Research Article

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Blueberries infected with the fungal pathogen *Colletotrichum fioriniae* release odors that repel *Drosophila suzukii*

Caitlin C Rering,[a*] Amanda Quadrel,[b] Pablo Urbaneja-Bernat,[c] John J Beck,[a] Yahel Ben-Zvi,[b] Fatemeh Khodadadi,[d] Srđan G Aćimović[e] and Cesar Rodriguez-Saona[b]

Abstract

BACKGROUND: Spotted-wing drosophila, *Drosophila suzukii*, is a serious pest of thin-skinned fruits. Alternative methods to control this pest are needed to reduce insecticide use, including new repellents. Previous research demonstrated that *D. suzukii* adults use odor cues to avoid blueberries infected with the fungal pathogen *Colletotrichum fioriniae*, which causes the disease anthracnose. To identify novel *D. suzukii* repellents, we investigated the volatile emission from experimentally-infected fruit, which were inoculated with *C. fioriniae* isolates in the laboratory, and from field-collected fruit, which were naturally infected and harvested from a field. We then tested the pathogen-induced volatiles on *D. suzukii* adult behavior.

RESULTS: Volatile emission was similar between all five *C. fioriniae* strains, with good agreement between experimentally-infected and field-collected berries. In total, 14 volatiles were found to be more abundant in infected *versus* uninfected fruit headspace. In multiple-choice bioassays, nine of the 14 volatiles elicited repellency responses from adult *D. suzukii*. These nine volatiles were further evaluated in dual choice assays, where all nine reduced fly capture by 43–96% compared to the control. The most repellent compounds tested were the esters ethyl butanoate and ethyl (*E*)-but-2-enoate, which were more or equally repellent to the known *D. suzukii* repellents 1-octen-3-ol, geosmin, and 2-pentylfuran. Dose–response assays identified concentration-dependent effects on *D. suzukii* repellency and oviposition when applied individually and consistent aversion observed across doses of a 1:1 blend.

CONCLUSION: We report two repellents from *C. fioriniae*-infected blueberries that could be useful semiochemicals for the behavioral manipulation of *D. suzukii* in the field.

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

Keywords: anthracnose; *Vaccinium* spp.; volatiles; spotted-wing drosophila; semiochemical

1 INTRODUCTION

Spotted-wing drosophila (*Drosophila suzukii* Matsumura, Diptera: Drosophilidae) is an invasive and highly destructive pest of berries and cherries.[1] Females possess a serrated ovipositor, allowing them to lay eggs on ripening, instead of rotting, fruit.[2,3] *Drosophila suzukii* is controlled by aggressive and unsustainable insecticide applications,[4] which can lead to resistance.[5–7] Alternative control methods, such as strategies that employ odors to manipulate insect behavior, are needed to reduce insecticide use. Previous attempts to manage *D. suzukii* using semiochemicals have achieved some success,[8–12] demonstrating the feasibility of this approach. However, new repellent chemicals could improve the approach by increasing aversion, enhancing specificity to *D. suzukii*, or by identifying more affordable chemicals to manufacture or source.

Like many insects, *D. suzukii* has important interactions with microbes and microbial volatiles that can be exploited to control the pest.[13] For example, *D. suzukii* adults are attracted to fermentation odors[14,15] and other microbial volatiles.[16] Though fermentation

* *Correspondence to: CC Rering, Chemistry Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, United States Department of Agriculture, 1700 SW 23rd Dr., Gainesville, FL 32608, USA. E-mail: caitlin.rering@usda.gov*

Caitlin C Rering and Amanda Quadrel authors should be considered joint first author.

a *Chemistry Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, United States Department of Agriculture, Gainesville, FL, USA*

b *Department of Entomology, Philip E. Marucci Center, Rutgers University, Chatsworth, NJ, USA*

c *Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Sustainable Plant Protection, Cabrils, Spain*

d *Department of Plant Pathology and Microbiology, University of California, Riverside, CA, USA*

e *Virginia Tech, School of Plant and Environmental Sciences, Alson H. Smith Jr. Agricultural Research and Extension Center, Winchester, VA, USA*

volatiles are used as lures in the field,[17–19] effective repellents are also needed for successful push–pull management. Currently, 1-octen-3-ol, geosmin, and 2-pentylfuran have been identified as promising *D. suzukii* repellents.[20–22] Odors from natural products from floral volatiles,[23] peppermint oil,[24] and hops[25] have also been investigated for repellency to *D. suzukii* with some promising initial results but limited success in the field. Because *D. suzukii* avoids fruits infected with certain phytopathogens like *Botrytis cinerea* Pers.[26] and *Colletotrichum fioriniae* (Marcelino & Gouli),[27] these fungi and their associated volatiles could provide valuable new repellent chemistries for its control.

Fungi from the genus *Colletotrichum* infect the fruit of many cultivated plant species, causing anthracnose and rot diseases that lead to significant economic losses worldwide.[28] *Colletotrichum fioriniae* is the causal disease agent of blueberry anthracnose in the Mid-Atlantic United States.[29] At least three *Colletotrichum* species have been described to cause insect mortality. For example, *C. fioriniae* attacks elongate hemlock scale (*Fiorinia externa* Ferris),[30] while *Colletotrichum nymphaeae* (previously known as *Colletotrichum gloeosporioides* f. sp. *ortheziidae*) infects citrus scale (*Orthezia praelonga*).[31] An unidentified species of the *Colletotrichum acutatum* complex was recently reported to cause mortality in the Asian chestnut gall wasp.[32] This points to the entomopathogenic potential of these fungi that could be useful to investigate for repellency or biocontrol.

To explore this potential, we examined the volatile emission of *C. fioriniae*-infected blueberries and the behavioral response of *D. suzukii* adults to these volatiles. We asked: (1) Do *C. fioriniae*-infected fruit have differing volatile emission than uninfected fruit? (2) How do *D. suzukii* adults respond to individual odors associated with *C. fioriniae* infection of blueberry? (3) Are volatiles from *C. fioriniae*-infected blueberries as effective at repelling *D. suzukii* as other known repellents? (4) How do *D. suzukii* respond to differing concentrations of *C. fioriniae*-induced volatiles? Ultimately, the goal of this study was to identify novel repellents for *D. suzukii*.

2 MATERIALS AND METHODS

2.1 Fungal isolates and inoculant preparation

Five *C. fioriniae* isolates originally isolated from apples in New York, USA were used in this experiment (Table 1; Khodadadi *et al.*[28]). Strains were maintained on potato dextrose agar and incubated in the dark at 28 °C. Inoculant solution was prepared by pouring sterile distilled water onto 7-day-old plates of *C. fioriniae* and gently scraped with an inoculation loop to liberate fungal spores from the agar. Spore suspension was then transferred from the plates, and cell density was determined by an automated cell counter (Bio-Rad TC20; Bio-Rad, Hercules, CA, USA). Final cell density in the inoculant solution was adjusted to 1×10^7 cells/mL. Inoculant solutions were placed in small spray bottles for use in experiments.

2.2 Sample preparation

Two sample types were analyzed, 'experimentally-infected' and 'field-collected' fruit. To confirm that experimentally-infected berries exhibited field realistic volatile emissions, infected and uninfected berries collected from the field were also analyzed. For experimentally-infected samples, store-bought organic blueberries (10–11 g, *c.* ten blueberries) were surface sterilized by submerging for 2 min each in first 10% bleach, then 70% ethanol, and finally sterile water. Berries were infected with the inoculant solution by liberally spraying fruit until runoff. Control samples were sprayed with sterile water. Three replicates were prepared for each strain of experimentally-infected samples.

Field-collected fruit were harvested from a commercial highbush blueberry (*Vaccinium corymbosum* L.) farm in Hammonton, NJ, USA. Fruit with visual signs of anthracnose infection (orange droplets seeping from berries) and control fruit with no signs of infection were shipped on ice in separate containers overnight to Gainesville, FL, USA for volatile analysis. Three replicates were analyzed for field-collected fruit; however, one control replicate was lost ($n = 2$ field-collected controls). All berry samples were sealed in 4 oz (118 mL) Ball® glass jars with custom-made PTFE (polytetrafluoroethylene) lids, which were fitted with a gas chromatograph septum as described in Rering *et al.*[33] Samples were incubated at room temperature on the laboratory bench before sampling.

2.3 Volatile analysis

Sample headspace was analyzed by gas chromatography–mass spectrometry (GC–MS), following the methods described in Rering *et al.*[33] Volatiles were collected from the experimentally-infected blueberries 2 and 4 days after inoculation. Incubation intervals were selected to coincide with pre-symptom and early symptom disease progression. Two days after inoculation, the odors of infected berries repelled *D. suzukii* in a previous study,[27] even though the fruit showed no visible signs of disease. At 4 days after inoculation, *c.* 20% of fruit in the samples showed early signs of infection, for example, minor droplet formation and sunken and discolored areas. To confirm that treatments were successful, we incubated all samples for 7 days to allow visual confirmation of infection (significant orange droplet accumulation on most or all fruit in treated samples). Field-collected samples were identified as infected by their presentation of disease symptoms and therefore collected at the early- to mid-symptom development stage and were analyzed 1 day after receipt.

