Universität für Bodenkultur Wien
University of Natural Resources
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Doctoral Dissertation

A yeast-based attract-and-kill formulation to control *Drosophila suzukii*

submitted by

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in partial fulfilment of the requirements for the academic degree

Doktor der Bodenkultur (Dr.nat.techn.)

Vienna, January 2022

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Affidative

I hereby declare that I have authored this dissertation independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included. Any contribution from colleagues is stated.

I further declare that this dissertation has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 14 January 2022 Urban SPITALER *(manu propria)*

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Acknowledgement

I would like to thank my supervisors Prof. Elisabeth H. Koschier and Silvia Schmidt for offering me the opportunity to work on this thesis, their helpful support during the project and for their valuable advice during my study. I would also like to thank Prof. Thomas Frank for the support I received during the last years.

I would like to thank the whole project team for the great collaboration. Many thanks go to my colleagues Carlo S. Cossu and Lorenz Delle Donne for keeping up a great working atmosphere and for their assistant and engagement during the entomological trials, which were performed at the Institute for Plant Health, Laimburg Research Centre, Italy. I would also like to thank Flavia Bianchi, Daniela Eisenstecken, and Peter Robatscher from the Laboratory for Flavours and Metabolites, Institute for Agricultural Chemistry and Food Quality, Laimburg Research Centre, Italy, for the chemical analyses of the yeast metabolites. My thanks also go to Irene Castellan, Claire Duménil, and Prof. Sergio Angeli from the Faculty of Science and Technology, Free University of Bozen-Bolzano, Italy for collecting and analyzing the volatile organic compounds. Furthermore, I would like to thank Guillermo Rehermann and Prof. Paul G. Becher from the Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Sweden, for performing the wind tunnel assays and behavioral trials in Sweden.

I am very grateful to the whole working group at the Laimburg Research Centre, Institute for Plant Health, for the technical support and great collaboration.

Finally, my biggest thanks go to my family, especially my partner Melanie and my son Paul, without whom I would not have been able to manage the whole study.

This work was funded by the European Regional Development Fund (ERDF) 2014–2020, project DROMYTAL (FESR1021, CUP H32F16000420009).

Table of contents

1 Preface

The insect pest *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), also known as the spotted wing drosophila (SWD), is a worldwide pest of several soft- and thin-skinned fruits (Walsh et al. 2011). Broad-spectrum insecticide application close to harvest is common for controlling *D. suzukii*. Therefore, development of alternative control strategies could contribute to a more sustainable pest management and fits the aim of integrated pest management. While for some insects, behavioral manipulation by use of attractive compounds is already part of control strategies, manipulation strategies should be further developed for *D. suzukii*. For this purpose, the present thesis investigates the role of yeasts in the biology, behavior, and nutrition of *D. suzukii*, for possible implementation in pest control. After detailed examination in the laboratory and greenhouse, an attract-and-kill formulation based on the yeast *Hanseniaspora uvarum* (Niehaus) and the insecticide spinosad was tested in the field to control *D. suzukii* in grapes.

The thesis consists of an introduction to the biology and the control of *D. suzukii*, as well as an overview of the results of four peer-reviewed publications. The first publication (Spitaler et al. 2020) addresses the impact of different yeast species and their chemical compounds on the fecundity, ingestion, and lifespan of *D. suzukii* adults. The second publication (Bianchi et al. 2020b) investigates the persistence of metabolites and volatile organic compounds that are present in the attract-and-kill formulation on treated grapevine leaves. The third publication (Rehermann et al. 2021) contains information about the attractivity of the attract-and-kill formulation when combined with various insecticides. The fourth publication (Spitaler et al. 2021) explores the efficacy of the attract-and-kill formulation in the greenhouse and in the vineyard.

In addition, two further works are listed as additional publications related to thesis. Those publications are not part of the thesis but were published within the scope of the present work. The additional publications contain a comparative analysis of the lipidome of five yeast species associated to *D. suzukii* (Bianchi et al. 2020a) and an overview of the implementation of insect chemical ecology in pest control (Mbaluto et al. 2020). Results of this thesis were also presented at the XI European Congress of Entomology in 2018 and at the Joint Meeting of the IOBC/WPRS Working Groups (PheroFIP 19) in 2019.

2 List of publications

2.1 Publications that compromise the main part of this cumulative dissertation

Spitaler U, Bianchi F, Eisenstecken D, Castellan I, Angeli S, Dordevic N, Robatscher P, Vogel RF, Koschier EH, Schmidt S (2020) Yeast species affects feeding and fitness of *Drosophila suzukii* adults. Journal of Pest Science 93:1295–1309. https://doi.org/10.1007/s10340-020-01266-y (open access).

Bianchi F, **Spitaler U**, Castellan I, Cossu CS, Brigadoi T, Duménil C, Angeli S, Robatscher P, Vogel RF, Schmidt S, Eisenstecken D (2020) Persistence of a yeast-based (*Hanseniaspora uvarum*) attract-and-kill formulation against *Drosophila suzukii* on grape leaves. Insects 11:810. https://doi.org/10.3390/insects11110810 (open access).

Rehermann G, **Spitaler U**, Sahle K, Cossu CS, Delle Donne L, Bianchi F, Eisenstecken D, Angeli S, Schmidt S, Becher PG (2021) Behavioral manipulation of *Drosophila suzukii* for pest control: high attraction to yeast enhances insecticide efficacy when applied on leaves. Pest Management Science. https://doi.org/10.1002/ps.6699 (open access).

Spitaler U, Cossu CS, Delle Donne L, Bianchi F, Rehermann G, Eisenstecken D, Castellan I, Duménil C, Angeli S, Robatscher P, Becher PG, Koschier EH, Schmidt S (2021) Field and greenhouse application of an attract-and-kill formulation based on the yeast *Hanseniaspora uvarum* and the insecticide spinosad to control *Drosophila suzukii* in grapes. Pest Management Science. https://doi.org/10.1002/ps.6748 (open access).

2.2 Additional publications related to the thesis

Bianchi F, **Spitaler U**, Robatscher P, Vogel RF, Schmidt S, Eisenstecken D (2020) Comparative lipidomics of different yeast species associated to *Drosophila suzukii*. Metabolites 10:352. https://doi.org/10.3390/metabo10090352 (open access).

Mbaluto CM, Ayelo PM, Duffy AG, Erdei AL, Tallon AK, Xia S, Caballero-Vidal G, **Spitaler U**, Szelényi MO, Duarte GA, Walker WB, Becher PG (2020) Insect chemical ecology: chemically mediated interactions and novel applications in agriculture. Arthropod-Plant Interactions:1–14. https://doi.org/10.1007/s11829-020-09791-4 (open access).

2.3 Congress participations

Spitaler U, Koschier E, Schmidt S (2018) Influence of dietary yeasts on the fecundity of adult spotted-wing drosophila (*Drosophila suzukii*) (Poster). XI European Congress of Entomology, Napoli (Italy). 2-6 July 2018.

Castellan I, **Spitaler U**, Schmidt S, Angeli S (2018) Identification of volatiles released by fruit-associated yeasts for the biocontrol of *Drosophila suzukii* (Poster). XI European Congress of Entomology, Napoli (Italy). 2-6 July 2018.

Spitaler U, Bianchi F, Castellan I, Rehermann G, Eisenstecken D, Becher PG, Angeli S, Schmidt S (2019) An innovative management approach for spotted wing drosophila (*Drosophila suzukii*) using an environmentally friendly attract-and-kill formulation. Joint Meeting of the IOBC/WPRS Working Groups (PheroFIP 19), Lisbon (Portugal). 20-25 January 2019.

2.4 Co-supervision of student thesis

Delle Donne, L (2020) Wirksamkeit und Persistenz von Hefe-Insektizid-Gemischen für die Formulierung eines Attract-and-Kill-Verfahrens gegen *Drosophila suzukii*. Master's thesis. University of Natural Resources and Life Sciences, Vienna.

3 Abstract (English)

Drosophila suzukii (Matsumura) is an invasive pest of berries, stone fruits, and grapes. The present thesis explores the effect of selected yeast species in the diet and on the behavior of *D. suzukii* adults. Knowledge of the yeast-*D. suzukii* interaction can help to improve control strategies against the insect pest by promoting the ingestion of attract-and-kill formulations. The tested yeast species influenced ingestion, fecundity, and mortality of *D. suzukii* adults. Among the tested yeasts, *Hanseniaspora uvarum* (Niehaus) was preferably ingested and increased the fecundity of *D. suzukii* females. Therefore, an attract-and-kill formulation based on *H. uvarum* and a suitable insecticide was applied to the foliage of grape plants in greenhouse and in vineyard trials to control *D. suzukii*. Furthermore, treated leaves from the vineyard were transferred to the laboratory to test the persistence of their effect on laboratory-reared *D. suzukii* flies. The exposure of *D. suzukii* flies to leaves treated with *H. uvarum* and insecticide in the field or greenhouse and transferred to a laboratory assay caused high mortality of *D. suzukii* adults and reduced the number of eggs laid on fruits. Application of the attract-and-kill formulation to the fruit-free canopy reduced *D. suzukii* infestation, comparable to applying insecticide to the whole plant. In comparison to conventional insecticide applications, the use of an attract-and-kill formulation prevented insecticide residues on the grapes by targeting the treatment to the canopy and decreasing the applied amount of insecticide per area without compromising control efficacy.

4 Kurzfassung (Deutsch)

Drosophila suzukii (Matsumura) ist ein invasiver Schädling, der vor allem Beeren, Steinobst und Trauben befällt. Die Auswirkungen von bestimmten Hefen in der Ernährung und auf das Verhalten von *D. suzukii* wurde in dieser Arbeit untersucht. Wissen über die Rolle der Hefen in der Nahrung von *D. suzukii* kann dazu beitragen, die Bekämpfungsstrategien zu verbessern, indem die Aufnahme der attract-und-kill (anlocken und abtöten) Formulierung gesteigert wird. Die getesteten Hefe-Arten beeinflussten die Nahrungsaufnahme, die Fruchtbarkeit und die Lebensdauer von *D. suzukii*-Fliegen. Dabei wurde *Hanseniaspora uvarum* (Niehaus) bevorzugt aufgenommen und steigerte die Fruchtbarkeit der Tiere. Aus diesem Grund wurde in Versuchen im Gewächshaus und im Weingarten, eine attract-und-kill Formulierung auf Basis von *H. uvarum* und einem geeigneten Insektizid auf das Laub appliziert, um *D. suzukii* zu bekämpfen. Behandelte Blätter wurden vom Feld ins Labor gebracht, um die Wirkung sowie die Persistenz an im Labor gezüchteten Tieren zu testen. Blätter, die im Feld oder im Gewächshaus mit *H. uvarum* und Insektizid behandelt und im Labor an *D. suzukii* getestet wurden, verursachten eine erhöhte Mortalität der *D. suzukii*-Fliegen und verringerten die Anzahl der auf den Früchten abgelegten Eier. Die Applikation der auf *H. uvarum* basierten attract-und-kill Formulierung auf die Laubwand reduzierte den Befall in gleichem Maße wie die Applikation des Insektizids auf die gesamte Pflanze. Im Vergleich zu herkömmlichen Insektizidanwendungen können durch die Verwendung der attract-und-kill Formulierung Insektizidrückstände auf den Trauben vermieden werden, da die Formulierung gezielt auf die Laubwand aufgebracht wird. Dabei gelang es die applizierte Insektizidmenge pro Fläche zu verringern, ohne dass die Wirkung beeinträchtigt wurde.

5 Introductory overview

5.1 The insect pest *Drosophila suzukii*

Drosophila species are small flies commonly known as vinegar flies. The genus *Drosophila* comprises about 1,500 species worldwide (Markow and O'Grady 2005). Of the known *Drosophila* species, *D. suzukii* is one of only a few species that are able to oviposit in ripe undamaged fruit (Lee et al. 2011). *Drosophila suzukii* is native to Asia and has spread throughout North America, South America, Africa and Europe (Asplen et al. 2015; Boughdad et al. 2021). In Europe, *D. suzukii* was first recorded in Spain in 2008 (Calabria et al. 2012; Cini et al. 2014).

The eggs of *D. suzukii* are milky-white and glossy with two filaments (Fig. 1a). Fresh hatched larvae are about 0.7 mm in length and develop through three instars to 3.5 mm before pupating (Fig. 1b). Pupae are brownish and about 3 mm in length and have anterior spiracles on both sides of the head (Fig. 1c). Adult *D. suzukii* flies have a yellow-brown thorax, black stripes on the abdomen, red eyes, and are 2-3 mm in length (Walsh et al. 2011). Female flies can be identified by the serrated ovipositor, which enables the females to oviposit in the skin of several fruits (Deprá et al. 2014) (Fig. 1d). Male flies can be identified by the black apical wing spots and the single row of combs on the first and second tarsal segment on the first pair of legs (Hauser 2011) (Fig. 1e). The total lifespan observed for *D. suzukii* flies range from 50 to 154 days (Emiljanowicz et al. 2014).

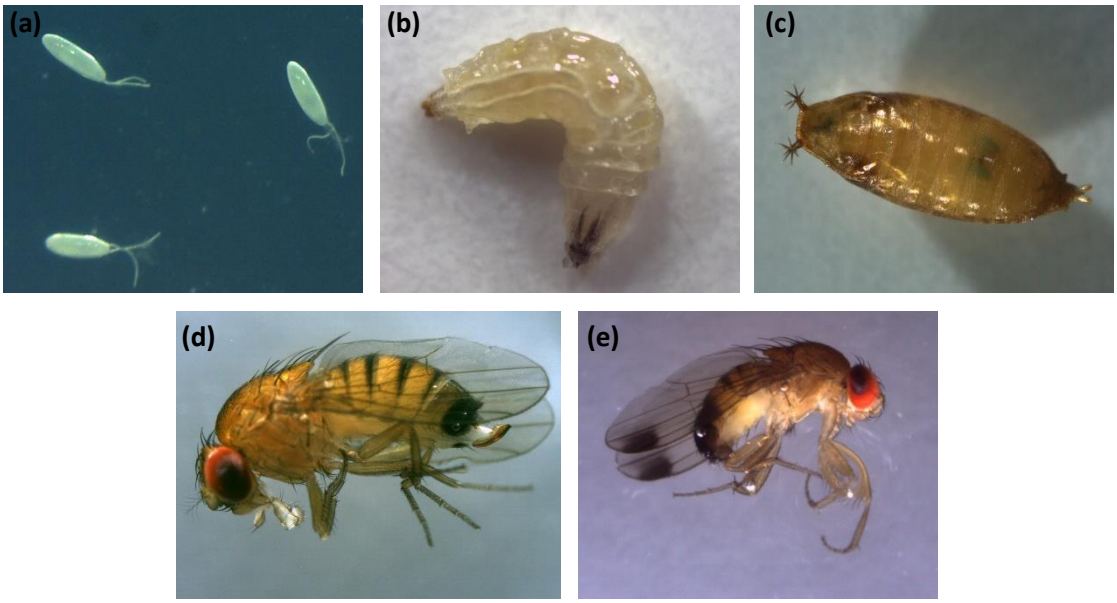

Figure 1. Life stages of *D. suzukii*: (a) eggs, (b) larvae, (c) pupae, (d) adult female, and (e) adult male (© U. Spitaler).

The behavior of *D. suzukii* to oviposit the eggs in healthy intact fruits in suitable stages of ripeness (Burrack et al. 2013; Kienzle et al. 2020) and their high reproductivity (Cini et al. 2012; Tochen et al. 2014) make it an important agricultural pest. Host plants are soft- and thin-skinned fruits, such as grapes, berry fruit and stone fruit, both wild and cultivated (Bellamy et al. 2013; Cai et al. 2019; Elsensohn and Loeb 2018; Mitsui et al. 2006). Damage due to *D. suzukii* infestation is caused by the larvae that feed inside the fruit and by microorganisms growing on the fruit pulp (Cini et al. 2012). In grapes, *D. suzukii* oviposition and larval development not only causes direct damage but also favors sour rot (Ioriatti et al. 2018). Especially the cultivation of the red grape variety Vernatsch (synonymous Trollinger, Schiava) was compromised by the first appearance of *D. suzukii* in Northern Italy in 2009 (Cini et al. 2012; Ioriatti et al. 2015; Ioriatti et al. 2018).

The control of *D. suzukii* typically relies on the application of insecticides and technical strategies, such as exclusion netting (Beers et al. 2011; Cini et al. 2012; Leach et al. 2016; Sial et al. 2019). Unfortunately, most insecticide applications result in undesired residues and cause problems with respect to marketing and consumer safety (Haviland and Beers 2012). Furthermore, application of not selective insecticides and pesticide resistances in *D. suzukii* have increased the need for alternative control methods to improve insecticide efficacy (Gress and Zalom 2019; Smirle et al. 2017). A promising approach to control *D. suzukii* could be the use of yeasts as an attractant and phagostimulant.

5.2 The interaction of *Drosophila suzukii* and yeasts

Volatile compounds emitted by the fruits (Abraham et al. 2015; Karageorgi et al. 2017; Liu et al. 2018) and the plants (Bolton et al. 2019; Piñero et al. 2019) or during yeast fermentation (Bing et al. 2018; Lasa et al. 2019a) lead *D. suzukii* flies to suitable fruits for oviposition and food sources for nutrition. In their habitat, *D. suzukii* adults and larvae feed on damaged fruits and other sugar containing sources, including the yeast flora naturally growing on sugar-rich substrate (Hamby and Becher 2016; Mitsui et al. 2010). In addition, yeasts were found to be attractive to *D. suzukii* (Burrack et al. 2015) and were repeatedly found to be associated with *D. suzukii* (Lewis et al. 2019). Larvae of *D. suzukii* benefit from yeast in their diet (Bellutti et al. 2018; Hardin et al. 2015; Lewis and Hamby 2019), but little is known about the effect of yeasts in the diet of *D. suzukii* adults.

Several yeast species, such as *Issatchenkia terricola, Metschnikowia pulcherrima, Saccharomycopsis vini, Clavispora santaluciae* and *Pichia kluyveri*, have been isolated from *D. suzukii* larvae and fruit damaged by

D. suzukii (Bellutti et al. 2018; Hamby et al. 2012; Lewis and Hamby 2019). The yeast *Hanseniaspora uvarum* (Niehaus) was frequently isolated from *D. suzukii* larvae and adults (Hamby et al. 2012; Knight et al. 2016; Lewis et al. 2019). It was also found that *D. suzukii* larvae would rather feed on *H. uvarum* than on alternative yeasts, such as *Saccharomyces cerevisiae* (Lewis and Hamby 2019). In laboratory assays, *H. uvarum* was the most attractive yeast towards *D. suzukii* adults compared to strains from *P. kluyveri*, *I. terricola*, *S. cerevisiae, Candida californica* and *Candida zemplinina* (Scheidler et al. 2015). Therefore, the effect of *H. uvarum* in the diet of *D. suzukii* adults and the possible use in pest control was studied in detail in this thesis.

5.3 Background on attract-and-kill strategies to control *Drosophila suzukii*

Semiochemicals provide additional control options to conventional pesticides (Mauchline et al. 2018). For example, the use of sex pheromones of Diptera, Lepidoptera, and Coleoptera species have been successfully used for mating disruption in pest control (Hillbur et al. 2005; Samietz et al. 2012).

Normally, insects are attracted to specific volatile compounds associated with a food source or other odor sources that are important in their biology (Davis et al. 2013). As a control strategy against *D. suzukii*, some yeasts were tested as adjuvants in insecticide sprays to increase ingestion of attract-and-kill formulations based on the association of attractive yeasts and an insecticide (Andreazza et al. 2017; Cowles et al. 2015; Knight et al. 2013; Knight et al. 2016; Noble et al. 2019; Roubos et al. 2019). For example, *H. uvarum* could be suitable for such a purpose because it is known to be attractive for *D. suzukii* flies (Scheidler et al. 2015) and it can increase ingestion compared to growth medium without yeast (Mori et al. 2017). Among the numerous insecticides that can be used against *D. suzukii* (Rosensteel and Sial 2017; Sial et al. 2019; Smirle et al. 2017; van Timmeren and Isaacs 2013), spinosad, which can be used in integrated as well as organic production (Sial et al. 2019), was proven to be effective against *D. suzukii* (Noble et al. 2019). For this reason, the insecticide spinosad was chosen as insecticidal toxic component of the attract-and-kill formulation.

Combining an insecticide with an attractant that guides the flies to the insect toxic bait might allow for the targeted application to the canopy while avoiding the fruit. Such a strategy could promote more sustainable and targeted chemical control and could reduce the amount of insecticide applied in the field and prevent residues on the fruit (Hamby and Becher 2016; Haviland and Beers 2012).

Successful attempts to develop an attract-and-kill strategy against *D. suzukii* were recently conducted with applications based on *H. uvarum* in strawberry and raspberry fields (Noble et al. 2021), *S. cerevisiae* and *Aureobasidium pullulans* in cherry orchards (Knight et al. 2016), and with a complex solution of unknown ingredients in combination with conventional treatments in blueberry and raspberry fields (Klick et al. 2019). Furthermore, control methods based on *H. uvarum* and insecticides were previously tested in laboratory trials and led to reduced oviposition and higher mortality of *D. suzukii* adults (Knight et al. 2016; Mori et al. 2017; Noble et al. 2019).

In *D. suzukii*, attraction to yeast is strain-specific (Lasa et al. 2019b). Therefore, an *H. uvarum* strain (LB-NB-2.2) that was isolated from feeding galleries of *D. suzukii* larvae in infested grape berries was used for the attract-and-kill formulation (Bellutti et al. 2018).

6 Objective

The present thesis aims to provide additional knowledge about the role of yeasts in the diet of *D. suzukii*. Data concerning their effect on attractivity, fecundity, feeding and mortality of *D. suzukii* adults could be used for the selection of suitable yeast species and for the establishment of targeted control strategies.

The observation of the persistence of volatile and other chemical compounds on leaf surfaces should provide knowledge about the duration of the control effect. Furthermore, different attract-and-kill formulations were tested to investigate if the addition of yeasts improves the efficacy of various insecticides.

In the vineyard, the objective was to determine if the application of the attract-and-kill formulation could restrict the spray application to the foliage and could reduce areal insecticide release without compromising the control efficacy compared to conventional treatment of the whole plant. In laboratory trials, *D. suzukii* flies were exposed to leaves collected in the field after treatment to obtain additional information about the effect in the vineyard. Different preservation methods for *H. uvarum* cultures were tested to develop a sustainable and cost-effective attract-and-kill formulation. Residual analyses were performed to provide information about the persistence of the applied insecticide.

7 Publication's summary

7.1 First publication: Yeast species affects feeding and fitness of *Drosophila suzukii* adults (Spitaler et al. 2020)

The first study investigated the effect of three *H. uvarum* strains and five yeast species [*I. terricola*, *M. pulcherrima*, *S. cerevisiae*, *S. vini*, and *Candida* sp. (later described as *Clavispora santaluciae* by Drumonde-Neves et al. 2020)] in the nutrition of *D. suzukii* adults. Since the substrate influences the availability of nutrients for *D. suzukii* in a yeast-based diet, microbiological cost-effective commercial media (potato dextrose agar and malt extract agar) were chosen. A significant difference in *D. suzukii* fecundity was observed between the yeast growing media with higher oviposition on potato dextrose agar compared to malt extract agar. In an oviposition assay (Fig. 2), *H. uvarum* and *S. vini* showed a positive effect on the fecundity, while *I. terricola*, *M. pulcherrima* and *C. santaluciae* had negative effects.

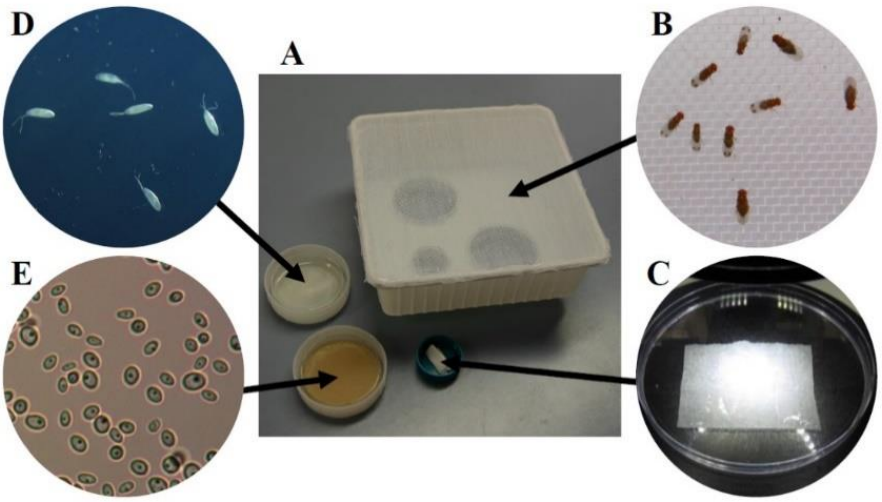

Figure 2. Cage design of the oviposition assay. (A) Experimental cages with three closeable openings with screw plugs on the bottom for changing the three components (Petri dish with agar, Petri dish with yeast culture, and a piece of paper towel with sucrose solution). (B) *D. suzukii* flies inside the cage sitting on the white mesh on the top of the box, (C) paper towel as sugar source, (D) *D. suzukii* eggs laid in water agar, and (E) yeast cells (Spitaler et al. 2020, supplementary material).

Intra- and extracellular compounds were analyzed for yeast cultures grown in potato dextrose broth, and the daily ingestion of different yeasts was measured with a modified capillary feeder assay (Ja et al. 2007)

(Fig. 3). *Drosophila suzukii* females feeding on *H. uvarum* and *S. vini* ingested a higher amount of yeast broth compared with the other yeast species tested. Furthermore, *H. uvarum* and *S. vini* led to a lower mortality of *D. suzukii* females compared to other yeasts. Interestingly, *S. vini* has rarely been mentioned as being associated with *D. suzukii* (Bellutti et al. 2018). *Hanseniaspora uvarum* is known to be attractive for *D. suzukii* larvae (Lewis and Hamby 2019) and adults (Scheidler et al. 2015). Based on the results, *D. suzukii* females also benefit from *H. uvarum* in their diet.

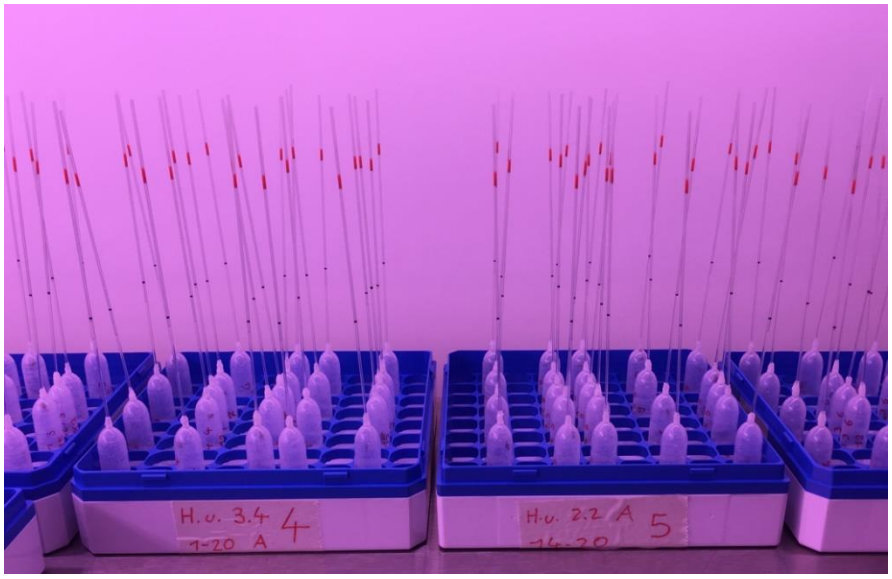

Figure 3. Capillary feeder assay to measure the daily ingestion of yeast broths. Single *D. suzukii* females were kept in Eppendorf tubes with the lid downward. The consumption of yeast broths was measured based on the liquid level inside the glass capillaries (© U. Spitaler).

The findings of the first study were useful for understanding the differences concerning the fecundity, ingestion, and mortality of *D. suzukii* adults fed with different yeast strains and provided relevant information for the development of attract-and-kill control strategies against *D. suzukii*. Additionally, the chemical characterization of yeast-based food provided insights concerning potentially phagostimulant components that may be exploited for pest management. The results showed for the first time that not only the aromatic compounds, but also differences in non-volatile metabolites of the yeasts play a role in the association between yeast and adult *D. suzukii*.

7.2 Second publication: Persistence of a yeast-based (*Hanseniaspora uvarum*) attract-and-kill formulation against *Drosophila suzukii* on grape leaves (Bianchi et al. 2020b)

The second study evaluated the efficacy and the persistence of the attract-and-kill formulation one day and one week after application on grapevine leaves. The attract-and-kill formulation consisted of the yeast *H. uvarum*, which was shown to be feeding stimulant (Spitaler et al. 2020) and the insecticide spinosad. Potted grape plants were cultivated in the greenhouse and different treatments were applied (Fig. 4). The treatments were insecticide-free potato dextrose broth, potato dextrose broth with spinosad and the attract-and-kill formulation based on *H. uvarum* and spinosad. Laboratory trials were performed to determine the efficacy of treated leaves in controlling *D. suzukii*. The concentrations of potential phagostimulants were assessed by quantitative measurement. Additionally, the volatile organic compounds released by plants treated with the attract-and-kill formulation were collected and compared to those emitted by untreated leaves.

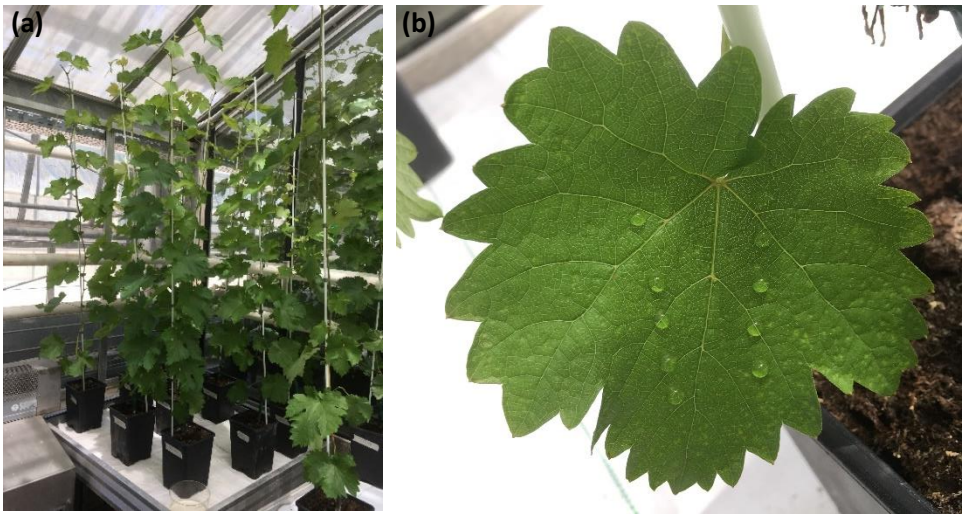

Figure 4. (a) Rooted grafted vines were grown for two months in the greenhouse to assess the persistence of the attract-and-kill formulation. (b) Leaf treated with 10 drops of 10 µL using a multichannel pipette (© U. Spitaler).

