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Original Article

Hippophae rhamnoides berry related *Pichia kudriavzevii* yeast volatiles modify behaviour of *Rhagoletis batava* flies

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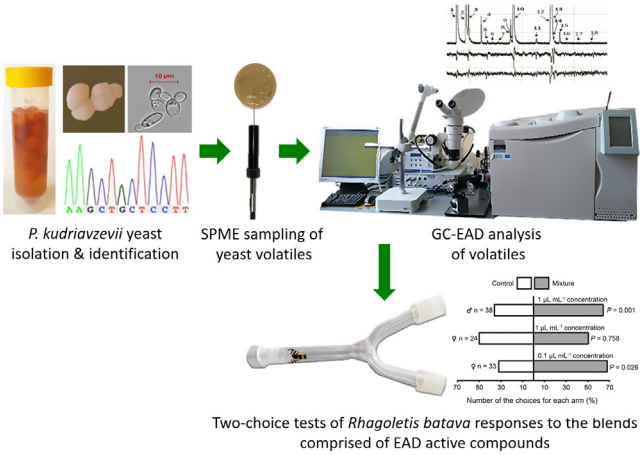
HIGHLIGHTS

- *Pichia kudriavzevii* yeasts were isolated from ripe *Hippophae rhamnoides* berries.
- Thirty-five yeast volatiles were identified from the headspace of *P. kudriavzevii*.
- Esters and alcohols contributed by 32% and 66% to the total blend amount.
- Ten of those volatiles elicited antenna responses of *Rhagoletis batava* flies.
- Mixture of synthetic olfactory active compounds attracted *R. batava* males and females.

GRAPHICAL ABSTRACT



Two-choice tests of *Rhagoletis batava* responses to the blends comprised of EAD active compounds

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ABSTRACT

Olfactory cues have a large impact on insect behaviour and fitness consequently showing potential in pest management. Yeast released volatiles are used by insects as olfactory cues for finding feeding and oviposition sites. The yeast strain SB-16-15 was isolated from spontaneous fermentation of *Hippophae rhamnoides* berries and identified as *Pichia kudriavzevii*. Thirty-nine volatiles were sampled from the headspace of *P. kudriavzevii* yeasts by solid phase micro extraction and identified by gas chromatography and mass spectrometry techniques. Ten of those volatiles elicited antennal responses of *Rhagoletis batava* flies, one of the most serious pest of *H. rhamnoides* berries. In the two-choice experiments, *R. batava* flies preferred the mixture composed of nine synthetic compounds analogous to electroanntenographic active volatiles released by the yeasts compare to the solvent control. Female flies were significantly attracted to the mixture at the concentration 0.1 $\mu L\,mL^{-1}$ and showed no preference to the mixture at the

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Gas chromatography-mass spectrometry
Gas chromatography–electroantenno graphic detection

concentration 1 μL mL^{-1} versus control while males reacted positively to the synthetic blend at the concentration 1 μL mL^{-1}. Herein, for the first time, behaviour modifying effect of *H. rhamnoides* berry related yeast volatiles was shown suggesting these semiochemicals have potential in use for monitoring *R. batava* flies.

Introduction

Sea buckthorn, *Hippophae rhamnoides* L. (Rosales: Elaeagnaceae) is a hardy, deciduous shrub native to Europe and Asia [1]. In Europe, *H. rhamnoides* is naturally distributed on river banks and coastal dunes along the Baltic Coast of Finland, Poland, Germany as well as along the Gulf of Bothnia in Sweden [1,2]. Due to drought resistance, rapid development of an extensive root system, capability of holding the soil on fragile slopes, ability to fix nitrogen due to association with nitrogen-fixing symbiotic Actinomycetes and conserve other essential nutrients, *H. rhamnoides* is an ideal plant for soil erosion control and land reclamation [1,3,4]. Sea buckthorn berries are among the most nutritious fruits known with vitamin C contents of 360 mg 100 g^{-1} of berries for the European subspecies *H. rhamnoides* [5] to 2500 mg 100 g^{-1} of berries for the Chinese subspecies *H. r. sinensis* [6]. Phytochemicals from berries and essential oil produced from berry pulp and seeds diminish inflammation, have antibacterial effect, relieve pain, promote regeneration of tissues, and have anticancer and radio-protective activity [1,7–11].

Sea buckthorn fly, *Rhagoletis batava* Hering (Diptera: Tephritidae), is the most harmful insect pest of sea buckthorn berries. In the years of mass development of *R. batava* flies without applying pest control means, damage reach 100% [12]. As far as we know *R. batava* damage sea buckthorn berries only, being a highly specialized pest. *R. batava* species was described in the Netherlands [13] and was known only in a few European countries causing no economically important damage to *H. rhamnoides*, however for a long time, this species has been a serious pest of sea-buckthorn in Western Siberia and Altai Region [12,14]. In 1970, Kolomiec has described the Siberian population of sea-buckthorn flies as a new subspecies, *Rhagoletis batava obscuriosa* Kol. On the basis of range expansion data of *R. batava* and notably increased damage level of *H. rhamnoides* berries in the new distribution areas of the flies, Stalažs and Balalaikins [15] have postulated that the more aggressive *R. b. obscuriosa* flies from Siberia is spreading through the eastern part of Russian Federation to Central and Western Europe. There are number of environment-friendly pest control strategies that could potentially be used to keep *R. batava* population below an economically significant level including natural limiting factors like parasites or entomopatogenic fungi; sterile insect technique; or methods based on application of semiochemicals, for example lure and kill, push-pull techniques and etc. [16–18]. Moreover, semiochemicals are widely used in monitoring programs including efficiency control of pest density regulating methods [16]. Unfortunately, up to our knowledge, no semiochemicals have been identified for *R. batava* species.