Headspace was sampled by solid-phase microextraction (SPME; Supelco, Bellefonte, PA, USA; 50/30 µm, 2 cm, divinylbenzene/

Table 1. *Colletotrichum fioriniae* strains used in this study and their GenBank accession numbers (Khodadadi *et al.*[28])

	GenBank accession number		
Strain	GAPDH	ITS	TUB2
ACFK3	MN689219	MN684827	MN689182
ACFK12	MN689227	MN684835	MN689190
ACFK25	MN689231	MN684839	MN689194
ACFK145	MN689233	MN684841	MN689196
ACFK299	MN689236	MN684844	MN689199

carboxen/polydimethylsiloxane) fibers, which were inserted through the gas chromatograph septum installed in the lid and allowed to collect volatiles for 15 min. Fibers were immediately injected in the GC inlet for 6 min at 230 °C to desorb volatiles which were separated by a DB-Wax column (60 m × 320 μm × 0.25 μm, J&W Scientific, Folsom, CA, USA). The MS was operated in positive scan mode with an electron ionization source. Technical replicates were also injected on a GC–MS outfitted with a non-polar DB-1 column (60 m × 320 μm × 0.25 μm) to assist in identification. After each sample was collected, the sample lid was removed in a biological safety cabinet for approximately 5 min to allow for gas exchange under sterile conditions.

Blueberry and fungal volatiles were identified by comparing sample and blank chromatograms. Compounds with similar abundance in both background (no fruit or *C. fioriniae*) and real samples were removed from the dataset. When compounds had elevated abundance in samples but were also present at lower levels in the background, sample peak areas were background subtracted.

Relative peak areas of a selected quantitative ion for each volatile were recorded using MassHunter Quantitative Analysis (B.07.01; Agilent Technologies, Palo Alto, CA, USA) and normalized by berry fresh weight. Qualifier ions were monitored to confirm compound identity. Compound identities were tentatively assigned based on their match to the National Institute of Standards and Technology (NIST) library and retention indices on polar and non-polar columns. Volatiles that were found to be emitted in higher amounts in anthracnose-infected fruits were confirmed by comparison to commercial standards. Identification results, quantitative and qualitative ions, and retention indices are described in Table 2.

2.4 Fly rearing

The *D. suzukii* colony used for experiments was initiated in 2013 and maintained on a standard Drosophila artificial diet[34,35] in a laboratory at Rutgers University (New Brunswick, NJ, USA) at 22 ± 2 °C, 55 ± 5% relative humidity (RH), and 16 h:8 h (light/dark) photoperiod. Wild flies were added every 2–3 years to the colony to help maintain genetic diversity. Male and female flies used in the experiments were 7–10 days old[20,36] and thus were sexually mature.[37] Flies were removed from the colony within 5 h from the start of the experiments.

2.5 Chemicals

For this study, we used the 14 differentially emitted compounds from anthracnose-infected blueberry fruit (Table 2). 3-Hydroxy-2-butanone (acetoin) (≥ 96%, CAS No. 513-86-0), styrene (> 98%, CAS No. 100-42-5), (*E*)-2-hexen-1-ol (96%, CAS No. 928-95-0), 2-methyl-1-butanol (> 99%, CAS No. 137-32-6), ethyl propanoate (99%, CAS No. 105-37-3), 2-methyl-1-propanol (analytical standard, CAS No. 78-83-1), diethyl carbonate (> 99%, CAS No. 105-58-8), 2-methylpropanal (> 99%, CAS No. 78-84-2), 3-methyl-1-butanol (analytical standard, CAS No. 123-51-3), ethyl 2-methylpropanoate (99%, CAS No. 97-62-1), ethyl butanoate (99%, CAS No. 105-54-4), ethyl (*E*)-but-2-enoate (99%, CAS No. 623-70-1), and 3-methyl-3-buten-1-ol (97%, CAS No. 763-32-6) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethyl 3-hydroxy-3-methylbutanoate (98%, CAS No. 18267-6-2) was purchased from AmBeed (Arlington Heights, IL, USA). We also used three known *D. suzukii* repellents purchased from Sigma-Aldrich: 1-octen-3-ol[21] (≥ 98%, CAS No. 3391-86-4), geosmin[22] (≥ 97%, CAS No. 16423-19-1), and 2-pentylfuran[20] (≥ 98%, CAS No. 3777-69-3).

2.6 Multiple choice bioassays #1: comparing *D. suzukii* responses to anthracnose-induced volatiles

To screen for behavioral effects among volatiles that were significantly different between anthracnose-infected and control fruit on *D. suzukii* adults, we conducted multiple choice bioassays as described by Cha *et al.*[20,36] Within a dome cage (60 cm width × 60 cm length × 60 cm height; MegaView Science Co., Taichung, Taiwan), 15 gated traps were positioned randomly in a circle (40 cm diameter) at equal distance with each trap 8.4 cm apart along the circumference (Fig. 1(A)). Each gated trap consisted of 50 mL polystyrene tubes (Fisher Scientific, Nazareth, PA, USA) covered with aluminum foil and sealed with Parafilm®, with a 4 mm-diameter hole in the center of the Parafilm to provide an entrance to flies. All traps had equal quantities (*c.* 5 g) of store-bought organic blueberry fruit ($n = 5$); fruit were sterilized with bleach before the study (10% bleach for 2 min, rinsed three times in distilled water). One of the 15 traps served as a control (with blueberry fruit only) and the remaining 14 traps had one of the anthracnose volatile compounds added to the blueberry fruit. The 14 compounds tested are listed in Section 2.5 earlier. Each volatile (50 μL, neat, equivalent to 39.5–50.5 mg or 342–573 μmol) was released from a 1 mL polyethylene vial (Globe Scientific Inc., Paramus, NJ, USA) with a piece of cotton placed inside each trap (control traps had vials with cotton but without the volatile). To provide flies with water during the experiment, a vial with a cotton ball soaked with deionized water was placed in the middle of the arena. Roughly 300 *D. suzukii* adults (1:1 male/female ratio) were released inside each arena, and the number and sex of flies inside the traps was recorded after 24 h. The experiment started at 1:00 p.m., was replicated four times, and was done in a laboratory under 22 ± 2 °C, 55 ± 5% RH, 16 h:8 h (light/dark).

2.7 Pairwise choice bioassays: evaluating *D. suzukii* response to putative anthracnose-emitted repellents

To investigate the effects of individual repellent compounds from anthracnose-infected fruit (identified from Section 2.6) on *D. suzukii* adults, we conducted pairwise choice bioassays as described by Feng *et al.*[38] and Urbaneja-Bernat *et al.*[27] These bioassays consisted of arenas using clear plastic cups (946 mL, 114 mm diameter, 127 mm height; Paper Mart, CA, USA) (Fig. 1(B)). Flies were given a choice between blueberry fruit without a repellent (control) and blueberry fruit with one of the repellent compounds (50 μL, neat, equivalent to 39.5–48.8 mg or 370–548 μmol). Repellents were added to a cotton plug in a 1 mL vial, as previously described. The nine compounds tested were: ethyl propanoate, 2-methyl-1-propanol, diethyl carbonate, 2-methylpropanal, 3-methyl-1-butanol, ethyl 2-methylpropanoate, ethyl butanoate, ethyl (*E*)-but-2-enoate, and 3-methyl-3 buten-1-ol (see Section 3, 'Results'). In addition, flies were given a choice between untreated fruit and a blank control to determine their attraction to fruit in the absence of other odors. The lid of each arena had an 80 mm diameter circular hole covered with a nylon mesh (anti-thrips insect screen, mesh size: 81 × 81; BioQuip, Rancho Dominguez, CA, USA) to provide ventilation while retaining flies (Fig. 1(B)). Two gated traps consisting of polystyrene vials (same as described earlier), one containing a repellent compound and one containing nothing, were placed inside each arena with equal quantities (*c.* 5 g) of blueberry fruit ($n = 5$) (stored-bought and sterilized as described earlier) (Fig. 1(B)). Each pair of traps in each choice arena was wrapped with aluminum foil and sealed with Parafilm with a 4-mm-diameter hole in the center of the Parafilm to provide an entrance for the flies, as

Table 2. Volatiles detected in *Colletotrichum fioriniae*-infected and control blueberries.

