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ORIGINAL PAPER

Assessment of a commercial spider venom peptide against spotted-wing Drosophila and interaction with adjuvants

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Abstract
Chemical control of insect pests in food crops is dominated by broad-spectrum insecticides from a few classes, and there is an urgent need for alternative modes of action. We examined the efficacy of a spider venom peptide, GS-omega/kappa-Hxtx-Hv1a (hereafter, Hv1a) for control of spotted-wing Drosophila and evaluated the importance of phagostimulants and adjuvants for its efficacy. Topical and residual activity of Hv1a was low, with only 17.5% of exposed adult *D. suzukii* dying after 72 h. In contrast, 100% adult mortality was observed after 24 h when three adjuvants were added to Hv1a. Survival of eggs of *D. suzukii* oviposited into blueberries was also reduced by exposure to Hv1a combined with the same adjuvants, indicating that Hv1a activity against *D. suzukii* in the laboratory, but requires penetration of the insect cuticle for efficacy. In a field trial in blueberries, Hv1a gave comparable control to phosmet, and significantly reduced infestation in fruit. This biopesticide adds a new mode of action to the options available for integrated pest management of this and other insect's pests.

Keywords Adjuvants · Blueberries · Biopesticide · *Drosophila*

Key message

- Commercially available Hv1a is active on both adults and eggs of *D. suzukii*.
- The efficacy of spider venom peptides can be increased by inclusion of commercially available adjuvant products.
- Spider venom could provide a new class of insecticides to improve control of insects

Introduction

Control of arthropod pests is necessary for food security, with insects and mites leading to the destruction of 10–14% of world's food supply (Oerke 2006; Pimentel 2009) and the transmission of human and animal pathogens (Lounibos 2002). Insecticides are one of the primary methods used for arthropod pest control, but they can also have negative non-target effects particularly those with broad-spectrum activity (Costa 2006; Smith and Stratton 1986) and also from restricted spectum and reduced risk insecticides (Guedes et al. 2016). Peptides derived from spider venom represent a potentially new source of insecticides, and their activity against arthropod pests has been explored in a number of studies (Fitches et al. 2012; King 2007; King and Hardy 2013; Tedford et al. 2004) and reviews (Herzig et al. 2014; Windley et al. 2012). King (2011) estimated there may be > 10 million bioactive spider venom compounds, with the vast majority expected to be insecticidal. These spider venoms are complex, consisting of many components dominated by disulfide-rich peptide neurotoxins, which impart the highest insecticidal activity (King and Hardy 2013). Spider venom peptides also act on a wide range of molecular targets including protease inhibitors, modulators of transient receptor potential channels, mechanosensitive channels, acid-sensing ion channels, ionotropic glutamate receptors, glutamate transporters, calcium-activated potassium (K_{Ca}) channels, voltage-gated sodium (Na_V) channels, voltage-gated calcium (Ca_V) channels and voltage-gated potassium (K_V)

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channels (King and Hardy 2013). The latter two targets in this list are currently not widely used in commercially available insecticides, and thus insecticides developed to exploit these molecular targets could be important for resistance management.

The vinegar fly, *Drosophila suzukii* (Diptera: Drosophilidae) Matsumura, commonly referred to as the spotted-wing *Drosophila*, is an invasive pest infesting small fruits and cherries, in the majority of temperate regions globally where fruit is produced (Asplen et al. 2015; Cini et al. 2014; Walsh et al. 2011). Whereas most Drosophila species are only of concern in overripe or damaged fruit, females of *Drosophila suzukii* possess a serrated ovipositor which allows them to oviposit in unripe or ripening fruit, leading the fruit to be unmarketable (Walsh et al. 2011). Multiple approaches to control *D. suzukii* have been investigated, including cultural (Leach et al. 2016, 2018) and biological (Rossi Stacconi et al. 2015; Wang et al. 2016; Becher et al. 2018; Garriga et al. 2018; Nikolouli et al. 2018; Yousef et al. 2018) controls among others. However, insecticides remain an important and widely used strategy for control. Increased use of insecticides has led to concerns about the development of resistance, particularly given the short generation time (Van Timmeren et al. 2017a). Additionally, the most effective insecticide classes for control of *D. suzukii* are the carbamate, organophosphate, pyrethroid and spinosyn insecticides (Van Timmeren and Isaacs 2013) and there is an urgent need for additional modes of action to control this pest. Insecticides that target both the adults and the egg and larval life stages of *D. suzukii* within the fruit are desirable as they lead to the most significant reduction in *D. suzukii* populations (Wise et al. 2015).

Pest control can be improved by increasing the uptake of insecticide into the insect's body. This can be done through greater ingestion, more rapid transportation through the gut barrier or penetration through the cuticle (Bonning and Chougule 2014; Cowles et al. 2015; Lewis 1980). Recent research has highlighted potential benefits of the inclusion of sugar or other sweeteners to act as phagostimulants to increase uptake of insecticides through feeding by adult flies (Cowles et al. 2015). In strawberries, the inclusion of sucrose in applications of spinosad resulted in a > 50% reduction in infestation by larvae, when compared to plots receiving spinosad only (Cowles et al. 2015). However, there was no such benefit when corn syrup was added to insecticides in a recent study in raspberries (Fanning et al. 2017). Enhanced penetration of ingested insecticides into the insect gut in the presence of *Bacillus thuringiensis* Berliner can also increase pesticide efficacy since it interacts with the insect gut to create pores that increase toxin uptake (Pardo-Lopez et al. 2012). Penetrating adjuvants formulated to be added to pesticides could also help improve efficacy since the cuticle can be a barrier to insecticide uptake. However, little research has been conducted to determine the effect of spray adjuvants on aiding penetration by venom proteins.

The first commercially available insecticide developed using an unmodified insecticidal spider venom peptide was recently approved for registration by the US EPA. This peptide GS-omega/kappa-Hxtx-Hv1a (hereafter, Hv1a) is isolated from the venom of *Hadronyche versuta* (Rainbow, 1914), a species of funnel-web spider native to Australia (Nicholson et al. 1994). Methods for synthesis of this peptide have been developed, with formulations being marketed for control of a range of pest insects, including the cabbage looper, *Trichoplusia ni* (Hübner) (Herzig et al. 2014). This biopesticide is exempt from US tolerance (Environmental Protection Agency 2014) and has no toxicity to honeybees (Nakasu et al. 2014). Another benefit of insecticidal peptides derived from spiders is their selectivity for insects and low mammalian toxicity (Fletcher et al. 1997; Tedford et al. 2004).