The goal of this study was to identify volatiles released by yeasts colonizing *H. rhamnoides* berries that could have behaviour modulating effect on *R. batava* flies. The aims have been formulated to determine whether: (i) some yeast species populating *H. rhamnoides* berries could be isolated and cultivated under laboratory conditions; (ii) some volatiles produce by yeast elicit electroantenographic responses in *R. batava* females and males; (iii) a blend comprised of synthetic compounds analogous to electroantenographic active volatiles have a behaviour modifying effect on *R. batava* flies under laboratory conditions.

Material and methods

Insects

Sea buckthorn flies used in laboratory studies, were collected in May 2018 as puparia in soil under sea buckthorn shrubs with damaged berries located in organic sea buckthorn plantation (global positioning system (GPS) coordinates: 55°15′12.179″N, 25°26′23.049″E) in Stacijava village, Molėtai district, Lithuania. Each puparium was separately placed in 14 mL glass vial containing wet 3 cm^2 filter paper inside and corked by foam stoppers. Vials were placed in a climate chamber "Fitotron" (Weiss Gallenkamp, UK) under 20–24 °C, 16L:8D (light:dark) photoperiod and 65–75% relative humidity. Two times a week 2–3 drops of water were added on a filter paper to maintain high humidity inside a vial. Emerged adults were kept in the same vials in walk-in climate room under 18–20 °C, natural day light photoperiod, 50–60% relative humidity and fed on 10% sugar solution in water.

Yeast isolation and identification

H. rhamnoides berries were sampled from the private farm located in the Vilnius region of Lithuania (GPS coordinates: 54°75′20.0″N, 25°27′99.6″E) in the mid-September 2016. Sea buckthorn berries were aseptically collected by using scissors cleaned with 70% ethanol, placed into sterile bags, transported to the laboratory and processed within 2 h after harvesting. Thirty grams of berries were placed into sterile tubes with 5% dextrose solution and kept for 15 days at a 18 °C temperature. Serial dilutions were made in a Ringer (Merck authorized distributor Biotecha UAB, Vilnius, Lithuania) solution, plated on yeast extract-peptone-dextrose (YPD)-agar plates (1% yeast extract, 1% peptone, 2% dextrose, 2% agar) [19] containing 50 μg mL^{-1} chloramphenicol and incubated for 2–3 days at 25 °C. Selected colonies were applied for morphological analysis and molecular identification.

DNA was isolated from fresh yeast culture (24 h) by using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's instructions. For identification of yeast, the regions between the 18S rRNA and 28S rRNA genes containing two non-coding internal transcribed spacers (ITS-A and ITS-B) separated by the 5.8S rRNA gene were polymerase chain reaction (PCR)-amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [20,21] and sequenced at Base Clear (Leiden, Netherlands). The obtained sequences were compared with those found in the Nucleotide Similarity Search database (NCBI BLAST +), FASTA network service at the European Bioinformatics Institute (EMBL-EBI, Cambridge, UK).

Chemicals

The compounds used in behaviour tests namely 3-methylbut-1-yl propionate (≥99% chemical purity), 3-methylbutan-1-ol (≥99% chemical purity), ethyl octanoate (≥99% chemical purity), 2-phenylethyl acetate (≥99% chemical purity) and 2-phenyl ethanol (≥99% chemical purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethyl propionate (99% chemical purity), 3-

methylbut-1-yl acetate (99% chemical purity), ethyl hexanoate (99% chemical purity) were purchased from Alfa Aesar (Ward Hill, MA, USA); ethyl acetate (≥99% chemical purity), hexane as a solvent (≥99% chemical purity) was obtained from Carl Roth (Karsruhe, Germany).

D (+)-Glucose monohydrate (obtained from Merck Millipore, Darmstadt, Germany), yeast extract, peptone and agar (purchased from Liofilchem, Rosetodegli Abruzzi, Italy) were used for YPD-agar medium preparation and yeast cultivation. To avoid bacterial contamination, YPD medium was supplemented with chloramphenicol (purchased from Applichem, Darmstadt, Germany).

Sampling of yeast-produced volatiles

The solid phase micro-extraction (SPME) technique [22] was used to sample the headspace of *P. kudriavzevii* yeast strain SB-16-15. The yeasts were cultivated in polystyrene Petri dishes (Ø 55 mm × 14 mm) poured with 14 mL of YPD-agar for 2 days at 25 °C. As control, YPD-agar plates were used for sampling background odours. Before each collection period, the routine purification of SPME fibres coated with polydimethylsiloxane-divinylbenzene polymer (DVB/PDMS, 65 μm coating layer thickness, Supelco, Pennsylvania, USA), was conducted at 240 °C for about 10 min in a GC injector. Afterwards, the needle of SPME syringe was pierced through a small hole made in a wall of a Petri dish just above the yeast culture; the fibre was pushed out from the needle and exposed to the headspace for 60 min at room temperature. After sampling was finished, the fibre was transferred to the injection port of gas chromatograph and volatiles were thermally desorbed from the fibre during 2 min.

Gas chromatography–electroantennogram detection and identification of volatiles

Coupled gas chromatography-electroantennogram detection (GC-EAD) was performed using a Clarus 500 gas chromatograph (PerkinElmer, Waltham, MA, USA) and the Synthech electroantennogram detection system (Hilversum, Netherlands) [23]. The GC injector and the detector temperatures were set at 240 °C. The oven temperature was maintained isothermally at 40 °C for 1 min, afterwards it was raised to 200 °C at a rate of 5 °C min^{-1}, then increased to 240 °C at a rate of 10 °C min^{-1}, and then maintained isothermally for 11 min. Hydrogen at the flow rate 1.5 mL min^{-1} was used as a carrier gas. Five antennae from the flies of each sex were used for the GC-EAD analysis. Relative amounts of the compounds were determined as areas under chromatographic peaks.