Class	Chemical	Mean peak area (± SE)				Retention index			
		Exp. infected	Exp. control	Field infected	Field control	Polar	Non-polar	Quant. ion	Qual. ion
Aldehyde	***2-Methylpropanal***[†]	34 (±14)[§]	0[§]	4984 (±2200)	17 (±17)	814	NA	72	41
	Acetaldehyde[‡]	4.39e4 (±3.7e3)	2.24e4 (±6.2e3)	2.75e5 (±1e5)	5.01e3 (±3.5e3)	NA	NA	44	NA
Alcohol	***2-Methyl-1-propanol***[†]	1.03e4 (±1.1e3)[§]	2112 (±1145)[§]	1.03e5 (2.6e4)	4085 (±3576)	1101	620	43	74
	2-Methyl-1-butanol[†]	1842 (±337)[§]	342 (±148)[§]	8.90e4 (±1.4e4)	464 (±242)	1210	721	57	70
	3-Methyl-1-butanol[†]	2186 (±420)[§]	569 (±205)[§]	3.74e4 (±1.2e4)	259 (±37)	1211	718	57	70
	3-Methyl-3-buten-1-ol[†]	90 (±13)[§]	0[§]	915 (±310)	0	1251	713	56	68
	(*E*)-2-Hexen-1-ol[†]	28 (±11)[§]	0[§]	32 (±17)	0	1409	850	57	67
	Isopropyl alcohol	611 (±90)	908 (±408)	0	146 (±146)	929	NA	59	NA
	Ethanol	1.46e6 (±6.7e4)	6.39e5 (±1.1e5)	1.27e6 (±3.7e5)	6.97e4 (±5.6e4)	936	NA	45	NA
	1-Propanol	984 (±60)	350 (±110)	1586 (±562)	0	1039	NA	59	42
	1-Methoxy-2-propanol	155 (±30)	446 (±53)	85 (±28)	542 (±118)	1133	ND	45	58
	1-Butanol	84 (±16)	81 (±46)	482 (±263)	12 (±12)	1149	649	56	41
	1-Hexanol	226 (±53)	371 (±286)	2190 (±923)	0	1357	854	56	69
	3-Hexen-1-ol	52 (±15)	31 (±31)	369 (±92)	0	1387	840	67	53
Benzenoid	**Styrene**[†]	8.52e4 (±2.6e4)[§]	7.01e3 (±3.0e3)[§]	9.05e3 (±2.4e3)	635 (±300)	1254	874	104	78
	Toluene	0	0	2042 (±307)	1534 (±232)	1039	752	91	65
	o-Cymene	278 (±54)	5969 (±5734)	129 (±43)	30 (±30)	1268	ND	119	134
	4-Ethenyl-1,2-dimethylbenzene[‡]	34 (±15)	19 (±19)	0	0	1436	ND	132	117
	1-(4-Methylphenyl)ethanol[‡]	176 (±32)	319 (±112)	87 (±87)	475 (±32)	1471	ND	121	93
	Ethyl benzoate	2109 (±374)	554 (±232)	2243 (±1897)	233 (±136)	1667	1144	105	77
	Benzyl alcohol	8 (±5)	0	641 (±58)	0	1876	1004	79	108
	2-Phenylethanol	7 (±7)	0	2518 (±630)	0	1912	ND	91	122
Carboxylic acid	2-Methyl-propanoic acid	0	0	1.08e4 (±9.4e3)	0	1570	ND	43	73
Ester	***Ethyl propanoate***[†]	2.17e4 (±3.3e3)[§]	2802 (±1034)[§]	1.2e4 (±3.4e3)	95 (±95)	956	694	57	102
	Ethyl 2-methylpropanoate[†]	6591 (±782)[§]	920 (±529)[§]	2594 (±696)	165 (±30)	965	743	116	41
	Ethyl butanoate[†]	8093 (±1045)[§]	774 (±242)[§]	1.04e4 (3.6e3)	163 (±69)	1036	783	71	88
	Diethyl carbonate[†]	4060 (±875)[§]	175 (±13)[§]	662 (±136)	0	1106	761	91	63
	Ethyl (E)-but-2-enoate[†]	1.25e4 (±1.5e3)[§]	1851 (±796)[§]	4842 (±1851)	0	1162	823	69	99
	Ethyl 3-hydroxy-3-methylbutanoate[†]	138 (±34)[§]	0[§]	3172 (±475)	26 (±26)	1413	932	59	43
	Methyl acetate	3051 (±363)	1605 (±715)	8854 (±3353)	118 (±118)	827	NA	74	59
	Ethyl acetate	1.12e5 (±1.6e4)	2.94e4 (±1.4e4)	8.52e4 (±2.0e4)	308 (±182)	888	608	61	70
	Methyl 2-methylbutanoate	417 (±25)	316 (±125)	4334 (±2932)	9896 (±8586)	1011	ND	88	57
	2-Methylpropyl acetate	461 (±56)	140 (±70)	5913 (±1662)	0	1014	756	56	43
	Methyl 3-methylbutanoate	1.13e4 (±1.4e3)	8.96e3 (±4.9e3)	3.18e4 (±1.8e4)	1.63e5 (±1.5e5)	1019	760	74	59
	Ethyl 2-methylbutanoate	2.92e4 (±3.5e3)	1.35e4 (±5e3)	7.14e4 (±2.5e4)	1.18e4 (±7.3e3)	1052	837	57	102
	Ethyl 3-methylbutanoate	4.34e5 (±3e4)	2.73e5 (1e5)	3.60e5 (±9.6e4)	1.07e5 (±5.1e4)	1068	839	88	57
	Ethyl 3-methylbut-3-enoate[‡]	1367 (±364)	1373 (±1187)	1.14e3 (±3.0e3)	53 (±21)	1225	904	83	128
	3-Methylbutyl acetate	646 (±116)	540 (±285)	3.86e4 (±2.0e4)	0	1122	861	43	70
	Ethyl 2-methylbut-2-enoate[‡]	216 (±33)	34 (±34)	438 (±122)	0	1161	ND	128	55
	2-Methylpropyl 3-methylbutanoate	126 (±38)	147 (±147)	1524 (±732)	62 (±62)	1190	992	85	57

Table 2. Continued

Class	Chemical	Mean peak area (± SE)				Retention index			
		Exp. infected	Exp. control	Field infected	Field control	Polar	Non-polar	Quant. ion	Qual. ion
	3-Methylbutyl 3-methylbutanoate	69 (±27)	37 (±37)	617 (±211)	0	1295	ND	70	55
	Ethyl 2-hydroxypentanoate‡	198 (±38)	74 (±74)	91 (±44)	49 (±49)	1427	950	73	55
	2-Nitroethyl propanoate‡	80 (±19)	0	3668 (±637)	0	1542	ND	45	57
Ketone	**3-Hydroxy-2-butanone (acetoin)†**	1.05e4 (±2.0e3)§	983 (±388)§	1.93e5 (±2.6e4)	299 (±215)	1285	681	45	88
	Acetone	1.49e4 (±1.2e3)	1.63e4 (±7.4e2)	2052 (±798)	130 (±7)	816	NA	58	
	2-Butanone	142 (±21)	27 (±19)	638 (±57)	88 (±60)	903	NA	72	57
	5-Ethyl-4-methylheptan-3-one‡	1527 (±195)	715 (±241)	3044 (±600)	310 (±110)	978	674	57	86
	2-Heptanone	0	0	867 (±64)	0	1180	ND	58	71
	6-Methyl-5-heptene-2-one	0	0	1092 (±157)	0	1337	ND	108	55
Monoterpene	α-Phellandrene	177 (±32)	823 (±534)	0	27 (±27)	1159	ND	93	105
	(*R*)-Limonene	1250 (±346)	3307 (±1870)	173 (±54)	115 (±23)	1196	1020	68	93
	(*S*)-Limonene	57 (±29)	216 (±103)	0	0	1202	ND	68	93
	β-Ocimene	101 (±59)	89 (±45)	364 (±102)	270 (±102)	1249	ND	93	79
	Terpinolene	162 (±60)	412 (±290)	0	0	1281	1078	121	93
	Linalool	95 (±25)	331 (±114)	185 (±42)	0	1550	1084	93	71
Monoterpenoid	Eucalyptol	132 (±18)	187 (±57)	410 (±276)	9 (±9)	1209	1019	108	154
	Dihydrocarvone‡	243 (±56)	272 (±176)	207 (±58)	0	1210	981	82	96
	Anhydrolinalool oxide	433 (±124)	628 (±522)	170 (±85)	0	1243	993	67	55
Other	Dimethyl sulfide‡	262 (±25)	197 (±78)	1403 (±417)	1673 (±384)	NA	NA	62	47
	2-Methylfuran	2028 (±236)	638 (±271)	7345 (±1916)	1842 (±818)	898	612	82	53
	Hexyl hydroperoxide‡	8 ± (5)	0	216 (±114)	0	1317	ND	56	41
Sesquiterpene	β-Caryophyllene	153 (±9)	137 (±35)	0	0	1598	1412	91	133
Unknown	Unknown (79 (100), 93 (64), 94 (48), 137 (44), 91 (34), 152 (28), 43 (25), 77 (24), 95 (14), 92 (13))	152 (±32)	235 (±136)	18 (±18)	0	1325	1058	79	93

Note: Experimentally (exp.) infected and exp. control fruit refer to fruit inoculated in the laboratory with pathogen or sterile water whereas field infected and control fruit were collected from the field, either displaying symptoms of anthracnose infection or not. Peak areas normalized by fresh weight are presented as mean ± standard error (SE). Peak areas were measured by integrating the quantitative (quant.) ion and monitoring relative abundance of the qualitative (qual.) ion. Compounds in bold typeface were significantly different between exp. infected and exp. control fruit. Data from day 4 is shown for exp. infected and control fruit. Italicized compounds were repellent *Drosophila suzukii* in bioassays. ND indicates a retention index value could not be calculated because the compound was not observed on this column, or that no suitable qual. ion was available. NA indicates a retention index value could not be calculated because it fell outside of the range of alkanes used.

† Compound identity confirmed by standard injection.