In the current study, we determined the effects of an insecticidal spider venom peptide GS-omega/kappa-Hxtx-Hv1a (Hv1a), for control of *D. suzukii* adults and eggs. The potential for ingestion of the insecticide to improve the efficacy against *D. suzukii* was determined, along with sucrose as a phagostimulant. The synergist *Bacillus thuringiensis* was also tested to explore greater uptake through the gut wall. The effects of adjuvants on the lethal concentration of Hv1a against *D. suzukii* were determined in a series of bioassays. Finally, the efficacy of Hv1a in the field was determined in a replicated trial in a blueberry planting.

Materials and methods

Laboratory bioassays

Spotted-wing *Drosophila* culture

Adults of *D. suzukii* for all experiments were from a colony established using flies reared out of fruit from multiple sites in Western Michigan. Sites were conventional, organic and unmanaged blueberry sites, and all collections were made in the autumn following harvest in 2015. Flies were cultured using a standard Drosophila diet, using a recipe from the Drosophila stock center consisting of cornmeal (62.5 g), white sugar (100 g), nutritional yeast flakes (35 g), agar (22.5 g), distilled water (1700 ml), propionic acid (8.85 ml), methyl paraben (1.65 g) and ethanol (19.65 ml). Flies were held in narrow vials (25 × 90 mm) (LabExpress, Ann Arbor, MI) and maintained in growth chambers at 25 °C, 16:8 light: dark photoperiod and 75% relative humidity. Newly emerged *D. suzukii* was collected 2 days before conducting bioassays; thus, adults used in bioassays ranged from 2 to 6 days old. Flies used to infest blueberries were 7–10 days old.

Spider venom peptide

The spider venom peptide tested in experiments was obtained from Vestaron Corporation, Kalamazoo, MI. The spider venom peptide GS-omega/kappa-Hxtx-Hv1a (Hv1a) is the active ingredient of the commercial compound Spear P (VST-006340-LC). In the following experiments, the peptide was tested using a concentration of 2% a.i. diluted in distilled water.

Topical and residual effect on adults

To assess the efficacy of Hv1a, a series of bioassays were conducted to demonstrate field-relevant exposure routes: topical exposure, residual exposure or a combination of topical and residual exposure. A list of the active ingredients and manufacturers of adjuvants used in the current study are in Table 1. Adult *D. suzukii* were topically treated with the following treatments of Hv1a and adjuvants: Hv1a (0.5%) (538 ml a.i./ha), Hv1a (0.5%) + WaterGuard (0.125%), Hv1a (0.5%) + MSO (0.125%), Hv1a (0.5%) + Silwet L-77 (0.125%) or zeta-cypermethrin (Mustang Maxx 0.8 EC, FMC Corporation, Philadelphia, PA) (27.98 g a.i./hectare) as a commercial standard or an untreated control of distilled water. All assays were conducted using 10 female *D. suzukii* per Petri dish, with each treatment replicated eight times. Treatments were applied using a Potter Spray Tower (Burkard Scientific, Uxbridge, UK) at 15 psi, and 2 ml of spray solution per replicate dish.

For the topical exposure assays, CO_2-anesthetized female *D. suzukii* held in Petri dishes were treated with the chemicals listed above. Following treatment, specimens were transferred to untreated Petri dishes and provided with a small portion (25 × 8 × 5 mm) of standard Drosophila diet for nutrition. For residual exposure, treatments were applied to the inside top and inside bottom of the Petri dishes to expose *D. suzukii* to a consistent dry residue across all surfaces. Following spray applications, Petri dishes were placed in a fume hood to dry for 1 h, and then CO_2-anesthetized *D. suzukii* were added along with a small piece of standard Drosophila diet. The third application method assessed was the combination of topical and residual exposure of *D. suzukii* to Hv1a. Flies were treated as described above in the topical exposure methods, and following treatment, specimens were transferred to Petri dishes treated as described in the residual exposure treatment. Flies in dishes were also provided a standard piece of diet to reduce starvation. Following applications, the Petri dishes were placed in a controlled environmental chamber at 25 °C and 16:8 light: dark cycle and 75% relative humidity. Assessments were conducted on specimens at 24, 48 and 72 h post-applications to determine whether they were alive, moribund or dead. Moribund flies were defined as flies were ones that were showing signs of toxicity such of toxicity such as twitching legs or slow, uneven movements.

Assessment of adjuvants on toxicity

A series of bioassays examined the effect of adjuvants (Table 1) on the effectiveness of Hv1a against adult *D. suzukii*. Hv1a solution at 0.5% was tested with and without WaterGuard (0.125%), Silwet L-77 (0.125%), Vintre (0.125%), LI-700 (0.125%), Load Up (0.125%), and LeafLife Widespread (0.125%) and an untreated control of distilled water.

The dose–response relationship of Hv1a against adults of *D. suzukii* was also determined in combination with adjuvants. For this experiment, three spray adjuvants: Silwet L-77 (0.125%), LI-700 (0.125%) and LeafLife Widespread (0.125%), were tested with Hv1a at 0.1, 0.2, 0.3, 0.4 or 0.5% ppt and an untreated control of distilled water. In total, six replicates of ten females were treated at each concentration for each treatment.

