California Cherry Research Review

January 10, 2018

Evelyn Costa Assembly Room
San Joaquin County – Office of the Agricultural Commissioner
2101 E. Earhart Avenue, #100, Stockton, California 95206

2017 Research Season Final Reports

CALIFORNIA CHERRY RESEARCH REVIEW
January 10, 2018

Evelyn Costa Assembly Room
San Joaquin County – Office of the Agricultural Commissioner
2101 E. Earhart Avenue, #100, Stockton, California 95206
Sponsored by the University of California and California Cherry Board

9:00	**Welcome** Nick Matteis, California Cherry Board
9:05	**Management and epidemiology of pre- and postharvest diseases of sweet cherry** Dr. Jim Adaskaveg, Dept. of Plant Pathology, UC Riverside
9:25	**Investigating the cause of sudden decline of sweet cherry in California** Dr. Florent Trouillas, Dept. of Plant Pathology, UC Davis, Kearney Agricultural Research & Extension Center, Parlier, CA
9:45	**Improved management of fungal canker diseases of sweet cherry** Dr. Florent Trouillas, Dept. of Plant Pathology, UC Davis, Kearney Agricultural Research & Extension Center, Parlier, CA
10:05	**Investigating biological controls to suppress spotted wing drosophila populations** Dr. Kent Daane, Dept. of ESPM, UC Berkeley and UC Kearney Agricultural Research & Extension Center, Parlier, CA
10:25	**Break**
10:45	**Engineered transgenic *Drosophila suzukii* for wild population suppression & eradication: Production, performance assessment and effective wild releases** Dr. Bruce Hay, Div. of Biology & Biological Engineering, Caltech
11:05	**Electronic sensors to capture spatiotemporal population density of SWD** Dr. Joanna Chiu, Dept. of Entomology, UC Davis
11:25	**Seasonal dynamics of non-structural carbohydrates (NSCs) in cherries** Dr. Anna Davidson, Dept. of Plant Sciences, UC Davis
11:45	**Existing approaches, challenges and possibilities for mechanical tree fruit and sweet cherry harvesting** Dr. Stavros Vougioukas, Dept. of Biological & Agricultural Engineering, UC Davis
12:05	**Lunch** (Courtesy of California Cherry Board)
1:30	**Adjourn**

CALIFORNIA CHERRY BOARD

2017-2018 RESEARCH COMMITTEE

MEMBER	PRIMARY REPRESENTATION AREA	INDUSTRY ACTIVITIES
NICK MATTEIS **(Staff)**	ALL	CCB (RESEARCH STAFF)
TYLER ROOD **(Staff)**	ALL	CCB (RESEARCH CO-OR)
RICH HANDEL (**Chair Emeritus)**	ALL	PACKER, GROWER, SHIPPER
JOE GRANT **(UCE, Advisor Emeritus)**	SAN JOAQUIN CO.	CCB (RESEARCH CO-OR)
ARNIE TOSO	SAN JOAQUIN CO.	CCB, GROWER
ANDREW DASSO, JR.	ALL	CCB, PACKER, GROWER, SHIPPER
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DONALD DRAKE	ALL	PACKER, GROWER, SHIPPER
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SCOTT BROWN **(Chair)**	SAN JOAQUIN CO.	CCB, GROWER, PACKER, SHIPPER