‡ Compound tentative identity confirmed by library match. Unmarked compound name indicates library and retention index match. An unidentified compound is listed with the ten most abundant fragments (relative abundance).

§ Indicates significant increase in abundance in exp. infected vs. exp. control fruit ($\alpha = 0.01$).

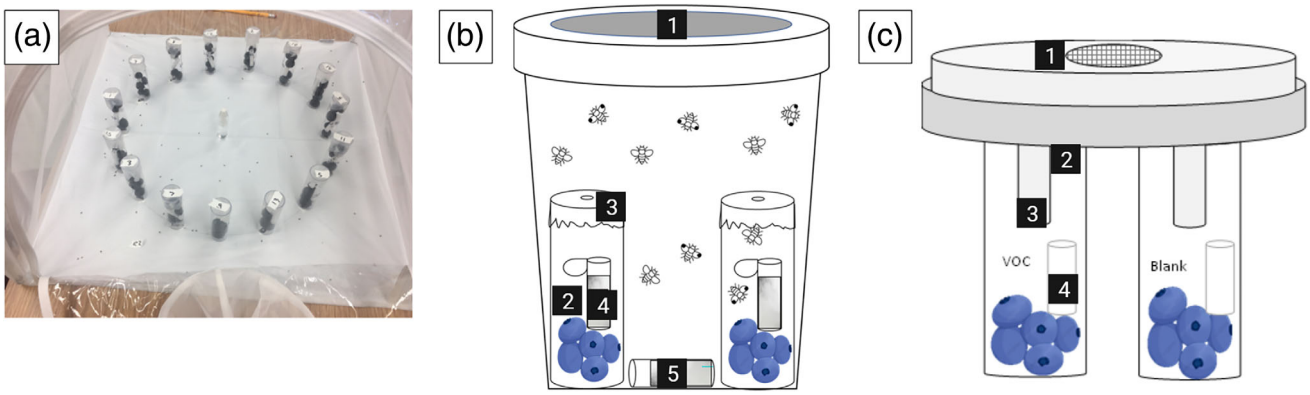

Figure 1. Bioassay arenas used in this study. (A) Multiple choice assays were conducted in arenas that consisted of a dome cage. Within the cage, gated traps containing blueberries and treatment (50 µL, neat of each volatile) were positioned in a circle at equal distance with each trap. A vial with moistened cotton in the center served as water source. (B) Pairwise choice bioassays were conducted in arenas that consisted of clear plastic cups (946 mL) modified with (1) mesh-covered opening for air circulation, (2) custom-made gated traps with blueberries and treatment inside, (3) Parafilm with a hole in the center provided an entrance for the flies, (4) 1 mL polyethylene vial containing the volatile treatment (50 µL, neat) or control (no volatile) applied to a piece of cotton, and (5) vial with moisturized cotton that served as water source. (C) Dose–response assays were conducted in two-choice arenas that consisted of clear Petri dishes (90 mm diameter × 15 mm high) with (1) mesh-covered opening for air circulation, (2) custom-made gated traps with blueberries and treatment inside, (3) micropipette tip entrances, and (4) 1-mL polyethylene vial containing the volatile treatment (neat) or control (no volatile) applied to a piece of cotton.

described earlier, and were placed vertically on opposite sides of the arenas. A vial with a cotton ball moistened with deionized water was placed in the center of each arena as a water source for the flies (Fig. 1(B)). Twenty *D. suzukii* (1:1 male/female ratio) were released inside each arena, and the number and sex of flies inside and outside the traps was counted after 24 h. All choice tests started at 1:00 p.m., were replicated five times, and were conducted under a fume hood which exchanged air at least once every 24 s, at 22 ± 2 °C, 60 ± 5% RH, and 16 h:8 h (light/dark).

2.8 Multiple choice bioassays #2: comparing *D. suzukii* responses to known and putative anthracnose-emitted repellents

We conducted additional multiple-choice assays, as described in Section 2.6, to compare the repellency of the two most effective repellents identified from anthracnose-infected fruit (ethyl butanoate and ethyl (*E*)-but-2-enoate; based on results from Sections 2.6 and 2.7) with three known *D. suzukii* repellents (1-octen-3-ol, geosmin, and 2-pentylfuran). Within each arena, six gated traps were positioned randomly in a circle (40 cm diameter) at equal distance with each trap 20 cm apart along the circumference. Each gated trap contained *c.* 5 g of store-bought organic blueberry fruit ($n = 5$), as described earlier. One of the six traps served as a control (with blueberry fruit only) and the remaining five traps had one of the repellents added to the blueberry fruit. Each volatile (50 µL, neat, equivalent to 41.9–50 mg or 274–402 µmol) was released from a 1 mL polyethylene vial with a piece of cotton placed inside each trap (control traps had vials with cotton but without the volatile). A vial with a cotton ball soaked with deionized water was placed in the middle of the arena. Roughly 150 *D. suzukii* adults (1:1 male/female ratio) were released inside each arena and the number and sex of flies inside treatment and control traps was recorded after 24 h. The experiment started at 1:00 p.m., was replicated six times, and was done in a laboratory under 22 ± 2 °C, 55 ± 5% RH, 16 h:8 h (light/dark).

2.9 Dose–response choice bioassay: evaluating *D. suzukii* choice and oviposition across a range of concentrations

Experiments were conducted to investigate *D. suzukii* responses to different doses of the two most repellent compounds identified from the pairwise choice bioassays described in section 2.7, ethyl butanoate and ethyl (*E*)-but-2-enoate. We also tested responses to a 1:1 blend of the volatiles, as they were emitted in roughly equal quantities in infected fruit headspace. Two-choice arenas were assembled as described by Prokopy *et al.*[39] with slight modifications (Fig. 1(C)). Each arena consisted of an enclosed Petri dish (90-mm diameter × 15-mm high) with two (10-mm diameter) holes bored into the lid, 40 mm apart. Disposable polyethylene micropipette tips were trimmed, fit snugly into each of the holes, and glued flush with the lid. The bottom of the Petri dish had a 20-mm hole bored into it and was sealed with nylon mesh for ventilation. During the assays, the arenas were oriented so that the pipette tips were facing downward and centered over two trap vials, one containing a volatile compound and one containing a blank control (see Fig. 1(C)). Each trap vial contained five (*c.* 5 g) store-bought organic blueberries as an attractive source. For each individual volatile compound, aliquots of neat chemicals (2.5, 5, 10, 25, and 50 µL) were pipetted onto a small piece of cotton inside a small polyethylene tube. For the 1:1 blend of compounds, equal amounts of each volatile compound were used, making the total volume of 5, 10, 20, 50, and 100 µL. Prior to the start of each assay, flies were starved for 24 h, with access only to distilled water. Ten sexually mature (7–10 days old) females were added to each choice arena. Once loaded into the arena, and after the arena was centered over the trap vials, the flies were allowed to make a choice for 2 h. After 2 h, the number of flies captured in each vial were counted and the berries in the trap vials were collected and observed for eggs. The assays were conducted under a fume hood which exchanged air at least once every 24 s, at 22 ± 2 °C, 60 ± 5% RH from 11:00 a.m. to 3:00 p.m., and were replicated eight times for each compound and dose combination.

2.10 Statistical analyses

2.10.1 Volatile analyses

Statistical analyses of the volatile relative abundances were carried out in R 4.2.2.[40] Peak areas were normalized by berry weight. Composition among samples was visualized with a nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities. Volatile emission was further investigated with permutational

multivariate analysis of variance (PERMANOVA) using time, inoculation treatment, and their interaction as effects with the *adonis* function in the vegan package.[41] Dispersion in the dissimilarity matrix based on the volatile emission data was compared between treatments and among collection days using the *betadisper* function. Differences in volatile emission between anthracnose-infected and control fruit were investigated using differential analysis based on the negative binomial distribution ($\alpha = 0.01$). A linear model of total volatile emissions (sum of volatile peak area divided by blueberry sample weight) with fixed effects of time, treatment, and their interaction was used to investigate patterns of volatile emission in samples. Statistical significance for linear model parameters was determined using analysis of variance (ANOVA).

2.10.2 Behavioral analyses

Data from multiple choice assays on the percent of male and female *D. suzukii* adults responding to each volatile were compared using ANOVA (Minitab version 17; Minitab Inc., State College, PA, USA). The ANOVA model included treatment, sex, and their interaction as fixed factors and block (replicate) as a random factor. A multi-comparison Tukey test (Minitab) was used to determine differences among treatments. Percent data were arcsine square-root transformed prior to ANOVA. Pairwise choice assays were analyzed using G-tests with William's correction. Non-responders were excluded from the statistical analyses. For dose–response bioassays, a repellency index was calculated as follows:

$$\text{Repellency index} = \frac{(n_{\text{control}} - n_{\text{volatile}})}{n_{\text{total}}}$$

where n_{control}, n_{volatile}, and n_{total} are the number of flies in the control tube, treatment tube, and total number of flies in the control and treatment tubes, respectively. Data on the repellency index were checked for normality (Anderson–Darling test) and equal variance (Levene's test) before analysis. The effect of dose on *D. suzukii* repellency index was analyzed using one-way ANOVA followed by Fisher least significant difference (LSD) test (Minitab). The effect of dose on *D. suzukii* oviposition was analyzed using generalized linear models (GLMs). The factors 'treatment' (repellent *versus* control), 'dose', and their interaction were used as fixed effects and were compared following Bonferroni *post hoc* tests. For data on oviposition, we used a Poisson distribution with a logitlink function using SPSS Statistics 23.0 (IBM Corp, Armonk, NY, USA).