Table 1 List of adjuvants, the principal ingredients and the manufacturers used in the current study

Adjuvant	Principal ingredients	Manufacturer
Leaf Life Widespread	Polyether-polymethylsiloxane-copolymer, polyether (100%)	Loveland Products INC., Greeley, CO
LI-700	Phosphatidylcholine, methylacetic acid and alkyl polyoxyethylene ether (80.0%)	Loveland Products INC., Greeley, CO
Load up	Alkyl phenol ethoxylate, propylene glycol, alkyl amine ethoxylate and sulfuric acid	J. R. Simplot Company, Lathrop, CA
MSO	Methylated vegetable oil, alcohol ethoxylate, phosphatidylcholine (56.1%)	Loveland Products INC., Greeley, CO
Silwet L-77	Polyalkyleneoxide-modified heptamethyltrisiloxane (99.5%)	Helena Chemical Company, Collierville, TN
Tri-Fol	2-Hydroxy-1,2,3-propanetricarboxylic acid (25.0%) and calcium chloride (9.0%)	Wilbur-Ellis. Fresno, CA
Vintre	Alcohol ethoxylate (8.92%)	ORO AGRI INC., Fresno, CA
WaterGuard	Tallowamine ethoxylate, nonylphenol ethoxylate propylene glycol, hydrogensulfate	J. R. Simplot Company, Lathrop, CA

In both experiments, adult *D. suzukii* were treated using the same methodology as the topical exposure treatments described above in the topical and residual bioassays. Treatments were applied to CO_2-anesthetized specimens of *D. suzukii* held in Petri dishes with the Potter spray tower set at 15 psi, and 2 ml of spray solution was applied to each replicate. The treatments were applied to 10 female *D. suzukii*, with each treatment replicated six times. Treated flies were transferred to untreated Petri dishes and provided with a small portion (25 × 8 × 5 mm) of standard Drosophila diet for nutrition. Following treatments, the flies were placed in a controlled environmental chamber at 25 °C and 16:8 light: dark cycle and 75% relative humidity. Mortality assessments were conducted on specimens at 24, 48 and 72 h post-applications to determine the number alive, moribund or dead.

Ingestion bioassays

To assess the effects of phagostimulants and a gut pathogen synergist on the efficacy of Hv1a against *D. suzukii*, adult flies were exposed to store-bought organic blueberries treated with Hv1a alone and in combination with sucrose (Mallinckrodt Baker, Inc. Philipsburg NJ) as a phagostimulant and *Bacillus thuringiensis* var. *israelensis* (Aquabac DF3000, Becker Microbial Products, Inc) as a potential synergist. Prior to exposure of adults to store-bought organic blueberries, the fruit was washed to remove potential insecticide residues and pathogens. Treatments were applied topically to ten blueberry fruits per Petri dish using a Potter spray tower, and a total of eight replicates were treated per chemical treatment. The following chemical treatments were evaluated: Hv1a (0.5%) + 5% (w/v) sucrose, Hv1a (0.5%) + 5% (w/v) sucrose + Aquabac (37.4 g/100L), Hv1a (0.5%) + 5% (w/v) sucrose + Aquabac (37.4 g/100L) + Silwet L-77. Untreated controls of distilled water, 5% (w/v) sucrose and 5% (w/v) sucrose + Aquabac (37.4 g/100L) were also tested. Following application of the treatments, berries were placed in a fume hood to allow residues to dry, and then berries were carefully transferred to 473-ml plastic deli cups (Gordon Food Services, Wyoming, MI) using forceps to avoid removal of residues. A small piece of Drosophila diet and a 4 cm length of moist dental wicking were also placed in each cup to ensure flies did not die of starvation or dehydration. Anesthetized adults of *D. suzukii*, five males and five females (2–7 days old), were introduced into each deli cup.

The flies were placed in environmental chambers and maintained at 25 °C and 16:8 light: dark cycle and 75% relative humidity. Mortality assessments were conducted on specimens at 24, 48 and 72 h post-application. At each point, the number of flies alive, moribund or dead was determined. Following the 72-h mortality assessments, adults of *D. suzukii* were removed from deli cups, the blueberries were retained in the environmental chamber for 7 days, after which the larval infestation of berries was assessed using a filter-based salt test (Van Timmeren et al. 2017b) with larvae being classified into small, medium and large size categories to correspond to the three instars.

Immature life stages bioassays

To determine the effect of Hv1a against eggs and early instar larvae of *D. suzukii* in blueberry fruit, store-bought organic blueberries were washed to remove potential insecticide residues and pathogens. Washed fruit was then exposed to 2–5-day-old adults of *D. suzukii* for 24 h, following which adults were removed to ensure that only the egg life and early instar larvae would be present in the fruit at the time of treatment. Infested blueberries were randomly selected and placed in replicates of 10 blueberries as seen in Fig. 1. Ten replicates of blueberries were then treated with Hv1a (0.5%), Hv1a (0.5%) + Silwet L-77, Hv1a (0.5%) + LI700, zeta-cypermethrin (Mustang Maxx 0.8 EC, FMC Corporation, Philadelphia, PA) (27.98 g a.i./hectare), distilled water, distilled water + Silwet L-77 or distilled water + LI700.

Treatments were applied using either a spray treatment or a dip treatment method. The spray treatments were applied using a Potter Spray Tower (Burkard Scientific) set at 15 psi and applying 2 ml of spray solution per replicate. Infested fruit assigned to the dip treatment was dipped into the spray solution for 10 s and then placed in a fume hood to dry. Following the treatments, the fruit was transferred to 473-ml plastic deli cups (Gordon Food Services, Wyoming, MI) with dental wicking, to prevent moisture buildup, and covered with a ventilated lid to prevent re-infestation post-treatment. The fruit was then placed in a growth chamber maintained at 25 °C and 16:8 day: night cycle and 75% relative humidity for 7 days. Following this, assessment of the number of larvae in each treatment was conducted using the modified salt test method (Van Timmeren et al. 2017b).

Field trial

A field trial was conducted to evaluate the efficacy of Hv1a alone and in combination with adjuvants, and these were compared to a standard insecticide applied against SWD in highbush blueberry, *Vaccinium corymbosum* L. The following positive and negative controls plus treatments with Hv1a were compared: untreated control (0.5%) (538 ml a.i./ha), Hv1a (0.5%) + MSO (0.125%), Hv1a (0.5%) + Silwet L-77 (0.125%) and phosmet (Imidan 70 WP, Gowan, Yuma, AZ) (422 g a.i./ha) + Tri-Fol (0.0625%) (Table 1). A wider list of treatments was reported in Wise et al. (2017). Two bush plots were established in a mature 'Jersey' variety planting (4 × 12 ft. spacing) located 4 miles west of Fennville, MI, USA (latitude 42.5951°: longitude − 86.1561°). Treatment