CALIFORNIA CHERRY BOARD -- Research Committee

2017-18 Proposal Research Projects Aprroved March 16, 2017

	Pre/ Post	Pathogen I.D.	Description	Status	Comments	Project Leader	Institution	Approved Funding
1	Pre	Sp. W. Drosophila	SWD attractants and yeast relationships	On-going	Development of lures, traps	Syed	Notre Dame	$ 31,000.00
2	Both	Diseases	Canker & Decay controls	On-going	Continuing annual work; Kasumin	Adaskaveg	UC Riverside	$ 51,000.00
3	Pre	Pathogen I.D.	Identifying decline casual agents	New	Fungal Pathogens	Trouillas	UC Cooperative Extension Kearney	$ 21,739.00
6	Pre	Sp. W. Drosophila	Population supression & eradication	On-going	Transgenic, *Medusa* allele	Akbari/Hay	Caltech/UC Riverside	$ 110,000.00
7	Pre	Sp. W. Drosophila	Population control	On-going	SWD natural parasites	Daane	UC Berkeley	$ 15,278.00
9	Pre	Sp. W. Drosophila	Oviposition deterrents	Ongoing	Evaluation of oviposition deterrent compounds	Rijal	UC Stanislaus	$ -
10	Both	Sp. W. Drosophila	Regulatory approval	New	A solution to regulatory approval of RNAi SWD for population control/erradication	Turpen	Technology Innovation Group	$ 15,500.00
								$ 244,517.00

CALIFORNIA CHERRY BOARD
2017 FINAL RESEARCH REPORTS

Annual Report - 2017

Prepared for the California Cherry Advisory Board

Project Title:	Management and Epidemiology of Pre- and Postharvest Foliar and Fruit Diseases of Sweet Cherry
Project Leader:	Dr. James E. Adaskaveg, Department of Plant Pathology, University of California, Riverside, CA 92521 (951) 827-7577
Cooperators:	Dr. H. Förster, D. Thompson, and L. Wade

SUMMARY

Research areas included evaluation of new blossom, preharvest, and postharvest treatments for the management of major foliar and fruit diseases of sweet cherry in California. We continued our efforts in managing bacterial canker, powdery mildew, blossom blights and fruit rots caused by *Monilinia* and *Botrytis* spp., as well as postharvest decays including brown rot, gray mold, Rhizopus rot, and Alternaria/Fusicladium (Cladosporium) rots.

1) Studies on bacterial canker caused by *Pseudomonas syringae* pv. *syringae*:
   a. In 2017 inoculation studies, kasugamycin was compared to the new bactericides zinc thiadiazole (ZTD) and DAS-1 (a copper activity-enhancing compound). Branches on cv. Coral Champagne were inoculated and treated with selected bactericides in the dormant period and evaluated for canker development in early spring. Canker size was significantly reduced by Kasumin and ZTD but not by copper DAS-1 or copper-ZTD treatments as compared to the control.
   b. Kasumin (active ingredient kasugamycin) is set for full registration on cherry in January 2018 based on the US EPA PRIA date. Oxytetracycline has been submitted to the EPA through the IR-4 program for registration on cherry with support from the registrants, the California Cherry Board, and other researchers in the North Central and Northeastern regions of the United States.
2) In powdery mildew studies, the disease developed at high incidence on leaves of water sprouts, but was low on new shoots on terminal branches. The incidence of fruit infections was low in 2017.
   a. In a trial in San Joaquin Co., the most effective treatments included SDHI (FG 7)-containing fungicides including Fontelis, pyraziflumid, Luna Sensation, and Merivon, selected DMI (FG 3)-containing fungicides such as Rhyme and Procure, as well as the experimental fungicides UC-2, EXP-AD, and -AF.
   b. Quintec continued to show reduced performance and was only effective in mixture with other FRAC Groups such as FG 7 (i.e., Fontelis). The natural product Terraneem was not effective in these studies.
3) For brown rot blossom blight, Rhyme and EXP-AF were highly effective as pre-infection treatments in laboratory studies. The biological treatments Botector, Serenade Opti, and two MB compounds also significantly reduced the disease from the control, but were less effective than the two fungicides. For gray mold blossom blight, none of the evaluated compounds was very effective, but Botector, Serenade Opti, and EXP-AF showed some reduction in disease.
4) Two field studies were conducted on the efficacy of preharvest fungicide treatments.
   a. Brown rot: In applications at 4- or 7-days PHI, Indar, Quash, Procure, the pre-mixture Quadris Top, and the experimental compounds UC-1, UC-2, UC-AD, and IL-5412 provided excellent protection in wound-inoculations of non-washed fruit. Indar, Luna Experience, UC-1, UC-2, EXP-AD, and IL-5412 were also highly effective on washed fruit. All of these treatments are based on FRAC Group 3 – DMI fungicides. When harvested fruit were non-wound drop-inoculated, most treatments including FG 7, FG 17, FG 19 compounds, and several pre-mixtures were highly effective on non-washed and washed fruit.