3 RESULTS

3.1 Volatile analysis

For all samples and treatments, a total of 63 volatiles were detected (Table 2), including many compounds that have been previously reported in blueberry[42–44] and fungal[45] headspace such as ethyl acetate, methyl butanoate, 2-phenylethanol, linalool, ethanol, and 2- and 3-methyl-1-butanol. Chemical classes detected include various aldehydes, alcohols, a carboxylic acid, esters, ketones, monoterpenes, monoterpenoids, and a sesquiterpene. Esters were the most frequently detected compound class.

Ordination of sample data via NMDS revealed a high degree of similarity among the five *C. fioriniae* strains used to infect fruit in the laboratory (Supporting Information Fig. S1). Because of this similarity, all samples inoculated with the pathogen were subsequently analyzed as a single group, that is, infected *versus* control.

Volatile emission differed between infected and control samples as visualized with NMDS (Fig. 2(A)) and further confirmed via PERMANOVA results (treatment: $F_{1,32} = 20.1$; $P < 0.001$). Time ($F_{1,32} = 49.7$; $P < 0.001$) and the interaction between time and treatment ($F_{1,32} = 6.4$; $P = 0.01$) were also significant. Total volatile emission was higher in infected than control fruit (treatment: $F_{1,32} = 34.5$; $P < 0.001$) and increased over time for both control and infected fruit (time: $F_{1,32} = 117$; $P < 0.001$; Fig. 2(B)), although a significant interaction between time and treatment (time × treatment: $F_{1,32} = 8.45$; $P = 0.006$) indicated that total volatile emission rose more rapidly in infected *versus* control fruit over time.

Differences between anthracnose-infected and control fruit were driven by higher emission of 14 volatiles, listed in Table 2 in bold typeface. They include an aldehyde (2-methylpropanal or isobutyraldehyde), a benzenoid (styrene), five alcohols (2-methyl-1-propanol, 3-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-3-buten-1-ol, (*E*)-2-hexen-1-ol), a ketone (3-hydroxy-2-butanone or acetoin), and six esters (ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, diethyl carbonate, ethyl (*E*)-but-2-enoate, ethyl 3-hydroxy-3-methylbutanoate). All 14 volatiles which differed between infected and control fruit were identified with a commercially available standard on two GC columns. No volatiles were found at higher quantities in control fruit headspace.

The 14 compounds identified in experimentally-infected fruit were also found to be elevated in the field-collected fruit (Table 2). Because field-collected fruit were riper and at a more advanced stage of infection than the experimentally-infected berries, field-collected fruit tended to have higher volatile emission regardless of infection status. Experimentally-infected fruit showed no visual signs of infection on day 2 and only subtle cues on day 4, whereas field fruit were identified as infected by visual cues. NMDS ordination of the field and laboratory collected fruit further indicated good agreement between experimental and field fruit (Fig. S2).

3.2 Multiple choice bioassays #1: comparing *D. suzukii* responses to anthracnose-induced volatiles

The 14 volatiles identified as more abundant in the headspace of infected berries were compared for their capacity to repel *D. suzukii* in a multi-choice bioassay. The behavioral response of *D. suzukii* adults was affected by treatment ($F_{14,87} = 15.92$; $P < 0.001$) and sex ($F_{1,87} = 5.51$; $P = 0.021$) but not by the interaction between treatment and sex ($F_{14,87} = 0.39$; $P = 0.975$), indicating that the effect of treatment was not influenced by sex. In the multiple-choice assay, nine volatiles reduced the number of flies captured relative to the control (Fig. 3): ethyl propanoate, 2-methyl-1-propanol, diethyl carbonate, 2-methylpropanal, 3-methyl-1-butanol, ethyl 2-methylpropanoate, ethyl butanoate, ethyl (*E*)-but-2-enoate, and 3-methyl-3-buten-1-ol. Three volatiles captured a similar number of flies as the control (2-methyl-1-butanol, (*E*)-2-hexen-1-ol, ethyl 3-hydroxy-3-methylbutanoate) and two compounds trapped a higher number of flies than the control (3-hyrdroxy-2-butanone, styrene). Differences according to fly sex were observed across all volatiles, with 30% more females caught than males.

3.3 Pairwise choice bioassays: evaluating *D. suzukii* response to putative anthracnose-emitted repellents

The nine volatiles screened in the multi-choice assay were then tested in dual choice bioassays. All nine showed significant

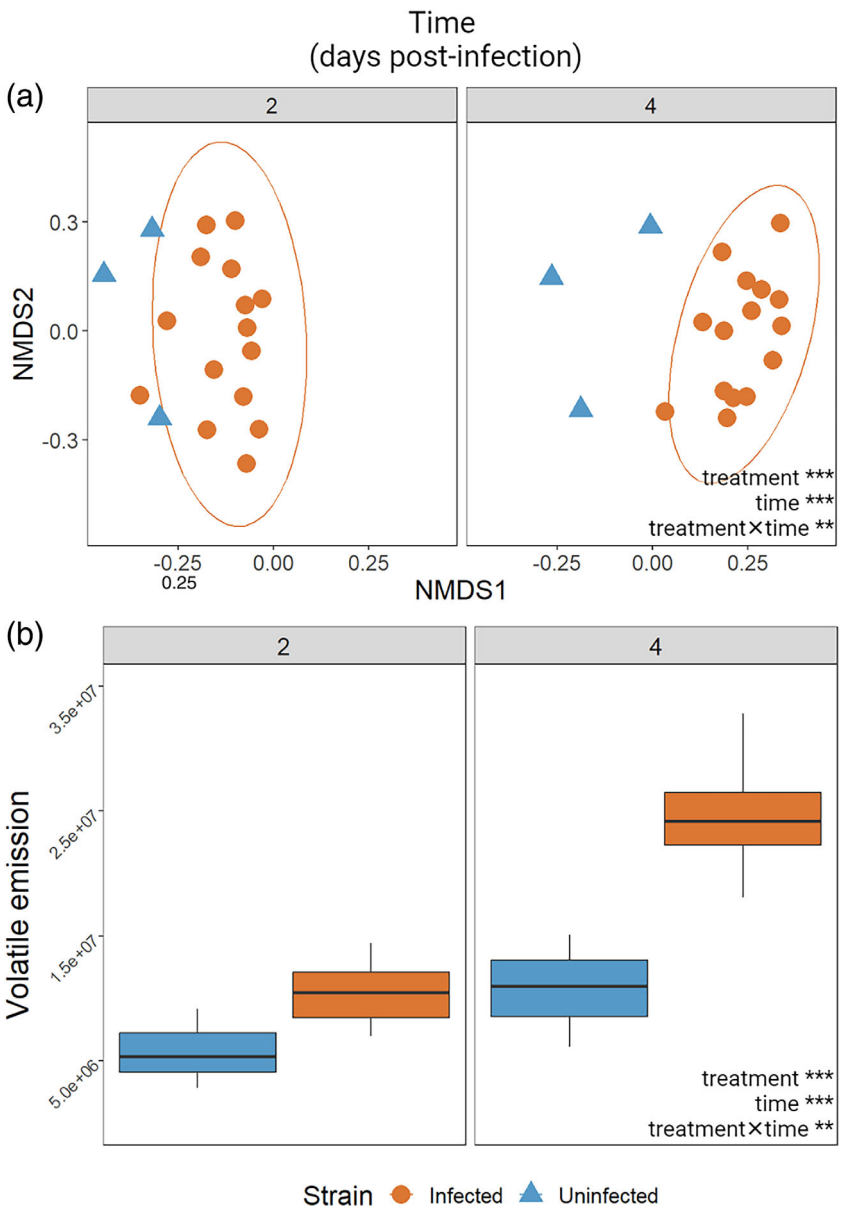

Figure 2. Volatile emission by fresh weight of *Colletotrichum fioriniae*-infected (orange) and uninfected (blue) fruit in experimentally-infected fruit samples. Panels indicate data summarized over day 2 and day 4. (A) A NMDS plot of volatile data with ellipses denoting 95% confidence limit for infected samples. (B) Total peak area of volatiles normalized by fruit weight. Boxplots display median value, first and third quartiles, and 95% confidence intervals.

repellency (Fig. 4). The two most repellent compounds were ethyl (*E*)-but-2-enoate and ethyl butanoate (> 90% repellency), followed by diethyl carbonate, ethyl 2-methylpropanoate, and 3-methyl-1-butanol (> 80% repellency). Both sexes responded similarly to all compounds.