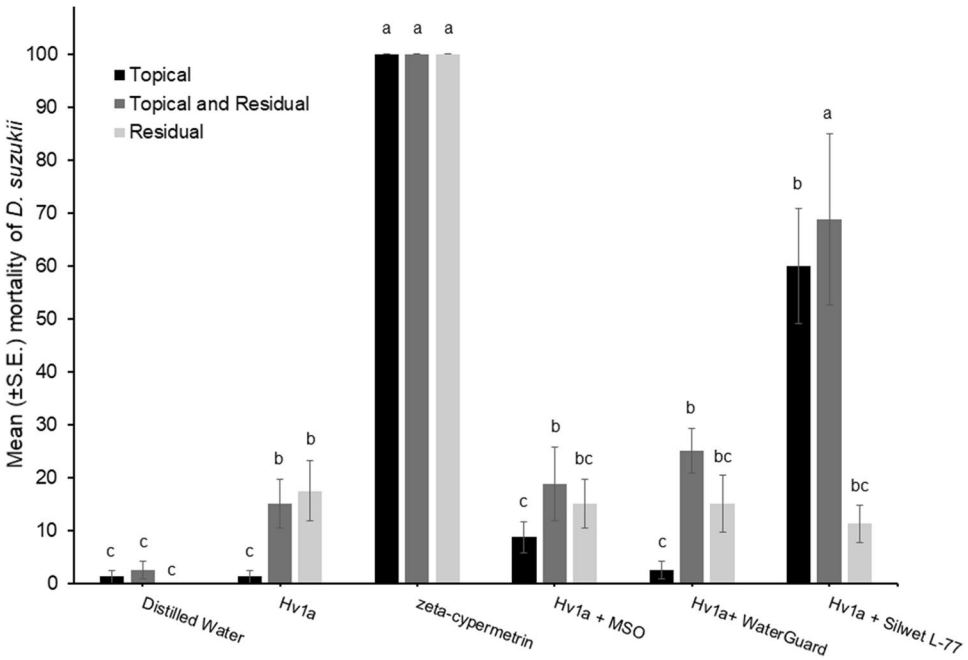

Fig. 1 Effect of Hv1a, with and without adjuvants, and zeta-cypermethrin on the mean (± S.E.) mortality of *D. suzukii* for topical and residual exposure at 72 h after treatment. Means comparisons were made among treatments within the same colored bars, and bars headed with the same letter within an exposure method are not significantly different ($P > 0.05$, ANOVA, Tukey's HSD test)

plots were replicated four times and set up in a randomized complete block design. Applications began on the July 20, 2016 on the date of the first trap catch for *D. suzukii* at the site and were re-applied on 7-d intervals for a period of 4 weeks. Treatments were applied with an FMC 1029 air-blast sprayer (Jonesboro, AR) calibrated to deliver 468 L/ha (50 gallons per acre) of diluent.

For the field trial, fruit infestation was assessed for *D. suzukii* on August 2, 9 and 16, using the Washington State Department of Agriculture brown sugar method (Yee 2014). Blueberries were lightly crushed in a solution of 840 g/l brown sugar. The larvae were allowed to float and counted. Data for infestation were standardized by weight in grams.

Statistical analysis

The data for adult fly mortality were arcsine transformed to meet the assumptions of normality and homogeneity of variances; these assumptions were tested using the Komologorv–Smirnov test and the Levene's test, respectively. A repeated measures analysis of variance (ANOVA) tested whether the treatments and the time since exposure significantly affected adult mortality. Tukey's honest significant difference (HSD) test was used for post hoc comparisons. Data on the infestation of berries were treated similarly to test for normality and homogeneity of variances. We used one-way ANOVA to test for significant effects of treatments ($P < 0.05$), and Tukey's HSD test was used for post hoc comparisons among the mean values. Berry infestation data were log (x + 1) transformed to meet the assumptions of normality and homogeneity of variances, followed by one-way ANOVA to test for significant effects of treatments ($P < 0.05$) and Tukey's HSD test for post hoc comparisons among means. Infestation data from the field trial were square root (x + 0.5) transformed and tested for normality and homogeneity of variances, followed by one-way ANOVA to test for significant effects of treatments ($P < 0.05$), and we used Tukey's HSD test for post hoc comparisons among means. All analyses were performed using R 3.3.2 (R Core Team 2016). All ANOVA were conducted using the 'car' package in R (Healy 2005), and for multiple post hoc comparisons, we used Tukey's HSD pairwise comparison test with the 'multcomp' package (Hothorn et al. 2008). Data transformations were used on data prior to analysis; however, untransformed mean and standard error values are reported in figure and tables.

Results

Laboratory bioassays

Topical and residual adult mortality

Mortality of *D. suzukii* differed greatly among the treatments after 72 h, when the flies were exposed using topical ($F_{5, 126} = 301.9$, $P < 2.0 \times 10^{-16}$), residual ($F_{5, 126} = 1308.3$, $P < 2.0 \times 10^{-16}$) and topical and residual ($F_{5, 126} = 113.3$, $P < 2.0 \times 10^{-16}$) forms of exposure (Fig. 1). The duration after exposure to treatments also had a significant effect on the level of mortality observed for the residual ($F_{2, 126} = 19.6$, $P < 2.0 \times 10^{-16}$) and topical and residual ($F_{2, 126} = 2.4$,

$P < 2.0 \times 10^{-16}$) treatment but not for the topical treatment ($F_{2, 126} = 0.4$, $P = 0.6386$). There was no interaction between treatment and the duration, for any exposure methods. The highest overall mortality of *D. suzukii* adults was from the zeta-cypermethrin, the commercial standard, with 100% mortality for all three forms of exposure. In treatments that included Hv1a, the highest mortality observed was 68.7% in adults exposed topically and to the residues of Hv1a in combination with Silwet L-77 (Fig. 1). This was significantly greater mortality ($P < 0.05$) than the control or other treatments including, Hv1a without adjuvants. Other adjuvants tested did not show as great an increase in efficacy as the Silwet L-77. Addition of WaterGuard and MSO provided maximum mortality of only 25.0 and 18.8%, respectively, in flies treated through both exposure routes. The data also indicated some residual activity associated with Hv1a alone, with 17.5% mortality after 72 h, which was significantly higher ($P < 0.05$) than the control (Fig. 1).