b. Gray mold: Ph-D, Elevate, Fontelis, pyraziflumid, pydiflumetofen, and several pre-mixtures were highly effective in non-wound inoculations of non-washed fruit. On washed fruit, the pre-mixtures Luna Experience, Merivon, EXP-AF, and EXP-AD numerically had the lowest incidence of gray mold. Thus, these studies identified new effective gray mold treatments.

5) Three commercial packingline studies with flooder or T-Jet applications were done for the evaluation of postharvest fungicides with emphasis on the recently registered bio-fungicide BioSpectra (natamycin), Chairman (a pre-mixture of fludioxonil and propiconazole), and Scholar (or other formulations of fludioxonil).

a. BioSpectra was mostly highly effective using both application methods against the major decays brown rot, gray mold, and Rhizopus rot when fruit were wound-inoculated before treatment, and brown rot was reduced to zero levels in most cases. This is in agreement with our previous studies. BioSpectra was also very effective in reducing the natural incidence of gray mold and Rhizopus rot and flooder applications were generally more effective. Efficacy against Alternaria/Fusicladium rots was reduced. Gray mold was generally not well controlled, when fruit were spray-inoculated after treatment. With increasing emphasis on food safety and consumer concerns, natamycin with 'exempt from tolerance status' will likely become an important component of postharvest decay management in the future. It will be best used in mixture with low rates of Scholar.

b. Scholar continued to perform very well against the major decays on inoculated fruit and natural incidence of gray mold and Rhizopus rot.

c. Chairman was highly effective against the three major decays after wound-inoculation, and reduced natural incidence of gray mold and Rhizopus rot to zero levels.

INTRODUCTION

Management of bacterial blast and canker. The main bacterial pathogen that causes blossom blast and cankers of woody tissue of sweet cherry and other stone fruit crops is *Pseudomonas syringae* pv. *syringae*, but other pathovars have been also associated with the disease namely *Pseudomonas syringae* pv. *morsprunorum*. Blossom blast develops after cold injury, and with subsequent infection, blossoms become dark to black in color, wilt, and die. The disease is more commonly found on early-blooming varieties or trees treated with rest-breaking treatments that experience cooler, wet environments in the spring. The disease can also occur on leaf and flower buds where it causes bud death; and on leaves and fruit where it causes spots and specks.

Based on our efforts, advances have been made in bacterial disease control with the identification and development of kasugamycin (commercial name: Kasumin) for fire blight management on pome fruit and other bacterial diseases of agronomic crops in the United States and elsewhere. This antibiotic is not used in animal or human medicine and the US-EPA registration for pome fruit was granted in 2014. Registration of Kasumin on sweet cherry in California is pending CDPR approval. In our studies using Kasumin for managing bacterial blast of sweet cherry, the disease was reduced in inoculation studies. The natural incidence of disease was also significantly reduced after commercial applications with Kasumin. Furthermore, using an increased rate of 200 ppm, kasugamycin was the only compound that consistently reduced the severity of bacterial canker of inoculated branches.

Our screening of compounds led to the identification of several other materials that look quite promising. We are also pursuing registration of oxytetracycline on cherry in California as dormant and bloom treatments. Oxytetracycline (Fireline, Mycoshield) was successfully accepted into the IR-4 program in Sept 2013 for residue trials on bacterial blast of cherry. Other compounds included the biocontrol Actinovate (fermentation product of *Streptomyces lydicus*) and Blossom Protect/Botector (*Aureobasidium pullulans*) that inconsistently reduced both the blossom and canker phase of the disease. In 2017, we focused on the evaluation of potentially copper activity-enhancing compounds in comparison to Kasumin for the management of bacterial canker of inoculated sweet cherry branches.