Adult *D. suzukii* were exposed to treated leaves in the laboratory and the mortality and oviposition was assessed (Fig. 5). The results showed that the addition of *H. uvarum* to spinosad increased mortality of *D. suzukii* flies and reduced the number of eggs laid. The observed mortality of *D. suzukii* flies was higher one

day after treatment, but the attract-and-kill formulation was still effective after one week under greenhouse conditions. Since mortality of *D. suzukii* flies can be related to attractiveness and feeding stimulation towards specific components, potential phagostimulants and attractants were analyzed. Several non-volatile compounds, including carbohydrates, sugar alcohols, amino acids, and organic acids, were found on the surface of treated leaves. The concentrations of the detected compounds generally decreased over time. Some of the compounds were previously reported as feeding stimulants for *D. suzukii* flies such as carbohydrates (Biolchini et al. 2017). Furthermore, numerous volatile organic compounds emitted by yeasts and the plant were detected, including benzaldehyde and 2-phenylethanol. Changes in the profile of volatile organic compounds emitted by the yeast treated leaves were observed over time.

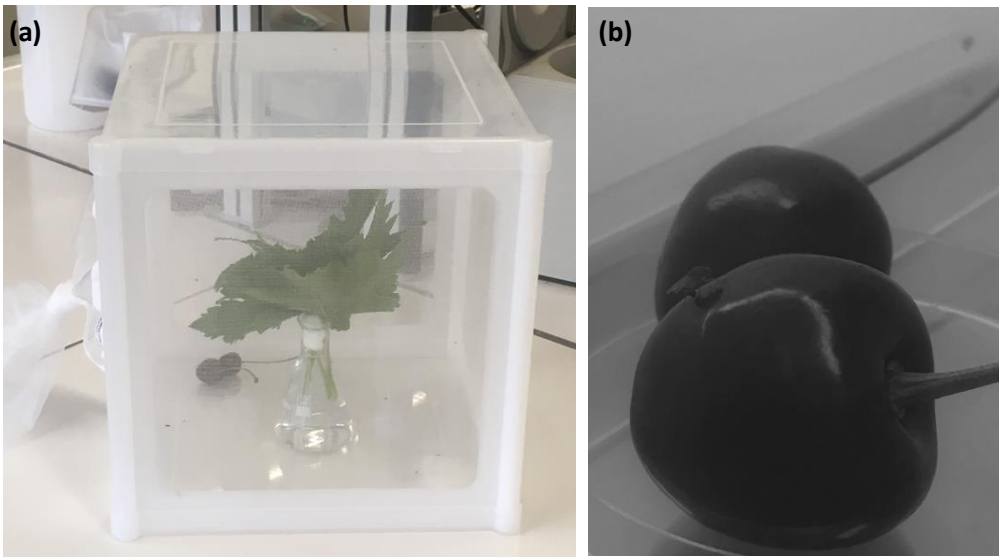

Figure 5. (a) Five grapevine leaves were exposed to *D. suzukii* flies in an insect cage together with cotton soaked in sucrose solution and two ripe cherries for oviposition. (b) *Drosophila suzukii* female laying eggs on a cherry (© U. Spitaler).

7.3 Third publication: Behavioral manipulation of *Drosophila suzukii* for pest control: high attraction to yeast enhances insecticide efficacy when applied on leaves (Rehermann et al. 2021)

The third study observed the attraction of *D. suzukii* females to the feeding stimulant yeast *H. uvarum* (Spitaler et al. 2020). The previous study showed that attractive volatile organic compounds are released by treated grapevine leaves (Bianchi et al. 2020b). The aim of this study was to test the attract-and-kill formulation under laboratory conditions, with spray-application on canopy but not on fruit. To evaluate the attractiveness of *H. uvarum* when applied on the surface of leaves, a two-choice set-up was performed and *D. suzukii* females were exposed to sprayed leaves and untreated leaves as alternative (Fig. 6a). To observe attraction behavior of *D. suzukii*, a wind tunnel assay was conducted with *D. suzukii* females. Upwind flight and landing at the odor source were recorded. Furthermore, three additional insecticides (acetamiprid, deltamethrin, tau-fluvalinate) were tested in combination with *H. uvarum* in order not to limit the applicability of the attract-and-kill formulation to the insecticide spinosad. Egg-laying and mortality of *D. suzukii* males and females were investigated when exposed to treated grapevine leaves (Fig. 6b).

The results showed that treatments with *H. uvarum* attracted *D. suzukii* when applied on leaves of grapevine, even in presence of untreated fruits. In a wind tunnel assay, all treatments containing *H. uvarum* alone or in combination with one of the insecticides showed that the addition of insecticide did not reduce *D. suzukii* attraction to *H. uvarum*. The addition of yeast increased the efficacy of all four insecticides. Yeast attraction was competitive to grape berries and improved insecticide effectiveness, suggesting that sprays covering canopy only could reduce residues on fruit without compromising control efficacy.

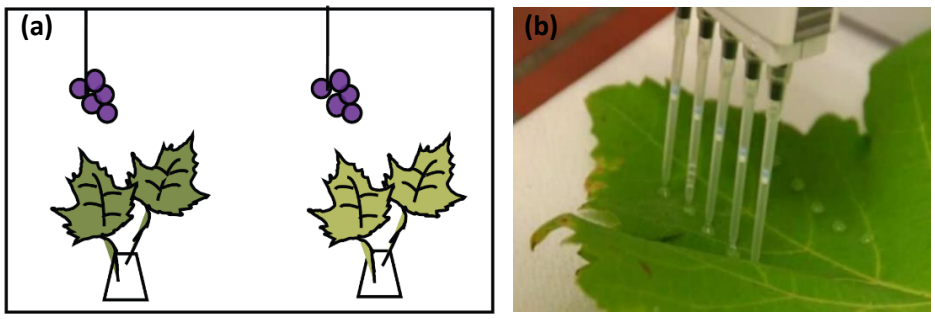

Figure 6. (a) Illustration of the two-choice set-up with grapes placed nearby untreated leaves (dark green) and leaves treated with the attract-and-kill formulation (light green) (Rehermann et al. 2021). (b) Different insecticides were applied in the laboratory using a multichannel pipette (© U. Spitaler).

7.4 Fourth publication: Field and greenhouse application of an attract-and-kill formulation based on the yeast *Hanseniaspora uvarum* and the insecticide spinosad to control *Drosophila suzukii* in grapes (Spitaler et al. 2021)

In the fourth study, field and greenhouse applications of an attract-and-kill formulation based on the yeast *H. uvarum* in combination with the field recommended dose of spinosad were tested. Yeast cultures were cultivated in the laboratory on potato dextrose broth (Fig. 7a). The field trials were performed in two vineyards cultivated according to the guidelines for integrated fruit production in South Tyrol, Italy. The grapes were cultivated using a pergola as the training method in 2019 (Fig. 7b) and with the single Guyot method in 2020. Two treatments were performed during the ripening phase of the grapes to test the attract-and-kill control strategy. Leaves treated in the field were also exposed to laboratory-reared *D. suzukii* flies to evaluate the efficacy of the field application. Analyses of residues were carried out to detect and better understand the persistence of the applied spinosad. For practical uses in agriculture, an issue could be the difficulty in obtaining a stable *H. uvarum*-based attract-and-kill formulation. For commercial use, a stable and dry product would simplify marketing and use by farmers. This study explores the effect of different procedures that can be used to conserve *H. uvarum*-based attract-and-kill formulations in greenhouse trials.

Figure 7. (a) Yeast cultures were cultivated at the Laimburg Research Centre in 4 L of potato dextrose broth in 6-L Erlenmeyer flasks closed with cotton and aluminum foil on magnetic stirrers. (b) Field trials were performed in vineyards that cultivate the local grape variety Vernatsch using a pergola as the training method (© U. Spitaler).

The results of the field application showed that targeted treatment of the foliage without spraying the grapes was possible for both training types. The attract-and-kill formulation reduced *D. suzukii* infestation compared to the unsprayed control and no differences in efficacy were observed compared to conventional spinosad application to the whole plant. Furthermore, the amount of spinosad applied per area in the attract-and-kill treatment was three times lower than that in the conventional treatment. The laboratory evaluation of the leaves showed that the *H. uvarum* bait was still effective one week after the first application and one week after the second application. The residue analyses revealed that a faster degradation of the insecticide occurred on the leaves treated with *H. uvarum* and spinosad, while the conventional spinosad treatment applied to the whole plant resulted in more residues on the leaves.

The supernatant of a centrifuged culture without *H. uvarum* cells showed a similar efficacy as the whole *H. uvarum* culture, and both retained their effect over two weeks. It can be assumed that commercial and storable attract-and-kill formulations should avoid the loss of the supernatant, which contains attractive and feeding stimulant compounds, while the preservation of living yeast cells seems to be less important. In contrast, freeze-dried *H. uvarum* pellet dissolved in water was less effective.

8 Conclusion and prospects

In conclusion, the yeast *H. uvarum* was successfully used as a component of an attract-and-kill formulation against *D. suzukii*. The advantages of the developed yeast-based control method in terms of sustainable control measures are associated with the lower amount of residual insecticide on the fruits at harvest and the reduced amount of insecticide applied in the field.

Further electrophysiological and behavioral studies may be helpful for the optimization of the efficacy of the attract-and-kill formulation and for understanding the role of single volatile and non-volatile compounds in the behavior of *D. suzukii*. Detailed knowledge of many compounds that are attractive or feeding stimulant could help to create an artificial and standardized product. Furthermore, the described compounds present in the yeast broth and the degradation of those compounds on the leaf surface can be used for the risk assessment of the attract-and-kill formulation. Generally, a decrease in the amounts of non-volatile compounds was observed over time, although numerous nutrients were still present on the plants one week after treatment. Additional experiments are necessary to proof the persistence of single compounds and if they could cause problems with respect to marketing and consumer safety. On the other hand, the yeast *H. uvarum* was evaluated with qualified presumption of safety as biological control agent intentionally added to food or feed by previous studies (Koutsoumanis et al. 2019). Furthermore, the yeast *H. uvarum* is naturally present on grapes, therefore its application likely would not interfere with winemaking and should be harmless (Albertin et al. 2015; Drumonde-Neves et al. 2017). Additional field experiments assessing the persistence of the insecticide on the leaves and testing of further easy-to-store products should be performed.

The application of the attract-and-kill formulation also induced the emission of volatile organic compounds by the treated grapevine leaves. Further studies focusing on the reaction of the plant to the treatment should be performed for other crops that are affected by *D. suzukii* such as berries and stone fruits.

Other open questions concern the efficacy of the attract-and-kill formulation against other insect pests, such as the European cherry fruit fly (*Rhagoletis cerasi*) and the impact of the attract-and-kill formulation on non-targeted insects (Böckmann et al. 2013). Furthermore, a targeted treatment of the foliage could support additional control strategies based on the release of parasitoids against *D. suzukii*.

In the light of all this, I think that the suggested attract-and-kill formulation is compatible with existing management practices and should therefore be considered for the implementation within organic and integrated pest management programs.

9 Publications in full text

The following pages contain the four publications of this thesis in full text.

Journal of Pest Science (2020) 93:1295–1309
https://doi.org/10.1007/s10340-020-01266-y

ORIGINAL PAPER

Yeast species affects feeding and fitness of *Drosophila suzukii* adults

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Received: 22 November 2019 / Revised: 13 July 2020 / Accepted: 18 July 2020 / Published online: 28 July 2020

Abstract

Yeasts play an important role in the life cycle and biology of the insect pest *Drosophila suzukii* (Matsumura), commonly known as the spotted wing drosophila (SWD). Adult and larvae of this species are known to feed and benefit from yeast in their diet. In addition, yeasts were found to be attractive to SWD and were repeatedly found to be associated with SWD. Among those, *Hanseniaspora uvarum* is the most commonly mentioned. The present study explores the chemical composition and the effects of three *H. uvarum* strains and five yeast species (*Saccharomyces cerevisiae, Candida* sp., *Issatchenkia terricola, Metschnikowia pulcherrima* and *Saccharomycopsis vini*) in the diet of SWD adults. The different yeast species used in this study influenced mortality, fecundity and ingestion by SWD females. *Hanseniaspora uvarum* and *S. vini* were preferably ingested and increased fecundity of SWD females. The intra- and extracellular concentrations of compounds, such as amino acids, carbohydrates, sugar alcohols and organic acids, produced or consumed by yeasts differed among the species. Knowledge of the interaction of different yeast species with SWD and specific differences in the profile of compounds of yeast can help to improve the development of control strategies against the insect pest by promoting the ingestion of attract-and-kill formulations based on the combinations of yeasts and an appropriate insecticide.

Keywords Fecundity · *Hanseniaspora uvarum* · Ingestion · Spotted wing drosophila · Yeast metabolites

Communicated note by Antonio Biondi

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10340-020-01266-y) contains supplementary material, which is available to authorized users.

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Key message

- Naturally occurring yeasts play an important role in SWD development.
- Mortality, fecundity and ingestion by SWD adults are influenced by the yeast species in their diet.
- The concentrations of nutritional compounds in the fermentation broth of different yeasts vary due to differences in the methods they produce or consume compounds.
- Chemical analysis of selected yeast cultures helps to understand the interaction between SWD and putative associated yeast species.

Introduction

Yeasts play an important role in the interaction with *Drosophila* flies. They constitute a food source and produce volatile compounds attractive to the insect. As previously shown in *Drosophila* flies, yeast communities are to some extent species specific (Chandler et al. 2012), and the density and

community structure of yeasts on damaged fruit is influenced by the flies (Stamps et al. 2012), which benefit from yeast in their diet (Becher et al. 2012).

Drosophila suzukii (Matsumura), also known as the spotted wing drosophila (SWD), is an invasive insect pest (Hauser 2011). The ability of SWD to lay eggs in healthy ripe fruits, their short generation time and high reproductivity (Tochen et al. 2014) make it an important pest for a wide range of small and stone fruits, both wild and cultivated (Bellamy et al. 2013; De Ros et al. 2013; Elsensohn and Loeb 2018; Lee et al. 2015; Mitsui et al. 2006). Oviposition and host fruit suitability for larval development are influenced by several factors, including fruit species preferences (Abraham et al. 2015; Bellamy et al. 2013; Cloonan et al. 2018; Lee et al. 2011); fruit characteristics, such as sugar content, pH or firmness (Arnó et al. 2016; Burrack et al. 2013; Lee et al. 2015); and protein and carbohydrate content (Hardin et al. 2015).

In their habitat, SWD flies feed on damaged fruits and other sugar sources, including the yeast flora naturally growing on sugar-rich reserves (Mitsui et al. 2010; Tochen et al. 2016; Walsh et al. 2011). In contrast to the common vinegar fly, *Drosophila melanogaster*, SWD females search for healthy intact soft-skinned fruits in suitable stages of ripeness to oviposit their eggs (Burrack et al. 2013; Lee et al. 2011; Mitsui et al. 2006). Searching for undamaged fruit or other food sources, the flies use signals, such as volatile compounds emitted by the fruits (Abraham et al. 2015; Liu et al. 2018; Revadi et al. 2015) and the leaves (Bolton et al. 2019; Keesey et al. 2015; Piñero et al. 2019) or fermenting products (Lasa et al. 2019b; Bing et al. 2018; Camargo and Phaff 1957; Steck et al. 2018). Damage to the fruit is caused by the SWD larvae and by microorganisms growing inside the infested fruit (Cini et al. 2012). The populations of yeasts and other microorganisms inside the damaged fruit are likely established through microorganisms present on adult SWD flies (Hamby and Becher 2016). *Hanseniaspora uvarum* was frequently isolated from *Drosophila* before (Chandler et al. 2012) as well as from SWD larvae and adults (Hamby et al. 2012; Knight et al. 2016; Lewis et al. 2019). It was also found that SWD larvae would rather feed on *H. uvarum* than on alternative yeasts, such as *S. cerevisiae* (Lewis and Hamby 2019). Several other yeast species, such as *Metschnikowia pulcherrima*, *Issatchenkia terricola* and *Pichia kluyveri*, have been isolated from SWD larvae and fruit damaged by SWD (Bellutti et al. 2018; Hamby et al. 2012; Lewis et al. 2019). In laboratory trapping assays, *H. uvarum* was the most attractive yeast for SWD adults compared to strains from *Pichia kluyveri*, *Candida californica*, *Issatchenkia terricola*, *Saccharomyces cerevisiae* and *Candida zemplinina* (Scheidler et al. 2015). Larvae of SWD benefit from yeast in their diet (Bellutti et al. 2018; Hardin et al. 2015; Lewis and Hamby 2019), but little is known about the effect of naturally occurring yeast species in the diet of SWD adults and the influence of yeasts on substrate food quality.

The present study focuses on the influence of yeast species on the diet of SWD adults to provide additional insights into the biology of this insect and to clarify the association between SWD adults and certain yeast species. Since yeasts could be exploited for the development of attract-and-kill strategies against SWD (Knight et al. 2015, Mori et al. 2016), additional data concerning their effect on fecundity, feeding and mortality of SWD adults can be useful for the establishment of focused control strategies. For this purpose, the fecundity over a long lifetime period of SWD fed with different yeasts was observed. Cultures of yeasts isolated from feeding tunnels of SWD larvae in infested grape berries were grown on two different commercial yeast culture media and offered as food sources to SWD flies to understand the influence of individual yeast species on SWD flies. To characterize the quality of the various nutrient sources and to explain the findings of the insect trials, the content of carbohydrates, amino acids, sugar alcohols and organic acids in the yeast fermentation broths was measured.

Materials and methods

Yeast material

Table 1 lists the yeasts used in this study. The yeast cultures except for *Saccharomyces cerevisiae* were isolated from feeding tunnels of SWD larvae found in infested grapes in South Tyrol in 2009 (Bellutti et al. 2018). *Saccharomyces cerevisiae* strain S288c is a conventional laboratory strain.

Yeast cultivation on solid media

For long-term storage, purified isolates were cultivated in chloramphenicol yeast glucose broth (5 g/L yeast extract (Merck, Germany), 20 g/L glucose (Sigma-Aldrich, Germany), 0.1 g/L chloramphenicol (Merck, Germany) and maintained frozen in 20% glycerol at -80 °C. The yeast cultures for the SWD assays were grown on Petri dishes (diameter 6 cm) on malt extract agar (MEA) (30 g/L malt extract, 3 g/L peptone from soymeal and 15 g/L agar; Merck, Italy) or potato dextrose agar (PDA) (4 g/L potato starch (from infusion), 20 g/L dextrose, 15 g/L agar; Difco™ Becton–Dickinson, France). To inoculate the Petri dishes, a loop full of yeast cells cultivated on MEA or PDA was transferred in a 2-mL Eppendorf tube filled with 1 mL 0.9% NaCl (Merck, Italy) solution and vortexed for 10 s at 1800 rpm. Then, 0.1 mL yeast cell suspension was pipetted into the Petri dishes with culture medium and spread evenly across the surface. Petri dishes containing culture medium only were inoculated with 0.1 mL 0.9%

Table 1 Yeasts used in this study

Yeast species	Strain	Accession number*	Abbreviation
Saccharomyces cerevisiae	S288c		S.c. S288c
Hanseniaspora uvarum	LB-NB-1.21	KP298009	H.u. 1.21
Hanseniaspora uvarum	LB-NB-2.2	MK567898	H.u. 2.2
Hanseniaspora uvarum	LB-NB-3.4	MK567905	H.u. 3.4
Issatchenkia/Pichia terricola	LB-NB-2.1	MK567903	I.t. 2.1
Metschnikowia pulcherrima	LB-NB-3.2	KP298012	M.p. 3.2
Saccharomycopsis vini	LB-NB-1.33	KP298011	S.v. 1.33
Candida sp.	LB-NB-3.3	KP298013	C.sp. 3.3

*The accession numbers were deposited in GenBank NCBI

NaCl as yeast-free control. All Petri dishes including the yeast-free control were kept at 22 °C, offered to the flies 48 to 72 h after inoculation and checked for contaminations prior to use. Forty-eight hours after inoculation, the yeast colonies covered the whole Petri dishes containing culture media.

Yeast cultivation in liquid medium

Yeasts were grown in 1 L PDB (24 g/L Difco™ Potato Dextrose Broth) at 25 °C, 120 rpm for 30 h in a 2-L Erlenmeyer flask closed with cotton and aluminum foil. The inoculum (1 mL) was prepared with a loop full of yeast cells cultivated on PDA, which was transferred to a 2-mL Eppendorf tube filled with 1 mL PDB and vortexed for 10 s at 1800 rpm. Preliminary trials showed that after 30 h all yeast cultures reached the stationary phase. Number of cells per mL (Fuchs Rosenthal counting chamber), optical density at 600 nm (OD_{600}) (Cary 60 UV–Vis, Agilent), pH value (pH meter, Crison GLP 21), dry weight (DW) of yeast pellet (centrifugation of fermentation broth, removal of the supernatant, drying of the pellet at 103 °C) and alcohol content (distillation) were measured after 30 h of growth. The values are shown in Table 2. The yeast fermentation broths were stored at –80 °C until use.

Insects

Rearing and all SWD assays were performed in the laboratory under controlled conditions (22 ± 1 °C, 75 ± 3% relative humidity, photoperiod of L16:D8). The mass rearing was refreshed on multiple occasions each year with pupae from various fruits from the field collected in South Tyrol, Italy. The larvae were reared on a *Drosophila suzukii* Cornmeal Diet ($DSCD_{(a)}$) with dry deactivated yeast and dry baker's yeast (Küchle GmbH & Co. KG, Günzburg, Germany) sprinkled on the surface (Bellutti et al. 2018). The rearing also contained 5% sucrose solution on cotton. Males and females that emerged from the pupal stage within 24 h were kept together in an insect cage with cotton soaked in 5% sucrose solution until they were used in the assays.

Oviposition assays

Cages used for the oviposition trials were made of white polystyrene boxes (CIB Verona, 18 cm long, 18 cm wide and 6 cm high) with three closable openings with screw plugs on the bottom of the box for changing the three components. The top was closed with a white mesh (mesh size 1 × 0.625 mm). The three components were (1) one Petri dish (diameter 6 cm) with culture medium or culture medium and yeasts culture (diets), (2) one Petri dish (diameter 6 cm)

Table 2 Cell concentration (cells/mL), optical density at 600 nm (OD_{600}), pH value, dry weight (DW) and alcohol content of yeast fermentation broth measured after 30 h of growth in potato dextrose broth (PDB)

Yeast	Cells/mL	OD_{600}	PH	DW pellet (mg/mL fermentation broth)	Alcohol (vol %)
S.c. S288c	7.28×10^7	1.94	4.13	1.30	1.01
H.u. 1.21	6.06×10^7	1.71	4.40	0.72	0.78
H.u. 2.2	3.80×10^7	1.59	4.24	0.55	0.93
H.u. 3.4	2.90×10^7	1.58	4.31	0.57	0.78
I.t. 2.1	1.59×10^7	1.96	4.25	1.47	0.71
M.p. 3.2	3.64×10^7	1.84	4.25	1.66	0.87
S.v. 1.33	n/a*	1.81	3.97	1.63	0.54
C.sp. 3.3	1.41×10^8	1.97	4.29	1.71	0.92

*Cell counting was not possible for the mycelial yeast *S. vini*

with water agar (15 g/L Agar–agar, Merck, Italy) covered with 0.1 mL 5% sucrose solution (sterilized by autoclaving) and (3) a piece (6 by 6 cm) of folded paper towel soaked in 1 mL 5% sucrose solution. The components were placed in matching lids to easily replace them daily. The cage design is shown in "Electronic Supplementary Material (Fig. S1)."

At day 0 of the oviposition assays, 10 male and 10 female SWD flies of known age (36 ± 12 h after emergence from pupal stage) were placed in the cages. Each cage was considered as a replicate. Dead flies were removed and not replaced; therefore, the number of flies decreased over the experimental period. The sex of the dead flies was determined, and the mortality was recorded. Different diets were tested simultaneously, and the replicates started at different time points. The daily oviposition was calculated as the total number of eggs laid per female on the three components.

Three different methods were used in the oviposition assays. In the malt extract agar assay (MEA assay), three yeast species (M.p. 3.2, C.sp. 3.3 and H.u. 3.4) were tested on MEA, and a yeast-free MEA served as control. In the MEA assay, the mortality of SWD adults and the number of eggs laid on the three components were counted daily over 50 days. Seven replicates of the MEA assay were performed. The yeast growth media assay (YGM assay) assessed the yeast culture media MEA and PDA to evaluate their suitability as a nutrient medium for SWD. The three components were changed daily, except on weekends, and the mortality of SWD adults and the number of eggs laid on the three components were measured over 30 days. Five replicates of the YGM assay were performed. In the potato dextrose agar assay (PDA assay), seven yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) were cultivated on PDA. The mortality of SWD adults and the number of eggs laid on the three components were measured daily over 30 days. Three replicates of the PDA assay were performed.

To evaluate whether the different yeasts or the yeast-free culture media impact oviposition, a linear mixed effect analysis was applied for all oviposition assays (Winter 2013). Yeasts or medium and day (without interaction term) were included in the model as fixed effects. The replicates, which started at different time points, were included in the model as random effects. The oviposition data entered the analyses as numbers of eggs laid per female and day. To avoid deviations from homoscedasticity or normality, squared root data transformation was performed for the dataset of the PDA assay and YGM assay, while a cubic root transformation was performed for the MEA assay. To find significant effects in the mean first occurrence of oviposition in the cages, a Kruskal–Wallis test was performed followed by a Wilcoxon rank sum test for pairwise comparison. The results were adjusted using FDR (false discovery rate) methodology. Survival curves were evaluated using the Kaplan–Meier method followed by a log rank test. P values were adjusted using FDR methodology.

Ingestion assay

The daily ingestion of different yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) grown in liquid medium (PDB) and yeast-less PDB were measured with a modified Capillary Feeder assay (CAFE assay) (Ja et al. 2007). The females were used in the CAFE assay 48 ± 12 h after their emergence from the pupal stage. Males and females hatched together, and the chosen females were only given water for 5 h before they entered the CAFE assay. For each tested fermentation broth, 20 females were kept individually in one Eppendorf tube (2-mL safe-lock tubes) with the lid positioned downward. Single flies were considered as replicates. Dead flies were not replaced; therefore, the number of flies decreased during the experiments due to the observed mortality. For air circulation, the Eppendorf tubes had three holes (diameter 1 mm) on the sides (at the 1.5-mL mark) and one hole at the bottom for insertion of a 10-µL glass capillary tube (Drummond Scientific Company, USA). The glass capillaries were held in place with a strip of parafilm wrapped around the capillary at 1 cm height. Every day, yeast fermentation broths were thawed at room temperature and mixed with a vortex mixer at 1800 rpm for 1 min. Ten microliters of yeast fermentation broth was offered through the capillary once a day. The daily consumption was measured in mm based on the liquid level and converted into µL. Inside the Eppendorf tube, an agar disk (diameter 8 mm, 15 g/L Agar–agar, Merck, Italy) placed in the lid provided an additional water source. The capillaries and agar disks were changed every 24 h, and ingestion and mortality were observed every 24 h over 4 days for single flies. For each solution, three Eppendorf tubes without SWD females were used daily to measure the evaporation rate. The daily evaporation was subtracted from the experimental readings. The mean evaporation was 1.6 ± 0.6 µL per day.

To identify significant effects in the daily consumption in the CAFE assay, a Kruskal–Wallis test was performed followed by a Wilcoxon rank sum test for pairwise comparison. The results were adjusted using FDR methodology. Survival was evaluated using the Kaplan–Meier method followed by a log rank test. P values were adjusted using FDR methodology.

Chemical analysis

For the analysis of metabolites, liquid chromatography mass spectrometry grade (LC–MS grade) solvents and reagents were used. Analytical standards of carbohydrates, organic acids, sugar alcohols and amino acids that were quantified

in PDB and in fermentation broth samples ("Electronic Supplementary Material (Table S1)") and isotope-labeled internal standards (IS) (DL-Phenylalanine-3,3-d2; L-Lysine-4,4,5,5-d4 hydrochloride; L-Glutamic acid-2,3,3,4,4-d5; and L-Alanine-2,3,3,3-d4) were purchased from Sigma-Aldrich (Germany).

Sample preparation: intracellular compounds

Samples were prepared as described by Boer et al. (2010), with further modifications. Ten milliliters of yeast fermentation broth was directly quenched in 20 mL of –80 °C methanol and centrifuged for 5 min at 4000 rpm in a –80 °C pre-chilled rotor in a centrifuge at –10 °C. After centrifugation, the supernatant was discarded, and 0.2 mL of 10 mg/L IS mix solution (DL-Phenylalanine-3,3-d2; L-Lysine-4,4,5,5-d4; L-Glutamic acid-2,3,3,4,4-d5; and L-Alanine-2,3,3,3-d4) plus 0.8 mL of –20 °C extraction solvent (acetonitrile:methanol:water = 2:2:1) were added to the pellet. Then, the sample was extracted for 15 min in an ultrasonic cold bath, and the temperature did not exceed 10 °C. The suspension was centrifuged, and the supernatant set aside. The pellet was re-extracted under the same conditions with 1 mL of extraction solvent for 15 min in ultrasonic bath. The suspension was again centrifuged, and the supernatants were pooled (total extraction volume 2 mL, IS final concentration 1 mg/L).

An aliquot of the extract was transferred to a high-performance liquid chromatography (HPLC) vial and directly analyzed for amino acid determination. One part of the extract (0.2 mL) was dried using a speed vac (Eppendorf Concentrator 5301, Eppendorf, Italy) and resuspended in 0.2 mL of milliQ water for the quantification of carbohydrates and sugar alcohols. Another part of the extract (0.5 mL) was dried using a speed vac and resuspended in 0.15 mL of milliQ water for the determination of organic acids.

Sample preparation: extracellular compounds and culture broth

For the analysis of extracellular metabolites, yeast fermentation broth and medium (PDB) were filtered using hydrophilic Surfactant-Free Cellulose Acetate (SFCA) filters (0.2 µm). One part of the filtered sample was diluted 1 to 10 with extraction solvent (acetonitrile:methanol:water = 2:2:1) after the addition of IS mix solution (final IS concentration 1 mg/L), and the amount of amino acids was measured.

Filtered yeast fermentation broth was diluted 1 to 10 with water for the analysis of carbohydrates and sugar alcohols. For filtered PDB, a 1 to 100 dilution was necessary given the high amount of glucose. For organic acids, filtered yeast fermentation and PDB were diluted 1 to 5 with water before analysis.

Analytical methods

The same yeast cultures used for the CAFE assay were used for the chemical characterization (Table 2). Analyses were performed in triplicates.