Assessment of adjuvants on toxicity

Topical applications of Hv1a in combination with Silwet L-77 resulted in significantly higher ($P < 0.05$, Tukey's HSD) mortality compared with other treatments containing Hv1a (Fig. 1). The additional bioassays examining the effect of the inclusion of an adjuvant with Hv1a at 5 ppt similarly resulted in significantly higher mortality at 24 ($F_{13, 70} = 42.71$, $P < 2.0 \times 10^{-16}$), 48 ($F_{13, 70} = 40.94$, $P < 2.0 \times 10^{-16}$) and 72 ($F_{13, 70} = 46.08$, $P < 2.0 \times 10^{-16}$) hours after treatment (Table 2). The addition of some adjuvants resulted in significantly higher mortality, especially Silwet L-77, LI-700 and LeafLife Widespread, all of which resulted in greater than 95% mortality when combined with Hv1a (Table 2). However, some of the mortality could be attributed to the adjuvants themselves, especially in the case of LI-700, which caused 80% mortality 72 h after topical application to *D. suzukii* (Table 2). In the case of Silwet L-77 and LeafLife Widespread, the mortality observed from the adjuvant only treatment was significantly lower than that observed in LI-700 after 72 h, 47 and 16%, respectively.

The inclusion of adjuvants also affected the concentration that provided 50 and 90% mortality (LC_{50} and LC_{90}) of the treated flies. In the absence of an adjuvant, the highest mortality from topically applied Hv1a was 10.8% at the 5 ppt concentration (Fig. 2). Treatments where adjuvants were added caused greater than 50% mortality at lower concentrations of 1 ppt or less. Ninety percent mortality was observed between 1 and 2 ppt for flies exposed to Hv1a in combination with Silwet L-77 and LI-700, and 4 ppt in flies treated with LeafLife Widespread.

Ingestion bioassays

There was no significant effect of the inclusion of sucrose or *B. thuringiensis* on mortality of *D. suzukii* after 24 h of exposure to treated blueberries. However, after 48 ($F_{5, 42} = 4.44$, $P = 2.40 \times 10^{-4}$) and 72 ($F_{5, 42} = 10.44$, $P = 2.40 \times 10^{-4}$) h, adults exposed to blueberries had significantly higher mortality in the Hv1a + sucrose, and the Hv1a + sucrose + Aquabac treatments, in which mortality was 17.5 and 12.5%, respectively, at 72 h after treatment (Table 3). These treatments had significantly higher mortality ($P < 0.05$) than the

Table 2 Effect of adjuvants on the efficacy of the Hv1a on the mean (± S.E.) percent mortality of adult *D. suzukii* in topical application bioassays

Treatment	24 h	48 h	72 h
Distilled water (D.I.)	0.0 ± 0.00c	3.33 ± 2.10c	3.33 ± 2.1d
VST-006340-LC	0.0 ± 0.00c	10.0 ± 4.47c	16.0 ± 5.09cd
D.I. + WaterGuard	0.0 ± 0.00c	10.0 ± 0.00c	10.0 ± 0.00cd
Hv1a + WaterGuard	20.0 ± 7.3c	36.6 ± 12.92c	46.6 ± 11.73c
D.I. + Silwet L-77	25.0 ± 5.62c	40.0 ± 6.32c	40.0 ± 6.32cd
Hv1a + Silwet L-77	98.3 ± 1.66a	98.3 ± 1.66ab	100.0 ± 0.00a
D.I. + Vintre	1.6 ± 1.60c	11.6 ± 4.01c	11.6 ± 4.01cd
Hv1a + Vintre	15.0 ± 3.41c	31.6 ± 4.77c	43.3 ± 6.60cd
D.I. + LI-700	61.6 ± 16.80b	80.0 ± 12.30b	80.0 ± 12.30b
Hv1a + LI700	98.3 ± 1.66a	100.0 ± 0.00a	100.0 ± 0.00a
D.I. + Load Up	0.0 ± 0.00c	3.3 ± 3.33c	3.3 ± 3.33d
Hv1a + Load Up	26.6 ± 6.14c	31.6 ± 7.03c	48.3 ± 7.92c
D.I. + Leaf Life	15.0 ± 7.63c	16.6 ± 7.14c	16.6 ± 7.14cd
Hv1a + Leaf Life	91.6 ± 4.77a	91.6 ± 4.77ab	96.6 ± 3.33ab
Statstic	$F_{13, 70} = 42.71$, $P < 2.0 \times 10^{-16}$	$F_{13, 70} = 40.94$, $P < 2.0 \times 10^{-16}$	$F_{13, 70} = 46.08$, $P < 2.0 \times 10^{-16}$

Means comparisons were made among treatments. Values in a column followed by the same letter are not significantly different ($P > 0.05$, ANOVA, Tukey's HSD test)

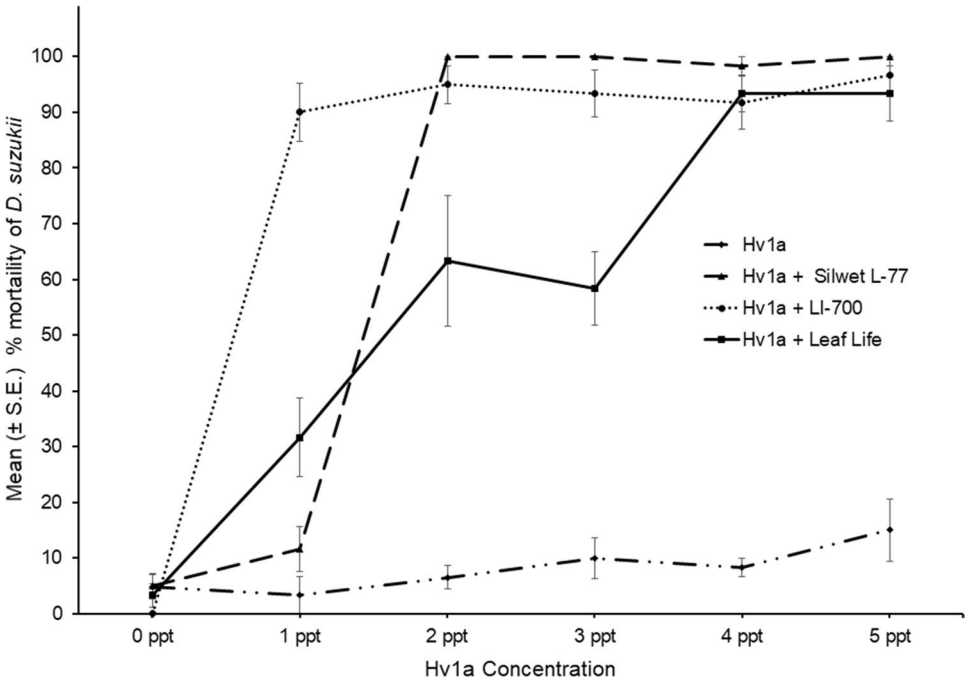

Fig. 2 Effect of adjuvants and different concentrations of Hv1a on the mean (±S.E.) % mortality of adult *D. suzukii* after 24 h of exposure in topical application bioassays