3.4 Multiple choice bioassays #2: comparing *D. suzukii* responses to known and putative anthracnose-emitted repellents

The two most repellent compounds, ethyl (*E*)-but-2-enoate and ethyl butanoate, were compared with previously discovered *D. suzukii* repellents, 1-octen-3-ol, geosmin, and 2-pentylfuran, for their capacity to repel *D. suzukii* in a multi-choice bioassay. The behavioral response of *D. suzukii* adults was affected by treatment ($F_{5,55} = 29.69$; $P < 0.001$) but not by sex ($F_{1,55} = 0.1$; $P = 0.756$) or by the interaction between treatment and sex ($F_{5,55} = 0.65$; $P = 0.663$). In the multiple-choice assay, all five repellents reduced the number of flies captured relative to the control (Fig. 5); however, there were differences in the strength of repellency among them (Fig. 5). The repellency of ethyl (*E*)-but-2-enoate and ethyl butanoate was comparable to or in some cases greater than that of the other known repellents (Fig. 5).

3.5 Dose–response choice bioassay: evaluating *D. suzukii* choice and oviposition across a range of concentrations

The dose of ethyl butanoate ($F_{5,42} = 6.3$; $P < 0.001$), ethyl (*E*)-but-2-enoate (($F_{5,42} = 9.34$; $P < 0.001$), and the 1:1 blend ($F_{5,42} = 2.82$; $P = 0.028$)) had a significant effect on the repellency index. For ethyl butanoate and ethyl (*E*)-but-2-enoate, the repellency index increased with increasing dose (Fig. 6). For the 1:1 blend, the repellency index for the 5, 10, 20, 50, and 100 µL doses was higher than the '0' dose or control (no compounds) but was similar across

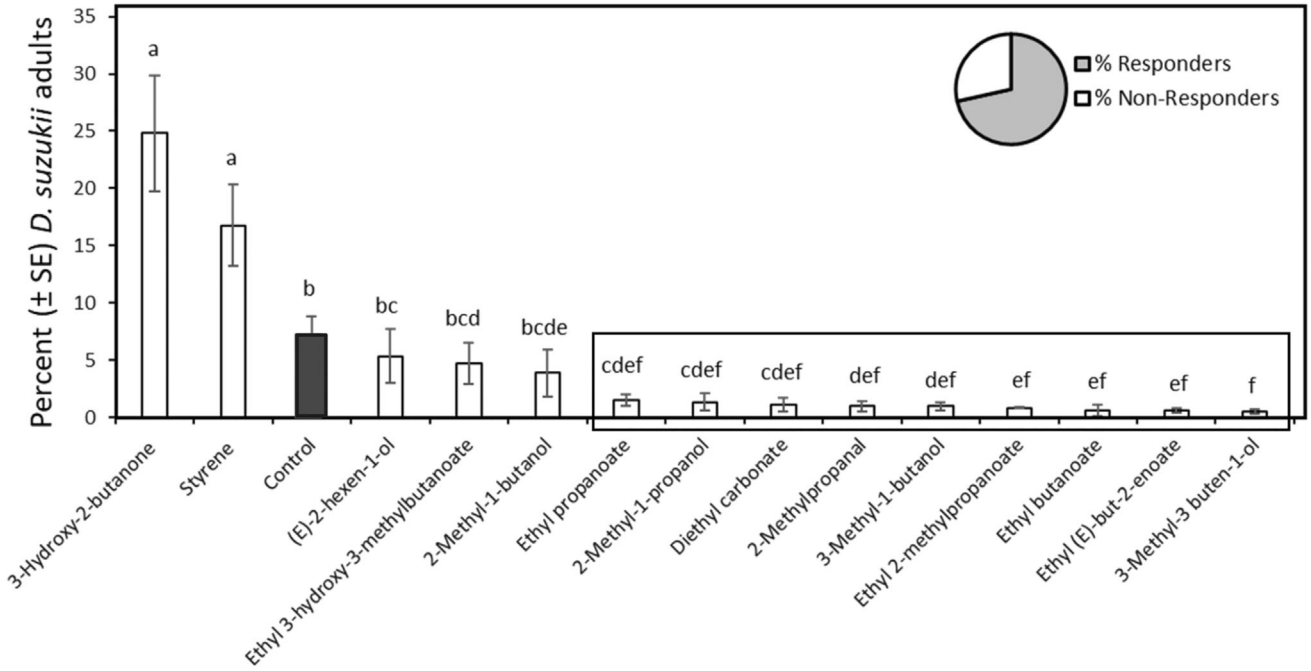

Figure 3. *Drosophila suzukii* adults captured in a multiple-choice bioassay with blueberries. Bars represent mean percent flies captured; error bars denote standard error (SE). Different letters on bars indicate significant differences by Tukey tests at $P \leq 0.05$. Statistical tests were based on arcsine square-root transformed data. Means from untransformed data are shown. Compounds within the box captured significantly fewer flies than the control (black bar). For each compound, 50 µL neat was spiked to cotton and placed inside a vial with *c.* 5 g fruit. The percent of responders and non-responders are presented in the circle at the top right of the figure. $n = 4$.

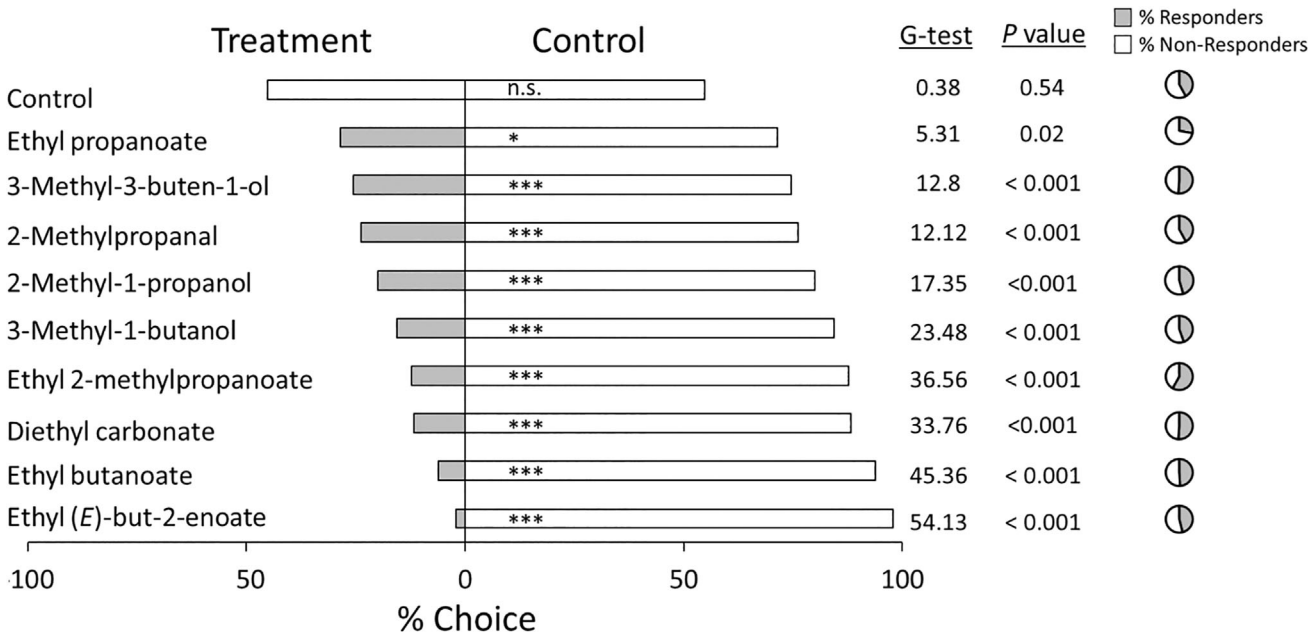

Figure 4. *Drosophila suzukii* adults captured in pairwise choice bioassays with blueberries. Bars represent mean percent flies captured. An asterisk indicates significant differences between treatments at $P \leq 0.05$; n.s., not significant ($P > 0.05$). The percent of responders and non-responders are presented in the circle at the right of the figure. $n = 5$. For each compound, 50 µL neat was spiked to cotton and placed inside a vial with *c.* 5 g fruit.

all doses tested (Fig. 6). When applied as single compounds, the highest doses of ethyl butanoate and ethyl (*E*)-but-2-enoate reached repellency index of *c.* 0.8, where approximately 80% of insects chose the control over the volatile-treated trap. Although the 1:1 blend also achieved repellency, a weaker effect was observed (repellency index *c.* 0.5 for all doses).