For the determination of amino acids, samples were analyzed using liquid chromatography electrospray ionization triple quadrupole mass spectrometry (UHPLC-QqQ, Dionex UltiMate 3000 UHPLC TSQ Quantiva, Thermo Fisher, US) in multiple reaction monitoring (MRM) mode. Separation procedures followed Paglia et al. (2012) with further modifications on a hydrophilic interaction chromatography (HILIC) column (Acquity BEH Amide 2.1 × 150 mm, 1.7 µm with ACQUITY UPLC BEH Amide VanGuard pre-column, 130 Å, 1.7 µm, 2.1 mm x 5 mm) at 45 °C. The HILIC solvents used included Solvent A (water with 0.1% formic acid (FA)) and Solvent B (acetonitrile (ACN) with 0.1% FA). Flow rate was 400 µL/min. The gradient was as follows: $t = 0$, 99% B; $t = 0.1$, 99% B; $t = 7$ min, 30% B; $t = 7.1$ min, 99% B; $t = 10$ min, 99% B. The autosampler temperature was 4 °C, and injection volume was 2 µL. For routine quality control and quantification, IS of four metabolites (DL-Phenylalanine-3,3-d2; L-Lysine-4,4,5,5-d4 hydrochloride; L-Glutamic acid-2,3,3,4,4-d5; and L-Alanine-2,3,3,3-d4) were spiked into the samples. Values are reported as mg/L and the amount was calculated with a calibration curve based on the ion ratio between each analyte and the relative IS used. The spray voltage was set at 3200 V when operating in positive ion mode and 3500 V in negative ion mode. Vaporizer temperature and ion transfer tube temperature were set at 275 °C and 325 °C, respectively.

Samples were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) for the quantification of carbohydrates and sugar alcohols and using a conductivity detector for organic acids (Dionex ICS 5000; Thermo Fisher, USA). Separation of carbohydrates and sugar alcohols was performed using a Dionex CarboPac PA10 analytical column (4 × 250 mm) and a Dionex CarboPac PA10 precolumn (4 × 50 mm). Separation was achieved by isocratic elution with a 40 mM sodium hydroxide solution (50% w/w; Sigma-Aldrich, Germany), and the column was regenerated using a 200 mM NaOH solution (50% w/w; Sigma-Aldrich, Germany) for 10 min. The flow rate was set at 1.2 mL/min, the column temperature at 30 °C and injection volume was 20 µL. The total run time was 30 min, and a pulsed amperometric detector was used to monitor the eluted carbohydrates and sugar alcohols. Organic acids were analyzed using a Dionex Ion Pac ATC-HC trap (9 × 75 mm) before a Dionex AG11-HC precolumn (4 × 50 mm) and a Dionex Ion Pac AS11-HC column (4 × 250 mm) coupled with a conductimetric detector. The column temperature was set at 30 °C, and the injection volume was 25 µL. Chromatographic conditions were based on

previous studies (Geng et al. 2008). For analysis of carbohydrates, sugar alcohol and organic acid quantitation of each compound was calculated based on the calibration curves of corresponding analytical standards.

Statistical analyses

All statistical analyses were performed with R (R Core Team 2019). Visual inspections of residual plots were performed for all models. Significance of growing medium or yeasts were obtained with likelihood ratio tests of the full model with the effect of the growing medium against the model without that effect.

Post hoc tests in linear mixed models were calculated by computing estimated marginal means (EMMs) for specified factors or factor combinations in the models (Searle et al. 1980), and P values were corrected for multiple comparisons using the Tukey method. The following R packages were used: lme4 (Bates et al. 2015) to fit the mixed models, lmerTest (Kuznetsova et al. 2017) for approximating degrees of freedom for the t and F tests of the models and emmeans (Lenth 2019) to perform the post hoc tests in the linear mixed models. The R package survival (Therneau and Grambsch 2000) was used to fit the survival data for the Kaplan–Meier method, and survminer (Kassambara and Kosinski 2018) was used to calculate the pairwise comparisons between group levels. Heatmaps of intra- and extracellular metabolites were generated using MetaboAnalyst 4.0 (Chong et al. 2019).

Results

Oviposition assay

The distribution of the eggs among the three components was considered as the result of the different surface structures. Only the Petri dish with water agar and the culture media without yeast exhibited a fruit-skin-like surface that allowed an easy insertion of the eggs with the serrated ovipositor of the female. In the MEA and PDA assays over all cages containing yeast cultures grown on MEA or PDA ($n = 21 + 21$), on average (± SD) 15.3 ± 15.4% of the eggs were laid into the yeast culture (1), 65.5 ± 22.0% into the water agar (2) and 19.2 ± 13.4% into the piece of folded paper (3). It was assumed that the different surfaces of the yeast colonies depending on the yeast species had no effect on the total oviposition given that most eggs were laid in the water agar in all yeast-containing cages. In the MEA and YGM assays overall cages containing a yeast-free MEA control ($n = 7 + 5$), on average (± SD) 32.9 ± 13.2% of the eggs were laid into the MEA (1), 32.3 ± 16.0% into the water agar (2) and 34.8 ± 17.5% into the paper towel (3). In the PDA and YGM assays containing a yeast-free PDA control ($n = 7 + 5$), on average (± SD) 70.5 ± 15.1% of the eggs were laid into the PDA (1), 21.2 ± 16.7% into the water agar (2) and 8.3 ± 8.8% into the paper towel (3).

Yeasts grown on MEA

In the MEA assay, SWD flies were fed with three selected yeasts (M.p. 3.2, C.sp. 3.3 and H.u. 3.4) grown on solid medium (MEA) and yeast-free MEA to evaluate the effect of the yeasts on fecundity and mortality of SWD adults. The average first occurrence of oviposition in the cages (± SD) was 4.64 ± 1.83 days after the flies entered the assay. No influence of the diets on the first occurrence of oviposition was detected ($\chi^2_{(3)} = 1.63$, $p = 0.653$). The average number of eggs laid per fly increased during the first 30 days, whereas only a slight increase was observed until the end of the test period, i.e., day 50 (Fig. 1). The different diets had a significant effect on the number of eggs laid over the test period (50 days) ($\chi^2_{(3)} = 963.19$, $p < 0.0001$). Significantly fewer eggs were laid by SWD females fed with MEA compared to all yeast cultures grown on MEA. Additionally, significant differences among the three yeast species were found, and H.u. 3.4 led to the highest oviposition.

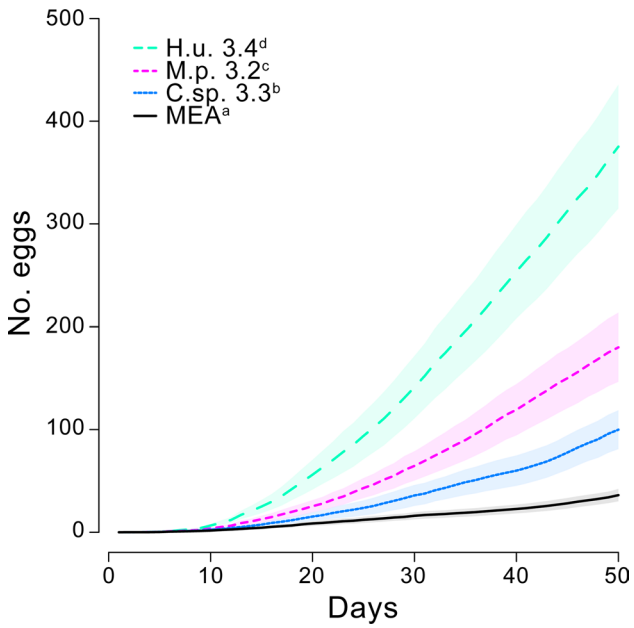

Fig. 1 Cumulative number (mean ± SE; $n = 7$) of eggs per SWD female laid in the MEA assay over a period of 50 days depending on the yeast species present on malt extract agar: without yeast (MEA), *Hanseniaspora uvarum* (H.u. 3.4), *Metschnikowia pulcherrima* (M.p. 3.2) and *Candida* sp. (C.sp. 3.3). Significant differences between total oviposition rates are indicated by sample names followed by different letters ($p < 0.05$) in the figure legend

Over all treatments, significantly more males (88.93%) than females (80.71%) survived the test period of 50 days ($\chi^2_{(1)}=7.29$, $p=0.007$). The different diets had no effect on the survivorship of SWD males ($\chi^2_{(3)}=7.50$, $p=0.058$) but influenced the survivorship of SWD females ($\chi^2_{(3)}=9{,}41$, $p=0.024$). No significant differences were found among diets after adjusting for multiple comparisons ($p<0.05$).

Yeast growth media without yeast

In the YGM assay, two yeast growth media, MEA and PDA, were compared to assess differences in their suitability as a nutrient medium for SWD and to evaluate their influence on oviposition and mortality of SWD adults. Yeast growth media used as SWD food affected female fecundity, and PDA led to significantly increased fecundity compared with MEA ($\chi^2_{(1)}=45.04$, $p<0.0001$) (Fig. 2). As also observed in the MEA assay, the egg laying started at a low level after an average (± SD) of 1.4 ± 0.55 days on PDA and 3.2 ± 1.30 days on MEA ($\chi^2_{(1)}=5.26$, $p=0.022$).

Overall, 84% of the females and 92% of the males survived until day 30 ($\chi^2_{(1)}=3.06$, $p=0.80$). The observed mortality was 88% for males and 80% for females on PDA and 96% for males and 88% for females on MEA. The different culture media showed no significant influence on the survivorship of SWD males ($\chi^2_{(1)}=2.20$, $p=0.138$) or females ($\chi^2_{(1)}=1.147$, $p=0.284$).

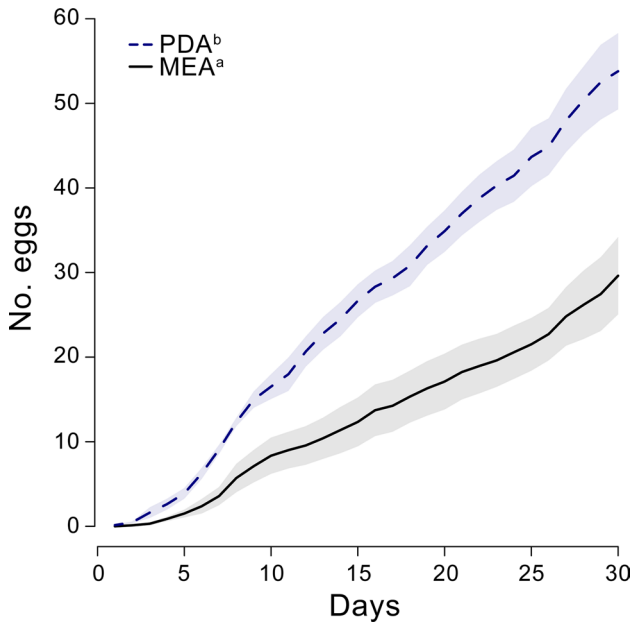

Fig. 2 Cumulative number (mean ± SE; $n=5$) of eggs per SWD female laid in the YGM assay over a period of 30 days on malt extract agar (MEA) or on potato dextrose agar (PDA). Significant differences between the diets are indicated by sample names followed by different letters ($p<0.05$) in the figure legend

Yeasts grown on PDA

After verifying the promoting effect of PDA on fecundity in comparison to MEA, the PDA assay tested eight different yeasts (S.c. S288c, H.u. 1.21, H.u. 2.2, H.u. 3.4, I.t. 2.1, M.p. 3.2, S.v. 1.33 and C.sp. 3.3) and yeast-free PDA over a period of 30 days. The first eggs were laid on average (± SD) 2 ± 0.93 days after the flies entered the assay. The effect of the diets on the first occurrence of oviposition was not significant ($\chi^2_{(7)}=6.065$, $p=0.532$). Significant differences in the egg laying curves were found between the different diets ($\chi^2_{(7)}=123.33$, $p<0.0001$) (Fig. 3). Greater than eighty percent of females (82.08%) and males (87.08%) survived until day 30 ($\chi^2_{(1)}=1.810$, $p=0.532$). The observed survival on the yeast grown on PDA ranged from 93.3% for males and 96.7% for females on H.u. 1.21 to 76.7% for males and 73.3% for females on H.u. 2.2. The different diets had no significant influences on the survival of SWD males ($\chi^2_{(7)}=4.16$, $p=0.760$), but a significant influence on females ($\chi^2_{(7)}=16.59$, $p=0.020$) was noted. Significant more females survived on H.u. 1.21 compared to I.t. 2.1 ($p=0.041$).

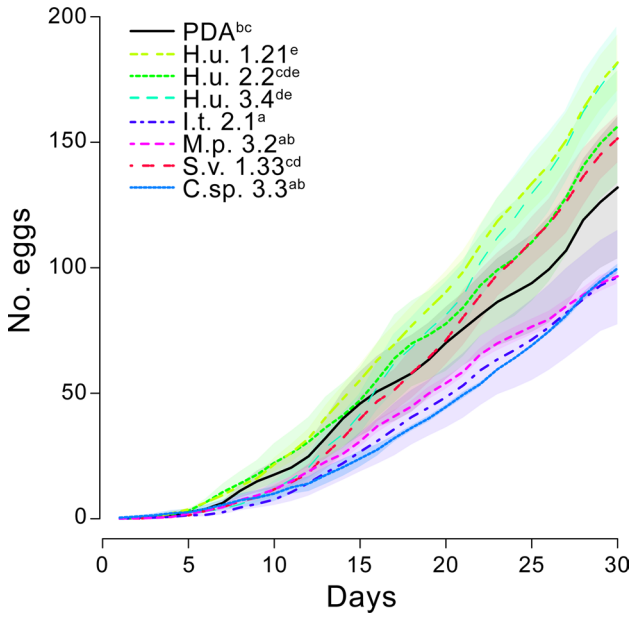

Fig. 3 Cumulative number (mean ± SE, $n=3$) of eggs per SWD female laid in the PDA assay over a period of 30 days depending on the yeast species present on potato dextrose agar without yeast (PDA) and one of three *Hanseniaspora uvarum* strains (H.u. 1.21, H.u. 2.2, H.u. 3.4), *Issatchenkia terricola* (I.t. 2.1), *Metschnikowia pulcherrima* (M.p. 3.2), *Saccharomycopsis vini* (S.v. 1.33) and *Candida* sp. (C.sp. 3.3). Significant differences between diets are indicated by sample names followed by different letters ($p<0.05$) in the figure legend

Ingestion assay

To evaluate the acceptance of the different yeast substrates by SWD females, the CAFE assay was performed to measure the daily ingestion over a four-day period. The ingested amount of yeast fermentation broths grown in PDB and yeast-free PDB showed an increasing trend over the four-day test period. Comparing the daily ingested amount of different yeast broths, except for day 1 ($\chi^2_{(8)} = 13.18$, $p = 0.106$), significant differences were found at day 2 ($\chi^2_{(8)} = 47.07$, $p < 0.001$), day 3 ($\chi^2_{(8)} = 19.47$, $p = 0.014$) and day 4 ($\chi^2_{(8)} = 23.28$, $p = 0.003$). At day 4, pairwise comparison showed no significant differences (Table 3). The mortality rates for the single days are also shown in Table 3.

Profile of intra- and extracellular compounds

Comparison of intra- and extracellular concentrations

Overall, 36 intracellular and 34 extracellular compounds were quantified in PDB and in the eight yeast fermentation broth samples listed in Table 2. Average values of intra- and extracellular compounds are summarized in "Electronic Supplementary Material (Table S2 and Table S3)," while heatmaps of the extracellular and intracellular concentrations of the compounds analyzed are reported in Fig. 4. Generally, extracellular compounds made the largest contribution to the total amount of nutrients, as they were present in much higher concentrations than intracellular compounds. Regarding amino acids and carbohydrates, the highest amount of these nutrients was found in the medium itself. While 18.34 g/L of glucose was found in PDB, extracellular concentrations in fermentation broth samples ranged from 0.01 g/L in S.c. S288c to 12.15 g/L in H.u. 1.21. The total extracellular amount of amino acids was 0.93 g/L in PDB, ranging from 0.25 g/L in I.t. 2.1 to 0.83 g/L in H.u. 3.4 in fermentation broth samples. Consumption of amino acids and carbohydrates by yeasts was observed since their concentrations decreased compared to those found in PDB with few exceptions. In addition, secretion of sugar alcohols and organic acids by yeasts was detected (Fig. 4a). Although clear differences in the profile of intracellular compounds were identified among yeasts (Fig. 4b), their concentrations were very low compared to extracellular compounds. This is not surprising since amino acids constitute building blocks of proteins, and glucose is clearly involved in sugar metabolism. Two of the organic acids, cis-aconitic acid and isocitric acid, could only be detected inside the cells and not in the extracellular environment.

Carbohydrate consumption and secretion of fermentation products

The extracellular concentrations of carbohydrates, sugar alcohols and organic acids in fermentation broths and PDB are shown in Fig. 5. The results show the consumption of carbohydrates and the secretion of metabolism and fermentation products, such as sugar alcohols and organic acids. The yeasts C.sp. 3.3 and S.c. S288c consumed almost all the available glucose within 30 h of growth.

Amino acid profile

The intra- and extracellular concentrations of amino acids found in fermentation broths and PDB are shown

Table 3 Mean daily ingestion per SWD female (µL ± SD) of yeast fermentation broth or PDB and daily mortality (% from the initial number ($n = 20$)) in the CAFE assay

Yeast	Day 1		Day 2		Day 3		Day 4	
	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)	Ingestion (µL)	Mortality (%)
PDB	0.27 ± 0.36^{a}	0	1.13 ± 1.19^{bc}	0	2.00 ± 1.48^{ab}	0	2.22 ± 1.09^{a}	0
S.c. S288c	0.64 ± 0.95^{a}	0	1.20 ± 1.32^{bc}	10	1.47 ± 1.27^{ab}	10	0.80 ± 0.79^{a}	50
H.u. 1.21	0.57 ± 0.56^{a}	0	1.60 ± 1.27^{cd}	0	1.94 ± 1.84^{ab}	0	3.33 ± 1.67^{a}	10
H.u. 2.2	0.71 ± 0.69^{a}	0	1.22 ± 1.30^{bc}	0	1.72 ± 1.16^{ab}	0	2.73 ± 1.59^{a}	5
H.u. 3.4	0.70 ± 1.01^{a}	0	2.25 ± 1.14^{d}	0	2.35 ± 1.49^{b}	0	2.78 ± 1.73^{a}	0
I.t. 2.1	0.43 ± 0.45^{a}	0	0.58 ± 0.66^{ab}	0	1.17 ± 1.19^{ab}	5	1.77 ± 1.53^{a}	20
M.p. 3.2	0.29 ± 0.42^{a}	0	0.29 ± 0.39^{a}	0	1.39 ± 1.66^{ab}	5	1.39 ± 1.49^{a}	45
S.v. 1.33	0.77 ± 0.75^{a}	0	0.91 ± 0.60^{bc}	0	1.44 ± 1.48^{ab}	0	3.11 ± 2.07^{a}	0
C.sp. 3.3	0.22 ± 0.28^{a}	0	0.63 ± 0.71^{ab}	0	0.81 ± 1.01^{a}	10	1.80 ± 0.80^{a}	60

Single females were considered as replicates. Due to mortality, the number of replicates decreases and varies over the test period. Significant differences between ingestion within the days are indicated by different letters ($p < 0.05$)

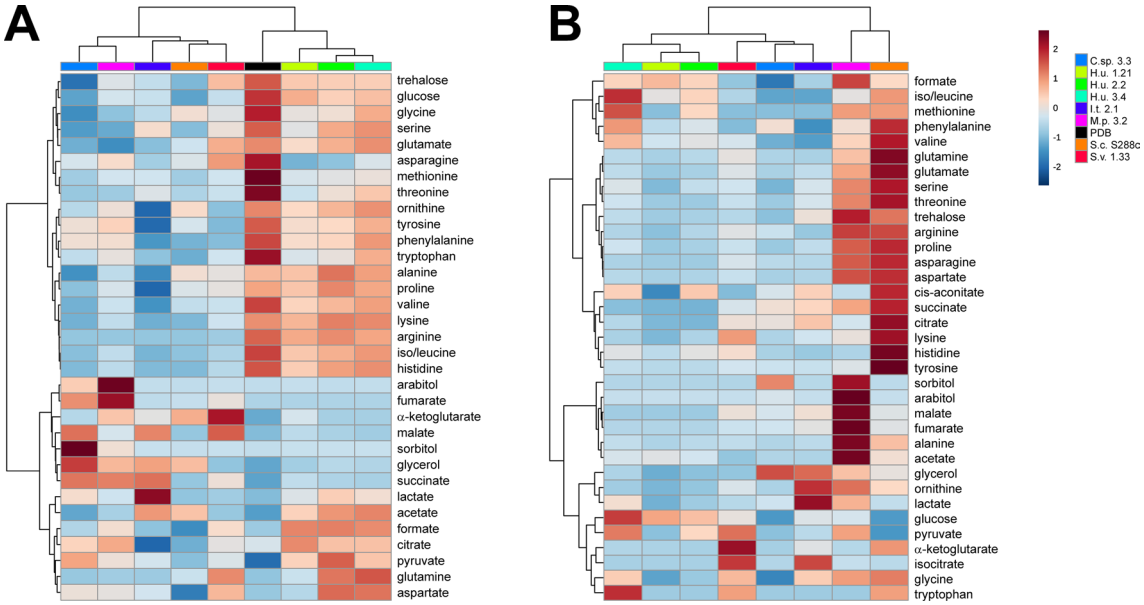

Fig. 4 Heatmaps of extracellular (**a**) and intracellular (**b**) compounds detected in the eight selected yeasts and in PDB. Concentrations of single compounds are displayed using a color scale ranging from red (higher amounts) to blue (lower amounts) as shown in the legend. Both rows and columns are clustered using Euclidean distances and a Ward clustering algorithm. Average values ($n=3$) for each sample are shown

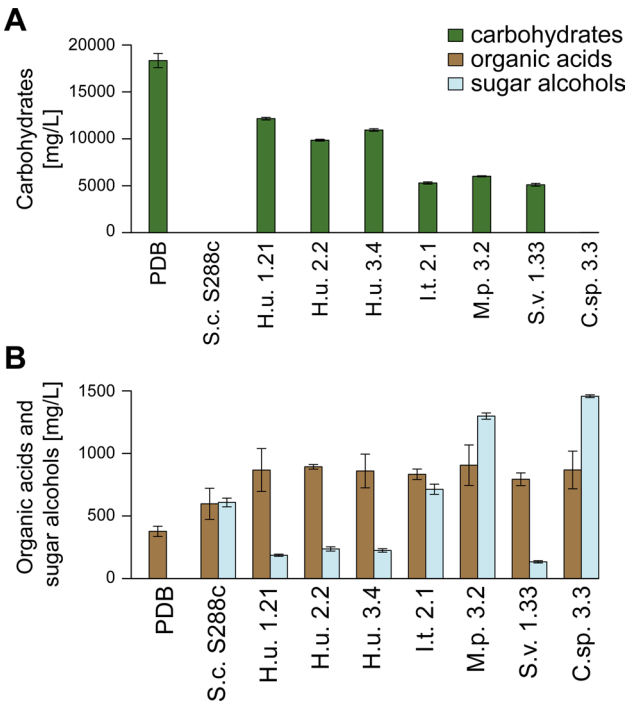

Fig. 5 Extracellular amount of total carbohydrates (**a**), organic acids and sugar alcohols (**b**) in the eight selected yeasts and in PDB. Values (mean ± SD) are reported as mg/L of fermentation or culture broth

in "Electronic Supplementary Material (Table S2 and Table S3)." Ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and arginine) are essential for *Drosophila* flies (Sang and King 1961). Regarding extracellular concentrations, PDB was rich in essential amino acids. The three *H. uvarum* strains consumed less amino acids than other yeasts. Some amino acids were lacking in three yeast fermentation broths. Specifically, a deficiency in extracellular concentrations of arginine, histidine and lysine was found in I.t. 2.1, while arginine, histidine and methionine were lacking in S.c. S288c and C.sp. 3.3. These deficient amino acids were present intracellularly, but methionine was present at low concentrations. In all samples, glutamic acid exhibited the greatest extracellular concentration among amino acids.

Relationships between extracellular compounds, ingestion and mortality

Various yeasts and PDB have different chemical profiles. Therefore, it is not possible to explain the results of this study based on linear correlations among single compounds and ingestion or mortality. However, some relationships were noted among ingestion, mortality and the chemical profile of different samples. The results of the CAFE assay and extracellular concentrations of glucose, total amino acids and glycerol are summarized in Fig. 6. Data on the consumption of females only, which survived the four-day experimental period, were included in the calculation of the total ingestion over four days ($n=6$ to 20). Significant differences were identified for the total ingestion over the four-day period ($\chi^2_{(8)} = 34.98$,

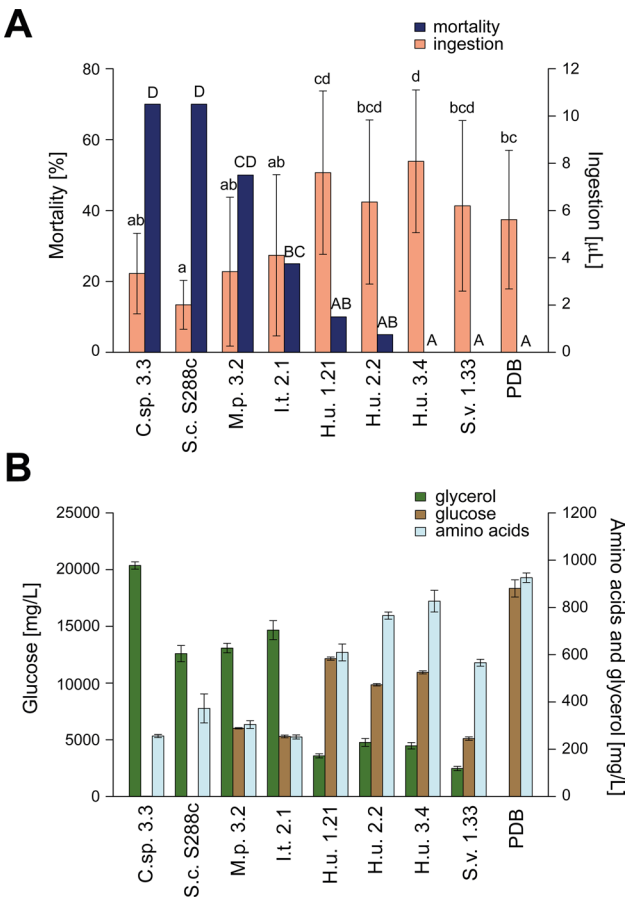

Fig. 6 Total ingestion per SWD female (mean ± SD) and mortality (%) of SWD females in the CAFE assay (**a**). Single females were considered as replicates ($n = 20$). Data on the ingestion only of females that survived the four-day experimental period were included in the calculation of the total ingestion over four days (resulting $n = 6$ to 20). Significant differences between diets are indicated by different letters ($p < 0.05$). Extracellular levels of glucose, glycerol and total amino acids in the eight selected yeasts and PDB (**b**). Values (mean ± SD) are reported as mg/L of fermentation or culture broth

$p < 0.001$). Different diets also significantly influenced the mortality over four days ($\chi^2_{(8)} = 78.967$, $p < 0.001$). The lowest mortality and the highest ingestion rate were observed for the three *H. uvarum* strains, S.v. 1.33 and PDB. Overall, a link between lower ingestion coupled with higher mortality could be observed (Fig. 6a), and an opposite relationship was observed between glucose and total amino acids versus glycerol concentration (Fig. 6b). Coherence between the supply of glucose and amino acids and increased ingestion and lower mortality as well as a link between increased glycerol levels and increased mortality are observed.

Discussion

The present study investigates the effect of different yeast species on the life history traits of SWD adults. The culture media PDA showed a positive effect on the fecundity of SWD compared with MEA, and an even increased fecundity rate was observed after inoculation of PDA with *H. uvarum* and S.v. 1.33, demonstrating generally positive effects of these two yeasts on SWD fecundity. Furthermore, SWD females feeding on *H. uvarum* or S.v. 1.33 ingested an increased amount of yeast broth compared with the other four yeast species tested, and a lower mortality of SWD females was observed. Relevant and phagostimulant compounds for SWD, such as carbohydrates and amino acids, were detected in PDB and in fermentation broths. Two yeasts, S.c. S288c and C.sp. 3.3, consume almost all available glucose within 30 h growth, reducing the amount available for SWD flies. *Hanseniaspora uvarum* tend to consume generally less nutrients within 30 h compared to the other species.

A trend toward different egg laying behaviors of *D. melanogaster* fed with different yeasts was previously reported by Anagnostou et al. (2010). The results of this study confirm their hypothesis for SWD and show that H.u. 3.4 cultivated on MEA leads to higher fecundity compared to C.sp. 3.3 and M.p. 3.2. Finding food sources rich in *H. uvarum* might offer an advantage for SWD females. A negative effect of *M. pulcherrima* on *Drosophila* larvae was reported by Anagnostou et al. (2010). On MEA without yeast, fecundity was very low, and all three yeasts exhibited increased oviposition. This study found that egg laying increases slowly over 50 days. A similar oviposition curve with a slow increase in egg laying was observed by Jaramillo et al. (2015).

Lasa et al. (2019a) showed that different growth media influence the attractiveness of yeasts to SWD. A significant difference in the fecundity was observed between yeast growing media with higher oviposition on PDA compared to MEA. In the fecundity assay on PDA, the three *H. uvarum* strains and S.v. 1.33 had positive influences on fecundity, while I.t. 2.1, M.p. 3.2 and C.sp. 3.3 had negative effects. A CAFE assay with the PDB growth medium was performed that included the model organism *Saccharomyces cerevisiae* strain S288c. The three *H. uvarum* strains and S.v. 1.33 led to a higher ingestion and lower mortality of SWD females compared to other yeasts. Interestingly, *S. vini* has rarely been mentioned as being associated with SWD (Bellutti et al. 2018). The results show that *S. vini* potentially represents a yeast with positive effects for SWD, recommending it for further studies. *Hanseniaspora uvarum* is attractive for SWD adults (Scheidler et al. 2015) and larvae (Lewis and Hamby 2019). Based on the

results of this study, SWD adult females also benefit from *H. uvarum* in their diet. Lewis and Hamby (2019) showed that larvae reared on *H. uvarum* reached a smaller adult body size compared to adults reared on diets prepared with *S. cerevisiae*, *P. kluyveri* or *I. terricola*. The reason may be different dietary requirements of larvae and adults.

To exclude the possible toxic effects of alcohol produced during the fermentation process, the alcohol content was measured in fermentation broths. Values ranged from a minimum of 0.54 vol % in S.v. 1.33 to a maximum of 1.01 vol % in S.c. S288c. The LD50 of *Drosophila* flies is generally greater than 1 vol % (Merçot et al. 1994; Chakir et al. 1993), and an influence on the results is therefore not likely.

Based on the availability of appropriate carbon, ammonium and nitrogen sources as well as the presence of specific amino acids in the extracellular environment, yeasts regulate their metabolism and growth (Ljungdahl and Daignan-Fornier 2012). Studies about the use of yeasts in control strategies are based on different methods, such as washing the cells from Petri dishes with different sugar-containing liquids (Noble et a. 2019), revitalizing active dry yeast with sugar solution (Knight et al. 2015; Roubos et al. 2019) and often adjusting the cell number (Mori et al. 2016). Since nutrients present in the substrate influence yeast metabolism and therefore the availability of nutrients for the SWD in a yeast-based diet, a microbiological cost-effective commercial medium (PDB) was chosen, and both intra- and extracellular compounds were analyzed separately.