Table 3 Effect of the inclusion of sucrose and *Bacillus thuringiensis* (Aquabac) with Hv1a on the on the mean (±S.E.) percent mortality and mean number of progeny of adult *D. suzukii* in ingestion bioassays

Treatment	% Mortality of adults			Mean number of progeny
	24 h	48 h	72 h	
Distilled water	0.0 ± 0.00	1.3 ± 1.25a	1.3 ± 1.25a	10.1 ± 1.38b
Distilled water + Sucrose	0.0 ± 0.00	1.3 ± 1.25a	1.3 ± 1.25a	13.2 ± 1.34b
VST-006340-LC + Sucrose	0.0 ± 0.00	8.8 ± 2.95b	17.5 ± 3.13b	16.3 ± 1.23b
VST-006340-LC + Sucrose + Aquabac + Silwett L-77	0.0 ± 0.00	3.8 ± 1.82a	12.5 ± 3.65b	14.8 ± 1.85b
VST-006340-LC + Sucrose + Aquabac	0.0 ± 0.00	0.0 ± 0.00a	3.8 ± 1.82a	16.6 ± 2.13a
Sucrose + Aquabac	0.0 ± 0.00	0.0 ± 0.00a	0.0 ± 0.00a	15.3 ± 0.86b
Statistics	ǂ	$F_{5,\ 42} = 4.448$ $P < 0.01$	$F_{5,\ 42} = 10.447$ $P < 0.001$	$F_{5,\ 42} = 10.447$ $P < 0.001$

Total values in a column followed by the same letter are not significantly different ($P > 0.05$, ANOVA, Tukey's HSD test)

ǂ No statistical comparison was performed as no mortality was recorded in any treatment

controls and other treatments and were at a similar level to those observed in the assessment of the residual effects (Fig. 1). This suggests that the inclusion of phagostimulants to increase ingestion will lead to only limited increases in mortality caused by Hv1a. Infestation by *D. suzukii* in berries treated with Hv1a alone was not significantly lower than the controls (Table 3), and none of the treatments reduced infestation compared to the distilled water control.

Effects on immature life stages

The number of live *D. suzukii* larvae in blueberries was significantly reduced by treatments applied using both the fruit dip ($F_{6,63} = 31.92$, $P < 2.0 \times 10^{-16}$) and topical application treatments ($F_{6,63} = 9.59$, $P = 1.7 \times 10^{-7}$). The lowest number of surviving larvae observed was in the commercial standard, with no larvae developing from the eggs in berries treated with zeta-cypermethrin. Fruit treated with Hv1a alone had an intermediate number of surviving larvae (Fig. 3). The inclusion of the adjuvants Silwet L-77 and LI-700 with Hv1a resulted in significantly lower ($P < 0.05$, Turkey's HSD) numbers of surviving larvae emerging from fruit dipped in the spray solution when compared to the distilled water control, leading to a 56 and 64% reduction, respectively. However, these treatments were not significantly lower than the H1va alone treatment, despite a

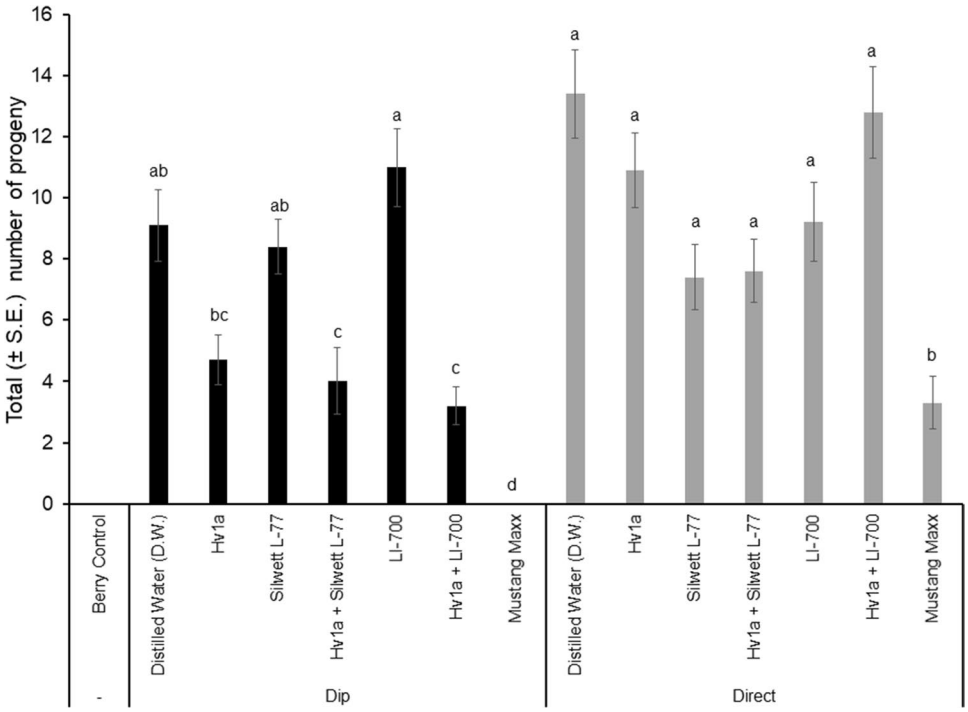

Fig. 3 Effect of Hv1a, with and without adjuvants, and zeta-cypermethrin on the mean (± S.E.) number of *D. suzukii* larvae in blueberries. The fruit was treated when the egg and early larva life stages of *D. suzukii* were present. Means comparisons were made among treatments within the same colored bars, and bars headed with the same letter within an exposure method are not significantly different ($P > 0.05$, ANOVA, Tukey's HSD test)

numerically lower surviving number of larvae emerging from dipped fruit. This trend was not as clear for infested fruit treated topically with spray solutions, where only fruit treated with zeta-cypermethrin showed a significant reduction in the number of surviving larvae.