Management of powdery mildew, blossom blight, and fruit rot. Powdery mildew of sweet cherry is an ongoing problem for growers in California especially in southern production areas. Leaves and fruit may be infected. In some export markets, powdery mildew is a quarantine disease and fruit for shipment may have to be certified as disease-free. With decreased powdery mildew sensitivity to Quintec, new, highly effective materials are being evaluated. Alternative fungicides that we evaluated over several years in our field trials on sweet cherry in California include the FG 3 (DMI) Procure (triflumizole), the FG 7 (SDHI) fungicides (e.g., fluopyram, fluxapyroxad, and penthiopyrad), and the pre-mixtures Luna Sensation (fluopyram/trifloxystrobin), Merivon (fluxapyroxad/pyraclostrobin) (FG 7/11), and Quadris Top (azoxystrobin/difenoconazole) (FG 3/11). Still, other new powdery mildew fungicides such as pyraziflumid, UC-2, and Syngenta's new pydiflumetofen and EXP-AD and -AF are being developed, and we are seeking their registration on cherry in California. This will allow alternatives to be used during bloom, petal fall, and preharvest.

For management of brown rot blossom blight and fruit rot of sweet cherry caused by *Monilinia fructicola* and *M. laxa* and Botrytis blossom blight and fruit rot caused by *Botrytis cinerea*, compounds of different modes of action (QoIs, DMIs, anilinopyrimidines, phenylpyrroles, hydroxyanilides, and SDHIs) have been evaluated by us over the years and were found to be effective. The pre-mixtures Quadris Top, Pristine, Merivon, Luna Experience, and Luna Sensation represent some of the top treatments along with tank mixtures of FG 7 and FG 3 fungicides. Still, more new fungicides are being developed. They generally belong to the same FRAC groups as previously registered compounds, but their activity against fungal pathogens is often different due to their different affinity to fungal target sites. Thus, some of the newer fungicides such as pydiflumetofen and UC-1 have extremely high in vitro activities. Thus, we continued to evaluate the efficacy, spectrum of activity, and persistence of residues of new fungicides, as well as the integration of these materials into a comprehensive management program. Information on the preventative and post-infection activity of fungicides is helping to develop our delayed bloom fungicide application model for improved timing in low to moderate disease pressure years and for optimizing fungicide treatments. Although DMI fungicides are highly effective against brown rot, they have to be complemented with other materials to obtain a high efficacy against gray mold.

Management of postharvest fruit decay with postharvest treatments. We are also continuing our efforts to provide effective and economical treatments for management of postharvest fruit decays such as brown rot, gray mold, Rhizopus rot, as well as powdery mildew lesions from field infections. Powdery mildew on fruit is a quarantine disease with selected trade partners and moreover, powdery mildew infections can be entryways for secondary infections by other fruit pathogens. Currently, six postharvest fungicides, Tebucon (the Elite replacement- Note: Tebucon label has changed to a maximum rate of 8 oz), Mentor (propiconazole), Scholar (fludioxonil), Penbotec (pyrimethanil), and the biofungicide BioSpectra (natamycin) are registered on sweet cherry. Additionally, the pre-mixture of fludioxonil and propiconazole Chairman is now available. In 2016, Judge (fenhexamid) was withdrawn from postharvest use. Natamycin (FG 49) is the first postharvest biofungicide and is exempt from tolerance on fruit crops in the United States. Penbotec is effective against brown rot and gray mold, whereas Scholar and BioSpectra are also active against Rhizopus rot. The DMI propiconazole (Mentor) is mainly effective against brown rot, but also against sour rot, a less common decay on sweet cherry. Chairman has the broadest spectrum of activity with controlling four decays. Of the four classes or FRAC groups (e.g., 3, 9, 12, and 49) registered, Tebucon and Mentor (FG 3) are not 'reduced-risk' fungicides. Scholar in 2011 and Penbotec in 2013 received Food Additive Tolerances in Japan, and the registrants of Mentor and BioSpectra have applied for FATs in Japan. Thus, continued studies on how to use these fungicides most efficiently for the Japanese export market are critical to the industry.