With few exceptions, the concentrations of extracellular compounds were considerably increased compared with the concentration of intracellular compounds. Overall nutrient consumption or secretion of products of yeast metabolism and fermentation was observed comparing PDB and fermentation broths. The fact that PDB is rich in the nutrients necessary for the development of SWD, such as carbohydrates and amino acids (Markow and O'Grady 2008; Tochen et al. 2016), explains its effectiveness as a food source as demonstrated in the results of the CAFE assay, even without the addition of yeast. The supply of a suitable energy source, such as carbohydrates, increases the appetite of SWD flies (Biolchini et al. 2017), and dietary glucose modulates appetite in *Drosophila* flies (Lebreton et al. 2014). Therefore, the availability of glucose in the diet of SWD is associated with the ingestion. Increased understanding of adult nutrient requirements and nutritional behavior can improve management of SWD (Mori et al. 2016; Tochen et al. 2016). Cowles et al. (2015) demonstrated how the addition of carbohydrates (sucrose) to insecticides targeting SWD enhanced lethality in field tests by increasing the food intake by SWD flies. The yeasts C.sp. 3.3 and S.c. S288c consume almost all available carbohydrates (glucose). The low ingestion of these two yeast fermentation broths in the CAFE assay could be due to the reduced amount of carbohydrates available for SWD compared to other yeasts and PDB. As a result of yeast metabolism, compounds, such as sugar alcohols and organic acids, are produced and secreted by microorganisms (Kayingo et al. 2001; Ljungdahl and Daignan-Fornier 2012). Some of these compounds, such as acetic acid, may affect the production of aromatic compounds, influencing the attractiveness to *Drosophila* (Erasmus et al. 2004; Vilela-Moura et al. 2011, Hamby and Becher, 2016). Ten amino acids (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and arginine) are essential for *Drosophila* flies (Sang and King 1961). Previous studies on *Drosophila* demonstrated how different amino acids can produce different appetitive larval responses, and no correlations with the essential/nonessential dietary requirements for amino acids or their chemical properties were observed (Croset et al. 2016). In contrast, other studies demonstrated that essential amino acid imbalances influence the larval food intake in *Drosophila* (Bjordal et al. 2014). Grandison et al. (2009) also associated the addition of essential amino acids to a dietary restriction diet of *Drosophila* with increased fecundity and decreased lifespan. On the other hand, little is known about the effect of essential and non-essential amino acids on the diet of SWD. In the present study, *H. uvarum* strains contain generally increased abundance of essential amino acids compared with other yeasts, while some yeasts consume specific essential amino acids. The yeast I.t. 2.1 consumes arginine, histidine and lysine, whereas a deficiency in extracellular arginine, histidine and methionine was found in S.c. S288c and C.sp. 3.3. Although yeasts are known to be a protein source in the diet of *Drosophila* (Bing et al. 2018; Camargo and Phaff 1957; Phaff et al. 1956; Steck et al. 2018; Yamada et al. 2015), a high mortality rate was found after four days in SWD fed with C.sp. 3.3 or S.c. S288c, which suggests that the lack of specific free essential amino acids can influence the fitness of the insect. Methionine, an essential amino acid known for its influence on fecundity and lifespan of *Drosophila* (Grandison et al. 2009; Lee et al. 2014; Schutz 2008), was consumed by the two yeasts species (C.sp. 3.3 and S.c. 288) associated with a high mortality in SWD. Glutamic acid was the most abundant amino acid in both PDB and fermentation broths. Although this amino acid is not essential in the *Drosophila* diet, previous studies demonstrated that three amino acidic compounds, glutamic acid, alanine and aspartic acid stimulate food consumption in *Drosophila* (Yang et al. 2018). *Hanseniaspora uvarum* consumed a smaller amount of important nutrients, such as carbohydrates and amino acids, from the culture medium. This finding could explain why increased ingestion by SWD fed with *H. uvarum* was observed. The highest mortality in the CAFE assay was found in SWD females fed

C.sp. 3.3 or S.c. S288c, and yeasts consumed most of the glucose and specific amino acids. Overall, amino acids and the glucose supply seem to be related to the promotion of ingestion and reduction of the mortality. On the other hand, glycerol appears to be associated with a lower survival rate in SWD. This compound also influences physiological and behavioral feeding responses in *Drosophila* (Koseki et al. 2004; Wisotsky et al. 2011). In addition, some nonnutritive sugar alcohol sweeteners, such as erythritol, show potential as a human-safe insecticide against SWD given their possible toxicity (Choi et al. 2017; Sampson et al. 2017). However, glycerol had a minimal effect on fly mortality (Díaz-Fleischer et al. 2019). Although not all compounds that were assessed were related with SWD, these data provide insight in the composition of naturally occurring yeasts grown in an artificial growth medium.

The biology of SWD makes it difficult to develop an effective control strategy (Cini et al. 2012; Sial et al. 2019) to avoid severe economic losses (De Ros et al. 2013). As a control strategy against SWD, suitable yeasts can be used as adjuvants in insecticide sprays to increase ingestion in attract-and-kill formulations based on the association of attractant yeasts and an insecticide (Andreazza et al. 2017; Knight et al. 2015; Noble et al. 2019; Roubos et al. 2019). To date, insecticides were registered against SWD, and technical strategies, such as exclusion netting, were applied to control this pest (Beers et al. 2011; Leach et al. 2016, Sial et al. 2019). Monitoring and control strategies based on acetic bacteria and yeast volatiles were developed (Lasa et al. 2019b; Cha et al. 2013; Iglesias et al. Iglesias et al. 2014). For example, *H. uvarum* is known to be attractive for SWD flies (Scheidler et al. 2015) and can increase ingestion compared to growth medium without yeast (Mori et al. 2016). Normally, insects are attracted to specific volatile compounds associated with a food source (Davis et al. 2013). Given its attractiveness, yeast could prove more suitable than odorless adjuvants, such as sucrose, which has already shown to increase the effectiveness of insecticides against SWD (Cowles et al. 2015). For example, *H. uvarum* is attractive for SWD flies (Scheidler et al. 2015) and increases ingestion compared to growth medium without yeast (Mori et al. 2016). This finding should be considered in view of development of attract-and-kill control strategies.

The findings of the present work are useful for understanding the differences in the fecundity, ingestion and mortality of SWD adults among yeast strains, providing relevant information for the development of attract-and-kill control strategies against SWD. Additionally, the chemical characterization of yeast-based food provides insights concerning potentially phagostimulant components that may be exploited for pest management, indicating that not only the aromatic compounds but also nonvolatile metabolites of the yeast play a role in the association between yeast and SWD.

Author contributions

US and SS performed the entomological assays. FB and DE performed the chemical analyses. US, SS, IC, EHK and SA designed the entomological research. FB, DE, PR and RFV designed the chemical research. FB and US analyzed the yeast broths and wrote the manuscript. ND contributed to statistical analyses. All authors reviewed and approved the final version of the manuscript and contributed to the interpretation of the data.

Acknowledgements The authors thank the Department of Innovation, Research and University of the Autonomous Province of Bozen/Bolzano for covering the Open Access publication costs. We also thank Christof Sanoll from the Laboratory for Wine and Beverages Analytics, Laimburg Research Centre for the analyses of the alcohol contents and Paul G. Becher and Guillermo Rehermann from the Swedish University of Agricultural Sciences for their helpful comments to the manuscript. This work is part of the project DROMYTAL (FESR1021, CUP H32F16000420009). The project DROMYTAL was funded by the European Regional Development Fund (ERDF) 2014-2020. ND was supported by the project "Incoming Researcher" (decree 334, 16.01.2019) of the Department of Innovation, Research and University of the Autonomous Province of Bozen/Bolzano.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard Ethical standards of institutional, national and international guidelines were considered and followed.

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Persistence of a Yeast-Based (*Hanseniaspora uvarum*) Attract-and-Kill Formulation against *Drosophila suzukii* on Grape Leaves

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Received: 23 October 2020; Accepted: 16 November 2020; Published: 18 November 2020

Simple Summary: *Drosophila suzukii* is an invasive pest species that feeds on yeast-laden fruits and is attracted to fermentation products. In nature, numerous yeast species are associated with *Drosophila suzukii*. Yeasts constitute a food source and produce volatile compounds attractive to the fly. The production of attractants and chemical compounds that stimulate feeding by *Drosophila suzukii* make the use of yeasts promising for the development of attract-and-kill formulations. In the present work, the efficacy and the persistence over a one-week period of a yeast-based attract-and-kill formulation was evaluated treating grape plants in a greenhouse. The efficacy was assessed by measuring the survival and oviposition rate of *Drosophila suzukii*. The concentrations or presence/absence of potential feeding stimulants and attractants were assessed by quantitative measurement of carbohydrates, sugar alcohols, amino acids, and volatile compounds. Results show that the formulation was still effective and that some of the chemical compounds monitored were still present on the surface of treated leaves one week after treatment, though changes in the chemical profiles were observed over this period.

Abstract: The production of phagostimulant and attractive volatile organic compounds (VOCs) by yeasts can be exploited to improve the efficacy of attract-and-kill formulations against the spotted wing drosophila (SWD). This study evaluated the persistence over one week of a yeast-based formulation under greenhouse conditions. Potted grape plants were treated with: (i) potato dextrose broth (PDB), (ii) PDB containing spinosad (PDB + S), and (iii) *H. uvarum* fermentation broth grown on PDB containing spinosad (H. u. + S). Laboratory trials were performed to determine the survival and the oviposition rate of SWD after exposure to treated leaves. Ion-exchange chromatography was performed to measure carbohydrates, sugar alcohols, and organic acids on leaf surfaces, while amino acids were assessed through liquid chromatography–mass-spectrometry. Additionally, the VOCs released by plants treated with *H.uvarum* were collected via closed-loop-stripping analysis and compared to those emitted by untreated leaves. A higher mortality was observed for adult SWDs

in contact with *H. uvarum* containing spinosad compared to PDB containing spinosad. Generally, a decrease in the amounts of non-volatile compounds was observed over time, though numerous nutrients were still present one week after treatment. The application of the yeast-based formulation induced the emission of VOCs by the treated leaves. The concentration of 2-phenylethanol, one of the main VOCs emitted by yeasts, decreased over time. These findings describe the presence of potential phagostimulants and compounds attractive to SWD in a yeast-based attract-and-kill formulation and demonstrate the efficacy of the formulation over one week.

Keywords: spotted wing drosophila; pest control; spinosad; metabolites; VOCs

1. Introduction

The spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura) is an important insect pest with a wide host range, including small soft fruit, stone fruit, and grapes [1,2]. The control of SWD in fruit cultivation relies usually on sprays of synthetic insecticides to reduce yield losses associated to SWD infestations [3]. Unfortunately, most insecticides are not selective and are facing severe limitations regarding pesticide residues in worldwide exports [4]. Yeasts were found to enhance the efficacy of insecticide treatments leading to a lower amount of insecticide needed to achieve a sufficient protection against the pest [5,6]. The possibility to exploit nutritional behavior and attractiveness induced by yeasts associated to SWD makes the use of these microorganisms a promising strategy for controlling SWD infestations [6–10]. One limit concerning the use of live microorganisms for control strategies is related to the microbial metabolic changes that occur in response to nutritional sources available in the medium [11]. Moreover, changes in the medium composition that occur during yeast growth and fermentation are reflected both in the loss of potentially phagostimulant compounds for SWD flies [12,13] and in modifications of the profile of volatile compounds [13,14], affecting the attractiveness to SWD [15]. For the development of an efficient attract-and-kill formulation, it is, therefore, important not only to select the appropriate species and growth medium that enhance the feeding stimulation and the attractiveness but also to ensure that the efficacy is maintained over time.

The present study aims at evaluating the persistence over time of a yeast-based attract-and-kill liquid formulation on grape plants in a greenhouse. *Hanseniaspora uvarum* was selected for the study because, among the numerous yeast species isolated from fruits infested by SWD, *Hanseniaspora* was reported as the predominant genus [16–19]. Additionally, *H. uvarum* was found to be more attractive to SWD adults and larvae in choice tests compared to other yeast species [20,21], as well as to promote the ingestion by SWD adults [12]. For the attract-and-kill formulation, spinosad was chosen as insecticide since it is allowed in viticulture, as well as in organic production, and is known to be very effective in toxic baits [6,10,22]. Grapevine leaves were treated with the yeast-based attract-and-kill formulation in a greenhouse. Mortality and oviposition assays were performed in the laboratory to evaluate the effects of the treatments on SWD adults over a one-week period. The persistence of yeast's metabolites and nutritional compounds on the leaves' surfaces, as well as volatile organic compounds (VOCs) emitted by the plants, were measured to evaluate their changes over time.

2. Materials and Methods

2.1. Insect Rearing

The SWD flies originated from different fruits in South Tyrol, Italy. The SWD flies were reared in a mass rearing on *Drosophila suzukii* cornmeal diet (DSCD(a) with dry deactivated yeast) and dry baker's yeast (RUF Lebensmittelwerk KG, Quakenbrück, Germany) sprinkled on the surface on which they fed and laid the eggs [18]. The rearing contained 5% sucrose solution on cotton as additional sugar and water source. The SWD larvae developed on the cornmeal diet. Female and male SWD adults

emerging from the pupal stage within three days were kept on cornmeal diet and sucrose solution in an insect cage (BugDorm—1, MegaView Science Co., Ltd., Taichung, Taiwan). When all flies were between five and eight days old, 20 female and 20 male SWD flies were placed together into one insect cage. Males were distinguished from females by the dark spot on the leading edge near the tip of each wing [1]. The insect cages were kept in climatic chambers at 22 ± 1 °C, with 65 ± 5% relative humidity and a photoperiod of L16:D8.

2.2. Yeast Cultivation

The yeast *Hanseniaspora uvarum* (strain: LB-NB-2.2, accession number GenBank NCBI: MK567898) was isolated from feeding tunnels of SWD larvae in infested grapes in South Tyrol, Italy [18]. Yeast cultures were grown under sterile conditions in 220 mL autoclaved potato dextrose broth (PDB; 4 g/L potato starch, 20 g/L dextrose, Difco™, Becton Dickinson, Le Pont de Claix, France) at 25 °C, 120 rpm for 30 h under light in a 250-mL Erlenmeyer flask closed with cotton and aluminum foil. The inoculum (0.5 mL) was prepared with a loop full of yeast cells cultivated on potato dextrose agar (4 g/L potato starch, 20 g/L dextrose, 15 g/L agar, Difco™, Becton Dickinson), which were transferred in a 2-mL Eppendorf tube filled with 1 mL PDB and vortexed for 10 s at 1800 rpm. After 30 h of growth, the yeasts reached the stationary phase. The number of cells per mL (Fuchs Rosenthal counting chamber, Assistent®, Sondheim vor der Rhön, Germany) was 6.4×10^7, optical density (OD) at 600 nm (Cary 60 UV-Vis, Agilent, Santa Clara, CA, United States) was 1.8, and the pH (pH meter, Crison GLP 21, Hach, Düsseldorf, Germany) of the fermentation broth was 4.13. The yeast fermentation broths and autoclaved PDB were stored at −80 °C and thawed at room temperature before use.

2.3. Grape Plants Cultivation

Rooted grafted vines of the local variety "Edelvernatsch Lb 43" on rootstock SO4 were potted in 4-L pots filled with standard soil (SP ED63 T coarsely, Einheitserde®, Sinntal-Altengronau, Germany). The plants were grown for two months in the greenhouse and treated once a week for 20 min against powdery mildew with vaporized sulfur using a sulfur burner. No sulfur treatments were performed during the experimental period. The temperature and the relative humidity in the greenhouse were monitored over the experimental period (Table S1).

2.4. Treatments

A summary of the experimental design is reported in Figure 1. Three different treatments were performed on grape leaves in the greenhouse for further laboratory trials of mortality and oviposition and for the analyses of non-volatile compounds: (i) insecticide-free PDB (PDB), (ii) insecticide-containing PDB (PDB + S), and (iii) insecticide-containing *H. uvarum* fermentation broth (H. u. + S). For the insecticide-containing samples, 11.32 µL/L Laser™ (480 g/L spinosad, Corteva Agriscience™, Milan, Italy) were added to *H. uvarum* fermentation broth or PDB with a resulting active ingredient (AI) of 5.43 mg spinosad per L. Each of the three different treatments were applied to ten plants at the same time. Ten leaves per plant were treated with 10 drops of 10 µL each using a multichannel pipette (Eppendorf Research Plus, Hamburg, Germany). Leaves belonging to five plants per treatment were ripped off one day after treatment (T1), while leaves belonging to other five plants were ripped off one week after treatment (T2). All treated leaves belonging to each of the five plants per timepoint were ripped off and used for further SWD assays and chemical analyses. For mortality and oviposition assays and analyses of non-volatile compounds, the same plants were used (five leaves belonging to one plant were used for assays and five for chemical analyses). Single plants were considered as replicates.

Since the amount of 10 drops was not sufficient for the detection of VOCs, for volatiles collection a slightly different treatment was performed. Six plants were treated with *H. uvarum* (H. u.), and each plant was considered as a replicate. Five leaves belonging to one plant were treated with 500 µL per leaf using an airbrush (Hansa 681, Harder & Steenbeck, Norderstedt, Germany) to cover the upper

surface. The volatile collections were performed one day before treatment (T0—VOCs), as soon as the formulation dried on the leaves surface (ca. 30 min after treatment) (T1—VOCs) and five days after treatment (T2—VOCs). The VOCs emitted by six untreated plants were collected and considered as a control.

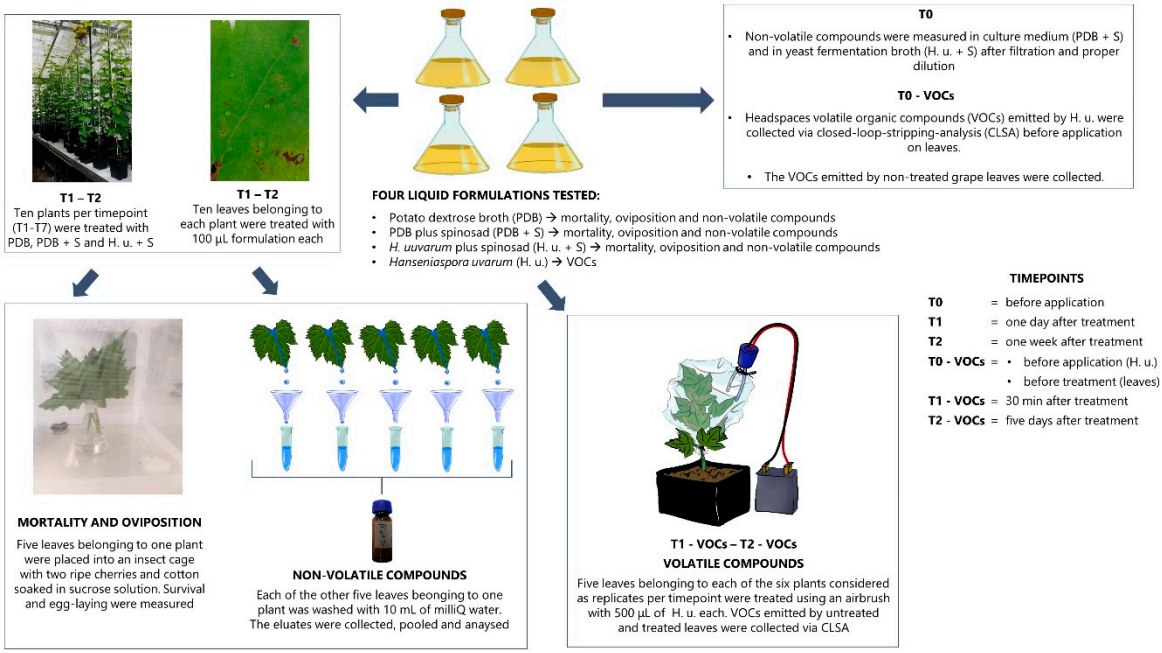

Figure 1. Experimental design of the treatments on potted grape plants in the greenhouse. A scheme of the assays and analyses performed is reported.

2.5. Mortality and Oviposition Assays

Five leaves belonging to the same plant were refreshed in an Erlenmeyer flask filled with tap water and closed with cotton to avoid the contact of the flies with water. The leaves were placed into the insect cage together with a small Petri dish (diameter 6 cm) containing cotton soaked in 10 mL of a 5% sucrose solution and two ripe cherries for oviposition. After 24 h and 48 h, the mortality of males and females was assessed, and the total number of eggs laid per cage was counted. The cherries were replaced by new cherries after 24 h. Single cages were used as replicates (n = 5). The insect cages were kept in climatic chambers at 22 ± 1 °C, with 65 ± 5% relative humidity and a photoperiod of L16:D8. The mortality was calculated as the total mortality over 24 h and 48 h and the oviposition as eggs laid during the first and the second 24 h.

2.6. Sample Preparation and Analysis of Chemical and Metabolic Compounds

For the analysis of metabolites, LC-MS grade solvents and reagents were used (VWR International Srl, Milan, Italy). Analytical standards of compounds under investigation were used for the quantitative analysis, and isotope-labeled internal standards (IS) of DL-Phenylalanine-3,3-d2, L-Lysine-4,4,5,5-d4 hydrochloride, L-Glutamic acid-2,3,3,4,4-d5, L-Alanine-2,3,3,3-d4 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were spiked into each sample for the analysis of amino acids.

Each leaf was washed with 10 mL of MilliQ water. The eluate from five leaves belonging to one plant were pooled and filtered (hydrophilic surfactant-free cellulose acetate filters, 0.2 µm). Single plants were used as replicates (n = 5). For carbohydrates and sugar alcohols, 1 mL of sample was transferred in a high performance liquid chromatography (HPLC) vial and analyzed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD, Dionex ICS 5000, Thermo Fisher, Waltham, MA, United States). For organic acids, 500 µL of sample were freeze dried, resuspended in 100 µL of MilliQ water and analyzed using high-performance anion-exchange

chromatography with conductivity detection (HPAE-CD, Dionex ICS 5000, Thermo Fisher, Waltham, MA, United States). For amino acids, 500 µL of sample were transferred in a HPLC vial containing 480 µL of acetonitrile and 20 µL of IS amino acids mix (50 mg/L). Samples were analyzed in liquid chromatography electrospray ionization triple quadrupole mass spectrometry (UHPLC-QqQ, Dionex UltiMate 3000 UHPLC TSQ Quantiva, Thermo Fisher, Waltham, MA, United States) in multiple reaction monitoring (MRM) mode. The liquid formulations were analyzed before their application on leaves (T0) after filtration and proper dilution. The analytical methods used were based on Spitaler et al. [12].

2.7. Volatile Compounds Collection and Characterization by CLSA-GC-MS

The VOCs were collected via closed-loop-stripping analysis (CLSA) and analyzed in gas chromatography–mass spectrometry (GC 7890A coupled with a MS 5975C Network, Agilent Technologies, Santa Clara, CA, USA). To reduce variation in chemical profiles due to the plant circadian rhythm, all collections were performed at a regular time between 12 p.m. and 3 p.m. The treated shoots were not covered during the five days between the first and the second collection. Untreated leaves were considered as a control. Plant materials were held in a VOC-bag (Cuki® oven bag, Cuki Cofresco S.r.l., Volpiano, Italy). Charcoal filtered air was pushed in via an inlet port at a rate of 500 mL/min while air was sucked out via an outlet port at a rate of 400 mL/min, creating a positive pressure in the bag for three hours. The airflow was maintained using a 12 V graphite vacuum pump (Fürgut, Tannheim, Germany) using Teflon tubes and ferrule connections. The outlet air passed through an adsorbent trap (glass tube, 6.5 × 0.55 × 0.26 cm) loaded with 1.5 mg activated charcoal (CLSA filter LR-type; Brechbühler AG, Schlieren, Switzerland). The VOCs were eluted from the adsorbent traps with 100 µL GC-grade dichloromethane (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and stored at −80 °C. Adsorbents were cleaned after each collection using three rinses with approximately 50 µL of HPLC-grade heptane, HPLC-grade methanol then GC-grade dichloromethane (all Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and baked 10 min at 160 °C. Two µL of extract were injected on a non-polar HP-5MS column (30 m × 0.25 mm ID, 0.25 µm film thickness, 7890A, Agilent Technologies, Santa Clara, CA, United States) in splitless mode when the inlet valve was at 280 °C. Helium was used as carrier gas at a flow rate of 1.2 mL/min and a velocity of 39.92 cm/s. The starting temperature of 50 °C was held for 1.5 min, followed by an increase of 7.5 °C/min until a temperature of 250 °C was reached and then held for 10 min.

VOCs emitted by the yeast fermentation broth were also collected via CLSA before their application on leaves (T0—VOCs). A double airflow pump system was used: charcoal-filtered air was pushed at rate of 1 L/min into a 250-mL Pyrex glass bottle containing 100 mL of yeast sample; simultaneously, CLSA filters (1.5 mg activated charcoal, LR-type, Brechbühler AG, Schlieren, Switzerland) fitted into the plastic lid of the glass bottle were connected to the outflow pump using a short Teflon tube, drawing out air at a rate of 0.4 L/min. The CLSA filters were then eluted with 100 µL of GC-grade dichloromethane solvent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in 1.1-mL GC glass vials (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and stored in a freezer at −80 °C until use for subsequent GC-MS analysis.

The chromatogram was recorded in the full scan mode m/z 20–400 amu, and the electron ionization was set at 70 eV and the ion source temperature at 250 °C. Data acquisition and analysis were carried out using ChemStation software (Agilent Technologies, Santa Clara, CA, United States). A commercially available mixture of n-alkane standards (nC8-nC40, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used to calculate the linear retention indices (LRI) [23]. Compounds were annotated initially by comparing their mass spectra with those in the databases NIST 14 (Gaithersburg, MD, USA) and Wiley7 (Wiley, Hoboken, NJ, USA). The identity of all compounds, with the exception of 1,8-cineole and trans-alpha-bergamotene, was confirmed by comparison with reference standards (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (Table 1).

2.8. Statistical Analysis

Statistical analyses were performed using the software R [24]. The mortality and oviposition data were analyzed with a one-way ANOVA. The equality of error variance was verified with a Levene's test. Multiple comparisons were performed with Bonferroni's procedure. To evaluate the variation of the concentration of each metabolite over time, a one-way ANOVA was applied using Tukey's post hoc test for pairwise comparison. Nonparametric tests (Wilcoxon statistic with Bonferroni's correction to adjust the significance level) were performed whenever at least one of the conditions to apply parametrical tests (normal distribution, variance homogeneity) was not satisfied. To evaluate if there was any significant difference between VOCs emitted by treated and non-treated leaves at two timepoints, all the compounds peak area means were compared using one-way ANOVA followed by post hoc Tukey's test. The distributions of data and residuals were verified graphically with the functions qqp and qplot from the R package ggplot2 [25]. Statistical significance was assessed at the level of $p < 0.05$.

3. Results and Discussion

3.1. Mortality and Oviposition Assessment One Day after Treatment (T1)

One day after treatment (T1), the mortality and the oviposition were affected by the different treatments (Figure 2). After 24 h ($F_{2, 7.673} = 35.481$, $p < 0.001$) and after 48 h ($F_{2, 12} = 122.00$, $p < 0.001$), a significant effect on the mortality of SWD flies was observed. Significantly more SWD died after exposure to H. u. + S compared with PDB or PDB + S ($p < 0.05$). This result confirms that the presence of yeast metabolites and VOCs is necessary to achieve a higher mortality rate compared to PDB + S. Within the first 24 h of exposure, the mortality of SWD flies in contact with H. u. + S lead to a 5.0-fold higher mortality compared to spinosad-free PDB. After 48 h of exposure, the treatment H. u. + S lead to a 4.3-fold higher mortality compared to PDB. Interestingly, no significant differences were observed between PDB and PDB + S ($p < 0.05$). This was surprising, since PDB contains glucose and sugars which were described as feeding stimulants for SWD in baits in combination with insecticides [7,26]. However, the absence of attractive fermentation products in PDB may prevent SWD flies from finding the insecticidal bait on the leaves. A similar ingestion by SWD females when fed with PDB and *H. uvarum* was observed in a previous study, where attractiveness did not play any role [12]. In this study, the flies had the possibility to feed on an additional sucrose solution in the cage and to move around in larger experimental cages. Therefore, based on the experimental setting, the flies could avoid contact with the insecticidal bait, meaning that emitted volatiles together with feeding stimulant compounds present in the yeast fermentation broth enhance the efficacy of the bait.

After 24 h of exposure to the treated leaves, the different formulations did not influence the oviposition ($F_{2, 12} = 0.158$, $p = 0.855$). Females of SWD oviposit eggs before reaching 5 days post-closure [27], and, when the fecund SWD flies entered the assay, some females immediately reached the fruits before getting in contact with the leaves. It was already shown that the mating status of SWD females influences the preference between fruits and fermentation volatiles. Seven-day-old mated females prefer strawberries, while virgin females prefer apple cider vinegar [28]. In the field, SWD females collected on fruit have more mature eggs in the ovaries than those collected in traps with fermentation baits [29]. The flies entered the assays five days after hatching and were already able to lay eggs. Therefore, it can be assumed that the females were able to lay eggs into the fruits present in the cages and that VOCs emitted by the fruits were attractive for the females before they get attracted by the VOCs emitted by yeasts. During the first 24 h, the females had time to get in contact with the bait. Therefore, a significant effect of the treatment became visible after the first 24 h ($F_{2, 12} = 9.621$, $p = 0.003$). Since oviposition was counted as the number of eggs laid per cage, these values reflect the influence of female mortality. A higher oviposition was observed comparing PDB treatment with PDB + S or H. u. + S ($p = 0.05$). No differences were observed between H. u. + S and PDB + S ($p < 0.05$). Even though there was no significant effect on the mortality comparing PDB and PDB + S, the lower

oviposition caused by spinosad in the PDB + S treatment compared to PDB could have been due to the contact of some flies with a sublethal dose of spinosad (Figure 2).