The identification of volatiles was carried out using a Shimadzu gas chromatograph GC-2010 coupled with Shimadzu mass selective detector MS-QP 2010 Plus (Kyoto, Japan) [23–25], The GC was equipped with Restek Stabil-Wax column (30 m × 0.25 mm × 0.25 μm, Bellefonte, PA, USA) and operated under the same conditions as described in the GC-EAD experiments, except that helium was used as the carrier gas at the flow rate 1.5 mL min^{-1}. The yeast-derived volatile compounds were identified by comparison of their mass spectral data and their retention indexes with the corresponding data available from NIST version 2.0 mass spectra search programme (National Institute of Standards and Technology, USA) and with those of synthetic standards for the compounds indicated in the Table 1 using the software GCMSsolution version 2.71 (Shimadzu, Kyoto, Japan).

Behaviour tests

A Y-tube olfactometer (14.5 cm main tube, 10 cm arms, 130° branching angle, 0.9 cm inner diameter) was used to test preference of flies to synthetic samples versus control [26]. Four 18 W tube type lamps (T8/840, Colourlux plus, NARVA, Germany) covered with white, mat, plastic shield (65 cm length, 42 cm width) at a distance of 23 cm were placed in front of the Y tube of the olfactometer. Positive phototaxis is characteristic for sea buckthorn flies and the light stimulated the insects to move towards the light source. The Y tube was held at a 10° angle upward from horizontal on a holder. The arms of the olfactometer were connected to separate glass tubes that contained the stimulus versus control. Clean air was pushed at a rate of 0.5°L°min^{-1} through each arm using a clean air delivery system CADS-4CPP (Sigma Scientific LLC, Micanopy, FL, USA).

Bioassays were conducted to test attraction of the blend comprised of ethyl acetate, ethyl propionate, 3-methylbutyl acetate, 3-methylbutyl propionate, 3-methylbutanol, ethyl hexanoate, ethyl octanoate, phenylethyl acetate and 2-phenyl ethanol at the ratio 42:2:30:1:8:1:4:5:7 against a control. This ratio was selected based on relative amounts of EAD-active headspace volatiles released by *P. kudriavzevii*. The blend was tested at 1 μL mL^{-1} and 0.1 μL mL^{-1} concentrations by dispensing 10 μL of the prepared solution onto a filter paper strip (5 × 40 mm). After 0.5 min of solvent evaporation, the filter paper strip was placed in the glass tube connected to one arm of the olfactometer. The same size filter paper was treated with 10 μL of hexane and after solvent evaporation was placed in the other arm serving as control. The olfactometer was dismantled and the glassware was cleaned with hexane after each test, soaked overnight in distilled water, and dried for 2 h in an oven by rising the oven temperature from 100 to 200 °C. Silicone parts of the Y-tube olfactometer were cleaned with hexane, soaked overnight in distilled water, and air dried or replaced between the tests.

Male and female adults between 2 and 5-day-old were used in the experiment. Flies were allowed feeding on the sucrose solution until used in olfactometer bioassays. Single fly was released into the Y olfactometer at the end of the main tube. Pre-choice duration, i.e. the time within which a fly must have reached the branch point, was 15 min. A fly was considered to have made a choice when the fly reached the distal end of the glass tube containing the stimulus or a solvent control irrespectively whether the fly switched arms or not before reaching the odour source. If the fly did not choose an arm within 15 min, it was considered as not making choice. The positions of the two Y-tube arms were reversed after every five tests. All insects were observed individually and used in a bioassay only once. Out of 106 flies tested 11 specimens (all of them were females) failed to make a choice (10.4%). The tests were carried out at 25 ± 2 °C, 60% RH, between 10.00 and 17.00 h local time.

Statistical analysis

Nonparametric Mann-Whitney U test (Statistica 6.0, StatSoft, Inc., Tulsa, OK, USA) was applied to evaluate differences of volatile amounts between yeast and control samples. Generalised linear mixed model (GLMM), logistic regression (glmer) with binomial distribution of dependent variable (fly choice: 0, 1) was used to determine whether the effects of independent variables such as the treatment (factorial: control and stimulus), and sex (factorial: male and female) were significant. Afterwards, we evaluated the effect of treatment on fly choice in each fly sex (male or female) and for each stimulus concentration separately. The effect of the stimulus concentration has only been evaluated in the female flies. In all models, experimental replication was treated as a random variable. Statistical evaluations were carried out with program R, version 3.5.1 and RStudio version 1.1.463 (R Core Team, 2017; RStudio Team, 2018).

Table 1
Pichia kudriavzevii yeast produced volatiles and their electroantennographic activity to *Rhagoletis batava* flies.

