Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2016

www.elsevier.com/locate/jbiosc

Polyamines in brown rice vinegar function as potent attractants for the spotted wing drosophila

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Received 24 May 2016; accepted 27 June 2016
Available online xxx

Vinegar produced by acetic acid bacteria is used as an attractant for fruit flies. Apple cider vinegar (ACV) and brown rice vinegar (BRV) are used as lures to detect *Drosophila suzukii* (also known as the spotted wing drosophila [SWD], a newly emerging invasive pest of soft-skinned fruits) and to capture *Drosophila melanogaster*, respectively. In the present study, we evaluated the attractiveness of BRV and ACV to SWD in laboratory trapping experiments using an upturned microcentrifuge tube with a pipette tip as a trap. We transferred SWD (approximately 20, 7–10 days old) to a glass vial containing a trap baited with BRV or ACV and counted the captured flies. BRV attracted more flies (52.88 ± 9.75%) than ACV (35.78 ± 7.47%) in 6 h. Based on high-performance liquid chromatography, we found that BRV contained greater amounts of putrescine (12.36 ± 0.44 μM) and spermidine (35.08 ± 4.34 μM) than ACV (putrescine, 0.31 ± 0.067 μM; spermidine, not detected). The attractiveness of ACV supplemented with putrescine (12 μM) and spermidine (35 μM) (68.56 ± 4.69%) was significantly higher than that of ACV, indicating that the enhanced attractiveness of BRV to SWD was accomplished by the additive effects of polyamines and other known attractive volatiles, such as acetic acid and acetoin. BRV is expected to be a powerful tool for the efficient management of SWD.

[**Key words:** *Drosophila suzukii*; Spotted wing drosophila; Polyamines; Brown rice vinegar; Apple cider vinegar; Acetic acid bacterium]

Drosophila suzukii, commonly known as the spotted wing drosophila (SWD) in the United States, is a globally invasive pest of various soft-skinned fruits (1–5). SWD is considered indigenous to Southeast Asia, and was first reported in Japan in 1916 (2). Since it invaded western countries in the late 2000s via international crop trade, SWD has spread widely throughout the Unites States, Canada, and Europe (4). Female SWD can lay eggs on healthy ripening fruits owing to a prominent serrated ovipositor, unlike most other drosophilids, such as *Drosophila melanogaster*, which mainly infest overripe and damaged fruits (6). Larvae of SWD feed on and destroy fruits, and wounds on the surface of fruits caused by oviposition provide access for secondary infestations by other insects and pathogens, resulting in accelerated decomposition and unmarketable products (1). SWD has extremely high fecundity and complete some dozen generations per year (3). Furthermore, SWD has a wide host range, from cultivars to wild fruiting plants, which enables the fly to survive almost everywhere (3). While some synthetic pesticides are applicable for SWD management, frequent applications might be required owing to the short generation time (7). These characteristics of SWD make containment or eradication quite difficult, and estimated economic losses have reached 500–700 million dollars and 3 million euros annually in the United States and Europe, respectively (1,8).

Because SWD causes substantial economic damage in the fruit industry, it has recently been intensively investigated. Ripening fruits emit high levels of carbon dioxide (CO_2) via active respiration (9) and *D. melanogaster* strongly avoids CO_2 (10,11). SWD does not exhibit an aversion to CO_2, even though it can detect CO_2 via olfactory receptor neurons orthologous to those of *D. melanogaster*, suggesting an evolutionary adaptation that allows SWD to infest ripening fruits (12). Transcriptome analyses have shown that short-day lengths and low temperatures induce drastic changes in the expression of genes involved in cold tolerance and reproductive diapause, enabling SWD to overwinter (13). Several physiological aspects of the pest fly have been clarified; however, integrated pest management (IPM) practices for SWD have yet to be established.

IPM comprises three steps: monitoring (detection of pest flies with traps), modeling (forecasting the current and future distribution of pest flies based on environmental factors), and management (the control of fly populations by implementing appropriate measures, such as pesticide application) (3). The initial monitoring step is very important for successful IPM since the rapid detection of pests is essential for cost-effective management practices (14). Minimum pesticide applications are also desirable to avoid the emergence of resistance and to protect beneficial insects and human health. Therefore, effective attractants for SWD are required.