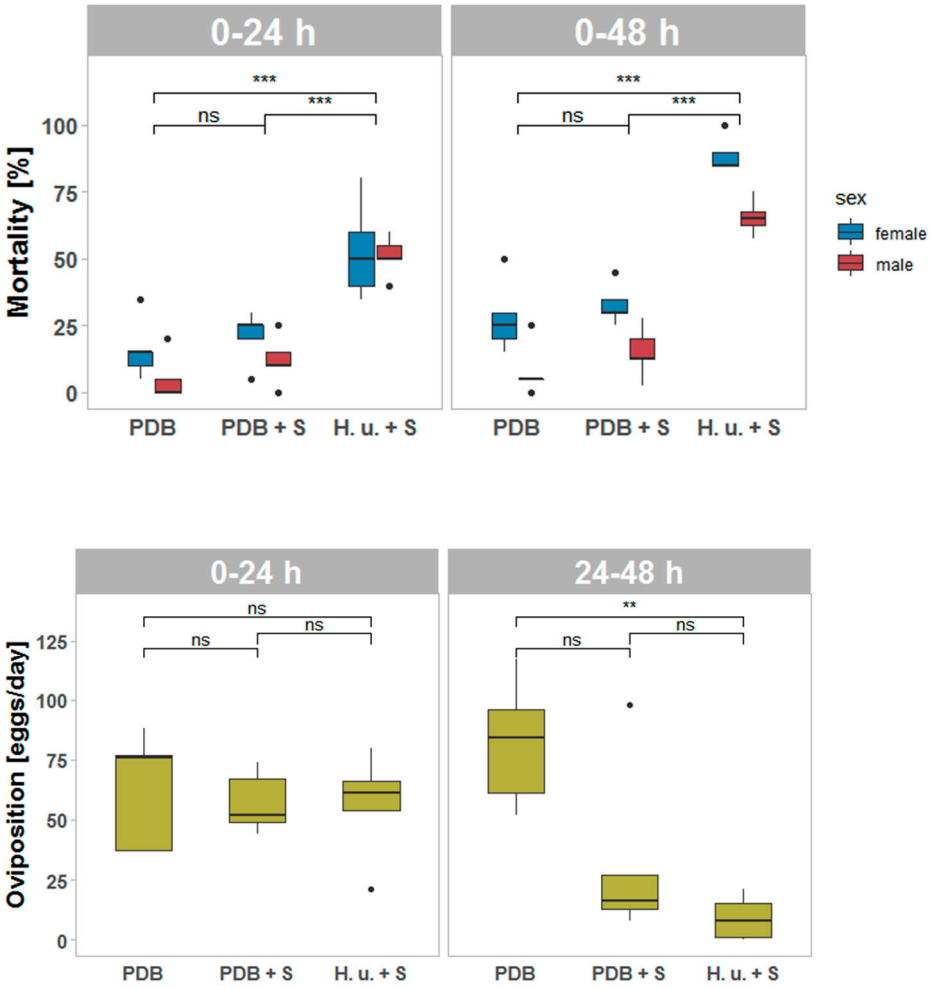

Figure 2. Mortality and oviposition (n = 5) of spotted wing drosophila (SWD) adults (20 males and 20 females) after the exposure to leaves treated with potato dextrose broth (PDB), PDB plus spinosad (PDB + S), or *H. uvarum* plus spinosad (H. u. + S) one day after treatment (T1). Mortality of males and females after 24 h and after 48 h of exposure. Oviposition after the first 24 h and between 24 and 48 h of exposure. Asterisks indicate significant differences between the treatments ($p < 0.05$). Not significant differences are reported, as well (ns). Outliers are indicated with dots. ≤0.01 = **; ≤0.001 = ***.

3.2. Mortality and Oviposition Assessment One Week after Treatment (T2)

The same methodology used for T1 was used for T2 to evaluate the effects after one week (Figure 3). A significant effect of the formulations on the mortality after 24 h ($F_{2, 6.190} = 694.376$, $p < 0.001$) and after 48 h ($F_{2, 7.151} = 131.912$, $p < 0.001$) of exposure to the treated leaves was observed. As observed at T1, there was also no significant difference in the mortality comparing flies exposed to PDB and PDB + S ($p < 0.05$) at T2, while H. u. + S lead to a significantly higher mortality compared to the previous two treatments ($p < 0.05$). No differences were observed in the number of eggs laid in the first 24 h ($F_{2, 12} = 3.239$, $p = 0.075$) as for T1. Over the second 24 h of exposure, the influence of the formulations on the oviposition was significant ($F_{2, 12} = 26.609$, $p < 0.001$). Lower oviposition was observed with the treatment H. u. + S compared to PDB or PDB + S ($p < 0.05$).

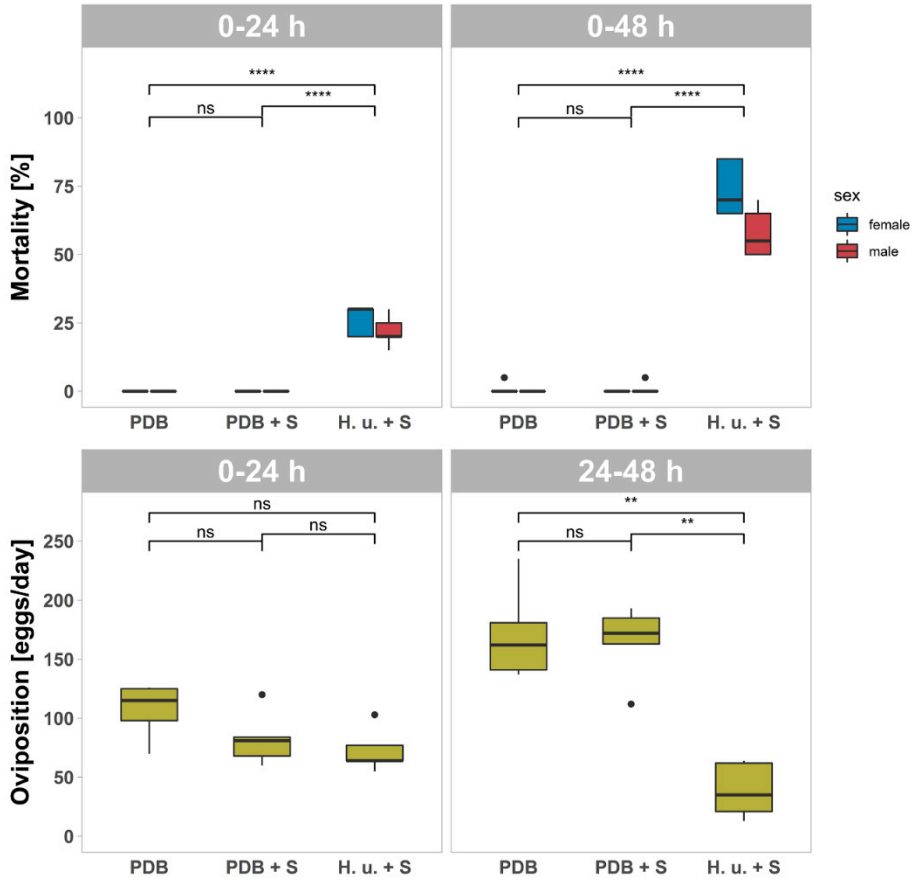

Figure 3. Mortality and Oviposition (±SD; $n = 5$) of SWD adults (20 males and 20 females) after the exposure to leaves treated with PDB, PDB plus spinosad (PDB + S), or *H. uvarum* plus spinosad (H. u. + S) one week after treatment (T2). Mortality of males and females after 24 h and after 48 h of exposure. Oviposition after the first 24 h and between 24 and 48 h of exposure. Asterisks indicate significant differences between the treatments ($p < 0.05$). Not significant differences are reported, as well (ns). Outliers are indicated with dots. ≤0.01 = **; ≤0.0001 = ****.

3.3. Carbohydrates and Sugar Alcohols

Two carbohydrates (glucose and trehalose) and two sugar alcohols (glycerol and arabitol) were found in the analyzed samples. The amount of carbohydrates and sugar alcohols in culture and fermentation broths (T0), as well as on leaves treated with PDB + S and H. u. + S (T1 and T2), are reported in Figure 4. The concentrations and the trend over time of the chemical compounds analyzed on leaves treated with insecticide-free PDB were very similar to those of leaves treated with PDB + S; therefore, the results of insecticide-free PDB treatment were not reported in the figures. Yeasts consumed glucose and produced sugar alcohols and trehalose. The concentration of glucose on the leaves treated with PDB + S and H. u. + S significantly decreased over time ($p < 0.05$). This reduction in the sugar's concentration, more evident in PDB + S but significant for both PDB + S and H. u. + S, may be a result of its biodegradation by epiphytic microorganisms populating the leaves' surface. As for glucose, the concentrations of trehalose and sugar alcohols significantly decreased over time ($p < 0.05$), except for glycerol in PDB + S (Figure 4). Exogenous trehalose is a carbon source for bacteria [30]; thus, biodegradation of this compound can occur. Lactic acid bacteria populate the grape surface, and many of them are able to use diverse sugars and sugar alcohols as substrate [31]. The rapid biodegradation, coupled with a photodegradation half-life of 6.8 h [32], explains the rapid reduction of the concentration of glycerol on the leaf surface within one day of exposure to light.

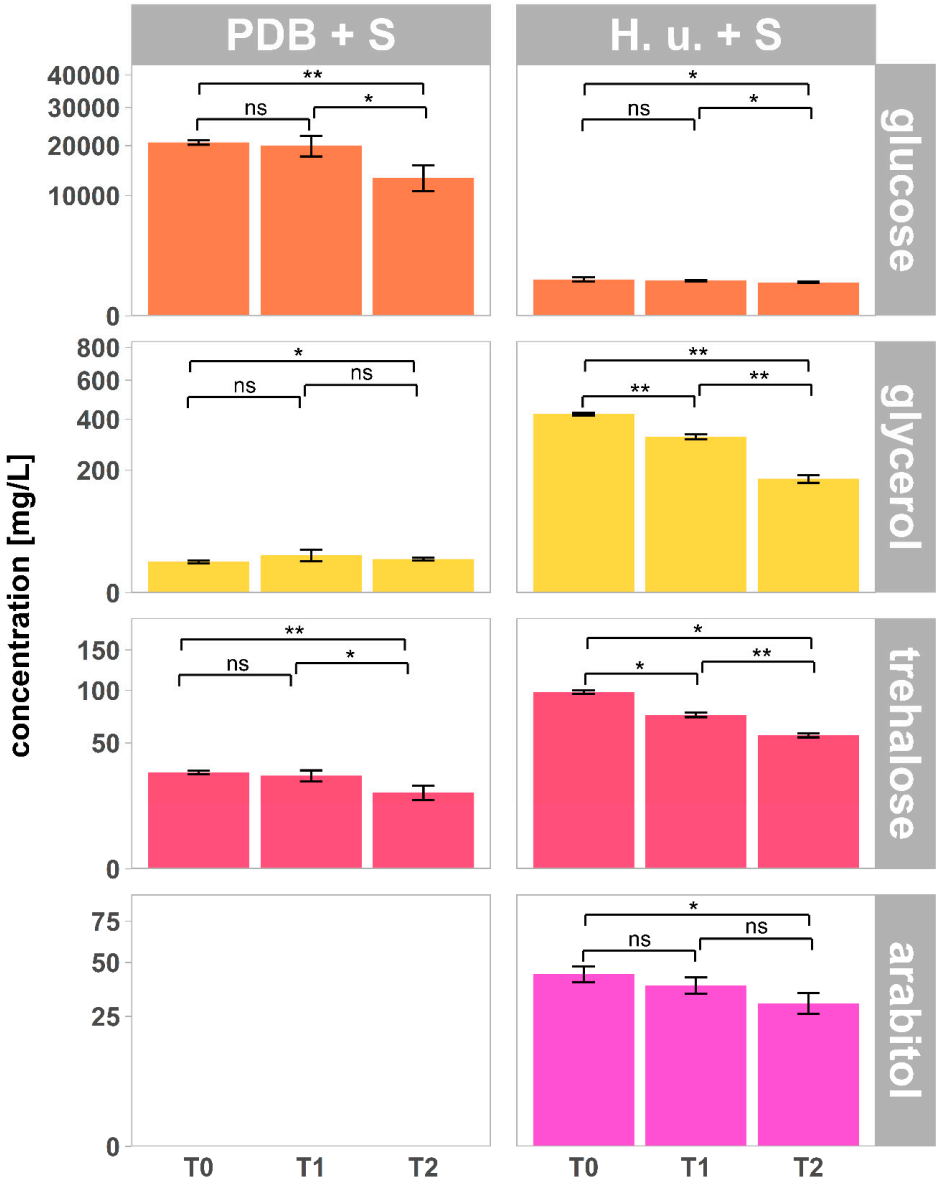

Figure 4. Concentration of glucose and sugar alcohols (mean ± SD; $n = 5$) in the culture broth PDB + S (T0), in the fermentation broth H. u. + S (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Arabitol was not detected at any of the timepoints in PDB + S. Asterisks indicate significant differences between timepoints for each treatment ($p < 0.05$). Not significant differences are reported, as well (ns). <0.05 = *; ≤0.01 = **.

Although a large amount of glucose was consumed by yeasts within 30 h of growth prior to the treatments, carbon sources suitable for SWD were still available one week after treatment. Concentrations of 173 mg/L glycerol, 30 mg/L arabitol, 56 mg/L trehalose, and 777 mg/L glucose were found on the surface of leaves treated with H. u. + S. Carbohydrates are known feeding stimulants in SWD flies [33]. Additionally, *Drosophila* flies possess gustatory receptors for trehalose [34], and this compound was found to elicit a response of sugar neurons [35]. Behavioral assays based on the proboscis extension reflex demonstrated that *Drosophila* flies extend proboscis to feed on glucose, trehalose, and glycerol [36], with the gene Gr64e conferring responsiveness to glycerol in *Drosophila* [37,38]. The lower amount of glucose in the yeast fermentation broth compared to PDB may, therefore, not necessarily be a limiting factor for the feeding acceptance of the attract-and-kill formulation by SWD, as long as it is coupled with the presence of sugar alcohols.

3.4. Amino Acids

Overall, 17 amino acids could be measured in all samples (Figure 5). The highest concentrations were found in PDB + S, whereas yeasts consumed amino acids (H. u. + S). As for carbohydrates and sugar alcohols, as soon as the formulation was applied on leaves surface, the concentrations of these compounds started to decrease over time in most of the cases, confirming, as discussed above, that biodegradation probably occurs. Few exceptions were found: the concentrations at the three timepoints of tyrosine in H. u. + S ($p = 0.879$), as well as those of glycine ($p = 0.078$), serine ($p = 0.293$), threonine ($p = 0.085$), and tyrosine ($p = 0.486$), in PDB + S did not change significantly. For glutamine, it was difficult to evaluate differences over time, since the concentrations in samples were found to be extremely variable among replicates, with values increasing over time in the case of H. u. + S. According to Spitaler et al. [12], glutamic acid was the most abundant amino acid present in PDB, as well as in yeast fermentation broth. Concentrations of 143 mg/L and of 255 mg/L of glutamic acids were found in H. u. + S fermentation broth and in PDB + S culture broth, respectively. This compound was reported as phagostimulant for *Drosophila* flies [39]. The availability of glutamic acid on leaves' surface up to seven days after treatment can be associated to the stimulation of the ingestion of the formulation by SWD. Although a reduction or loss over time of some essential amino acids for SWD, including methionine, threonine, and tryptophan, was observed in H. u. + S, the yeast-based attract-and-kill formulation was found to have a strong effect on SWD survival up to one week after treatment. This may indicate either that the low amount of these compounds does not influence the feeding acceptance of a food source for SWD flies, or that their lack is compensated by the biosynthesis of macromolecules by yeasts.

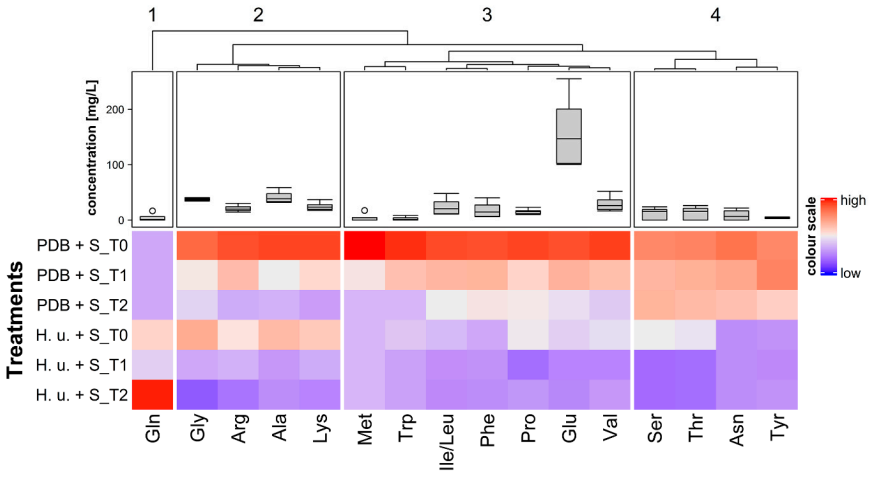

Figure 5. Heatmap of the concentration of amino acids in PDB + S and H. u. + S fermentation broth (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Three letter codes were used to indicate amino acids. Boxplots show the concentration of amino acids over all six treatments. Clustering of the amino acids was performed using Ward method with Euclidian distance, and the split was based on the k-means algorithm (k = 4).

3.5. Organic Acids

The concentrations of seven organic acids in the samples analyzed are reported in Figure 6. As expected, the amounts at T0 of succinate, acetate, pyruvate, and malate, which are products of the yeast metabolism, were much higher in H. u. + S compared to PDB + S. The concentrations of citrate and formate at T0 were instead similar between the two. Citrate was the most abundant organic acid with concentrations of 328 mg/L and 334 mg/L in *H. uvarum* fermentation broth and PDB culture broth, respectively. As sugar alcohols, these compounds constitute a product of the yeast metabolism. A decreasing trend in the concentrations of most of the organic acids was observed, with few exceptions. Time had no influence on malate in H. u. + S ($p = 0.264$) and on formate ($p = 0.170$)

and acetate ($p = 0.403$) in PDB + S. The decrease or increase of the concentrations of organic acids can again be a result of the biodegradation. In addition, based on the chemical characteristics of acetate, it is expected that, after drying on the leaf surface, this compound would be mainly present as a vapor in the ambient atmosphere [32], as confirmed by the highly significant reduction of its concentration at T1 in H. u. + S. The high volatility of formate too [40] resulted in a reduction of its concentration on the surface of leaves already one day after treatment with H. u. + S. The rapid degradation of pyruvate already at T1 after treatment with H. u. + S can be explained as a result of the photolysis in presence of sunlight [41].

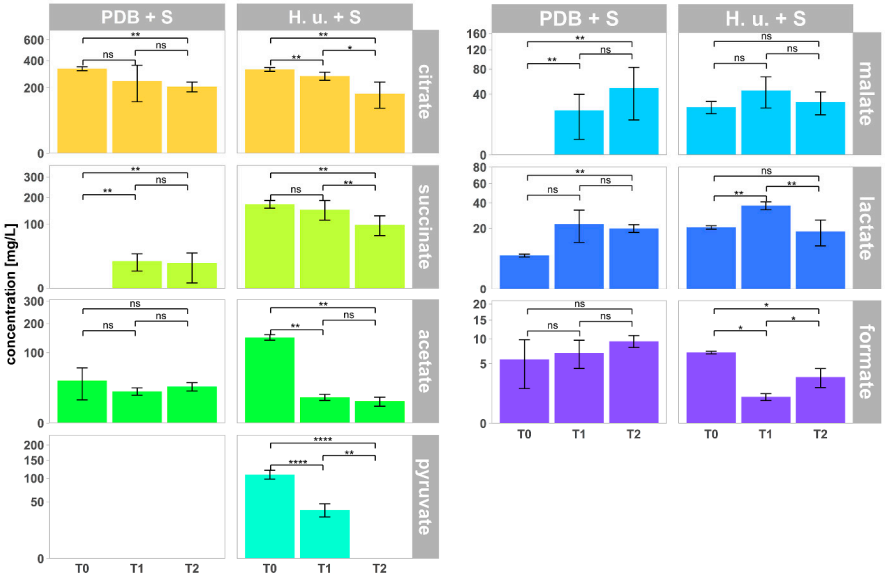

Figure 6. Concentration of organic acids (mean ± SD; $n = 5$) in the culture broth PDB + S (T0), in the fermentation broth H. u. + S (T0) and on the surface of leaves treated with PDB + S and H. u. + S collected one day (T1) and one week (T2) after treatment. Pyruvate was not detected at any of the timepoints in PDB + S. Succinate and malate were not detected at T0 in PDB + S. Asterisks indicate significant differences between timepoints for each treatment ($p < 0.05$). Not significant differences are reported, as well (ns). <0.05 = *; ≤0.01 = **; ≤0.0001 = ****.

Previous studies showed that *Drosophila* flies tend to reject too acidic food and to have adverse responses to carboxylic acids, including acetic acid and citric acid [42–44]. In addition, sweet perceiving neurons were found to be inhibited by acid taste. However, an increase in sugar concentration allowed to overcome food rejection [42]. Therefore, an appropriate combination of sugar and acid concentrations is crucial to favor the acceptance of a food source by *Drosophila* flies.

3.6. VOCs

The VOCs composition of headspaces from *H. uvarum* culture and *H. uvarum*-treated grapevine leaves was assessed (Figure 7 and Table 1). The yeast culture alone released mostly benzaldehyde and 2-phenylethanol. The latter was also found in the headspace of grapevine leaves, along with benzaldehyde, (*Z*)-3-hexenyl butyrate, beta-caryophyllene, trans-α-bergamotene, (*E*,*E*)-alpha-farnesene, and VOCs known to be emitted by grapes, such as germacrene D [45]; by grapevines, like humulene [46]; and by other plants, like 1,8-cineole [47], the latter being the most abundant compound released by the non-treated grapevine leaves. After application of *H. uvarum* on the leaves, the volatile profiles changed. The two main compounds detected in *H. uvarum* (benzaldehyde, 2-phenylethanol) were significantly more abundant in the headspace from treated leaves collected 30 min after treatment (T1—VOCs) and after five days (T2—VOCs). In addition, the release of compounds not detected in the grapevine leaves headspaces was induced just after the treatment with *H. uvarum*, including

octanoic acid, 2-phenylethyl acetate, methyl salicylate, and (*E*)-4,8-dimethylnona-1,3,7-triene, and were still released after five days but in lower amounts. On the contrary, the compounds indole, linalool (unidentified isomer), and (*E,E*)-alpha-farnesene release were significantly increased after treatment and five days later. The latter was the most abundant VOC released by the treated grapevine leaves five days after application. In total, one aldehyde, one alcohol, one acetate, one acid, one green leaf volatile, two aromatic compounds, and eight terpenes were the characterized volatiles. Most of the terpenes detected in the volatile profiles also showed significant difference among non-treated and *H. uvarum*-treated grapevine leaves.

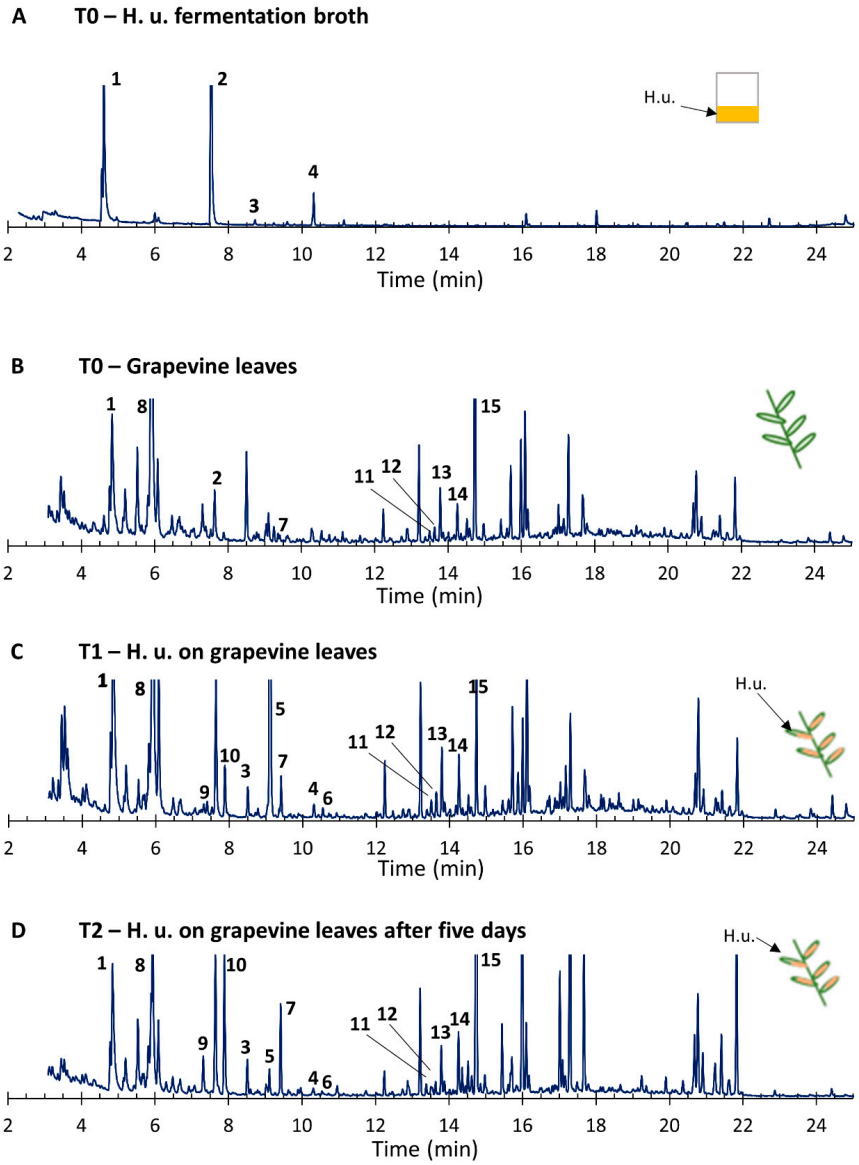

Figure 7. Chromatogram from headspace extracts of H. u. fermentation broth (**A**), grapevine leaves (**B**), grapevine leaves with H. u. 30 min after application (**C**), grapevine leaves with H. u. 5 days after application (**D**). All headspaces were collected with close loop stripping analysis (CLSA) for 3 h. Chemicals identified from H. u. are reported: 1. benzaldehyde; 2. 2-phenylethanol; 3. octanoic acid; 4. 2-phenylethyl acetate; 5. methyl salicylate; 6. indole; 7. (*Z*)-3-hexenyl butyrate; 8. 1,8-cineole; 9. linalool; 10. (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT); 11. beta-caryophyllene; 12. humulene; 13. germacrene D; 14. trans-alpha-bergamotene; 15. (*E,E*)- alpha–farnesene. All graphs are on the same scale showing the total ion chromatogram peaks in time.

Table 1. Total Ion Chromatogram (TIC) peak area of volatile organic compounds (VOCs) from yeast-treated and non-treated grapevine leaves collected by Closed Loop Stripping Analysis (CLSA) (n = 6). Average amounts of VOCs measured in TIC † are indicated. The following abbreviations are used: Linear Retention Index (LRI), Green Leaf Volatiles (GLVs), (E)-4,8-dimethylnona-1,3,7-triene (DMNT), not detected (nd). Significant differences ($p < 0.05$) are reported using different letters and asterisks (≥0.05 = ns; <0.05 = *; ≤0.01 = **; ≤0.001 = ***).

No	Compound	LRI ∀ on HP-5MS	Reference LRI	Grapevine Leaves (T0)	Grapevine Leaves + *H. uvarum* (T1)	Grapevine Leaves + *H. uvarum* (T2)	Significance (ANOVA p Value, df = 2, 15)
ALDEHYDES 1	Benzaldehyde α	961	965	0.33 ± 0.28 ^{a}	2.85 ± 1.35 ^{b}	1.20 ± 1.07 ^{ab}	F = 8.039, 0.004 **
ALCOHOLS 2	2-phenylethanol α	1114	1116	0.18 ± 0.20 ^{a}	1.09 ± 0.45 ^{b}	0.48 ± 0.36 ^{a}	F = 8.648, 0.004 **
ACIDS 3	octanoic acid α	1171	1175	nd ^{a}	0.70 ± 0.30 ^{b}	0.53 ± 0.39 ^{b}	F = 8.431, 0.003 **
ACETATES 4	2-phenylethyl acetate α	1258	1265	nd ^{a}	1.48 ± 0.60 ^{b}	0.96 ± 0.63 ^{b}	F = 11.27, 0.001 **
AROMATICS							
5	methyl salicylate α	1193	1190	nd ^{a}	12.73 ± 9.00 ^{b}	2.25 ± 2.17 ^{a}	F = 14.45, $p < 0.001$ ***
6	Indole α	1291	1288	nd ^{a}	0.57 ± 0.24 ^{ab}	1.82 ± 1.85 ^{b}	F = 3.75, 0.047 *
GLVs 7	(Z)-3-hexenyl butyrate α	1188	1180	0.06 ± 0.08	2.63 ± 2.39	3.87 ± 4.99	F = 1.846, ns
TERPENES							
8	1,8-cineole	1030	1030	7.80 ± 4.37	24.95 ± 14.86	15.98 ± 12.13	F = 2.851, ns
9	Linalool α	1103	1101	nd ^{a}	1.53 ± 0.88 ^{ab}	2.79 ± 1.54 ^{b}	F = 9.299, 0.002 **
10	DMNT α	1117	1105	nd ^{a}	9.07 ± 4.70 ^{b}	7.51 ± 5.32 ^{b}	F = 6.996, 0.007 **
11	beta-caryophyllene α	1418	1418	0.20 ± 0.15 ^{a}	8.90 ± 3.83 ^{b}	5.80 ± 5.39 ^{ab}	F = 6.659, 0.008 **
12	Humulene α	1452	1440	0.29 ± 0.11 ^{a}	5.11 ± 1.94 ^{b}	3.49 ± 2.66 ^{b}	F = 8.334, 0.004 **
13	germacrene D α	1480	1480	0.80 ± 0.46 ^{a}	4.61 ± 2.08 ^{b}	3.35 ± 2.41 ^{b}	F = 5.478, 0.016 *
14	trans-alpha-bergamotene	1495	1496	0.15 ± 0.22	1.99 ± 0.62	3.63 ± 3.66	F = 3.284, ns
15	(*E*,*E*)-alpha-farnesene α	1508	1500	0.13 ± 0.11 ^{a}	25.02 ± 13.40 ^{a}	136.25 ± 115.62 ^{b}	F = 5.816, 0.013 *

∀ Linear Retention Indices as calculated from experimental retention times; † The amount in TIC of each compound in the yeast cultures is the mean peak area of six replicates; mean ± standard deviation, divided by 10^6 (in TIC/3 h) of volatile compounds calculated from six replicates. α VOCs confirmed by comparison with reference standards.

4. Conclusions

Besides being more attractive to SWD adults and larvae compared to other yeast species [20,21,48], *H. uvarum* has been reported to have a beneficial effects in the diet of SWD larvae and to promote the ingestion by SWD adults [12,18]. Based on the aforementioned findings, in the present work, potted grape plants in a greenhouse were treated with an attract-and-kill formulation based on *H. uvarum* fermentation broth containing spinosad. The efficacy and persistence over time of the formulation were evaluated. The addition of the selected yeast to the insecticide resulted in increased mortality of SWD flies and reduced egg-laying. The effect on the survival of SWD was stronger one day after treatment, but the formulation was still effective after one week. Since mortality of SWD flies can be related to attractiveness or feeding stimulation towards specific components of the formulation, potential phagostimulants and attractants were analyzed, in order to determine whether changes in their composition were correlated with the efficacy of the formulation. Several non-volatile compounds, including carbohydrates, sugar alcohols, amino acids, and organic acids, were found in the formulation, the concentrations of which generally decreased over time. Many of these compounds are reported as feeding stimulants for SWD flies, indicating that it is worth investigating the chemical composition of SWD food to improve attract-and-kill control strategies. Numerous VOCs emitted by yeasts and induced in the plant after the treatment were detected, and changes in the VOCs profile over time were observed. Further electrophysiological and behavioral studies may be helpful for the optimization of an effective attract and kill formulation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/11/810/s1, Table S1: Temperature (°C) and relative humidity (%) registered over the experimental period.