No	Compound	RI	CAS No	Group	ID	Amount		EAD	
						Control	Yeast	Female	Male
1	Ethyl acetate[32]	898	141-78-6	E	L, RI	0.06 ± 0.02	11.17 ± 6.09*	4 (5)	4 (5)
2	Ethanol[32]	902	64-17-5	OH	L, RI	0.10 ± 0.02	11.50 ± 2.04**	NR	NR
3	Ethyl propionate[32]	915	1105-37-3	E	RC	0.01 ± 0.00	1.07 ± 0.09***	5 (5)	5 (5)
4	2-Methylprop-1-yl acetate[32]	985	110-19-0	E	RC	–	0.41 ± 0.16	NR	NR
5	Ethyl butanoate[32]	1013	105-54-4	E	RC	–	0.14 ± 0.03	5 (5)	4 (5)
6	Ethyl 2-methylbutanoate	1033	7452-79-1	E	RC	–	0.07 ± 0.01	NR	NR
7	But-1-yl acetate	1063	123-86-4	E	L, RI	0.10 ± 0.02	0.02 ± 0,01**	NR	NR
8	2-Methylpropan-1-ol[32]	1095	78-83-1	OH	L, RI	–	1.07 ± 0.02	NR	NR
9	Unknown	1099					0.24 ± 0.07	NR	NR
10	3-Methylbut-1-yl acetate[32]	1105	123-92-2	E	RC	0.10 ± 0.07-	9.04 ± 2.55*	4(5)	5 (5)
11	3-Methylbut-1-yl propionate[32]	1176	105-68-0	E	RC	–	0.22 ± 0.01	4 (5)	5 (5)
12	2-Methylbutan-1-ol[32]	1207	137-32-6	OH	RC	–	4.17 ± 0.21	NR	NR
13	3-Methylbutan-1-ol[32]	1213	123-51-3	OH	RC	0.21 ± 0.11	20.11 ± 1.49***	5 (5)	5 (5)
14	Ethyl hexanoate	1224	123-66-0	E	RC	–	0.26 ± 0.07	5 (5)	5 (5)
15	Styrene	1238	100-42-5	AR		0.14 ± 0.06	0.25 ± 0.09 ns	NR	NR
16	3-Methylbutyl 2-methylbutanoate	1258	27625-35-0	E	RC	0.01 ± 0.001	0.04 ± 0.001***	NR	NR
17	3-Methylbutyl 3-methylbutanoate	1287	659-70-1	E	RC	0.06 ± 0.03	0.02 ± 0.02 ns	NR	NR
18	2,5-Dimethyl pyrazine	1316	123-32-0	O	L, RI	0.09 ± 0.05	0.05 ± 0.01 ns	NR	NR
19	Heptyl acetate	1382	112-06-1	E	L, RI	–	0.01 ± 0.001	NR	NR
20	Ethyl octanoate	1430	106-32-1	E	RC	–	0.45 ± 0.09	5 (5)	5 (5)
21	Acetic acid[32]	1449	64-19-7	AC	L, RI	–	0.03 ± 0.001	NR	NR
22	3-Methylbut-1-yl hexanoate	1456	2198-61-0	E	RC	–	0.03 ± 0.01	NR	NR
23	2-Ethylhexan-1-ol	1488	104-76-7	OH	RC	0.12 ± 0.09	0.04 ± 0.01 ns	NR	NR
24	2-Methylpropionic acid	1562	79-31-2	AC	L, RI	–	0.20 ± 0.05	NR	NR
25	Unknown	1620					0.06 ± 0.01	NR	NR
26	Butanoic acid[32]	1634	107-92-6	AC	L, RI	–	0.58 ± 0.14	NR	NR
27	Unknown	1655					0.1 ± 0.02	NR	NR
28	3-Methylbutanoic acid[32]	1703	503-74-2	AC	L, RI	–	0.05 ± 0.01	NR	NR
29	Ethyl 2-phenylethanoate	1752	101-97-3	E	L, RI	–	0.01 ± 0.001	NR	NR
30	Methoxy-phenyl-oxime	1767		O	L, RI	0.03 ± 0.01	0.11 ± 0.01**	NR	NR
31	2-Phenylethyl acetate[32]	1795	103-45-7	E	RC	–	1.71 ± 1.1	2 (5)	4 (5)
32	3-Methylbutyl decanoate	1839	2306-91-4	E	L, RI	–	0.12 ± 0.01	NR	NR
33	2-Phenyl propionate	1858	12270-3	E	L, RI	–	0.13 ± 0.02	NR	NR
34	2-Phenyl ethanol[32]	1894	60-12-8	OH	RC	0.03 ± 0.01	15.44 ± 2.22**	4 (5)	3 (5)
35	2-Phenyl 3-methylbutanoate	1982	140-26-1	E	L, RI	–	0.01 ± 0.002	NR	NR
36	2-Pentadecanone	2014	2345-28-0	K	L, RI	–	0.03 ± 0.01	NR	NR
37	Octanoic acid	2044	124-07-2	AC	L, RI	–	0.01 ± 0.002	NR	NR
38	2-Hexadecanone	2119	18787-63-8	K	L, RI	–	0.03 ± 0.01	NR	NR
39	2-Heptadecanone	2224	2922-51-2	K	L, RI	–	0.05 ± 0.02	NR	NR

No. is a number of compound as indicated in the Fig. 1; a superscript following compound name indicates the reference reporting that the compound was identified from a volatile blend released by *P. kudriavzevii* yeast; RI – retention index (polar DB-Wax fused silica capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness); CAS No – chemical abstract service number; Group – group of chemical compound; ID – identification; EAD – electroanntenographic detection; AR – aromatic; AC – acid; E – ester; OH – alcohol, K – ketone; O – other compound; L – NIST and MassFinder3 libraries; RC – reference compound; all values in the columns headed Amount are the absolute amounts expressed as areas under the chromatographic peaks and have to be read as numbers times 100,000; ns – not-significant according to nonparametric Mann-Whitney *U* test; values in the columns headed EAD represent number of antennae which responded to the compound and values in the brackets indicates how many time the compound was tested; NR – no response.
*** $P < 0.001$.
** $P < 0.01$.
* $P < 0.05$.

Results and discussion

Pichia kudriavzevii yeast and their odours

Based on Next generation sequencing (NGS) results, the vast majority of sea buckthorn-associated fungal microorganisms and yeasts were described as unidentified (87.9%) and at the species level they were assigned to uncultured fungi [21]. Application of fermentation-based enrichment and cultivation techniques revealed that about 68% of the cultured yeast population obtained from the tested sample of *H. rhamnoides* berries, collected in early autumn of 2016, was composed of *P. kudriavzevii* yeast. The sequence identity match of isolated yeast strain SB-16-15 was 100% compare to that of *Pichia kudriavzevii* culture B-WHX-12-19 in GenBank (Fig. 1). To our knowledge, there are no data published dealing with direct isolation and analysis of cultivable yeasts associated with *H. rhamnoides* berries. *P. kudriavzevii* yeast have been found in significant amounts on the berries of various grape cultivars [27–29] on apple, pear and plum fruits [30] as well as on cherry fruits [31].

Gas chromatographic – mass spectrometric analyses revealed 39 compounds 35 of which were exclusively present or occur in significantly large amounts in the headspace samples obtained from *P. kudriavzevii* yeast compare to those of blank samples. The yeast released volatiles were dominated by 19 esters followed by 6 alcohols and 5 volatile fatty acids as well as by 3 ketones, 3 unknown compounds and 2 other type substances. Quantitatively, esters and alcohols contributed by 32% and 66% to the total blend amount, respectively. Five compounds, namely, ethyl acetate, ethanol, 3-methylbut-1-yl acetate, 3-methylbutan-1-ol and 2-phenyl ethanol were released at the largest quantity and contributed to 85% of total blend amount (Table 1).