Field trial

There was no significant difference ($F_{4,15} = 0.95$, $P = 0.46$) between treatments in larval infestation in the first assessment of the trial; however, treatments differed significantly in the second ($F_{4,15} = 22.89$, $P = 3.06 \times 10^{-6}$) and third ($F_{4,15} = 38.18$, $P = 1.07 \times 10^{-7}$) assessments. In the second assessment, all treatments significantly ($P < 0.05$) reduced the larval infestation in treated fruit when compared to the untreated control, with no significant difference between treatments (Fig. 4). In the third assessment of the trial, again all treatments significantly ($P < 0.05$) reduced the larval infestation in treated fruit when compared to the untreated control. The lowest infestation was detected in fruit treated with Hv1a and phosmet + Tri-Fol, in the case of phosmet + Tri-Fol, this was significantly ($P < 0.05$) lower than all other treatments with the exception of Hv1a (Fig. 4).

Discussion

There is an urgent need for new insecticide classes to help control invasive pests that have proven challenging to manage using existing integrated pest management programs (Haye et al. 2016). This includes *D. suzukii,* which has caused hundreds of millions of dollars of lost revenue to berry and cherry growers in recent years (Farnsworth et al. 2017; Goodhue et al. 2011). This study indicates that Hv1a is an effective new biopesticide with potential to control this pest, while also providing a new mode of action (Nakasu et al. 2014). It is also the first study to demonstrate insecticidal activity of a spider venom peptide against an insect via topical activity, which was previously considered unlikely (King and Hardy 2013). However, the results indicate the need for a penetrating adjuvant when using Hv1a to achieve the potential of this active ingredient. This is probably because the adjuvants assist in transportation of the active ingredient into the hemolymph of *D. suzukii* and we expect a similar synergism against other insects. Interestingly, the different adjuvants tested had varying effects on the performance of Hv1a, with the greatest benefit seen from LI-700, with Silwet L-77, Load Up and Leaf Life. These adjuvants may aid in Hv1a transportation into the hemolymph of insects through paracellular transport as suggested by Audsley et al. (2007) and Herzig et al. (2014). There was also some inherent toxicity of the adjuvants tested in this study to *D. suzukii*, particularly from the organosilicates. The mortality caused by adjuvants was the highest after 72 h in LI-700 (80%), Silwet L-77 (40%) and Leaf Life (17%) treatments. In assessing the effects of including an adjuvant with Hv1a, the result for mortality 24 h after topical treatment highlights the greatest synergism particularly in the Hv1a + Silwet L-77 and Hv1a + Leaf Life treatments, where there was an increase in mortality of 73 and 77%,

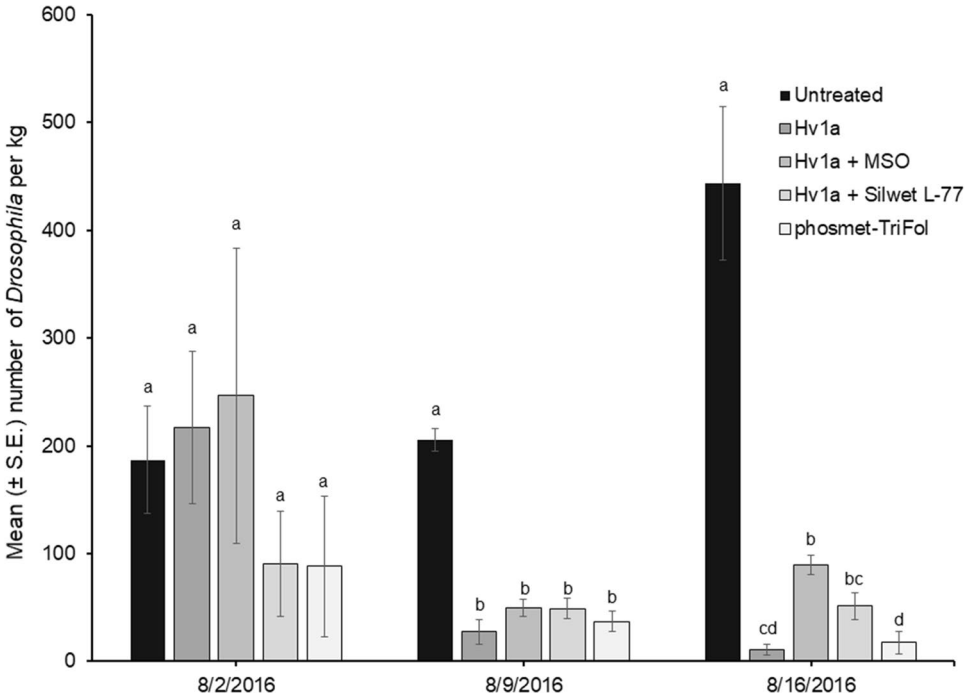

Fig. 4 Field performance of Hv1a, with and without adjuvants, and phosmet on the mean (± S.E.) number of *D. suzukii* larvae in blueberries. Means comparisons were made among treatments within each date, bars headed with the same letter within an exposure method are not significantly different ($P > 0.05$, ANOVA, Tukey's HSD test)

respectively, above the associated adjuvant controls. The synergism was not as evident in the Hv1a + LI-700 treatment, where there was only 37% higher mortality above the adjuvant control, resulting from the high mortality in the adjuvant control (Table 2). These observations are consistent with recent research that examined the non-target effect of organosilicates and decreased survival of honeybees (Chen et al. 2018).