Author Contributions: Conceptualization, F.B., U.S., D.E., S.S., I.C. and S.A.; methodology, F.B., U.S., I.C., D.E., S.S. and S.A.; formal analysis, F.B., T.B., U.S., I.C., C.D. and C.S.C.; investigation, F.B., T.B., U.S., I.C., D.E., S.S. and S.A.; data curation, F.B., U.S., I.C., D.E., S.S. and S.A.; writing—original draft preparation, F.B., U.S. and I.C.; writing—review and editing, F.B., U.S., D.E., S.S., I.C., S.A., P.R., R.F.V., C.D., T.B. and C.S.C.; supervision, D.E., R.F.V. and S.A.; project administration, D.E., S.S. and S.A.; funding acquisition, D.E., S.S. and S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Regional Development Fund (ERDF) 2014-2020 (project Dromytal, FESR1021, CUP H32F16000420009). Laimburg Research Centre is financed by the Autonomous Province of Bolzano.

Acknowledgments: The authors would like to thank Roberto Bianchi for the graphical support. Kathrin Plunger and Cristina Gadotti are gratefully acknowledged for administrative support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Research Article

Received: 29 May 2021 Revised: 25 October 2021 Accepted article published: 29 October 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6699

Behavioral manipulation of *Drosophila suzukii* for pest control: high attraction to yeast enhances insecticide efficacy when applied on leaves

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Abstract

BACKGROUND: The invasive pest, *Drosophila suzukii* attacks fresh soft-skinned fruit. Broad-spectrum insecticides are implemented for control but there is a need to reduce environmental risks and insecticide residues on fruits. *Hanseniaspora uvarum* is a yeast frequently found on ripe fruits and associated with *D. suzukii*. We aim to exploit the ecological association and attraction of *D. suzukii* to *H. uvarum* by developing an attract-and-kill strategy, with spray-application on canopy but not fruit. We therefore investigated *D. suzukii* attraction, egg-laying and mortality when exposed to insecticidal yeast-based formulations.

RESULTS: *Hanseniaspora uvarum* strongly attracted *D. suzukii* when applied on leaves of grapevine, *Vitis vinifera*. Notably, this attractiveness was competitive to ripe grape berries that were susceptible to *D. suzukii* infestation. Moreover, adding *H. uvarum* enhanced the efficacy of insecticidal formulations against *D. suzukii*. Flies exposed to leaves treated with yeast-insecticide formulations showed higher mortality and laid a lower number of eggs compared to flies exposed to insecticide alone. In a wind tunnel, all treatments containing *H. uvarum* alone or in combination with insecticides, caused similar upwind flight and landing at the odor source, which provides evidence that the addition of insecticide did not reduce *D. suzukii* attraction to yeast.

CONCLUSION: *Hanseniaspora uvarum* can be used to manipulate the behavior of *D. suzukii* by attracting flies to insecticide formulations. Yeast attraction is competitive to grape berries and improves insecticide effectiveness, suggesting that sprays covering canopy only, could reduce residues on fruit without compromising management efficacy.

Supporting information may be found in the online version of this article.

Keywords: integrated pest management; semiochemicals; spinosad; spotted wing drosophila; viticulture

1 INTRODUCTION

Integrated pest management (IPM) in cropping systems aim at the optimization of preventive actions to maintain pest pressure below the economic damage threshold, while minimizing the use of chemical pesticides when control is required.[1] Manipulation of insect pest behavior is a management option directed towards the aims of IPM. Behavioral manipulation stimulates or inhibits a behavior, or changes its expression,[2] in order to negatively impact a pest's performance and life cycle, and consequently to reduce crop damage.[3] While for certain crops behavioral manipulation of pests by use of pheromones or plant volatiles is already part of management strategies,[4,5] manipulation methods could in general be further developed and diversified for application in additional cropping systems.

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Microbial semiochemicals, in addition to plant volatiles and pheromones, affect insect behavior and should therefore be exploited in the development of new pest control methods.[5–8] Yeast volatiles attract insects of several orders, which most likely represents a conserved trait among phylogenetically diverse yeasts.[9] Consequently, yeasts are promising agents for application in insect pest control.

The spotted wing drosophila, *Drosophila suzukii* (Matsumura), is a worldwide spreading pest that can lay eggs in fruit close to harvest.[10] Development of preventive measures[11] and biological control[12,13] contribute to a sustainable pest management, while pesticide application is still common for controlling *D. suzukii* in various crops.[14–17] Despite existing pre-harvest regulations, pesticide residues cause problems with respect to marketing and consumer safety.[18–21] Moreover, application of broad-spectrum insecticides,[14,22,23] together with recent findings on insecticide resistance in *D. suzukii*[16,24,25] have increased the need for alternative methods and improving insecticide efficacy.

A promising approach to meet this need is the use of attractants or phagostimulants to control *D. suzukii*.[26–31] In fruit production, formulations of insecticides with attractants allow application without the need to spray the fruit. One option is to target the canopy only, but little is known about the behavioral response of *D. suzukii* to such treatments (but see[31,32]).

Yeast has potential for being used both as attractant and phagostimulant.[33–35] Interestingly, *D. suzukii* is closely associated with yeasts[36,37] which suggests to exploit the relationship between yeast and fly to lure *D. suzukii* to toxic baits.[34] *Hanseniaspora uvarum* (Niehaus), an apiculate yeast, is the predominant yeast species associated with *D. suzukii*.[36] The presence of *H. uvarum* in infested fruit, larval gut and frass reveals this yeast as a food resource for *D. suzukii*. In addition, a strong olfactory response and attraction of *D. suzukii* towards *H. uvarum* semiochemicals[38] and increased fecundity of females fed with the yeast,[39] suggests a strong and specific ecological relation between *D. suzukii* and *H. uvarum*. A range of field studies confirmed attraction to traps baited with *H. uvarum*.[40–42] Not surprisingly, *H. uvarum* has been suggested for the development of attract-and-kill formulations against *D. suzukii*.[31,35,43] Moreover, *H. uvarum* suppresses plant pathogens[44] and was evaluated with qualified presumption of safety as biological control agent intentionally added to food or feed.[45]

In viticulture, *D. suzukii* causes significant problems in the cultivation of certain *Vitis vinifera* (grapevine) varieties, such as Vernatsch (synonymous Trollinger, Schiava) traditionally grown in parts of Italy, Austria and Germany.[46–49] Saliently, *H. uvarum* is one of the main yeasts associated with *V. vinifera* and is commonly found on the grape-surface and during early stages of wine fermentation.[50–52]

Viticulture is characterized by intensive pesticide use.[53,54] With focus on grapevine, we hypothesized that *H. uvarum* in combination with insecticides will induce *D. suzukii* odor-driven attraction when applied on grapevine leaves and improve insecticide efficacy by increasing fly mortality and reducing fruit infestation. Thus, we assayed the attraction of *D. suzukii* to *H. uvarum* applied on grapevine leaves in comparison to untreated leaves and grape berries. Moreover, testing up-wind flight in a wind tunnel, we investigated the possible change of *D. suzukii* attraction after blending *H. uvarum* with insecticide. Finally, in formulation with yeast, four commercial products were assayed for their insecticidal effects in comparison to formulations without yeast. The obtained results provide a foundation for the development of attract-and-kill strategies to control *D. suzukii* based on the natural association and interaction between insect and yeast.

2 MATERIALS AND METHODS

2.1 Yeast culture and formulation

Hanseniaspora uvarum (strain: LB-NB-2.2; accession number GenBank NCBI: MK567898), was isolated from feeding grooves of *D. suzukii* infested grapes in South Tyrol, province of Bolzano, Italy.[37] Cultures of *H. uvarum* were grown on PDA (Difco, Potato Dextrose Agar: 39 g L^{-1}) plates. For liquid cultures, PDB (Difco, Potato Dextrose Broth: 24 g L^{-1}) was inoculated with single colonies and incubated at 25°C for 24–30 h on an incubator shaker (Stuart Scientific). A freeze-dried stock culture of *H. uvarum* was stored at −80°C and used for generating fresh cultures on PDA.

2.2 Flies

Drosophila suzukii flies from infested fruit collected in South Tyrol in 2019 were used to establish laboratory fly rearings. For attraction assays, flies were reared and tested at the Swedish University of Agricultural Sciences (SLU), Alnarp, and for assaying mortality and egg-laying, flies were reared and tested at the Laimburg Research Center Ora, Italy. At SLU, flies were reared on a Bloomington drosophila cornmeal diet (BDSC Cornmeal Food) at 22–24°C and maintained at 50–65% R.H., under a 12:12 h L:D (light: dark) photoperiod. Adult flies were kept in 30-mL rearing vials on fresh diet, closed with a cotton ball. Mated females were used for testing attraction in cages (two choices between differently treated grapevine leaves) and up-wind flight behavior (wind tunnel). In preparation for behavioral assays, newly emerged flies were anesthetized with CO_2 and sexed. Females and males were kept separate in rearing vials on fresh diet. For obtaining mated females, virgin females and males of similar age were grouped in a rearing vial during the peak hours of their sexual activity[55] within the 2nd and 3rd h of the photophase. Mating couples were isolated and after finishing copulation, females were kept alone or in groups of 10 until the wind tunnel or cage attraction experiment, respectively (see below for further details). Individuals at Laimburg were maintained at similar conditions with slight modifications: flies were reared in insect cages (W47.5 × D47.5 × H93.0 cm, BugDorm – 4 M4590, MegaView Science Co., Ltd., Taichung, Taiwan) on *D. suzukii* cornmeal diet (DSCD(a) containing dry deactivated yeast) with dry baker's yeast (RUF Lebensmittelwerk KG, Quakenbrück, Germany) and additional 5% sugar solution,[37] at 16:8 h L:D photoperiod. To determine the insecticidal activity of yeast-insecticide formulations, *D. suzukii* males and females were kept together from emergence until reaching an age of 5–8 days. Then, groups of 20 males and 20 females were placed together in an insect cage for testing egg-laying behavior and mortality (see below for further details).

2.3 Plant and fruit material

The leaves and grapes used for behavioral assays were of *Vitis vinifera* L., variety Regent, locally grown without pesticide application at the vineyard at SLU, Alnarp (55°39′37.9″N 13°05′07.3″E) in September 2019. Leaves were picked in the morning and transferred to the laboratory about 2 h before the start of the attraction assays. Leaves were chosen to be similar in size and coloration for treatment and control. The berries were picked at the first day of the experimental period and thereafter stored in a fridge for consecutive use during 10 days. The grapes were ripe, firm, undamaged, blue in color and attached to a cluster. Preliminary

experiments confirmed that *D. suzukii* was able to infest and develop in Regent and *D. suzukii* flies were found emerging from Regent berries collected at Alnarp in 2020 (for details see Supporting information). Yeast-insecticide formulation efficacy was tested on *V. vinifera* leaves of the variety Vernatsch, from a vineyard cultivated according to the guidelines for integrated fruit production in South Tyrol (46°23′04.8″N 11°17′10.6″E). Non-treated blueberries (*Vaccinium corymbosum*) from organic production were used as substrate for egg-laying assessment.

2.4 Attractiveness of grape leaves sprayed with *H. uvarum*

To evaluate the odor attractiveness of *H. uvarum* when applied on the surface of green plant leaves, a two-choice set-up was designed and mated *D. suzukii* females were exposed to vine leaves sprayed with *H. uvarum* and untreated vine leaves as alternative. For treatment, two fresh *V. vinifera* leaves were sprayed with 250 µL of *H. uvarum* grown in liquid PDB and left to dry for 1.5 h before testing attraction in comparison to two untreated leaves. The experimental arena consisted of a rectangular cuboid (Plexiglas: W66 × D33 × H33 cm). In the top of the cuboid, two closable openings allowed to introduce the green plant material and the experimental flies. In the morning of the experimental day, the leaves were placed with their stems into Erlenmeyer flask (10.5 cm high, 100-mL, VWR) filled with water; the opening around the stems of the leaves closed with cotton wool. The flasks with the treatment and the control leaves were placed in opposite ends of the cage, 5 cm from the sidewall, at equal distance (approximately 16 cm) to the front and the back of the cage. The distance between the treatments was about 46 cm. Ten mated females were tested (n = 18 replicates). For acclimatization, mated females were kept starved in plastic dishes with mesh lids (diameter 10 × H4 cm) inside and at the center of the experimental arena for 18–22 h before the experiments started. The experiments were conducted for 2 h. For the first hour the flies were checked every 10 min and the position of the flies in the cage was observed and recorded (positions: treated leaves, control leaves, elsewhere). After the sixth observation, at 60 min, the flies were left for one more hour and then a final observation was recorded (sketch of experimental set-up, Fig. 1(A)).

In a second experiment, the set-up was slightly modified by adding two clusters of five grape berries (*V. vinifera*., Regent) into the cage, one on each side above the treated and untreated vine leaves, respectively. The clusters were connected to metal wires fastened with paper tape at the roof of the cage (sketch of experimental set-up, Fig. 1(B)). As before, the position of the flies was recorded (treated leaves, control leaves, berries on treatment side, berries on untreated side, elsewhere) every 10 min for 1 h and at the end of the experiment that is, after 2 h (n = 20 replicates).

As supplementary experiment, in order to detangle the attraction of *D. suzukii* females to the formulation of medium and yeast, we tested the preference of groups of 10 mated females (n = 15 replicates) to grapevine leaves sprayed with *H. uvarum versus* PDB. This was conducted with the same procedure as in the first experiment (*i.e.*, with grapevine leaves only), but in smaller cages (W30 × D30 × H30 cm, BugDorm – 1, MegaView Science Co., Ltd., Taichung, Taiwan).

2.5 Efficacy of *H. uvarum* formulations with different insecticides

To evaluate the efficacy of insecticides in formulation with *H. uvarum*, we conducted a mortality and oviposition bioassay by treating freshly picked *V. vinifera* leaves (Vernatsch) and offering (untreated) *V. corymbosum* berries together with water agar as egg-laying substrate. In a first experiment, insecticide-yeast formulations were compared to aqueous insecticide solutions and untreated leaves in separate experimental cages (W30 × D30 × H30 cm, BugDorm – 1, MegaView Science Co., Ltd., Taichung, Taiwan) (n = 5 per treatment). In total, four insecticides toxic for *D. suzukii* and allowed for IPM in Italian viticulture, were tested: (i) 15 mg L^{-1} deltamethrin (Decis EVO, Bayer CropScience S.r.l.), (ii) 100 mg L^{-1} acetamiprid (Epik SL, Sipcam Italia S.p.A.), (iii) 120 mg L^{-1} spinosad (Laser, Dow AgroSciences Italia S.r.l.) and (iv) 720 mg L^{-1} tau-Fluvalinate (Mavrik 20 EW, Adama Italia S.r.l.). The applied doses were established according to the manufacturer's instructions for viticulture in Italy. This allowed to test the efficacy of insecticides in combination with *H. uvarum* culture at the concentrations used in vineyards. For each insecticide-yeast-formulation, 100 µL (10 droplets of 10 µL volume) were applied onto individual leaves and dried at room temperature for approximately 2 h. Similarly, the four different insecticides were applied on leaves at the same dose as before but in 100 µL of distilled water instead of yeast culture, and untreated leaves were used for control tests. For each test, five leaves were placed with their stems into an Erlenmeyer flask filled with water and the opening around the stems was closed with cotton. Leaves were then exposed to groups of 20 female and 20 male *D. suzukii* per experimental cage. A 5% sugar solution supplied on cotton in a small Petri dish (diameter 6 cm) served as water and energy source for the flies. Each cage contained an additional Petri dish with water agar (diameter 9 cm, 15 g L^{-1} agar) on which four blueberries were placed. Egg-laying was quantified from the number of eggs laid on agar and berries together. The blueberries and the agar substrates were removed and replaced by a new set after 24 h. In a second experiment, the four *H. uvarum*-insecticide mixtures were tested like before and in addition *H. uvarum* was tested as pure culture (10 droplets of 10 µL volume) applied on leaves (n = 5 per treatment). For both experiments adult mortality and the number of laid eggs was counted after 24 h and 48 h. Experiments were performed at similar laboratory conditions as insects were reared (*ca*. 22°C, 65 ± 5% R.H., 16:8 h L:D photoperiod).

2.6 Flight attraction behavior to *V. vinifera* leaves with *H. uvarum*-insecticide formulations

To assess odor-mediated flight attraction behavior of *D. suzukii* towards *H. uvarum* blended with insecticides, wind tunnel experiments were conducted with the same equipment (glass wind tunnel system with D100 × H30 × W30 cm flight section), but slightly modified protocol as described earlier.[35,56] Mated females, 4–6 days old, starved 6–8 h prior testing, were released individually at the down-wind end of the tunnel and exposed for 5 min to a main air stream (0.3 m s^{-1}) carrying a plume of stimulus odor. The stimulus was delivered in charcoal filtered air (0.3 L min^{-1}) that was blown through a wash bottle containing the test material described below. The scented airstream was, *via* a Pasteur pipette, vertically injected at the up-wind end into the wind tunnel onto an 18 cm high, 38 mm diameter horizontal platform of aluminum, from which it diffused down-wind as an odor plume.

Three different treatments and two controls were placed into different wash bottles to test their headspace emissions for *D. suzukii* attraction: (1) *H. uvarum* applied on *V. vinifera* leaves, (2) *H. uvarum* blended with spinosad applied on *V. vinifera* leaves, and (3) *H. uvarum* blended with deltamethrin applied on *V. vinifera* leaves. (4) *Vitis vinifera* leaves and (5) *H. uvarum* applied

on filter paper were tested to control for leaf and yeast volatiles, respectively. For the insecticide treatments, two leaves were sprayed, each with 250 µL of a formulation of yeast culture and spinosad (5.4 mg L^{-1}, treatment (2), comparable to doses applied in other studies,[31,35]) or deltamethrin (7.5 mg L^{-1}, treatment (3)), respectively. Sprayed leaves were dried for 1.5 h at room temperature prior testing. Similarly, *H. uvarum* was applied on leaves for treatment (1) or on filter paper for control (5). Flies were recorded for upwind flight and landing at the odor source (*i.e.*, on top of the aluminum platform or the tip of the pipette injecting the scented air). In total, 50 individual females were tested for attraction towards each treatment. Experiments were performed at similar conditions as insects were reared, 1.5–2 h prior the onset of the scotophase.

2.7 Data analysis

Analyses were performed using R statistical software.[57] To evaluate odor-driven attraction of *D. suzukii* females to *H. uvarum* applied on plant leaves over time, a mixed linear model (MLM) fitted with a Gaussian error distribution (R software package 'lme4') was performed. The preferred substrate where *D. suzukii* females were observed when exposed to yeast-treated leaves in the presence of grape berries was analyzed with a mixed effects generalized linear model (GLMM) fitted with a binomial error distribution. A Tukey´s contrast test (R software package 'multcomp') was used for pairwise comparison between treated and untreated green leaves and the grapes placed nearby each treatment, respectively. The effects of the treated leaves on the mortality and oviposition of *D. suzukii* adults were evaluated with a GLM fitted with a binomial and Poisson distribution, respectively. A chi-square test (R software package 'car') followed by Tukey's contrast analysis was used to estimate the significance of fixed effects and for pairwise comparison of treatments, respectively. The up-wind flight towards yeast odors in the wind tunnel was modeled with a GLM fitted with a binomial error distribution followed by a Tukey's contrast pairwise comparison between the different treatments. Models were selected, based on Akaike information criterion values. Residuals were analyzed to verify the distribution of the errors. For further details, see supporting information (Table S1 and S2). Figures were drawn using 'Tidyverse' (R software package 'tidyverse').

3 RESULTS

3.1 Attractiveness of grape leaves sprayed with *H. uvarum*

Mated *D. suzukii* females were significantly more attracted to *V. vinifera* leaves sprayed on the surface with *H. uvarum* compared to untreated leaves, and this preference significantly increased along the experimental time (MLM: F = 42.18, df = 231,

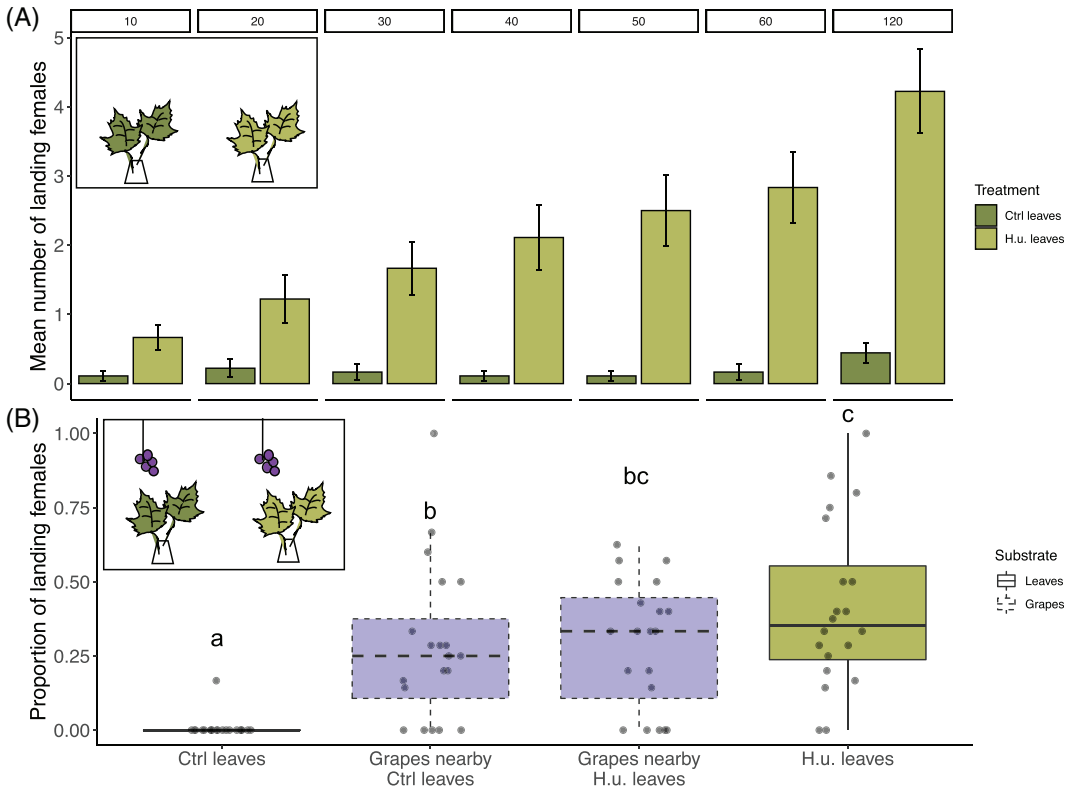

Figure 1. (A) Preference (Mean ± standard error of the mean) of 10 mated *Drosophila suzukii* females (n = 18) when given the choice between *Vitis vinifera* leaves treated with *Hanseniaspora uvarum* yeast (H.u. leaves, light green) or untreated leaves (Ctrl leaves, dark green) along the experimental period of 120 min (after 10, 20, 30, 40, 50, 60 and 120 min). Significantly more females landed on H.u. leaves compared to Ctrl leaves ($P < 0.05$) with a significant increase of preference over time ($P < 0.001$). (B) Preference (after 120 min) of 10 mated *D. suzukii* females (n = 20) when exposed to *V. vinifera* leaves treated with *H. uvarum* (light green), untreated leaves (dark green) and grapes (purple) placed nearby the treated and untreated leaves. Leaves sprayed with yeast were as attractive as the grapes. The boxes represent the interquartile range divided by the median, and whiskers represent the data within 1.5× the interquartile range. Dots represent the data distribution of the individual replicates. Different letters above boxes describe significant difference after multiple comparisons of means. Scheme of the experimental design at the top left corner of each plot illustrating treated and untreated leaves, and grape berries in B.

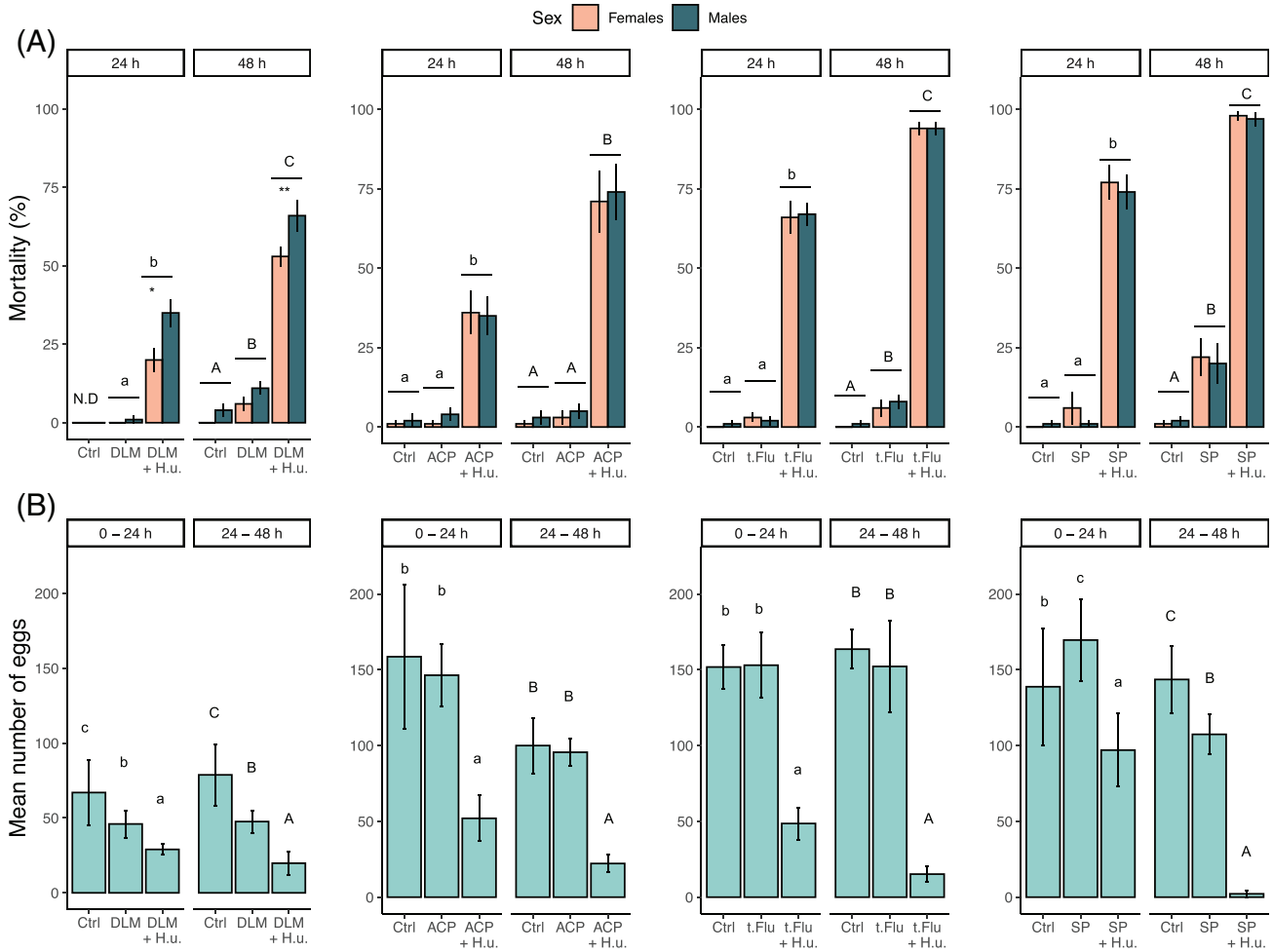

Figure 2. Effect of *Hanseniaspora uvarum*-insecticide formulations on *Drosophila suzukii* mortality (A) and oviposition (B) for 20 females and 20 males per replicate, (n = 5). (A) Cumulative mean percentage of female (light coral) and male (dark cyan) mortality ± standard error of the mean after 24 h and 48 h of continuous exposure to *Vitis vinifera* untreated leaves (Ctrl), insecticide-treated leaves (acetamiprid = ACP, deltamethrin = DLM, tau-Fluvalinate = t.Flu, spinosad = SP) and leaves treated with formulations of insecticide and *H. uvarum* (abbreviated insecticide name + H.u.). Both at 24 h and at 48 h leaves treated with formulations of insecticide and *H. uvarum* caused a significantly higher mortality in both sexes compared to those exposed to insecticide alone or control leaves ($P < 0.01$). (B) A significantly lower number of eggs (Mean ± standard error of the mean) was laid both during the first 24 h as well as from 24–48 h, when flies were exposed to any of the *H. uvarum*-insecticide formulations in comparison to control or the insecticides without yeast. Different letters denote significant differences between treatments ($P < 0.05$, lowercase at 24 h, uppercase at 48 h). Asterisks denote significant differences in mortality between sexes (* $P < 0.05$, ** $P < 0.01$). N.D. means No-Dead flies and data were therefore excluded from data analysis.

$P < 0.0001$). After 120 min, 42.2% (76 individuals in total) of the experimental flies were found on *H. uvarum*-treated leaves compared to only 4.4% (eight in total) on the control leaves (Fig. 1 (A)). When given the choice between *V. vinifera* leaves sprayed with *H. uvarum* or the growth medium alone (PDB), flies were again significantly more attracted to *H. uvarum* compared to PDB-treated leaves (GLMM binomial distribution: F = 12.71, df = 27, $P < 0.001$; Fig. S1). Interestingly, when grapes were placed nearby to the treated and untreated leaves (drawing Fig. 1(B)), females still preferred to land and stay significantly more on the *Hanseniaspora*-treated leaves compared to the untreated ones (GLMM binomial distribution: F = 8.52, df = 75; Multiple Comparison of Means (MCM): Z = 4.41, $P < 0.0001$). Yeast-sprayed leaves were as attractive for *D. suzukii* mated females as the grapes placed above (GLMM binomial distribution, MCM: Z = 1.86, $P = 0.22$), but more attractive than the grapes on the untreated side of the experimental cage (GLMM binomial distribution, MCM: Z = 2.96, $P < 0.05$).