The same compounds released at the large quantities by kudriavzevii yeast cultivated in malt extract broth medium were reported by Wu et al [32], except 3-methylbut-1-yl acetate comprising 3% of total content while in our analysis the ester

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Pk SB-16-15        1   GATAGTACTACACTGCGTGAGCGGACGAAACAACAACACCTAAAATGTGGAATATAGCAT  60
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19     9   GATAGTACTACACTGCGTGAGCGGACGAAACAACAACACCTAAAATGTGGAATATAGCAT  68

Pk SB-16-15       61   ATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACGGATCTCTTGGT  120
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19    69   ATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACGGATCTCTTGGT  128

Pk SB-16-15      121   TCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATCGTG  180
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   129   TCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATCGTG  188

Pk SB-16-15      181   AATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGCATGCCTGTTT  240
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   189   AATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGCATGCCTGTTT  248

Pk SB-16-15      241   GAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGACGACGTGTAA  300
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   249   GAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGACGACGTGTAA  308

Pk SB-16-15      301   AGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGCGAACTAGACtt  360
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   309   AGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGCGAACTAGACTT  368

Pk SB-16-15      361   tttttCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAAAAGCTCGACCT  420
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   369   TTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAAAAGCTCGACCT  428

Pk SB-16-15      421   CAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA  460
                       ||||||||||||||||||||||||||||||||||||||||
Pk B-WHX-12-19   429   CAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA  468
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Fig. 1. Identification of *P. kudriavzevii* SB-16-15 strain. Similarity of SB-15-16 to *Pichia kudriavzevii* culture B-WHX-12-19 based on sequences of internal transcribed spacer 1 and 2 including 5.8S ribosomal RNA gene.

contributed by 18% to the total composition of the blend. Taking into consideration biosynthetic origin of the compounds [22] produced by *P. kudriavzevii*, the volatiles derived from the carbohydrate catabolism and fermentation pathway have been detected, for example, ethanol, acetic acid and ethyl acetate; from the fatty acids biosynthesis and degradation pathway, for example, octanoic acid, ethyl hexanoate and ethyl octanoate; and from amino acid synthesis and degradation pathway like 2-methylbutan-1-ol, 3-methylbutanoic acid and 2-phenyl ethanol, while volatiles produced by terpene biosynthetic pathway have not been detected.

Electroantennographic activity of yeast-produced volatiles

GC–EAD analyses of *P. kudriavzevii* headspace collections (n = 10) showed that antennae of *R. batava* flies were able to detect 10 volatile compounds in total (Fig. 2).

There were no qualitative differences detected in EAD responses between females and males. Chemical structure and EAD activity comparison showed that all ten EAD active compounds elicited antenna responses of both females and males in nearly all replicates tested, except two aromatic compounds, namely 2-phenylethyl acetate and 2-phenyl ethanol which showed activity in two of five male and in three of five female antennae recordings, respectively. Moreover, the compounds 3-methylbut-1-yl acetate and 3-methylbutan-1-ol bearing a methyl group at the carbon 3, showed clear EAD activity, while the presence of methyl moiety closer to a functional group, i.e. at the carbon 2 in the structurally similar compounds, i.e. 2-methylbut-1-yl acetate and 2-methylbutan-1-ol, obliterated EAD activity.

As olfaction is essential sensory modality in insects used to find suitable food sources, oviposition sites and mates [33], ten volatiles present in the headspace of *P. kudriavzevii* yeasts which elicited

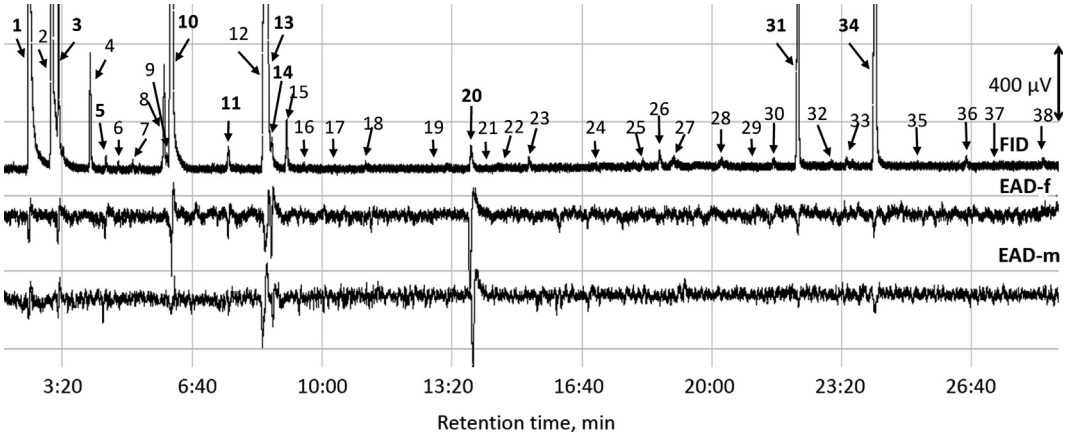

Fig. 2. Representative flame-ionization (FID) and electroantennogram detector (EAD) recordings of antennal responses of *Rhagoletis batava* male and female flies to the *Pichia kudriavzevii* yeast produced volatiles. Name of the compounds indicated by numbers are presented in the Table 1.

EAD responses of *R. batava* flies, indicate that yeasts may have ability to modulate searching behaviour of flies towards feeding and oviposition sites. The EAD responses of flies to yeasts' volatiles are the best studied in the family Drosophilidae [34–37] showing that yeast species complexes specific for drosophila hosts and for host plant berries' development stages could be resolved solely based on volatile constituents [38,39] which provides base for observed preference for certain host-yeast complex affecting performance of flies. Preference for certain host-yeast complexes leads to specialisation and niche separation which in turn have impact on a sensory specialization for odours enhancing suitable habitat detection [34,36,38]. The olfactory-chemosensory part of associations between Tephritidae flies including those of the genus *Rhagoletis* and yeasts has received far less attention despite that *Rhagoletis* flies are considered direct pests of economic importance.