Both repellent compounds, ethyl butanoate (Wald's $\chi^2 = 90.567$; $P < 0.001$), ethyl (*E*)-but-2-enoate (Wald's $\chi^2 = 71.299$; $P < 0.001$), and their 1:1 blend (Wald's $\chi^2 = 115.492$; $P < 0.001$), reduced *D. suzukii* oviposition; however, this was affected by dose (significant treatment × dose interaction; ethyl butanoate: Wald's $\chi^2 = 79.073$; $P < 0.001$; ethyl (*E*)-but-2-enoate: Wald's $\chi^2 = 61.444$; $P < 0.001$;

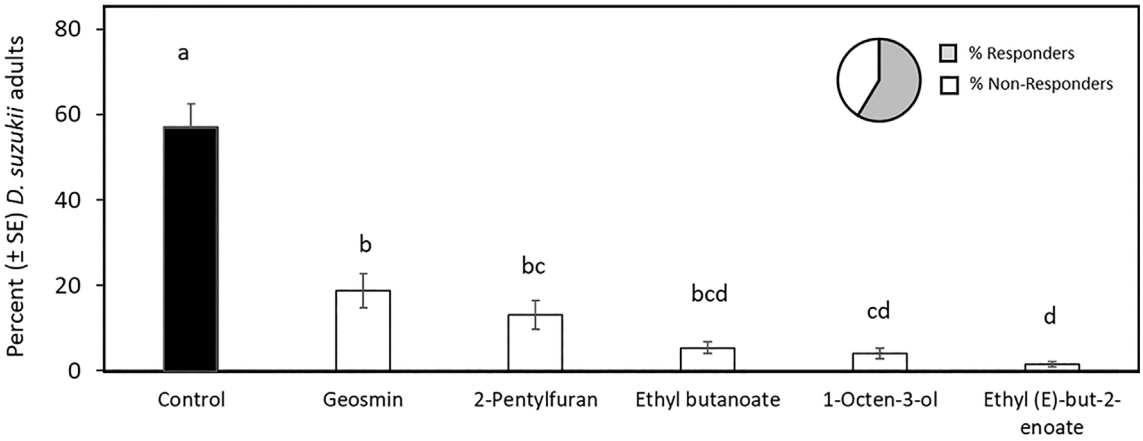

Figure 5. *Drosophila suzukii* adults captured in a multiple-choice bioassay with known and putative repellents. Bars represent mean percent flies captured; error bars denote standard error (SE). Different letters on bars indicate significant differences by Tukey tests at $P \leq 0.05$. Statistical tests were based on arcsine square-root transformed data. Means from untransformed data are shown. The percent of responders and non-responders are presented in the circle at the top right of the figure. $n = 6$. For each compound, 50 µL neat was spiked to cotton and placed inside a vial with *c.* 5 g fruit.

1:1 blend: Wald's $\chi^2 = 121.858$; $P < 0.001$). For both compounds and the 1:1 blend, there was no difference between treatments in the control or '0' dose, but all other doses reduced oviposition (Fig. 7). There was also a significant dose effect (ethyl butanoate: Wald's $\chi^2 = 64.307$; $P < 0.001$; ethyl (*E*)-but-2-enoate: Wald's $\chi^2 = 71.194$; $P < 0.001$; 1:1 blend: Wald's $\chi^2 = 121.009$; $P < 0.001$). Females laid the most eggs in the '0' dose (i.e., in the absence of repellents) and laid fewer eggs with increasing doses of ethyl butanoate and ethyl (*E*)-but-2-enoate, while similar number of eggs were laid across all doses of the 1:1 blend (Fig. 7). Here, even at low doses the number of eggs per trap was lower when the blend was applied (0–1 egg per trap at 5 µL), whereas more eggs were observed in comparable doses of individual compounds (3–4 eggs at 2.5 and 5 µL).

4 DISCUSSION

To characterize potential *D. suzukii* repellents, we identified the volatiles emitted from anthracnose-infected blueberries, finding that *C. fioriniae* infection increased the emission of 14 volatiles, many of which are associated with ripening blueberries,[46–48] like ethyl butanoate, a putative repellent identified here. Furthermore, although volatile abundance increased over time in both infected and control fruit as they matured, emission increased more rapidly in infected fruit. Since *D. suzukii* prefers to oviposit in ripening as opposed to overripe or rotting fruit[3,49] and *D. suzukii* flies are known to be very sensitive to fluctuations in fruit ripening odors,[50] this may explain their aversion to high concentrations of specific volatiles as reported here.

Next, using a series of laboratory bioassays, we selected two volatiles that exhibited remarkable repellency for further study. The first, ethyl butanoate, has been shown to elicit an antennal response in *D. suzukii*[51] and has been previously identified as a *D. suzukii* repellent,[15,52] and the other, ethyl (*E*)-but-2-enoate, has not been previously identified as a repellent yet has a highly correlated chemical structure. The performance of these volatiles in laboratory bioassays indicated comparable repellency to the known *D. suzukii* repellents 1-octen-3-ol, geosmin, and 2-pentylfuran.

We further explored the response of *D. suzukii* to varying amounts of ethyl butanoate and ethyl (*E*)-but-2-enoate individually and in a 1:1 mixture, as dose-dependent responses of *D. suzukii* to aversive stimuli have been identified in other studies.[22] As expected, we found *D. suzukii* response to the individual volatiles was sensitive to differing doses. However, the 1:1 blend did not elicit dose-dependent behavior in *D. suzukii*. It is possible that since the blend contains two highly structurally similar volatiles that may act on the same *D. suzukii* receptors, the combined effects of these volatiles saturate the inhibitory or avoidant response of the insects such that increasing amounts cannot elicit further response.

Interestingly, although the individual volatiles seemed to elicit a stronger repellent effect, especially when high doses were used, strong oviposition deterrence was observed with the blend, even at the lowest dose tested. The blend therefore could prove a more useful tool in preventing infestation in the field, where it can be challenging to maintain very high concentrations of volatiles over the course of a growing season. Especially for ethyl butanoate and ethyl (*E*)-but-2-enoate, which are more volatile than the other *D. suzukii* repellents geosmin, 2-pentylfuran, and 1-octen-3-ol, maintaining sufficient emission rates in the field will be critical to potential successful deployment of these repellents. A variety of techniques have been used to facilitate the slow release of odors in the field that could be applied to these repellents including nanoencapsulation,[53] incorporation into an inert matrix, for example, Specialized Pheromone and Lure Application Technology (SPLAT®),[21] and aerosol diffusers which periodically puff volatiles into field.[54]

The use of chemical repellents for crop protection from *D. suzukii* infestation has achieved reduction in infestation rates in raspberries in glasshouse and field studies of cultivated raspberry.[11,12,21,22,54] Although repellents on their own are not likely to eradicate *D. suzukii* infestations, they could be used to supplement foliar insecticide applications, potentially reducing their frequency and thereby reducing environmental contamination and the risk of pest resistance to insecticides. Additionally, the efficacy of repellents can be increased by pairing them with attractants as in 'push–pull' strategies that aim to divert pests away from crops via repellents ('push') while enticing them to a physical or chemical trap that kills the pests ('pull'). Push–pull strategies have been successful in reducing *D. suzukii* infestation in the field, though efficacy was variable over the course of the growing season.[12]

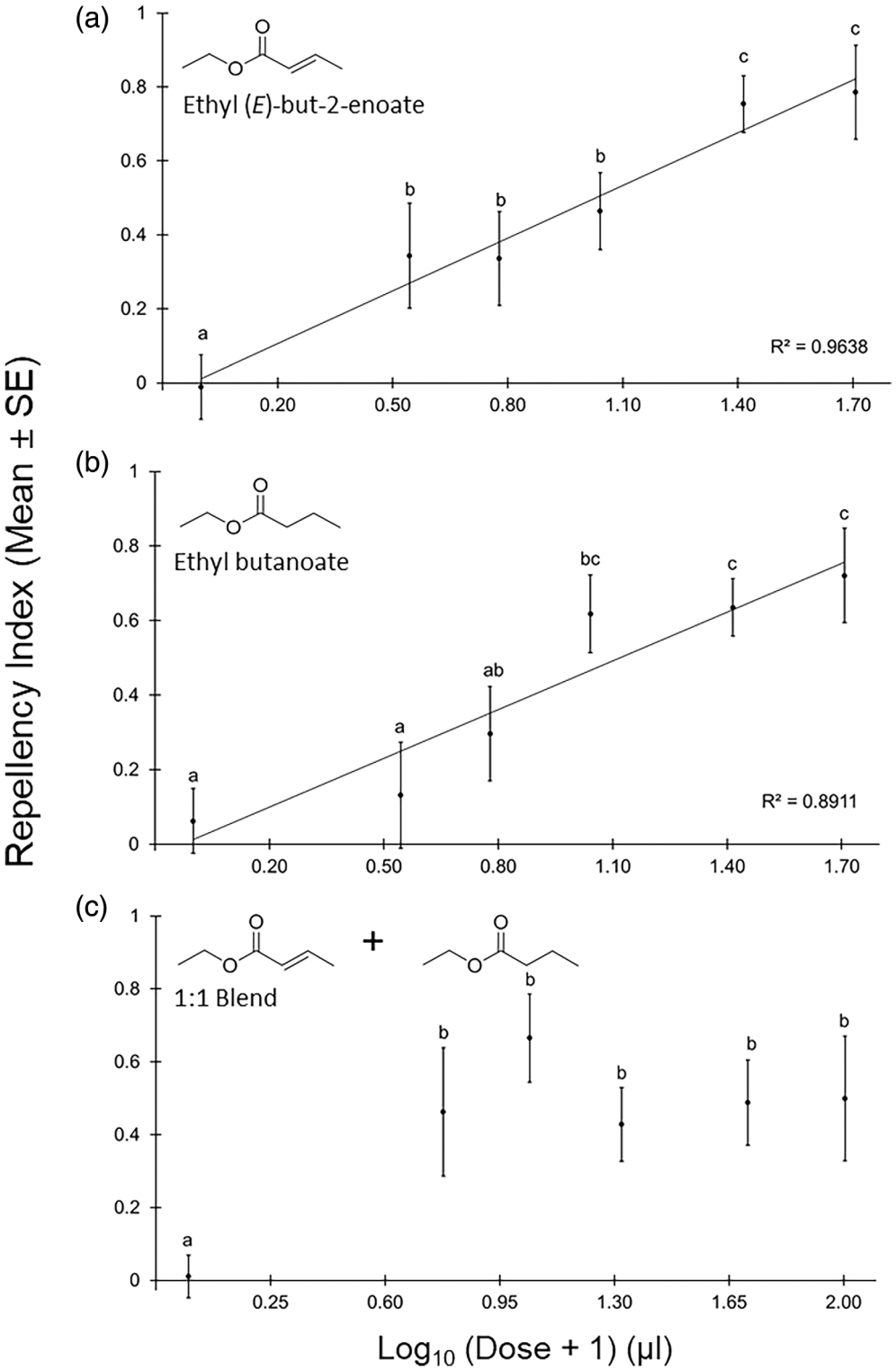

Figure 6. Dose–response effects of (A) ethyl (*E*)-but-2-enoate, (B) ethyl butanoate, and (C) a 1:1 blend of both compounds on the repellency index of *Drosophila suzukii*. The repellency index was calculated as (number of flies in the control – number of flies in the treatment)/total number of responding flies. Different letters indicate significant differences among doses. Percent non-responders = 31.8 ± 1.6%, $n = 8$.