3.2 Efficacy of *H. uvarum* formulations with different insecticides

In the first experiment, the addition of *H. uvarum* into insecticide formulations when applied onto green *V. vinifera* leaves significantly increased the mortality of *D. suzukii* individuals in both sexes (Fig. 2(A)) and reduced the total number of eggs laid (Fig. 2(B)), compared to the application of insecticide alone or untreated leaves. This was true for the four tested yeast-insecticide formulations at 24 h and the differences were even clearer at 48 h after exposure (Mortality: GLM binomial distribution, df = 55, $P < 0.001$; Oviposition: GLM Poisson distribution, df = 12, $P < 0.01$; for further details see supporting information, Table S2). Most yeast-insecticide formulations affected female and male mortality similarly ($P > 0.05$), but not deltamethrin-*H. uvarum* (DLM + H.u.) for which mortality in males (percentage mean ± standard error of the mean = 66.0 ± 4.9%) was significantly higher than in females (53.0 ± 3.0%) ($P < 0.05$). At the end of the experiment, on average 59.5 ± 3.0% of flies exposed

Table 1. Comparison of *Hanseniaspora uvarum*-insecticide formulations for their effects on *Drosophila suzukii* mortality and oviposition. Cumulative mean number (± standard error, SE) and percentage of *D. suzukii* adult mortality by *H. uvarum*-insecticide formulations after 24 h and 48 h of continuous exposure to treated *Vitis vinifera* leaves. Total numbers of eggs laid at the end of the experiment are represented by means (±SE). A total of five leaves per treatment was applied with 100 µL of formulation per leaf

Treatment formulation	Dead flies after 24 h exposure		Dead flies after 48 h exposure		Total eggs laid
	Mean (SE)	Percentage	Mean (SE)	Percentage	Mean (SE)
H. uvarum	0.0[†]	0	0.1 (0.1)[a]	0.5	311.0 (45.3)[a]
Acetamiprid + *H. uvarum*	8.4 (1.2)[a]	42	15.2 (0.8)[b]	76	82.2 (13.4)[b]
Deltamethrin + *H.uvarum*	11.5 (1.0)[b]	58	17.0 (0.6)[b]	85	77.6 (14.5)[b]
tau-Fluvalinate + *H. uvarum*	15.2 (0.5)[c]	76	19.0 (0.3)[c]	95	62.2 (16.3)[c]
Spinosad + *H. uvarum*	15.9 (0.5)[d]	80	19.9 (0.1)[c]	100	57.0 (7.5) [c]
	GLM binomial distribution		GLM binomial distribution		GLM Poisson distribution
	Res. df = 35		Res. df = 45		Res. df = 20
	$X^2 = 79.6, P < 2.2e\text{-}16$ ***		$X^2 = 706.6, P < 2.2e\text{-}16$ ***		$X^2 = 1578.5, P < 2.2e\text{-}16$ ***

Mortality data were analyzed using a generalized linear model (GLM) with binomial distribution and number of eggs laid using a GLM Poisson distribution. Treatment formulation was the fix effect (n = 5) in all models. Column means followed by a different letter denote significant differences between treatments after pairwise comparison ($P < 0.05$). See Table S1 for model details.
[†] *H. uvarum* treatment was excluded from analyses due to the absence of dead flies in all replicates in the first 24 h.

to DLM + H.u. died compared to 8.5 ± 1.5% on DLM alone, or 3.5 ± 2.5% on untreated leaves (Ctrl). Acetamiprid-*H. uvarum* (ACP + H.u.) caused on average a higher mortality (72.5 ± 6.1%) than ACP (4.0 ± 1.5%) and Ctrl (2.0 ± 1.1%). Finally, tau-Fluvalinate-*H. uvarum* (t-Flu + H.u.) and spinosad-*H. uvarum* (SP + H.u.) formulation treatments showed the highest efficacy, killing on average 97.5 ± 1.1% and 94.0 ± 1.3% of the experimental flies, respectively. In the second experiment, direct comparison of the four insecticide-*H. uvarum* formulations showed a similar pattern as in the first experiment; SP + H.u. and t-Flu + H.u. treatments were the most effective, followed by DLM + H.u. and ACP + H.u. (Table 1). Notably, only 1 out of 200 flies exposed to control H.u. leaves died after 48 h, denoting that no negative impact on flies was caused by *H. uvarum*.

3.3 Flight attraction behavior to *V. vinifera* leaves with *H. uvarum*-insecticide formulations

While *V. vinifera* leaf volatiles induced up-wind flight of 10% (5 out of 50 flies), the application of *H. uvarum* (250 µL) on the surface of the leaves significantly increased attraction resulting in 44% (22 flies) *D. suzukii* females flying up-wind and landing at the odor source within the 5 min test period (GLMM, MCM: Z = 3.55, $P < 0.01$). Notably, the response of flies to odors emitted by *H. uvarum* applied on filter paper (38%, 19 flies) was similar as to

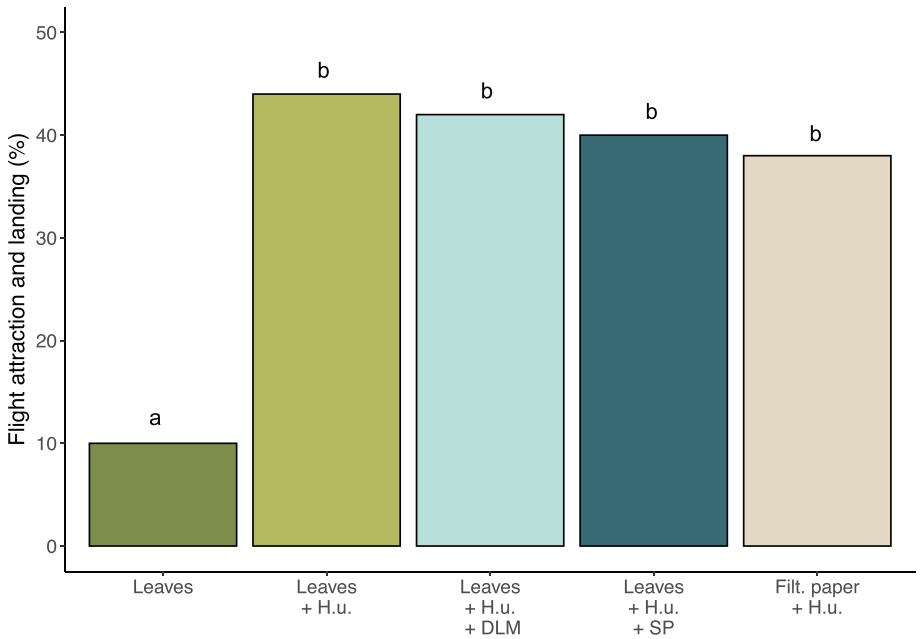

Figure 3. Up-wind flight and landing at the odor source of mated *Drosophila suzukii* females towards headspace volatiles of *Vitis vinifera* leaves (Leaves), leaves sprayed with *Hanseniaspora uvarum* (Leaves + H.u.), leaves sprayed with *H. uvarum* in combination with the insecticides deltamethrin (DLM) or spinosad (SP), and filter paper sprayed with *H. uvarum*. The presence of *H. uvarum*, either alone or in combinations with insecticides increases flight attraction significantly in comparison to untreated green leaves ($P < 0.05$). Different letters illustrate significant differences in response to the treatments. In total, 50 flies for each treatment were tested.

odors emitted by *H. uvarum* applied on leaves (GLMM, MCM: $Z = 0.61$, $P = 0.97$). Furthermore, insecticide formulations of spinosad and *H. uvarum* (40%, 20 flies) or deltamethrin (42%, 21 flies) combined with *H. uvarum* were similar attractive as the *H. uvarum* spray without insecticide (GLMM, MCM: $P > 0.05$).

4 DISCUSSION

The spread of *D. suzukii* causes damage across fruit plantations in many parts of the world.[10,48,49] We studied the impact of yeast in formulation with insecticides on *D. suzukii* attraction in the context of plant leaves to generate a foundation for future field application of a new attract-and-kill strategy.

Our results demonstrate that *H. uvarum* volatiles have a strong capacity to manipulate *D. suzukii* behavior when applied onto plant leaves. In laboratory assays, *D. suzukii* flies were highly attracted to *H. uvarum* sprayed on leaves of *V. vinifera* compared to the yeast growth medium. What is more, mated *D. suzukii* females preferred leaves treated with *H. uvarum* relative to untreated leaves and this preference was competitive to grape berries that were susceptible to *D. suzukii* infestation (Fig. 1). Neither in cage experiments nor wind tunnel tests with mated females we could see a strong attraction to grapevine leaves except when *H. uvarum* was sprayed onto the surface.

Previous research has shown that *H. uvarum* represents a valuable food resource for the flies.[39] In our assay, females were arrested and stayed feeding on *H. uvarum* after landing on the grapevine leaves (Fig. 1(A) and supporting video, SV1). This result is in line with earlier findings showing that shortly after mating, females increased their feeding activity on yeast, possibly to maximize nutrient allocation for egg production.[35] Intriguingly, even in the presence of ripe grape berries, female flies were still attracted in a similar manner to leaves with *H. uvarum* as to fruit (Fig. 1(B)), thus yeast disrupted host infestation at least temporarily. Stronger attraction to yeast than to fruit odors was earlier shown for *D. melanogaster*.[58]

Remarkably, volatiles emitted from *H. uvarum* culture triggered strong up-wind flight in *D. suzukii* even when applied at µL-amounts and dried on the treated leaf surface before testing. In a previous study, we used headspace emitted from 50 mL of fresh culture[35] unaware of the high sensitivity of *D. suzukii* that we now revealed towards a different strain of *H. uvarum*. More than that, in the current study we show that even after addition of insecticide, *H. uvarum* attracted similarly strong as the pure yeast culture. In other words, insecticides blended with *H. uvarum* did not affect the sensitivity and attraction of flies compared to *H. uvarum* alone (Fig. 3). So far experimental evidence about the ability and sensitivity of flies to respond with up-wind flight attraction to yeast-insecticide formulations was lacking. Furthermore, *H. uvarum* applied on filter paper was sufficient to induce *D. suzukii* up-wind flight. Hence, volatiles of *H. uvarum* induce flight attraction even in absence of background odors such as leaf volatiles.

Our study shows that *H. uvarum* acts as an attractant that stimulates the contact with the insecticidal food-bait and enhances insecticide efficacy; when flies were exposed to treated grape leaves, all four tested insecticides combined with *H. uvarum* led to increased mortality and reduced egg-laying as compared to treatments with insecticide alone or exposure to untreated control leaves (Fig. 2).

Noteworthy, manipulative attraction or phagostimulation of *D. suzukii* has been shown in earlier studies even for application of non-nutritive baits as well as traps without insecticides.[26,28,30] Similarly, the attraction to *H. uvarum* offers options for manipulation different to the here studied insecticide-based attract-and-kill.[40–42]

Moreover, in agreement with our work, earlier studies have shown that the addition of yeast to insecticide can lead to increased *D. suzukii* adult and larval mortality, and reduce egg-laying.[31,33,35,43] However, studies on yeast-based attract-and-kill for control of *D. suzukii* need to be compared with caution as test protocols differ substantially, for example with respect to yeast species and strains (*e.g.*: *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *H. uvarum*), different types and concentrations of insecticides, and differing substrates (*e.g.*: acrylic, glass, leaves, fruits) across diverse crops, in both laboratory and field trials. Keeping this in mind, it is understandable that the efficiency of attract-and-kill on the *D. suzukii* target appears variable. For example, the addition of *S. cerevisiae* and sugar to spinosad significantly increased fly mortality and reduced larval density, but not the number of eggs laid in fruit compared with the addition of sugar only.[33] In contrast, Roubos *et al*. (2019)[59] found no improvement when adding *S. cerevisiae* to spinosad formulations for control of *D. suzukii* neither in laboratory nor field experiments. In the case of acetamiprid-formulations contrary to our results, Noble *et al*. (2019)[43] did not find improvement of efficacy. In our work, direct comparison of four insecticide-*H. uvarum* formulations showed that the treatment containing acetamiprid was the least effective (Table 1). With regards to *D. suzukii*, deltamethrin, to our knowledge, was previously only tested alone[17,60] or in combination with the insecticide imidacloprid[16] in laboratory studies on insecticide efficacy and resistance, and no studies were found addressing tau-Fluvalinate effects.

Spinosad is a commonly used and effective insecticide for management of *D. suzukii* also in organic fruit production[14,61] and formulations with *H. uvarum* and spinosad have previously been shown to increase *D. suzukii* mortality and negatively impact fruit infestation or egg-laying.[31,35,43] Spinosad is thus a strong candidate for the development of attract-and-kill strategies. Moreover, spinosad is especially active by ingestion which makes formulation with a phagostimulatory yeast even more compelling.[39] More efficient uptake of spinosad by the flies would furthermore counteract absorption of active compound by the plant material.[62]

Finally, using attractants to lure *D. suzukii* to insecticide reduces the need of comprehensive spray coverage and might allow reduction of spray volumes and drift. Optimized, a reduced spray coverage could result in excluding the fruit from insecticide application and thus minimize pesticide residues on the harvested crop. Developing strategies of targeted insecticide application is timely with respect to current advance of precision technology in viticulture.[63–65]

Overall, our data suggest high efficacy of our novel yeast-insecticide formulations and application methods targeting specifically green-plant leaves rather than the whole plant with consumable fruit. Our data show that *H. uvarum*-based attract-and-kill is compatible with different kinds of insecticides, facilitating resistance management by rotating products with different modes of action.[61] The corroborated concept of yeast-based behavioral manipulation could be applied even in strategies different to attract-and-kill. For example, *H. uvarum* volatiles might be used to attract *D. suzukii* to baits for monitoring or mass trapping.

In summary, the collective findings provide a foundation for the design of a behavioral management strategy targeting *D. suzukii*.

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Formulations with the highly attractive *H. uvarum* enhance insecticide efficacy even when applied on leaves, not fruit, and might allow to decrease spray volumes, coverage of the plant and chemical residues on the crop. All-importantly, we think that the suggested approach is complementary to existing management practices and should therefore be evaluated for implementation within IPM programs. Thus, field experiments assessing the efficacy of *H. uvarum*-insecticide formulations as well as insecticide residues on the crop after spray application on canopy exclusively, is the logical next step in the development of the suggested *D. suzukii* management method.

ACKNOWLEDGEMENTS

The authors wish to thank the European Regional Development Fund (ERDF) 2014-2020 for funding DROMYTAL project (FESR1021, CUP H32F16000420009) to conduct the research. GR was in addition funded by Uruguayan National Agency for Research and Innovation (ANII Fellowship POS_EXT_2016_1_134106). PGB was in addition supported by the Swedish Research Council Formas (Grant 2015-1221) and the SLU Centre for Biological Control (CBC). Advice in data analysis by Franca Stábile is acknowledged.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad DigitalRepository at https://datadryad.org/stash.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Research Article

Received: 9 August 2021 Revised: 27 November 2021 Accepted article published: 2 December 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6748

Field and greenhouse application of an attract-and-kill formulation based on the yeast *Hanseniaspora uvarum* and the insecticide spinosad to control *Drosophila suzukii* in grapes

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Abstract

BACKGROUND: The invasive insect *Drosophila suzukii* (Matsumura) is an important pest of several red grape varieties. The yeast *Hanseniaspora uvarum* (Niehaus), which is associated with *D. suzukii*, strongly attracts flies and stimulates them to feed on yeast-laden food. In the present study, a formulation based on *H. uvarum* culture with spinosad insecticide was applied to the foliage of vineyards and control of *D. suzukii* was compared to applying spinosad to the whole plant. After successful *H. uvarum* and insecticide application in the vineyard, we tested additional *H. uvarum*-based formulations with spinosad in a greenhouse to determine their capacity to control *D. suzukii*.

RESULTS: Application of the *H. uvarum*-spinosad formulation at 36.4 g of spinosad per hectare reduced the *D. suzukii* field infestation at the same rate as applying 120 g of spinosad per hectare and prevented spinosad residues on grapes. Leaves treated with *H. uvarum* and spinosad in the field and transferred to a laboratory assay caused high mortality to flies and reduced the number of eggs laid on fruits. Formulations with spinosad applied in the greenhouse showed that both *H. uvarum* culture and the yeast cell-free supernatant of a centrifuged culture increased fly mortality and reduced the number of eggs laid compared to the unsprayed control.

CONCLUSION: In comparison to typical spinosad spray applications, the use of *H. uvarum* in combination with spinosad as an attract-and-kill formulation against *D. suzukii* reduces pesticide residues on the fruits by targeting the treatment to the canopy and decreasing the amount of insecticide per hectare without compromising control efficacy.

Keywords: grapes; integrated pest management; invasive fruit pest; precision agriculture; spotted wing drosophila

1 INTRODUCTION

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), also known as spotted wing drosophila, is an important insect pest of soft- and thin-skinned fruit crops, including berries, stone fruit and grapes.[1] The control of *D. suzukii* usually relies on the application of insecticides to the whole plant to reduce yield losses.[2] Unfortunately, most insecticides result in fruit residues and are not selective.[3] New strategies based on insect semiochemicals could reduce the amount of insecticide applied in the field and prevent residues that remain on the fruit.[3,4] Combining insecticide with an attractant that guides the flies to the insect toxic bait might allow for the targeted application to the canopy while avoiding the fruit.[4,5] Such a strategy could promote more sustainable and targeted chemical control. Successful attempts to

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develop an attract-and-kill strategy against *D. suzukii* were previously conducted with formulations based on *Saccharomyces cerevisiae* and *Aureobasidium pullulans* in cherry orchards and with a complex formulation of unknown ingredients in combination with conventional treatments in blueberry and raspberry fields.[6,7]

For *D. suzukii*, yeasts are considered suitable lures for attract-and-kill control strategies since they act as feeding stimulants[8,9] and are an important source of nutrients for this pest.[4,10,11] One of the most relevant yeasts is *Hanseniaspora uvarum* (Niehaus), which was found in *D. suzukii*-infested grapes and raspberry fruits,[12,13] as well as in *D. suzukii* adults and larvae.[6,11,14] The yeast *H. uvarum* is more attractive and phagostimulatory toward *D. suzukii* than other investigated yeast species.[9,10,15] Furthermore, *H. uvarum* is naturally present on grapes, therefore its presence likely would not interfere with winemaking.[16,17] Control methods based on *H. uvarum* and insecticides were previously tested in the laboratory and greenhouse, and led to reduced oviposition and higher mortality of *D. suzukii* adults.[5,6,8,18,19]

Attraction to yeast is strain-specific,[20] therefore an *H. uvarum* strain that has been extensively studied and is attractive to *D. suzukii* was used in the present study. The *H. uvarum* strain LB-NB-2.2 was isolated from feeding galleries of *D. suzukii* larvae in infested grape berries of the variety Vernatsch in South Tyrol in 2012.[12] This strain was previously shown to act as a feeding stimulant and attractant for *D. suzukii* females, and it was successfully used as an attractive component in control strategies in greenhouse assays.[5,10,19] Furthermore, the intra- and extracellular concentrations of compounds such as amino acids, carbohydrates, sugar alcohols, organic acids and lipids for the culturing of this *H. uvarum* strain grown in liquid medium were previously characterized,[10,21] and the persistence of nutritional and volatile compounds on the surface of grape leaves of potted plants treated with an attract-and-kill formulation based on this *H. uvarum* strain was described.[5]

Among the numerous insecticides that can be used against *D. suzukii*,[22–26] some have been tested in combination with *H. uvarum*.[6,8,19] Spinosad, which can be used in integrated and organic production,[22] was proven to be effective against *D. suzukii* based on laboratory and greenhouse trials.[5,18] Therefore, this insecticide was chosen in combination with the yeast *H. uvarum* LB-NB-2.2 for our study.

The soft-skinned red grape variety Vernatsch (alternative names Schiava in Italy, Trollinger in Austria and Germany), which is used for winemaking, has a lower penetration resistance against *D. suzukii* oviposition than other grape cultivars.[27,28] The dispersal of *D. suzukii* has compromised the cultivation of Vernatsch since the first appearance of this pest in 2009.[2] Great damage due to *D. suzukii* infestation occurs, especially when the penetration resistance of the berry decreases before harvest and when temperatures are mild and precipitation occurs.[29,30]

Our objective was to determine whether the combined application of *H. uvarum* and spinosad in vineyards could restrict the spray application to the foliage and reduce areal insecticide release and residues on grapes without compromising the control efficacy relative to conventional treatment of the whole plant. In the laboratory, *D. suzukii* flies were exposed to leaves collected in the field after treatment to obtain additional information about its effect in the vineyard. Furthermore, this study explores the effect of different *H. uvarum* formulations that can be used for storage of yeasts to develop sustainable and cost-effective attract-and-kill strategies. Residual analyses were performed to better understand the persistence of the applied insecticide.

2 MATERIALS AND METHODS

2.1 Yeast cultures and formulations

All assays were performed with the yeast *H. uvarum* (strain LB-NB-2.2, accession number GenBank NCBI: MK567898). This *H. uvarum* strain was isolated in 2012 from *D. suzukii*-infested grapes.[12]

The first *H. uvarum* culture was industrially manufactured by Agrifutur srl (Alfianello, Italy) in a 40-L fermenter under aerobic conditions on potato dextrose broth (4 g L^{-1} peptone from potato, 20 g L^{-1} dextrose) at 25 °C for 30 h, and it had a pH value of 4.1 and a cell density of 4.8×10^7 cells per mL. The second *H. uvarum* culture was cultivated in the laboratory at the Laimburg Research Centre in 4 L of potato dextrose broth (24 g L^{-1}; Difco, Becton–Dickinson, Le Pont de Claix, France) at 25 °C for 30 h in 6-L Erlenmeyer flasks closed with cotton and aluminum foil on magnetic stirrers at 300 rpm, and it had a pH value of 4.0 and a cell density of 7.1×10^7 cells per mL. For both cultures, media were inoculated with yeast cells grown on potato dextrose agar [4 g L^{-1} potato starch (from infusion), 20 g L^{-1} dextrose, 15 g L^{-1} agar; Difco, Becton Dickinson, Le Pont de Claix, France]. Both yeast cultures were used undiluted.

For the greenhouse assay, the industrially manufactured *H. uvarum* culture was preserved in three different formulations by Agrifutur srl. The formulations were *H. uvarum* culture without modifications before storage, *H. uvarum* supernatant obtained by centrifugation of the entire culture at 4000 rpm, and *H. uvarum* pellets obtained by centrifugation at 4000 rpm and subsequent freeze-drying. All cultures or formulations were stored at −80 °C and thawed overnight at room temperature before use. The freeze-dried *H. uvarum* pellets were diluted to the initial volume with distilled and autoclaved water after thawing.

2.2 Insects

A laboratory colony of *D. suzukii* in insect cages (BugDorm – 4M4590; MegaView Science Co., Ltd, Taichung, Taiwan) was maintained at 22 ± 1 °C, 65 ± 5% relative humidity, and 16 h photoperiod. The *D. suzukii* flies originated from infested fruits in South Tyrol, Italy and were reared on a *D. suzukii* cornmeal diet (previously designated DSCD(a) containing dry deactivated yeast) with living dry baker's yeast (RUF Lebensmittelwerk KG, Quakenbrück, Germany) sprinkled over the surface.[12] The flies were also provided with a 5% sugar solution on cotton. Males and females that hatched together over 3 days were fed a cornmeal diet and sugar solution until the start of the experiment. When the flies reached an age of 5–8 days after emergence from the pupal stage, 20 females and 20 males were placed together in an insect cage (BugDorm – 1; MegaView Science Co., Ltd).

2.3 Vineyard trials

2.3.1 Field application

The field trials were performed in two vineyards that cultivate the local grape (*Vitis vinifera*) variety Vernatsch according to the guidelines for integrated fruit production in South Tyrol, Italy: at Schlossleiten (46°23′04.8″ N, 11°17′10.6″ E), the grapes were cultivated using a pergola as the training method in 2019 and at Piglon (46°21′46.4″ N, 11°17′21.0″ E), the grapes were cultivated with the single Guyot method in 2020 (Fig. 1). The experimental design consisted of three blocks, each containing one plot per treatment. The plots consisted of three rows and were 130 m^2 in 2019 and 120 m^2 in 2020. The plots were oriented adjacent to each other and perpendicular to the bordering edge of a forest, a *D. suzukii* infestation pressure point observed in previous years.

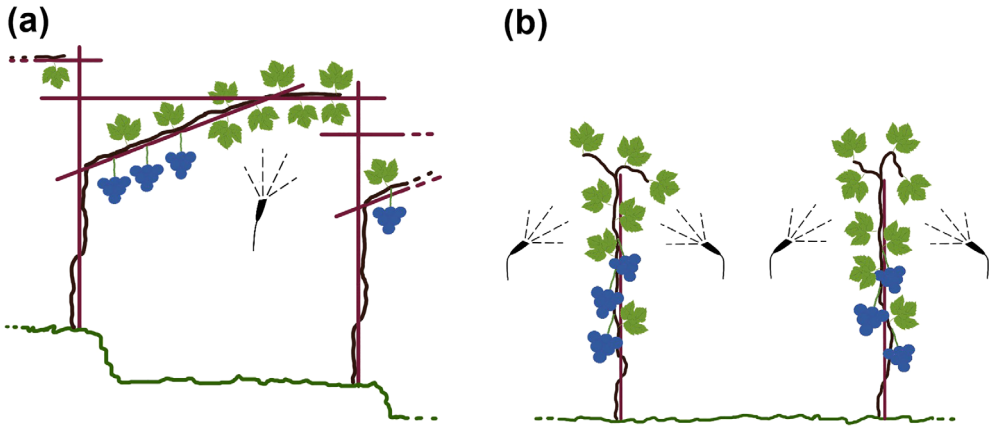

Figure 1. Illustration of the grapes trained (a) on a pergola and (b) with the Guyot method. The black spray patterns illustrate the targeted treatment of the foliage for both training types.

The vineyard trials in 2019 and 2020 were performed between the end of August and the end of September.

The treatments were an unsprayed control, a conventional spinosad treatment applied to the whole plant and thawed *H. uvarum* culture + spinosad applied on the portion of the fruit-free canopy. The *H. uvarum* culture applied in 2019 was industrially manufactured by Agrifutur srl, while the *H. uvarum* culture applied in 2020 was produced in the laboratory at the Laimburg Research Centre.

The conventional spinosad treatment contained 0.12 g spinosad (Laser, Dow AgroSciences Italia S.r.l., Milan, Italy; 480 g of spinosad per liter of product) per liter of water and was used at a spray rate of 1000 L of water per hectare. The applied amount resulted in 120 g of spinosad per hectare. The spinosad treatment was applied with a trailed airblast sprayer (AP 2/28 with axial fan; Lochmann GmbH, Italy) at 5.5 bar and 6 km h^{-1} through 12 black Albuz ATR 80° hollow cone nozzles (Agrotop Gmbh, Obertraubling, Germany) in 2019 and at 5.5 bar and 6.5 km h^{-1} through 12 red Albuz ADI 110° flat fan nozzles (Agrotop Gmbh) in 2020. On the pergola and on the Guyot system, one spinosad treatment was applied to both sides of each row.

The *H. uvarum* + spinosad treatment contained 0.1584 g of spinosad (0.33 mL of Laser) per liter of *H. uvarum* culture and was used at a spray rate of 230 L of yeast culture per hectare for both training systems. The applied amount resulted in 36.48 g of spinosad per hectare, which was added after thawing and was applied with an electric knapsack sprayer equipped with an anti-drift fan nozzle CVI 110° green (Serena EL 16 LT; Italdifra Agricultural Tools S.r.l., Francofonte, Italy) at 2.5 bar. The manual application with the knapsack sprayer allowed a more precise treatment of the fruit-free canopy. The amount of *H. uvarum* applied was previously tested to avoid dripping from the leaves to the ground. Targeted treatment of the fruit-free canopy with *H. uvarum* + spinosad was possible for both training types (Fig. 1). On the pergola, one treatment was applied from below, and on the Guyot system, one treatment was applied on both sides of the plant. The treated area of the canopy had a width of approximately 80 cm.

The concentrations and volumes used resulted in an applied amount of approximately 0.012 g of spinosad per m^2 of treated area for the spinosad treatment and for the *H. uvarum* + spinosad treatment. The dates of application, the leaf sampling dates for the laboratory efficacy evaluation in 2019, the two leaf sampling dates and grape sampling dates for the residual analyses in 2020, and the harvest dates are in Fig. 2(a). In 2019, the second treatment was applied after an increase in *D. suzukii* infestation of grapes was observed. The harvest date was 28 September in 2019 and 23 September in 2020, which took into account the 15-day pre-harvest interval of the insecticide and the maturity of the grapes.

Grape samples were collected from the central row of the plot to minimize border effects. Ten samples were collected during the test period in 2019 and nine samples were collected in 2020. Each sample consisted of 50 single, blue and ripe intact berries that were cut off randomly with the berry stalk. The number of infested grape berries was counted in the laboratory with a stereomicroscope (Leica MZ 6, Leica Microsystem Srl, Milan, Italy). Eggs were visible on the surface of the grape skin by viewing the oviposition hole and two milky-white filaments protruding out of the egg. The *D. suzukii* infestation at each timepoint was recorded as the percentage of grape berries with at least one *D. suzukii* egg. Meteorological data were obtained from the meteorological station of the Laimburg Research Centre (46°22′56.8″N, 11°17′19.5″E).

Spinosad residues were determined through liquid chromatography–tandem mass spectrometry (LC–MS/MS) as milligrams of spinosad (sum of spinosyns A and D) per kilogram of leaves or grapes during the field trial performed in 2020 following the European standard method for the analysis of pesticide residues (UNI EN 15662:2018)[31] at the Laimburg Research Centre. One sample consisted of 17 leaves without leafstalk or approximately 300 g of grape berries. The samples were collected evenly in the central row of each block.