Behavioural responses of Rhagoletis batava flies to the blend of electroanntenographic active compounds

Statistical data evaluation by GLMM revealed that the independent variable treatment (control and stimulus) significantly influenced the fly choice ($\chi^2 = 11.24$, $P < 0.001$; β estimate control = 0.39; OR ± SE = 0.61 ± 0.21; β estimate treatment = 0.59; OR ± SE = 0.41 ± 0.21). However, the independent variable sex (male and female) had no significant effect on fly choice ($\chi^2 = 0.03$, $P = 0.85$; β estimate female = 0.24; OR ± SE = 0.76 ± 0.56; β estimate male = 0.06; OR ± SE = 0.94 ± 0.32). In the two-choice experiments, *R. batava* males preferred the mixture composed of nine synthetic compounds which were analogous to EAD active volatiles released by *P. kudriavzevii* yeasts compare to the control ($\chi^2 = 10.563$; $P = 0.001$; β estimate stimulus = 0.773; OR ± SE = 0.23 ± 0.35), whereas female flies showed no preference to the nine component mixture presented at 1 μL mL^{-1} ($\chi^2 = 0.095$; $P = 0.758$; β estimate stimulus = 0.182; OR ± SE = 0.82 ± 0.59) but were attracted to the mixture at the concentration 0.1 μL mL^{-1} ($\chi^2 = 4.972$; $P = 0.026$; β estimate stimulus = 0.559; OR ± SE = 0.44 ± 0.36) (Fig. 3).

Paleontology and molecular evolutionary biology data suggest that yeasts and insects have coexisted for 300–400 Ma years [40,41] long before the origin of angiosperms, which evolved 125–150 Ma years [42]. The long lasting coexisting between yeasts and insect resulted in the widespread and diverse nature of the association ranging from pathogenic and parasitic to mutualistic interactions, wherein the latter relationships are based on yeasts as a food for the insects and the insects as a vector for the yeasts [43–45]. Yeasts produce variety of odours [22] and substantially

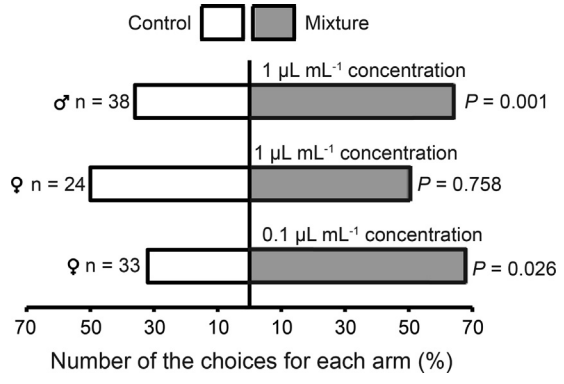

Fig. 3. Preference of *Rhagoletis batava* males and females to the mixture composed of synthetic compounds versus control. The synthetic compounds were analogous to EAD active volatiles released by *P. kudriavzevii* yeasts. The mixture was composed of ethyl acetate, ethyl propionate, 3-methylbutyl acetate, 3-methylbutyl propionate, 3-methylbutanol, ethyl hexanoate, ethyl octanoate, phenylethyl acetate and 2-phenyl ethanol at the ratio 42:2:30:1:8:1:4:5:7.

contribute to volatile chemical profile of a habitat affecting insect search for resources [46]. Behaviour modifying effect of volatiles released by yeasts have been observed in different insect orders [45,47–50], while behavioural activity of berry and fruit populating yeast odours was mostly reported in dipterans with major focus on drosophilid flies mainly from the genus *Drosophilla* [34,38,45,46]. Scarce information is available for flies from the family Tephritidae [51–53].

Our results showed that both males and females positively responded to the mixture of nine EAD active yeast volatiles in the two-choice experiment under laboratory conditions. Notably, females were attracted to the lower concentration of the blend compare with males most probably due to differences in response to olfactory cues eliciting search for oviposition site compare to that mediating food source finding. *P. kudriavzevii* were characterised as well-fermenting yeasts [54,55] and were more abundant on damaged than on intact grape berries [27]. We assume that *P. kudriavzevii* yeast produced odours could indicate to flies easier access to berry interior and higher nutrient quality due to presence of yeasts for adult feeding and less suitability for larval development.

Odour-mediated interactions between yeasts and insect are complex and diverse ranging from attractive to repellent depending on whether mutualistic or harmful yeasts inhabited a substrate. There is growing experimental evidence that yeast volatiles can be successfully used in integrated pest management programmes [56–58]. Our present work revealed that the blend composed of nine EAD active yeast volatiles attracted *R. batava* flies under laboratory conditions providing background for further optimisation of the blend and development of semiochemical based trap for monitoring and control of this pest in *H. rhamnoides* orchards.

Conclusions

P. kudriavzevii yeast inhabiting ripe *H. rhamnoides* berries have been successfully isolated and cultivated. Thirty-nine yeast associated volatiles were sampled and 35 identified from the headspace of *P. kudriavzevii*. Ten of those volatiles elicited antenna responses of *R. batava* flies. Nine component mixture comprised of synthetic compounds analogous to EAD active volatiles at the concentration 1 μL mL^{-1} significantly attracted males while the females showed significant preference for the mixture at the lower 0.1 μL mL^{-1} concentration. The behaviour modifying effect of the mixture indicates an application potential of EAD active volatiles in pest management programs of *R. batava* flies.

Compliance with Ethics requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

Authors declare no conflict of interest.