By contrast, a combination of Hv1a with either *Bacillus thuringiensis*, that was expected to damage the gut lining or sucrose which was expected to increase ingestion, showed no evidence of greater mortality when either was combined with Hv1a. While adults of *D. suzukii* were supplied with a small portion of diet was provided to reduce control mortality and acted as an alternative food source, this was standard across all treatment. The failure of the *B. thuringiensis* to increase the uptake in these experiments could be due to its specificity that can require insect-specific binding sites (Zhang et al. 2005). The *B. thuringiensis* var. *israelensis* used in this experiment is designed to control mosquito larvae in water. Previous research has shown that insecticidal spider venom peptides have low toxicity when ingested due to slow rates of absorption through the midgut, as previously seen in similar disulfide-rich peptides from scorpion and snake venoms (Casartelli et al. 2005). The infusion of Hv1a to a protein that is capable of moving across the insect gut epithelium by transcytosis might lead to increased activity of the peptide against *D. suzukii*. The infusion of insecticidal peptides and protein to enhance absorption through insect guts has previously been reviewed by Bonning and Chougule (2014). The infusion of insecticidal spider venom peptides to a plant lectin agglutinin, derived from the snowdrop *Galanthus nivalis* L., has previously been shown to increase the oral uptake and activity of both Hv1a and the insecticidal spider venom peptides U2-SGTX-Sf1a (SFI1), with a dramatic improvement in efficacy by fusion to the N-terminus of *Galanthus nivalis* agglutinin. Second instar cabbage moths, *Mamestra brassicae* L., fed on cabbage leaves coated with 0.2% Hv1a–GNA fusion protein suffered 85% mortality after 10 days, compared to less than 20% on untreated leaves.

In addition to the control of adult flies, our bioassays with infested blueberries indicate that Hv1a has some toxicity on the immature stages of this pest in the fruit post-infestation, also termed curative activity (Wise and Whalon 2009). This activity was aided by the addition of particular adjuvants. Curative activity has previously been shown for a number of conventional insecticides for immature stages of *D. suzukii* (Wise et al. 2015), and this can provide the combined action of targeting adult and immature stages, with greater effects on their populations. Another aspect of Hv1a's activity not explored in this study is the potential for embryonic lethality due to its activity on the Ca_V channels of flies. Mutations to the Ca_V channels in *Drosophila melanogaster* Meigen led to death in the late embryo stage because they lack vigorous hatching movements to enable eclosion to adults (Eberl et al. 1998). The curative activity observed in our laboratory bioassays could also explain the efficacy of the Hv1a treatments observed in the field trial. While the Hv1a alone did not have the lowest number of larvae surviving in treatments, it was also not significantly different from the other treatment that

included Hv1a. In the field trial, the Hv1a provided comparable control of *D. suzukii* to phosmet, which is a broad-spectrum organophosphate insecticide. In the field, trial by the third assessment (8/16/16), the Hv1a only treatment had numerically the lowest infestation was significantly lower than the untreated control and Hv1a + MSO treatments. These results suggest that the inclusion of an adjuvant with Hv1a might not be as necessary as suggested by the laboratory bioassays.

Development of resistance to groups of insecticides with the same mode of action also represents a significant threat to their long-term use for pest control. The major classes of chemical insecticides act on only six molecular targets in the insect nervous system (Graf, 1993; Tedford et al. 2004; Lai and Su 2011; Smith et al. 2013). There are currently 232 cases of resistance in species of arthropods to one or more pesticides (Mota-Sanchez and Wise 2017), and so the search for insecticides with new modes of action is critically important for future effective pest control and food security (Smith et al. 2013; Tedford et al. 2004). Spider venom peptides offer multiple modes of action and have a number of unique properties including high potency when delivered into the hemocoel, rapid speed of kill, lack of vertebrate toxicity, relatively low production costs and activity against a wide range of crop pests and disease vectors (King and Hardy 2013). Peptides like Hv1a tested here have potential in providing control options against arthropod pests that have already developed resistance to one or more classes of insecticide. Even if the molecular target of the peptide is the same as the insecticide that resistance has been observed, toxins in these peptides act at sites different from those targeted by chemical insecticides (King and Hardy 2013). An example of this was demonstrated by McCutchen et al. (1997) who showed that even though the scorpion toxin AaIT and pyrethroids both target Na_V channels; a pyrethroid-resistant strain of the tobacco budworm *Heliothis virescens* (Fabricius) was more susceptible than nonresistant strains to a recombinant baculovirus expressing AaIT. Additionally, the transgenes encoding these peptides could be used to engineer insect-resistant plants (Cao et al. 2010; Jiang et al. 1995; Khan et al. 2006) and to enhance entomopathogens (Wang and St Leger 2007). One disadvantage of these peptides has been their apparent lack of contact activity, but as this study demonstrates for *D. suzukii*, this be solved through the addition of an adjuvant prior to application, whether in the formulation or as a tank mix. The combination of Hv1a and synergistic spray adjuvants for controlling arthropod pests in other crops is understudied, but there is great potential for further development of this class of biopesticides if the need for their movement into their insect target can be resolved.

Further research into the potential for control of *D. suzukii* should include studies on how spray adjuvants facilitate improved toxicity. Understanding whether this is through increased physical transport of the peptide through the cuticle, changes to the pH or other chemical characteristics of the spray solution or some combination will help further development of this class of biopesticides. In the laboratory and field, greater testing should be focused on the lethal and sublethal effects to beneficial insects (Desneux et al. 2007; Biondi et al. 2013; Guedes et al. 2016), including the *Drosophila* parasitoids native to newly invaded areas (Mazzetto et al. 2016; Wang et al. 2016) and natural enemies of *D. suzukii* from its native range (Daane et al. 2016; Rossi Stacconi et al. 2018). In addition to the non-target effects on beneficial insects and parasitoids of *D. suzukii*, exploitation of the effect of pollinators need, to be conducted. While Hv1a has previously been screened for its effects on the European honeybee *Apis mellifera* L., with both acute and chronic exposure and a combination of injection, and by oral and contact bioassays, using adult forager honeybees (Nakasu et al. 2014). In these experiments, survival was only slightly affected by ingestion or injection of Hv1a what had been fused with a protein GNA (*Galanthus nivalis* agglutinin). Bees fed acute (100 mg bee^{-1}) or chronic (0.35 mg ml^{-1}) doses of Hv1a/GNA and trained in an olfactory learning task had similar rates of learning and memory to no-pesticide controls. Larvae were unaffected, being able to degrade Hv1a/GNA. The response of native pollinators has yet to be determined. Additional peptides, with novel modes of action, derived from spider venoms should also be tested in combinations with commercially available spray adjuvants to determine whether adjuvants can similarly increase the insecticidal activity of these peptides.

Author contributions

PF and RI designed and conducted the laboratory bioassays and JW, RI and AVW designed and conducted the field trials. PF did the statistical analysis and wrote the manuscript. All authors contributed to the critical evaluation of the manuscript, read and approved it.

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Compliance with ethical standards

Human and animal rights statement All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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