2.3.2 *Efficacy evaluation of the field application in the laboratory*

Leaves from the field trial in 2019 were sampled randomly in the central row of the three plots from the treated canopy to observe the effect of the treatments on *D. suzukii* males and females in the laboratory. Samples were taken 1 and 7 days after the first application (leaf sampling 1 and 2) and 1 and 7 days after the second application (leaf sampling 3 and 4) (Fig. 2(a)). In the laboratory, five leaves from the same treatment were placed with the stalk in a 100-mL Erlenmeyer flask filled with tap water. The Erlenmeyer flask opening was closed around the stalk with cotton and then placed in an insect cage. The cage also contained three undamaged and untreated grape berries from the control plots on a

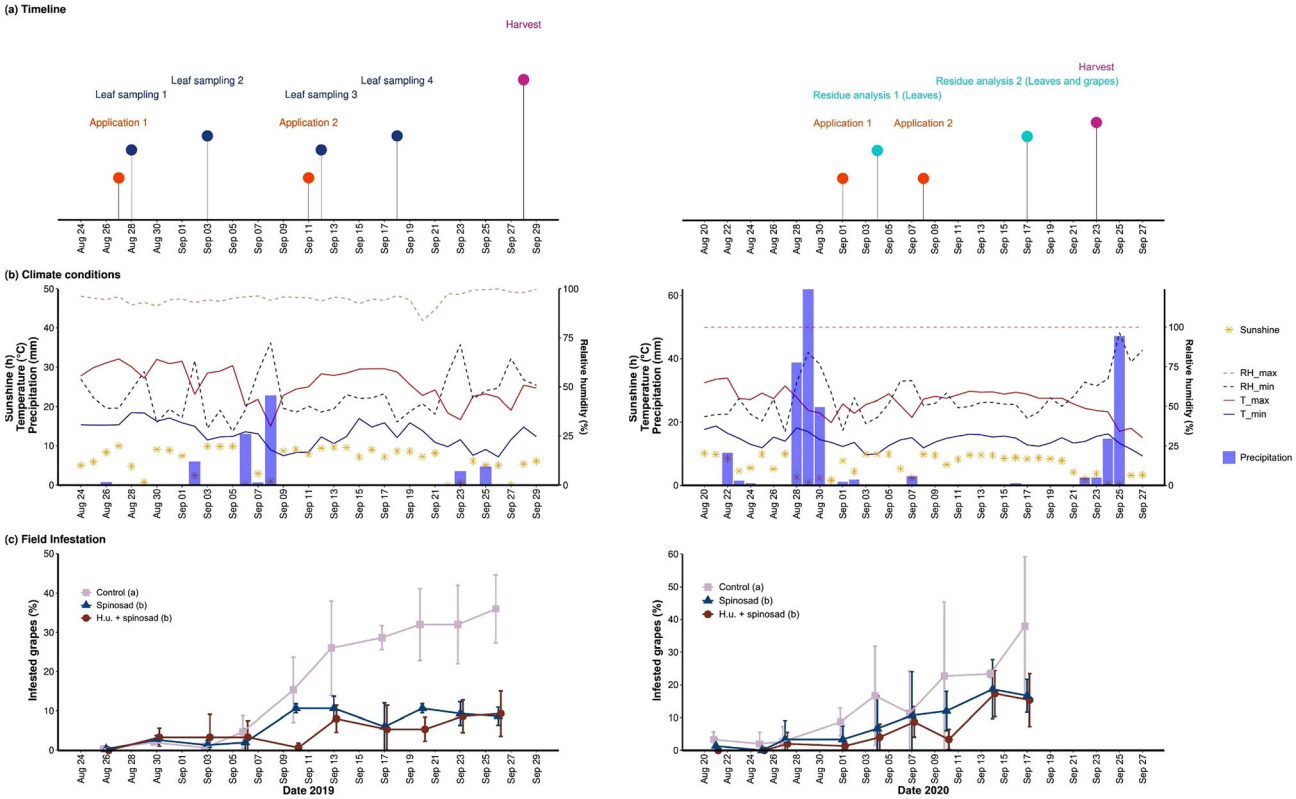

Figure 2. Effect of spinosad application with and without *H. uvarum* bait on *D. suzukii* field infestation of grapes trained with a pergola in 2019 (left) and with the Guyot method in 2020 (right). (a) Timeline with timepoints for the applications, leaf sampling for laboratory trials in 2019 (four samplings), leaf and grape sampling for spinosad residue analyses in 2020 (two samplings) and harvest. (b) Hours of sunshine, maximum and minimum relative humidity (RH), maximum and minimum temperature (T) and daily precipitation during the field trial. (c) Effect of the treatments on the mean *D. suzukii* infestation (% infested grapes ± SD). The treatments included an unsprayed control (Control), conventional spinosad treatment of the whole plant (Spinosad) and *H. uvarum* culture with spinosad treatment applied to the fruit-free zone (H.u. + spinosad). The applied spinosad amounts were 120 g per hectare for the conventional spinosad treatment and 36.4 g per hectare for *H. uvarum* with spinosad. Treatment names followed by different lowercase letters in brackets denote significant differences in infestation between the treatments ($P < 0.05$, $n = 3$).

Petri dish (diameter 9 cm, polystyrene) with water agar (15 g L^{-1} agar-agar; Merck, Darmstadt, Germany) for oviposition and cotton soaked in 10 mL of 5% sucrose solution in a small Petri dish (diameter 6 cm, polystyrene). Water agar served as an additional oviposition substrate. Twenty *D. suzukii* females and 20 males were released in the cage for 48 h. After 24 h, the berries and the water agar were replaced to count the eggs, and dead flies were removed and counted. Mortality was evaluated as the percentage of the initial number of flies, and the oviposition rate was evaluated as the number of eggs laid on the grapes and on the water agar per cage. The cages were kept under the same conditions as the *D. suzukii* rearing and arranged in a completely randomized design. Single cages were used as replicates ($n = 6$).

2.4 Comparison of *H. uvarum* formulations in the greenhouse

Rooted grafted vines of the variety Vernatsch (Clone: Edelvernatsch Lb 43, Rootstock: SO4) were potted in 4-L pots filled with standard soil (SP ED63 T coarsely; Einheitserde, Sinntal-Altengronau, Germany). The plants were grown for 2 months in the greenhouse and treated once a week for 20 min with vaporized sulfur against powdery mildew using a sulfur burner. No sulfur treatments were performed during the assay in May 2020. The mean temperature and relative humidity in the greenhouse during the assay were 21.8 °C (min. 17.3 °C, max. 29.8 °C) and 85.2% (min. 41.9%, max. 100%), respectively.

The formulations preserved in different ways by Agrifutur srl were used in this assay. Five different treatments were applied to the vines: freeze-dried *H. uvarum* pellets dissolved in water, water + spinosad, *H. uvarum* culture + spinosad, *H. uvarum* supernatant + spinosad and freeze-dried *H. uvarum* pellets dissolved in water + spinosad. The treatments with spinosad contained 5.43 mg of spinosad per liter of solution (11.3 µL of Laser per liter of solution), which was added after thawing and shortly before application. The chosen spinosad concentration was based on previous studies.[5,8]

Each treatment was applied to 11 plants to evaluate its effect on *D. suzukii* flies and to measure the spinosad residue. Per plant, 10 leaves were marked at the stalk with a twist tie before treatment. The treatment consisted of 10 drops to 10 µL per leaf using a multichannel pipette (5–100 µL; Eppendorf Research Plus, Hamburg, Germany). One day, 7 days and 14 days after treatment, 25 leaves treated in the same way were randomly removed from the 11 plants and transferred to the laboratory.

In the laboratory, five leaves treated in the same way were immediately pooled and placed with the stalk in a 100-mL Erlenmeyer flask filled with tap water. The opening around the stalk was closed with cotton. After that, the flask with the five leaves was placed in an insect cage. Twenty male and 20 female flies were exposed to the five leaves for 48 h. The cages also contained four nontreated blueberries (*Vaccinium corymbosum*) from organic production on a Petri dish (diameter 9 cm, polystyrene) with water agar (15 g L^{-1} agar-agar; Merck, Darmstadt,

Germany) for oviposition and cotton soaked in 10 mL of 5% sucrose solution in a small Petri dish (diameter 6 cm, polystyrene) as a water and energy source. The blueberries were washed under cool running tap water for approximately 1 min and dried with a paper towel before use. The cages were kept under the same conditions as the *D. suzukii* rearing and arranged in a completely randomized design. Single cages served as replicates ($n = 5$). After 24 h, the berries and the water agar were replaced to count the eggs, and dead flies were removed and counted. After 48 h of exposure, mortality was evaluated as a percent of the initial number of flies, and oviposition was evaluated as the number of eggs per cage.

To measure the spinosad residue amount on the leaves, one sample per treatment consisting of 10 leaves (one leaf per plant) from different positions was cut off without leafstalk 1 day, 7 days and 14 days after treatment. Samples were stored for no more than 1 month at −80 °C until analysis. The spinosad residues were analyzed by the same method as described above.

2.5 Statistical analyses

The *D. suzukii* infestation in the field over the entire experimental period was analyzed with a linear mixed-effects model. The treatments were input to the model as fixed effects, while the sampling date and block were input as random effects. Tukey's pairwise comparisons were performed for the treatments.

The *D. suzukii* mortality and number of eggs laid per cage in the assays testing the efficacy of the field treatment in the laboratory and in the assays comparing the different *H. uvarum* formulations in the greenhouse were evaluated independently for each time point. Data were analyzed with a generalized linear model fitted with a gamma distribution. Datasets with zero values were $x + 1$ transformed to allow the use of a gamma distribution. The treatment and the sex of the flies entered the model as fixed effects. Models were chosen based on Akaike information criterion values, and residuals were analyzed to verify the distribution of the errors. Tukey's pairwise comparisons were performed for the treatments.

All statistical analyses were prepared with R version 4.0.2 (The R Foundation for Statistical Computing http://www.R-project.org).

3 RESULTS

3.1 Vineyard trials

3.1.1 *Efficacy of the* H. uvarum *treatment in a vineyard trained with a pergola in 2019*

In 2019, the temperature and rainfall were typical for the region in August and September (Fig. 2(b)). Some rainfall was recorded after the second application and before harvest. Most days were characterized by sunshine and maximum temperatures above 25 °C.

A significant effect of the treatments on *D. suzukii* infestation over the entire experimental period occurred ($F_{2,86.82} = 31.344$, $P < 0.001$) (Fig. 2(c)). The treatment of the whole plant with spinosad and the treatment of the foliage with *H. uvarum* + spinosad reduced the *D. suzukii* field infestation significantly compared to the unsprayed control ($P < 0.001$). No differences were observed between the spinosad treatment and *H. uvarum* + spinosad treatment ($P = 0.683$).

Table 1. Spinosad residues (mean mg/kg ± SD) on leaves and grapes sampled during the field trial and trained with the Guyot system in 2020 ($n = 3$)

	Spinosad (mg/kg)		
Treatment	Leaves Sep 04	Leaves Sep 17	Grapes Sep 17
Control	<0.01	<0.01	<0.01
Sp	1.31 ± 0.40	0.54 ± 0.20	0.05 ± 0.02
H.u. + Sp	0.16 ± 0.10	0.25 ± 0.07	<0.01

The treatments were an unsprayed control (Control), a spinosad treatment applied to the whole plant (Sp) and *H. uvarum* with spinosad treatment applied in the fruit-free zone (H.u. + Sp).

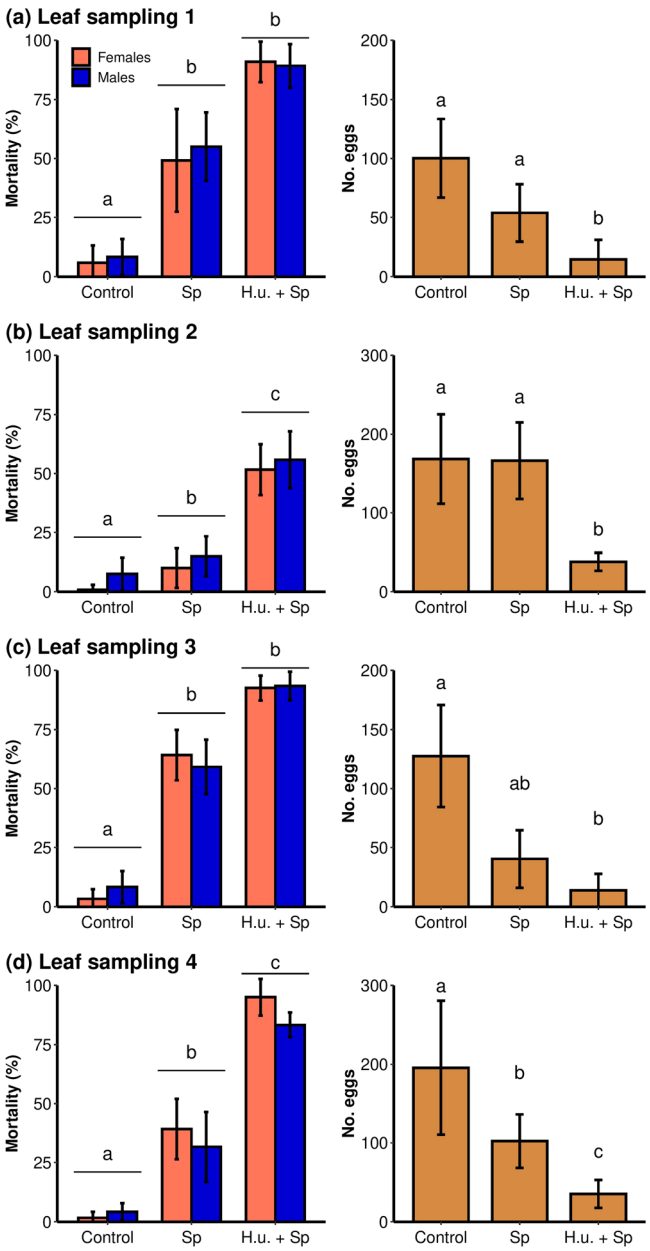

Figure 3. Effect of different treatments applied in the vineyard. Mean *D. suzukii* female and male mortality ± SD (left) and mean number of eggs laid per cage ± SD (right) during 48 h of exposure to leaves collected (a) 1 day and (b) 7 days after a first application and (c) 1 day and (d) 7 days after a second application. The treatments were applied in a vineyard with a pergola training system in 2019 and included unsprayed control (Control), spinosad in water (Sp) and *H. uvarum* culture with spinosad (H.u. + Sp). Different letters denote significant differences in *D. suzukii* mortality or number of eggs laid between the treatments ($P < 0.05$, $n = 6$).

3.1.2 *Efficacy of the* H. uvarum *treatment in a vineyard trained with the Guyot method in 2020*

The experimental period in 2020 was characterized by intense rainfall over 3 days before the first application and low precipitation after the first application until harvest. Furthermore, most days were characterized by sunshine and maximum temperatures above 25 °C (Fig. 2(b)). Some *D. suzukii* infestation was already present before the first application was applied, and the *D. suzukii* infestation showed a constant increase in all treatments until harvest (Fig. 2(c)). The treatments had a significant effect on *D. suzukii* infestation ($F_{2,77.25} = 10.9$, $P < 0.001$). The foliage treatment with *H. uvarum* + spinosad significantly reduced *D. suzukii* infestation compared to the unsprayed control ($P < 0.001$). Treatment of the whole plant with spinosad also significantly reduced *D. suzukii* infestation compared to the unsprayed control ($P = 0.003$). No differences in efficacy were observed between the spinosad treatment and *H. uvarum* + spinosad treatment ($P = 0.444$).

The residue analyses showed that a lower amount of spinosad was present on the leaves treated with *H. uvarum* + spinosad, while the spinosad treatment resulted in more spinosad residue on the leaves (Table 1). In the unsprayed control treatment, no spinosad residues were found. Furthermore, no residues were detected on the untreated grapes from the control treatment and on the untreated grapes from the *H. uvarum* + spinosad treatment.

3.1.3 *Efficacy evaluation of the field application in the laboratory*

No significant differences between *D. suzukii* male and female mortality were observed (leaf sampling 1: $F_{1,35} = 0.038$, $P = 0.847$; leaf sampling 2: $F_{1,35} = 0.399$, $P = 0.532$; leaf sampling

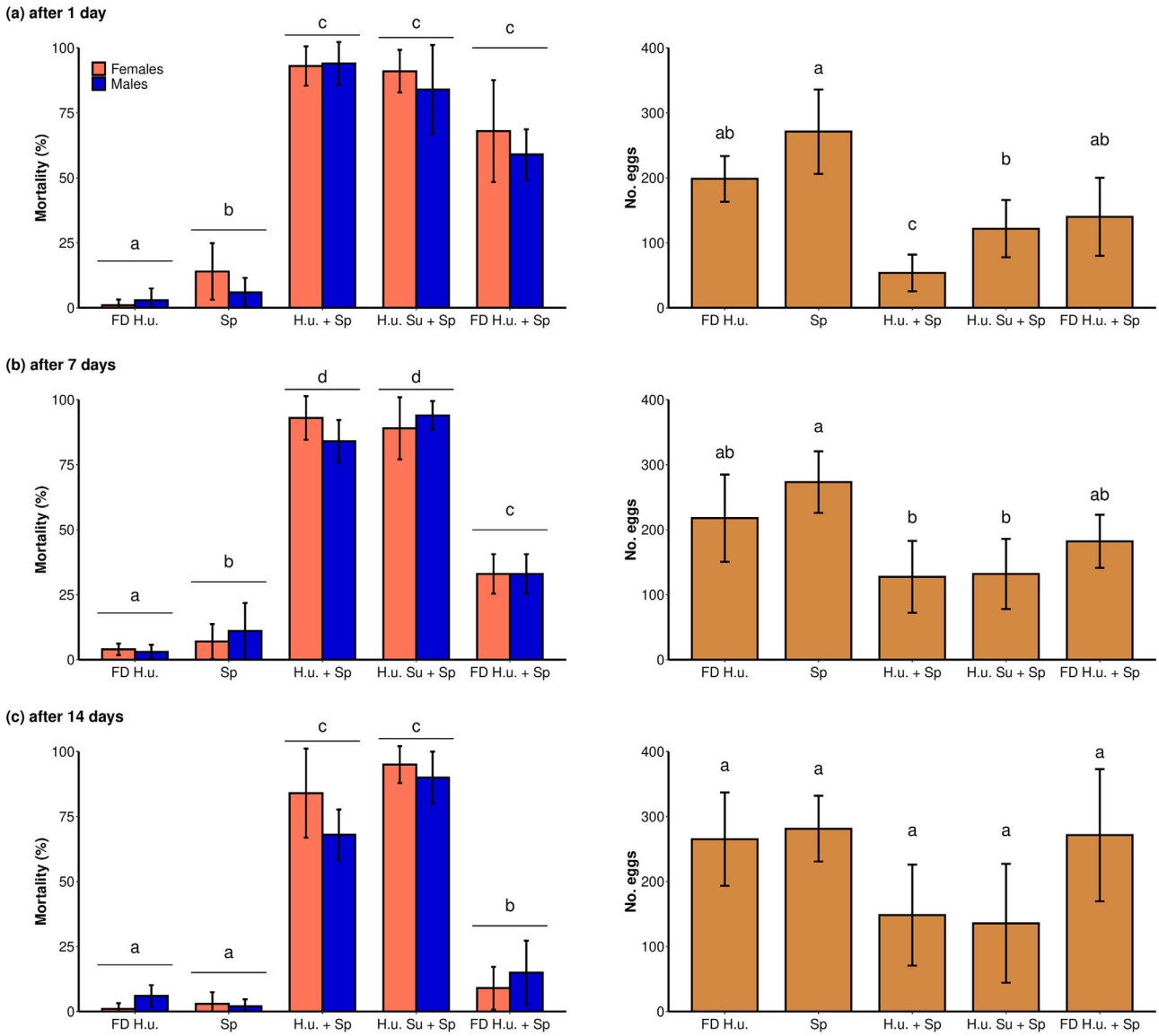

Figure 4. Effect of leaves treated with different *H. uvarum* formulations in the greenhouse. Mean *D. suzukii* female and male mortality ± SD (left) and mean number of eggs laid per cage ± SD (right) during 48 h of exposure to leaves collected 1 day (a), 7 days (b) or 14 days (c) after application. The treatments were applied to vine plants in the greenhouse and included an insecticide-free formulation prepared from freeze-dried *H. uvarum* pellets (FD H.u.), spinosad in water (Sp) and one of three *H. uvarum* formulations with spinosad. The formulations were *H. uvarum* culture + spinosad (H.u. + Sp), *H. uvarum* supernatant + spinosad (H.u. Su + Sp) and a formulation made from freeze-dried *H. uvarum* pellets and water + spinosad (FD H.u. + Sp). Different letters denote significant differences in *D. suzukii* mortality or number of eggs laid between the treatments ($P < 0.05$, $n = 5$).

3: $F_{1,35} = 0.001$, $P = 0.981$; leaf sampling 4: $F_{1,35} = 0.2934$, $P = 0.592$). The different treatments had a significant effect on the mortality of *D. suzukii* adults (leaf sampling 1: $F_{2,35} = 45.792$, $P < 0.001$; leaf sampling 2: $F_{2,35} = 27.228$, $P < 0.001$; leaf sampling 3: $F_{2,35} = 60.99$, $P < 0.001$; leaf sampling 4: $F_{2,35} = 74.072$, $P < 0.001$) and on the number of eggs laid (leaf sampling 1: $F_{2,17} = 9.999$, $P = 0.002$; leaf sampling 2: $F_{2,17} = 1.406$, $P < 0.001$; leaf sampling 3: $F_{2,17} = 13.733$, $P < 0.001$; leaf sampling 4: $F_{2,17} = 21.438$, $P < 0.001$) (Fig. 3).

For the leaves collected 1 day after the first application (leaf sampling 1; Fig. 3(a)), both spinosad and *H. uvarum* + spinosad caused mortality over 50% and reduced the number of eggs laid by 46.7% or 83.2%, respectively. One week after application, *H. uvarum* + spinosad caused mortality of 53.8% while the spinosad treatment caused mortality of 12.5%; moreover, a significant influence on the eggs laid was not observed for spinosad without *H. uvarum* (leaf sampling 2; Fig. 3(b)). The leaves sampled after the second application (leaf sampling 3; Fig. 3(c)) confirmed the results observed 1 day after the first application. Additionally, 1 week after the second application (leaf sampling 4; Fig. 3(d)), *H. uvarum* + spinosad caused significant higher mortality and reduced oviposition compared to the control or the spinosad treatment.

3.2 Comparison of the *H. uvarum* formulations in the greenhouse

No significant differences were found in mortality between males and females (after 1 day: $F_{1,49} = 0.121$, $P = 0.73$; after 7 days: $F_{1,49} = 0.001$, $P = 0.98$; after 14 days: $F_{1,49} = 0.039$, $P = 0.844$). The different treatments had a significant effect on the mortality of *D. suzukii* adults (after 1 day: $F_{4,49} = 35.565$, $P < 0.001$; after 7 days: $F_{4,49} = 61.14$, $P < 0.001$; after 14 days: $F_{4,49} = 38.771$, $P < 0.001$) and on the number of eggs laid (after 1 day: $F_{4,24} = 12.274$, $P < 0.001$; after 7 days: $F_{4,24} = 5.027$, $P = 0.006$; after 14 days: $F_{4,24} = 3.128$, $P = 0.038$) (Fig. 4).

Over the 2-week experimental period, all three *H. uvarum* formulations with spinosad increased mortality and reduced oviposition more than the spinosad treatment without *H. uvarum*. The spinosad treatment caused a low but significantly higher fly mortality at 1 day (10%) and 7 days (9%) after application compared to the insecticide-free control (2%) (Fig. 4(a),(b)). Over the whole test period, *H. uvarum* culture + spinosad and *H. uvarum* supernatant + spinosad were the most effective formulations and still resulted in over 75% mortality after 2 weeks (Fig. 4(c)). In contrast, the formulation prepared with freeze-dried *H. uvarum* pellets, water + spinosad showed a significantly lower mortality after 1 and 2 weeks compared to the other two *H. uvarum* formulations.

Analyses of the spinosad residues on the leaves from the different treatments revealed high variability (Table 2). A trend toward higher degradation of spinosad was observed in the formulation prepared of freeze-dried *H. uvarum* pellets.

Table 2. Spinosad residues on leaves collected 1 day (T1), 7 days (T7) and 14 days (T14) after applying the different treatments ($n = 1$)

	Spinosad (mg/kg)		
Treatment	T1	T7	T14
FD H.u.	<0.01	<0.01	<0.01
Sp	NA[a]	0.41	0.34
H.u. + Sp	1.29	0.78	0.35
H.u. Su + Sp	0.62	0.36	0.29
FD H.u. + Sp	0.64	0.13	0.06

[a] Not available due to a measurement error.
The treatments were a formulation made from freeze-dried *H. uvarum* pellets and water (FD H.u.), water + spinosad (Sp), *H. uvarum* culture + spinosad (H.u. + Sp), *H. uvarum* supernatant + spinosad (H.u. Su + Sp) and the formulation made of freeze-dried *H. uvarum* pellets and water + spinosad (FD H.u. + Sp).

4 DISCUSSION

In field trials over 2 years, yeast cultures of *H. uvarum* with spinosad were compared with spinosad applications to control *D. suzukii* in vineyards. The results showed that targeted treatment of the foliage without spraying the berries of the grapevines was possible for both the pergola and Guyot methods, and as efficient pest control as the conventional insecticide treatment of the whole plant. Compared to the application of spinosad on the whole plant, the treatment based on *H. uvarum* with the addition of spinosad that was applied only on the foliage did not leave spinosad residue on the fruits at harvest. Furthermore, the amount of spinosad applied in the *H. uvarum* treatment was 36.48 g of spinosad per hectare, which was approximately three times lower than that in the conventional treatment with 120 g of spinosad per hectare. Based on these results, the proposed pest control strategy based on *H. uvarum* and spinosad could be a practicable alternative to typical insecticide applications.

H. uvarum bait with spinosad targets highly mobile adults. Control of adult *D. suzukii* is important because flies immigrating into vineyards from noncrop hosts cause initial infestations.[32–34] Since *D. suzukii* females are more attracted to the fruit than to the grapevine leaves, a promising strategy is the application of the bait evenly on the foliage, thus creating a multitude of attractive points to reduce the attraction to the fruit and increase the attraction to *H. uvarum*-treated leaves.[19,35] After attraction, the flies readily come into contact with the insecticide as they stay on the leaves to feed. Another aspect is the persistence of the attractiveness of the bait and the insecticidal effect of the insecticide. The evaluation of the leaves in the laboratory showed that the *H. uvarum* bait was still effective after 1 week and up to 2 weeks with two applications. Since spinosad loses some effect after 1 week,[22] the treatments should be applied at intervals of 1 week to 2 weeks based on the *D. suzukii* infestation, predicted rainfall and precipitation quantity after application, which could affect the efficacy by washing off the *H. uvarum* bait and the insecticide. The results from the laboratory showed the higher efficacy of spinosad in the *H. uvarum* treatment and confirmed that *H. uvarum*-treated leaves were indeed more effective.[18] Since the applied dose of active ingredient per area treated zone was not reduced in the *H. uvarum* treatment, no negative effects on resistance development are to be expected. As different insecticides can be used in combination with *H. uvarum*,[18,19] exchanging the active ingredients could also reduce the risk of developing resistance, such as to spinosad,[36,37] and allow for the application of this control strategy to crops for which spinosad is not registered.

For practical uses in agriculture, an issue could be the difficulty in obtaining a stable *H. uvarum*-based formulation.[19,20] For commercial use, a stable and dry product would simplify marketing and use by farmers. Freeze-dried *H. uvarum* pellets were tested since smaller volumes reduce the costs of storage and transport. A second possibility would be the elimination of *H. uvarum* cells. The storage of a sterile product without living cells does not require any special preservation to maintain vitality, and the cells

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can deposit in the sprayer. In this study, the supernatant without *H. uvarum* cells had a similar efficacy as the whole *H. uvarum* culture (both stored at −80 °C before application) and both retained their effect over 2 weeks. This finding was not surprising since the largest part of the yeast metabolites in a similarly grown culture of *H. uvarum* was in the supernatant and not in the yeast cells.[10] The freeze-dried *H. uvarum* pellets dissolved in water lost some efficacy. Although a prior centrifugation step reduced the effort and energy requirements compared to freeze-drying the entire *H. uvarum* culture, it also probably caused the loss of important components in the supernatant. Furthermore, leaves treated with freeze-dried *H. uvarum* pellets dissolved in water with spinosad showed the lowest spinosad residues after 14 days. Numerous factors affect the stability of spinosad, such as photolysis and biotic degradation.[38] Further studies are necessary to determine the reasons for the differences in spinosad residues observed under the experimental conditions reported in this study.

In addition to the necessary improvement of the formulation, new emerging precision technologies could simplify the implementation of the proposed targeted treatment strategy for fruit-free canopies and reduce the drift of the yeast formulation onto weeds and surrounding vegetation, and thus the hazards to non-target organisms.[39] On the pergola, the treatment can be applied by one application from below to the canopy, while with the Guyot system, the treatment can be applied from both sides to the canopy. On the Guyot system, the grapes are below the treated canopy, therefore the probability of dripping from the treated canopy to the grapes is higher. No spinosad residues were found on the grapes, therefore it can be assumed that at an application rate of 230 L of yeast culture per hectare, no notable dripping to the fruit occurred. In the pergola system, the canopy- and grape-containing zones are not on top of each other, which reduces the risk of dripping on the grapes. Therefore, residues on the grapes are less likely. For small-scale field applications, the spraying of *H. uvarum* with spinosad bait using a knapsack sprayer can provide an alternative for the control of *D. suzukii*. Advantages result from the applicability in different training systems and manual application, which allows for the easy and selective treatment of the canopy. Further improvements should focus on the development of spraying equipment for large-scale vineyards, which allows for fast and precise application due to the automatic limitation of the application to the grape-free canopy.

5 CONCLUSION

The yeast *H. uvarum* can be used for attract-and-kill control strategies against *D. suzukii* under the conditions proposed in this study. The advantages of this method in terms of sustainable control measures are associated with the lower amount of residual spinosad on the fruits and the reduced amount of insecticide applied in the environment. Commercial and storable formulations based on *H. uvarum* should avoid the loss of the supernatant, which contains attractive and feeding stimulant compounds, while the preservation of living yeast cells seems to be less important. Further studies are needed to explore the efficacy of this technique on other fruit crops and to develop a stable, easy-to-store and ready-to-use product.

ACKNOWLEDGEMENTS

We thank Andrea Lentola (Laboratory for Residues and Contaminants, Laimburg Research Centre) for the analyses of the spinosad residues, Werner Pichler (Agrobusiness Laimburg) for maintaining the vineyards and assistance during field treatments, and Roberto Kron Morelli (Agrifutur srl) for providing yeast cultures. The present work was funded by the European Regional Development Fund (ERDF) 2014–2020, project DROMYTAL (FESR1021, CUP H32F16000420009). PGB was also supported by the Swedish Research Council Formas (Grant 2015–1221) and the SLU Centre for Biological Control (CBC). Open-access publication costs were covered by the Department of Innovation, Research, University and Museums of the Autonomous Province Bozen/Bolzano.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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11 Curriculum vitae

Dipl.-Ing. Dipl.-Ing. Urban Spitaler, BSc

Born in Bozen, South Tyrol, Italy on January 17, 1991

Education

2017 to present **Doctoral studies of Natural Resources and Life Sciences**
University of Natural Resources and Life Sciences, Vienna (Austria)

2012 to 2016 **Master's program Crop Sciences**
University of Natural Resources and Life Sciences, Vienna (Austria)

Master's thesis at the Institute of Plant Breeding in cooperation with the Institute of Chemistry of Renewable Resources: „Quantitative Analyse des Anthocyan- und Phenolgehaltes von Weizen (*Triticum aestivum* L.) mittels UV/Vis-Spektroskopie und HPTLC"

2012 to 2016 **Master's program Phytomedicine**
University of Natural Resources and Life Sciences, Vienna (Austria)

Master's thesis at the Institute of Plant Protection in cooperation with the Laimburg Research Centre: „Influence of dietary yeasts on the fecundity and oviposition of adult spotted-wing drosophila (*Drosophila suzukii*; Diptera: Drosophilidae)"

2010 to 2012 **Bachelor's program Agricultural Sciences**
University of Natural Resources and Life Sciences, Vienna (Austria)

Bachelor's thesis at the Institute of Viticulture and Pomology: „Goji – Neuer Hype um alte Frucht: Reelle Chancen für den österreichischen Obstbau?"

2005 to 2010 **Technical School of Agriculture, Auer (Italy)**

Employment

2017 to present **Laimburg Research Centre (Italy)**
Employee at the Institute for Plant Health

2014 to 2015 **University of Natural Resources and Life Sciences, Vienna (Austria)**
Student assistant at the "Pflanzenschutz-Übungen" and "Symbionten und Pathogene in der Rhizosphäre"