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References

[1] Li TSC, Schroeder WR. Sea buckthorn (*Hippophae rhamnoides* L.): a multipurpose plant. HortTechnology 1996;6:370–80.
[2] Swenson U, Bartish IV. Taxonomic synopsis of *Hippophae* (Elaeagnaceae). Nord J Bot 2002;22:369–74.
[3] Zhou X, Tian L, Zhang JF, Ma LN, Li XJ, Tian CJ. Rhizospheric fungi and their link with the nitrogen-fixing Frankia harbored in host plant *Hippophae rhamnoides* L. J Basic Microbiol 2017;57:1055–64.
[4] Zhang N, Jiang DM, Jiang SY. Study of drought resistance of thirteen sand-fixing plants in Horqin Sand Land. China. Int J Agric Biol 2018;20:1717–24.
[5] Yao YM, Tigerstedt PMA, Joy P. Variation of vitamin-C concentration and character correlation between and within natural Sea Buckthorn (*Hippophae-rhamnoides* L) populations. Acta Agric Scand Sect B-Soil Plant Sci 1992;42:12–7.
[6] Yao YM, Tigerstedt PMA. Genetic diversity in *Hippophae* L and its use in plant-breeding. Euphytica 1994;77:165–9.
[7] Olas B, Skalski B. Ulanowska K. The Anticancer Activity of Sea Buckthorn *Elaeagnus rhamnoides* (L.) A. Nelson. Front Pharmacol 2018;9:e232.
[8] Olas B. The beneficial health aspects of sea buckthorn (*Elaeagnus rhamnoides* (L.) A.Nelson) oil. J Ethnopharmacol 2018;213:183–90.
[9] Shi J, Wang L, Lu Y, Ji Y, Wang YQ, Dong K, et al. Protective effects of seabuckthorn pulp and seed oils against radiation-induced acute intestinal injury. J Radiat Res 2017;58:24–32.
[10] Ulanowska K, Skalski B, Olas B. Sea-buckthorn (*Hippophae rhamnoides* L.) as a source of compounds with antitumor and radioprotective activity. Postepy Hig Med Dosw 2018;72:240–52.
[11] Bahramsoltani R, Farzaei MH, Rahimi R. Medicinal plants and their natural components as future drugs for the treatment of burn wounds: an integrative review. Arch Dermatol Res 2014;306:601–17.
[12] Shamanskaya LD. Bioecology of the sea-buckthorn fly (Rhagoletis batava obscuriosa Kol.) and pest control treatment in Altai. In: Kauppinen S, Petruneva E, eds. Producing Sea Buckthorn of High Quality. Helsinki: 2015; Natural Resources Institute Finland; 2015. p. 7–20.
[13] Hering EM. Zwei neue palaarktische *Rhagoletis* (Dipt., Trypet.). Stuttgarter Beiträge zur. Naturkunde 1958;7:1–4.
[14] Kolomiec NG. New subspecies of Rhagoletis batava Hg. (Diptera, Tephritidae) from Siberia. Novye i maloizvestnye vidy fauny Sibiri 1970; 3: 40-52.
[15] Stalažs A, Balalaikins M. Country checklist of *Rhagoletis* Loew (Diptera: Tephritidae) for Europe, with focus on *R. batava* and its recent range expansion. Proc Latvian Acad Sci B 2017;71:103–10.
[16] Howse PE, Stevens IDR, Jones OT. Insect pheromones and their use in pest management. New York: Chapman and Hall; 1998.
[17] Murali-Baskaran RK, Sharma KC, Kaushal P, Kumar J, Parthiban P, Senthil-Nathan S, et al. Role of kairomone in biological control of crop pests-A review. Physiol Mol Plant Pathol 2018;101:3–15.
[18] Sarles L, Verhaeghe A, Francis F, Verheggen FJ. Semiochemicals of *Rhagoletis* fruit flies: Potential for integrated pest management. Crop Prot 2015;78:114–8.
[19] Sherman F. Getting started with yeast. Meth Enzymol 2002;350:3–41.
[20] Vepštaitė-Monstavičė I, Lukša J, Stanevičienė R, Strazdaitė-Žielienė Ž, Yurchenko V, Serva S, et al. Distribution of apple and blackcurrant microbiota in Lithuania and the Czech Republic. Microbiol Res 2018;206:1–8.
[21] Lukša J, Vepštaitė-Monstavičė I, Yurchenko V, Serva S, Servienė E. High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota – A pilot study. Food Res Int 2018;111:597–606.
[22] Ebert BE, Halbfeld C, Blank LM. Exploration and exploitation of the yeast volatilome. Curr Metabol 2017;5:102–8.
[23] Blažytė-Čereškienė L, Apšegaitė V, Radžiutė S, Mozūraitis R, Būda V, Pečiulytė D. Electrophysiological and behavioural responses of *Ips typographus* (L.) to trans-4-thujanol—a host tree volatile compound. Ann For Sci 2016;73:247–56.
[24] Heath RR, Dueben BD. Analytical and preparative gas Chromatography. In: Millar JG, Haynes KF, editors. Methods in chemical ecology: chemical methods. Norwell: Kluwer Academic Publishers; 2000. p. 85–126.
[25] Webster FX, Millar JG, Kiemle DJ. Mass spectrometry. In: Millar JG, Haynes KF, editors. Methods in chemical ecology: chemical methods. Norwell: Kluwer Academic Publishers; 2000. p. 127–52.
[26] Hare DJ. Bioassay methods with terrestrial invertebrates. In: Millar JG, Haynes KF, editors. Methods in chemical ecology: bioassay methods. Norwell: Kluwer Academic Publishers; 2000. p. 212–70.
[27] Nemcova K, Breierova E, Vadkertiova R, Molnarova J. The diversity of yeasts associated with grapes and musts of the Strekov winegrowing region, Slovakia. Folia Microbiol 2015;60:103–9.
[28] Raspor P, Milek DM, Polanc J, Mozina SS, Cadez N. Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int J Food Microbiol 2006;109:97–102.
[29] Barata A, Malfeito-Ferreira M, Loureiro V. The microbial ecology of wine grape berries. Int J Food Microbiol 2012;153:243–59.
