment	Day 1		Day 2		Day 3		Day 4	
1%	0		0		33.33		60.0	
5%	0		0		50.0		33.33	
10%	0		0		100		50.0	
Control 1	76.92		86.49		83.33		80.0	
Control	80.95		92.5		82.86		80.0	

Control 1 – water; Control 2 – acetone; SD – standard deviation; $\chi 2$ – chi-square test; df – degrees freedom; F – female; M – male.

In the composition of anise essential oil, the most dominant compound was trans-anethole with an average content of 93.43%. Methyl chavicol is the second most abundant compound with a percentage of 3.46%. Other compounds are found in a percentage of less than 2%.

Tab 2. Chemical composition of anise essential oil

Compound	**RI$_{exp}$**	**RI^{a,b}**	**Relative content (%)**	**IM^{c}**
			Anise	
linalool	1098	1095	0.87	RI^{a}, MS
methyl chavicol	1198	1195	3.46	RI^{a}, MS
cis- anethole	1251	1249	0.13	RI^{a}, MS
trans- anethole	1285	1285	93.43	RI^{a}, MS
anisyl methyl ketone	1384	1380	Tr	RI^{a}, MS
trans- caryophyllene	1415	1417	0.31	RI^{a}, MS
trans-*α*-bergamotene	1432	1432	0.37	RI^{a}, MS
foeniculin	1676	1676	1.42	RI^{a}, MS

t-trace (<0.1); RI a,b—retention index from the literature; a—Adams 2205 database; b—EssentialOil 4a database; RI—retention indices calculated from retention times in relation to those of a series of n-alkanes C8-C40 on a 30 m DB-5 capillary column; MS—mass spectra; IM c—identification method was MS based on a comparison with the Adams 2205 and EssentialOil 4a databases.

The mosquitoes *Anopheles stephensi* (Liston*), Culex pipiens* (L.), *Aedes aegypti* (L.), and the housefly *Musca domestica* (L.) are the *Diptera* species commonly studied in biological tests involving anise essential oil (*Pimpinella anisum*) (Prajapati et al., 2005; Chantawee & Soonwera, 2018; Kimbaris et al., 2012; Laojun et al., 2020). *Illicium verum* (Hook.f.) i *P. anisum* are distinct plants with varying characteristics, yet they share a remarkably similar taste, aroma, and chemical composition. Star anise, also known as *I. verum*, is a woody plant indigenous to China and Vietnam. In contrast, *P. anisum* is an herbaceous annual plant that originates from the Eastern Mediterranean region and Southwest Asia (Tepe & Tepe, 2015). The essential oil of *I. verum* was assessed in a contact test on adults of *D. suzukii*, where it showed an insecticidal effect even at lower applied concentrations (LC50 - 1.9 µL mL−1) (de Souza et al., 2022). This study assessed the repellent effect of anise (*P. anisum*) essential oil in a multiple-choice test, revealing that even berries treated with the lowest concentration (1%) showed a low number of laid eggs. Trans-anethole is the predominant compound found in anise essential oil, with varying percentages (Shojaii & Abdollahi, 2012) that align with the findings of this study. This compound is believed to be responsible for the deterrent effect observed, as its deterrent properties on certain storage pests have been previously confirmed (Alkan & Ertürk, 2020). The mode of action of botanical insecticides based on essential oils is complex and unique to each compound, typically involving a synergistic effect of multiple compounds simultaneously. The individual components of essential oils play a crucial role in influencing their impact on insects. Considering their chemical composition and insecticidal effect, both anise essential oils could be a prominent candidate for the use as biological control against *D. suzukii*.

Conclusion

Understanding the mode of action of essential oils remains a challenge in practical implementation in the field. Further research is crucial to explore essential oil formulation processes, to ensure the insecticidal efficacy, stability of their active components in the field and economical price. Our study showed that the anise (*P. anisum*) essential oil has a different effect on *D. suzukii* female adults in a multiple choice test for the assessment of egg-laying behaviour, even at the lowest concentration applied (1%), since recorded laying activity was very low. There is no available data on the deterrent effect of anise essential oil on spotted wing drosophila, which makes this study the first one for laboratory assessment of anise on *D. suzukii* oviposition. Our findings suggest that anise essential oil can serve as an alternative to chemical insecticides. However, additional studies on the safety of botanical insecticides for non-target organisms are necessary for the practical use of plant essential oils, especially in organic agriculture.

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Испитивање утицаја есенцијалних уља *Pimpinella anisum* на понашање *Drosophila suzukii* при полагању јаја: Приступ тестом вишеструког избора

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Сажетак

Drosophila suzukii, азијска воћна мушица, значајна је штеточина коштичавог, а нарочито бобичастог воћа. Ова штеточина се налази на карантинској EPPO-A2 листи. Има веома мало природних непријатеља на европском континенту што јој је омогућило брзо ширење, чиме је добила статус значајне штеточине у пољопривредној производњи јер доводи до великих економских губитака у производњи воћа са меканом покожицом плода. Азијска воћна мушица је најштетнија када су плодови у фази зрења. Примена хемијских метода сузбијања у том периоду носи са собом висок ризик од појаве остатака пестицида у плодовима. Како би се превазишао овај проблем, потребно је пронаћи алтернативне мере сузбијања. Једна од могућих алтернативних стратегија, укључује употребу биоактивних једињења биљног порекла. Све више се раде истраживања у оквиру испитивање етарских уља са потенцијалним инсектицидним својствима. Циљ овог истраживања је испитивање утицаја етарског уља аниса (*Pimpinella anisum* L.) на понашање и овипозицију имага *D. suzukii*, примењеног у три различите концентрације у multiple-choice огледу. Такође, праћена је и еклозија одраслих јединки из третираних бобица. Применом GC-MC хроматографије одређен је хемијски састав етерског уља, како би се лакше могао разумети потенцијални механизам деловања. Етарско уље аниса испољило је високу ефикасност у примењеном тесту са вишеструким избором јер је при све три примењене концентрације (1%, 5% и 10%) забележен мањи број положених јаја у односу на контролу (вода и ацетон). Резултати овог истраживања представљају основу за даље истраживање и формулисање потенцијалног биоинсектицида, као алтернатива хемијским инсектицидима, који би био погодан за коришћење у у конвенционалној, интегалној и органској биљној производњи.

Кључне ријечи: Drosophila suzukii, етерична уља, овипозиција, биоинсектицид, еколошки прихватљиво

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