We observed high variability in the response rates of *D. suzukii* in some of our assays, particularly in the pairwise choice assay, where some treatments had *c*. 50% non-responders, or flies who made no choice between treatment and control. We have observed similar variability in the past.[55] This variability could be linked to several factors. First, we used store-bought fruit in all experiments, so it is very likely different cultivars were used. Some of the cultivars could emit less attractive volatiles blends that reduced response rates. Second, although we initiated assays within a specific time window to try to control for effects of time

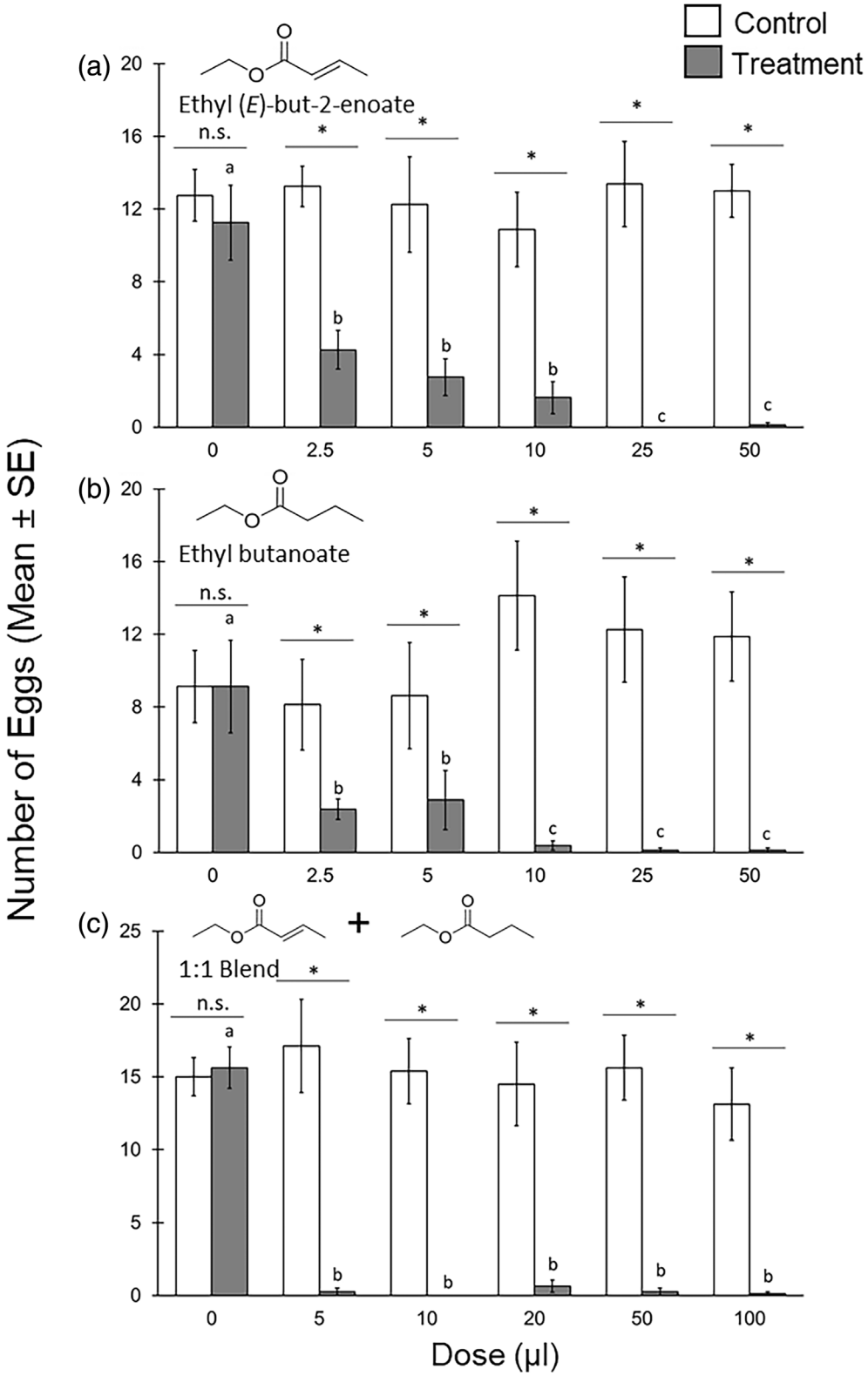

Figure 7. Dose–response effects of (A) ethyl (*E*)-but-2-enoate, (B) ethyl butanoate, and (C) a 1:1 blend of both compounds on the number of eggs laid by *Drosophila suzukii*. Different letters indicate significant differences among doses. An asterisk indicates significant differences between the control and the treatment for each dose; n.s. indicates no significant differences between the control and treatment. Percent non-responders = 31.8 ± 1.6%, $n = 8$.

of day between assays, *D. suzukii* activity are known to exhibit diurnal fluctuations with increases in activity at dawn and dusk[56] and so variation in experiment initiation may have contributed to the response rate. Third, although we attempted to mitigate the potential for volatiles to interact with one another in the bioassays by conducting bioassays in fume hoods, especially with the assays

where multiple volatiles were screened simultaneously, fly response could have been impacted by the mixture of compounds present in arenas and had a disorienting effect. Finally, although we attempted to standardize experimental conditions, fluctuations in room temperature and humidity, as well as the physiological state of flies could have impacted fly behavior. To account for these differences, we used replicate as a random factor in our analysis. Despite the variability in percent of responders observed in some assays, the consistency of the effects observed across the multiple bioassays presented here suggest that the compounds function as repellents.

In most assays, no differences in capture rate were identified between the sexes. However, in multiple choice bioassay #1, we observed higher capture rates of females than males. According to previous studies,[27] only *D. suzukii* females were repelled by volatiles from anthracnose-infected fruit, likely because they are seeking suitable oviposition sites. Driven by mating behavior, it is possible that *D. suzukii* males may follow females into traps, thereby influencing the number of flies captured and apparent response to a stimulus. Although male-specific responses to anthracnose-induced volatiles were not investigated here, we observed strong repellency and oviposition deterrence in the dose–response assays where only adult females were tested.

Though it was our aim to identify repellents for *D. suzukii* in this study, two attractive volatiles were also identified. The first, 3-hydroxy-2-butanone (acetoin), is a ubiquitous fermentation volatile already known to attract *D. suzukii* adults.[14,15] The second volatile, styrene, has previously been found attractive to other flies[57] and has been detected at higher emission levels when blueberries are infected by phytopathogens,[58] but until now *D. suzukii* response to this volatile was unknown. It would be interesting to evaluate whether incorporation of styrene into lures could boost their effectiveness.

5 CONCLUSION

The current study is the first to identify promising repellents for *D. suzukii* from pathogen-infected fruit. Although previous studies showed that pathogen infection can repel *D. suzukii*,[26,27] the repellent compounds were not identified. By analyzing the volatile emission of blueberries infected with *C. fioriniae*, we tentatively identified 11 novel *D. suzukii* semiochemicals, nine repellents and two attractants. The esters ethyl butanoate and ethyl (*E*)-but-2-enoate were the most repellent compounds, performing similarly to known *D. suzukii* repellents 1-octen-3-ol, geosmin, and 2-pentylfuran in laboratory tests. Further testing is needed to evaluate *D. suzukii* response in the field, however, the strong repellency and oviposition deterrence exhibited by ethyl butanoate and ethyl (*E*)-but-2-enoate suggests that these volatiles could be useful in modulating behavior of this destructive pest.

AUTHOR CONTRIBUTIONS

CR-S, CCR and PU-B conceived of the study. FK supplied the fungal isolates and advised culturing and inoculation procedures. CCR designed the volatile analysis methods and analyzed volatile data. JJB and CCR collected volatile data. CR-S and AQ designed bioassays and analyzed behavioral data. AQ and YB-Z collected bioassay data. CCR, CR-S and PU-B performed statistical analyses. The first draft of the manuscript was written by CCR, and all authors contributed to and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

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

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