[30] Vadkertiova R, Molnarova J, Vranova D, Slavikova E. Yeasts and yeast-like organisms associated with fruits and blossoms of different fruit trees. Can J Microbiol 2012;58:1344–52.
[31] Stollarova V. The presence of yeasts and yeast-like microorganisms on cherry fruits (*Cerasus-avium* (L) moench). Biologia. 1982;37:1115–20.
[32] Wu ZF, Sun L, Zhang X, Shen XQ, Weng PF. Quantitative analysis of predominant yeasts and volatile compounds in the process of pickled wax gourd. CyTA-J Food 2016;14:92–100.
[33] Louis M, Schoonhoven LM, van Loon JJA, Dicke M. Insect-Plant Biology. USA: Oxford University Press; 2005.
[34] Versace E, Eriksson A, Rocchi F, Castellan I, Sgado P, Haase A. Physiological and behavioral responses in *Drosophila melanogaster* to odorants present at different plant maturation stages. Physiol Behav 2016;163:322–31.
[35] Cha DH, Adams T, Rogg H, Landolt PJ. Identification and field evaluation of fermentation volatiles from wine and vinegar that mediate attraction of Spotted Wing Drosophila, *Drosophila suzukii*. J Chem Ecol 2012;38:1419–31.
[36] Date P, Dweck HKM, Stensmyr MC, Shann J, Hansson BS, Rollmann SM. Divergence in olfactory host plant preference in d-mojavensis in response to cactus host use. PLoS ONE 2013;8:e70027.
[37] Crowley-Gall A, Date P, Han C, Rhodes N, Andolfatto P, Layne JE, et al. Population differences in olfaction accompany host shift in *Drosophila mojavensis*. Proc R Soc B-Biol Sci 2016;283:e20161562.
[38] Scheidler NH, Liu C, Hamby KA, Zalom FG, Syed Z. Volatile codes: Correlation of olfactory signals and reception in *Drosophila*-yeast chemical communication. Sci Rep 2015;5:e14059.
[39] Arguello JR, Sellanes C, Lou YR, Raguso RA. Can yeast (*S. cerevisiae*) metabolic volatiles provide polymorphic signaling? PLoS ONE 2013;8:e70219.
[40] Rolland T, Dujon B. Yeasty clocks: Dating genomic changes in yeasts. C R Biol 2011;334:620–8.
[41] Nel A, Roques P, Nel P, Prokin AA, Bourgoin T, Prokop J, et al. The earliest known holometabolous insects. Nature 2013;503:257–61.
[42] Clarke JT, Warnock RCM, Donoghue PCJ. Establishing a time-scale for plant evolution. New Phytol 2011;192:266–301.
[43] Janson EM, Stireman JO, Singer MS, Abbot P. Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution 2008;62:997–1012.
[44] Christiaens JF, Franco LM, Cools TL, De Meester L, Michiels J, Wenseleers T, et al. The fungal aroma gene ATF1 promotes dispersal of yeast cells through insect vectors. Cell Reports 2014;9:425–32.
[45] Ganter PF. Yeast and invertebrate associations. In: Augusto RC, Gábor P, editors. Biodiversity and ecophysiology of yeasts. Berlin: Springer; 2006. p. 303–70.
[46] Becher PG, Flick G, Rozpedowska E, Schmidt A, Hagman A, Lebreton S, et al. Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. Funct Ecol 2012;26:822–8.
[47] Witzgall P, Proffit M, Rozpedowska E, Becher PG, Andreadis S, Coracini M, et al. "This is not an apple"-yeast mutualism in codling moth. J Chem Ecol 2012;38:949–57.
[48] Six DL. The bark beetle holobiont: why microbes matter. J Chem Ecol 2013;39:989–1002.
[49] Madden AA, Epps MJ, Fukami T, Irwin RE, Sheppard J, Sorger DM, et al. The ecology of insect-yeast relationships and its relevance to human industry. Proc R Soc B-Biol Sci 2018;285:e20172733.
[50] Tasin M, Herrera SL, Knight AL, Barros-Parada W, Contreras EF, Pertot I. Volatiles of grape inoculated with microorganisms: modulation of grapevine moth oviposition and field attraction. Microb Ecol 2018;76:751–61.
[51] Mesquita PRR, Magalhaes-Junior JT, Cruz MA, Novais HO, Santos JRJ, Carvalho SL, et al. Sources of protein as food baits for *Anastrepha obliqua* (Diptera: Tephritidae): tests in a wind tunnel and the field. Fla Entomol 2018;101:20–4.
[52] Leblanc L, Vargas RI, Rubinoff D. Captures of pest fruit flies (Diptera: Tephritidae) and nontarget insects in biolure and torula yeast traps in Hawaii. Environ Entomol 2010;39:1626–30.
[53] Piper AM, Farnier K, Linder T, Speight R, Cunningham JP. Two gut-associated yeasts in a tephritid fruit fly have contrasting effects on adult attraction and larval survival. J Chem Ecol 2017;43:891–901.
[54] Sharma AK, Singh PN, Sawant SD. Evaluation of fermentation efficiency of yeast strains and their effect on quality of young wines. Indian J Microbiol 2012;52:495–9.
[55] Nyanga LK, Nout MJR, Smid EJ, Boekhout T, Zwietering MH. Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. Int J Food Microbiol 2013;166:426–32.
[56] Mori BA, Whitener AB, Leinweber Y, Revadi S, Beers EH, Witzgall P, et al. Enhanced yeast feeding following mating facilitates control of the invasive fruit pest *Drosophila suzukii*. J Appl Ecol 2017;54:170–7.
[57] Hamby KA, Becher PG. Current knowledge of interactions between *Drosophila suzukii* and microbes, and their potential utility for pest management. J Pest Sci 2016;89:621–30.
[58] Holighaus G, Rohlfs M. Fungal allelochemicals in insect pest management. Appl Microbiol Biotechnol 2016;100:5681–9.