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.06.014

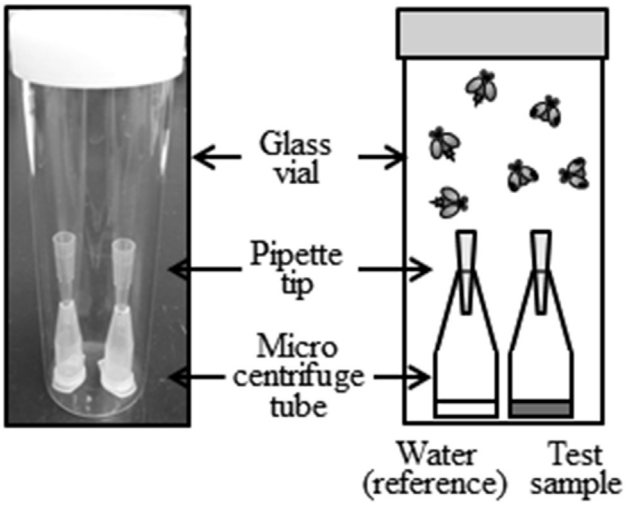

FIG. 1. Trap design. Trapping assays were performed as described previously (24,25), with slight modifications. Briefly, an upturned microcentrifuge tube with a pipette tip was used as a trap. Test samples and distilled water were applied to the upturned lid of the inverted tube. Bred flies were transferred to a glass vial with traps, and the number of flies captured in each trap was counted.

For monitoring, a variety of fermented foods, especially apple cider vinegar (ACV) and wine, are conventionally used as baits for SWD traps in the United States (3). Cha et al. demonstrated that four chemicals released from ACV and wine (i.e., acetic acid, acetoin, ethanol, and methionol) are key olfactory signals for SWD attraction (15,16). In our previous studies, we constructed a metabolically engineered acetic acid bacterium overproducing acetoin and related metabolites via specific gene disruption (17,18). The culture supernatant of the engineered strain captured significantly more *D. melanogaster* than that of the parental strain, suggesting that the enhanced attractiveness was achieved by the synergistic effects of acetic acid, acetoin, isobutyric acid, and unidentified metabolites (19). The Caribbean fruit fly, which attacks several tropical and subtropical fruits, is strongly attracted to putrescine (20), a polyamine that is essential for numerous cellular processes in all organisms, from bacteria to humans (21–23). Lures composed of putrescine and ammonium bicarbonate have been utilized for crop protection (20).

In Japan, brown rice vinegar (BRV), produced from brown (unpolished) rice, is widely used as an ingredient in various commercial flytraps for household use because it exerts a potent attractiveness to *D. melanogaster*, which is considered an indicator of unsanitary conditions. Our objective was to develop effective flytraps with higher attractiveness to SWD than those commonly used in agricultural fields. We evaluated the attractiveness of BRV and ACV to SWD in laboratory trapping assays. We also quantified polyamines in vinegars and investigated their effects on SWD attraction.

MATERIALS AND METHODS

Breeding of flies *D. suzukii* (SWD) strain E-15001 was obtained from the Ehime-Fly Drosophila Stocks of Ehime University, Ehime, Japan. Cornmeal medium, which was composed of 40 g/l yeast extract (Nihon Seiyaku, Tokyo, Japan), 90 g/l cornmeal, 100 g/l sucrose, 3 ml/l propionic acid, and 9 g/l agar, was used as a feed for SWD. The flies were transferred to a glass vial (40 mm [diameter] × 130 mm [height]) baited with 25 ml of the cornmeal medium and capped with a cotton plug, and reared at 20°C for approximately three weeks. When pupae formed, adult flies were moved to glass vials containing fresh cornmeal medium to maintain the fly population. Newly eclosed flies were collected every 24 or 48 h and further reared on fresh cornmeal medium for 7–10 days before they were used for subsequent trapping assays.

Trapping assays using chemicals as attractants Trapping assays were performed in accordance with previous methods (24,25), with slight modifications. A 1.5-ml microcentrifuge tube was cut 3 mm from the tapered end. A 200-μl pipette tip was cut 14 mm from the narrow end to create a hole with an inner diameter of 2 mm and inserted into the inverted centrifuge tube. Test chemical solutions (125 μl), i.e., acetic acid, acetoin, isobutyric acid, putrescine, spermidine, and spermine, were applied to the upturned lid of the microcentrifuge tube as attractants (Fig. 1). The concentrations of acetic acid (0.5% [wt/vol]), acetoin (0.1% [wt/vol]), and isobutyric acid (0.01% [wt/vol]) were determined based on our previous study (19). The trap containing 125 μl of distilled water was used as a reference (reference trap). The chemically baited trap and the reference trap were placed at the bottom of a glass vial (40 mm [diameter] × 130 mm [height]) (Fig. 1). Approximately 20 flies (7–10 days old) were starved for 20 h in a glass vial containing two Kimwipes moistened with 3 ml of distilled water and were then transferred to the vial with traps. All assays were performed at 20°C under dark conditions to minimize fly responses caused by innate characteristics, such as phototaxis (26). To examine whether the test chemicals were attractive to SWD, flies captured in the traps were counted after 24-h assay periods. As a negative control, assays were performed using the trapping device with two reference traps. The attractiveness of the test chemicals was estimated as the ratio of the numbers of flies captured in traps to total flies used (%). Experiments were conducted in triplicate, and the position of trapping devices was randomized for each assay. Mean attractiveness values of the test chemicals were compared with that of distilled water and statistically analyzed by *t*-tests using *P*-value thresholds of 0.05, 0.01, or 0.001.

Trapping assays using vinegars as attractants Flies and trapping devices were prepared as described above. BRV (Marukan Vinegar Co. Ltd., Kobe, Japan) and ACV (Marukan Vinegar Co. Ltd.) were diluted ten times with distilled water because undiluted vinegars were rather repulsive to SWD in our small assay system. After starved flies were transferred to the vial with the trap baited with 10% (vol/vol) vinegar and the reference trap, individuals captured in traps were monitored every hour for six hours. The attractiveness of vinegar was estimated as the ratio of the numbers of flies captured in traps to total flies used (%). Assays were conducted in triplicate, and the position of trapping devices was randomized for each assay. Mean attractiveness values were analyzed using *t*-tests with a *P*-value threshold of 0.05.

Quantification of volatile compounds, organic acids, and polyamines in vinegars The concentrations of acetic acid, acetoin, and ethanol in vinegars were measured using a GC-2014 gas chromatograph (GC; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a packed column (PEG20M 10%, Shincarbon A 60/80, 2.1 m by 3.2 mm; Shinwa Chemical, Kyoto, Japan). The column oven temperature was maintained at 60°C for 3 min, and then programmed to increase at a rate of 10°C per min up to 200°C (27).

Organic acids in vinegars were quantified by high-performance liquid chromatography (HPLC; Organic Acid Analysis System Prominence; Shimadzu) on an ion-exclusion column (Shim-pack SCR-102H; Shimadzu). The column was equilibrated with a mobile phase (5 mM *p*-toluenesulfonate; Wako Pure Chemicals, Osaka, Japan) at a flow rate of 0.8 ml per min at 40°C. Eluted organic acids were automatically mixed with a reaction buffer consisting of 5 mM *p*-toluenesulfonate, 100 μM ethylenediaminetetraacetic acid (EDTA; Dojindo Molecular technologies, Kumamoto, Japan), and 20 mM Bis-Tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane; Dojindo Molecular Technologies] at a flow rate of 0.8 ml per min at 40°C, and were detected with an electrical conductivity detector (CDD-10AVP; Shimadzu) (19). All samples were filtered with a 0.45-μm-pore-size filter (Millex LH filter; Millipore, Bedford, MA, USA) before injection.

Polyamines in vinegars were analyzed by HPLC as described previously (28). To extract polyamines in vinegar, 50 μl of 10% (wt/vol) trichloroacetic acid (TCA; Wako Pure Chemicals) was added to 500 μl of vinegar and mixed well by vortexing. The mixture was centrifuged, and the resultant supernatant was collected. As an internal standard, caldopentamine was added to the supernatant at a final concentration of 10 μM. The supernatant (100 μl) was filtered with a 0.45-μm filter (Millipore), and then analyzed by HPLC on a CK-10S cation-exchange column (8.0 mm inner diameter × 50 mm; GL Science, Tokyo, Japan). The column was equilibrated with a mobile phase [100 mM potassium citrate monohydrate, 1.7 M KCl, 650 mM 2-propanol, 2.4 mM polyoxyethylene(23)lauryl ether (Brij 35; Wako Pure Chemicals), and 65 mM HCl] at a flow rate of 1.0 ml per min at 70°C. The eluted polyamines were automatically mixed with a reaction buffer comprising 400 mM boric acid, 400 mM NaOH, 4.9 mM Brij 35, 7.5 mM *o*-phthalaldehyde, 171 mM ethanol, and 28 mM 2-mercaptoethanol at a flow rate of 0.5 ml per min at 70°C and monitored with a fluorescence detector (GL-7453A; GL Science).

RESULTS

Trapping assays using chemicals as attractants The laboratory assay system illustrated in Fig. 1 is mainly used to investigate behavioral responses of *D. melanogaster* to various stimuli, including attractants, repellents, and toxicants (24,25). We first confirmed that the system was applicable for SWD trapping

Please cite this article in press as: Akasaka, N., et al., Polyamines in brown rice vinegar function as potent attractants for the spotted wing drosophila, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.06.014