Objectives

1. Evaluate new products against bacterial blast in flower inoculation studies and against canker in twig inoculation studies. (Cooperate with C. Ingels).
   a. Biologicals/natural products (e.g., Actinovate, polyoxin-D, Blossom Protect).
   b. Antibiotics – kasugamycin, oxytetracycline – large-scale trials once federally registered; improve penetration into plant tissue.

c. Continue to evaluate wound susceptibility of branches and antibiotic protection over time to prevent bacterial canker. We will also look at low temperature conditions during bloom that favor blast.

2. Evaluate, under field conditions, bloom and preharvest applications of new compounds (e.g., Fontelis), premixtures (e.g., Luna Sensation, Merivon, Quadris Top), pydiflumetofen, EXP-AD, and -AF, as well as UC-1 and UC-2, pyraziflumid, IL-54111, and biologicals for control of brown rot and Botrytis blossom blight, powdery mildew, and pre- and postharvest brown rot and gray mold fruit decay.
   a. Evaluate new powdery mildew fungicides polyoxin-D, pydiflumetofen, SDHI compounds (fluopyram, fluxapyroxad, penthiopyrad), and premixtures (e.g. EXP-AD, -AF) using different rates and timings and develop a powdery mildew fungicide program that integrates new materials with single- and multi-site mildew fungicides.
      - Develop base-line data for pydiflumetofen and other new chemicals.
   b. Evaluate new brown rot and gray mold materials including new DMIs, SDHIs (fluopyram, fluxapyroxad, penthiopyrad), pre-mixtures, polyoxin-D (Ph-D, Oso), and pydiflumetofen.

3. Evaluate new fungicides as postharvest treatments and develop cost-effective application methods:
   a. Continue to evaluate Scholar, Mentor, as well as Scholar-Mentor, and Scholar mixtures with natamycin due to their approved or pending food additive tolerance (FAT) in Japan.
   b. Continue to develop EC_{50} values, baseline sensitivities, and monitor resistance in target pathogen populations to newly developed fungicides.
   c. Continue to evaluate 'exempt from tolerance' materials (BioSpectra).

MATERIALS AND METHODS

Evaluation of treatments for control of bacterial canker. In winter of 2017, the bark of 2-year-old twigs of Coral cherry trees was puncture-wounded using a 12-gauge needle (3 wounds per twig). Wounds were sprayed with bactericides to run-off using a hand sprayer, allowed to air-dry, and spray-inoculated with a copper-resistant strain of *Pseudomonas syringae* pv. *syringae* (2×10^8 cfu/ml). Treatments included ChampION^{++} by itself or mixed with the copper activity-enhancing compounds DAS1 or ZTD, ZTD by itself, and Kasumin. In May, inoculated branches were sampled and evaluated for the severity of canker formation by measuring canker length (in mm). Data were analyzed using analysis of variance and mean separation procedures of SAS 9.4.

Evaluation of new fungicides for control of powdery mildew of sweet cherry. A field trial in San Joaquin Co. was conducted to evaluate fungicides for powdery mildew control. Treatments were done on 3-16-17 for protection from primary inoculum (ascospores from overwintering chasmothecia), and were followed by two additional treatments on 4-5 and 4-26-17 (early fruit development) for protection from secondary infection from conidia. Single fungicides, pre-mixtures, and two rotation programs were evaluated (Fig. 2). The incidence of powdery mildew was evaluated on 20 leaves from four random shoots each from inside the tree or from the outer tree perimeter for each of the four single-tree replications on 6-6-17. Severity was rated using the following scale: 0 = healthy, 1 = 1-3 lesions, 2 = <25%, 3 = up to 50%, 4 = >50% of leaf area affected. Data were analyzed using analysis of variance and mean separation procedures of SAS 9.4.

Evaluation of new fungicides for control of brown rot and Botrytis blossom blight and fruit decay. Laboratory experiments were conducted to evaluate the pre-infection activity of fungicides against brown rot and gray mold blossom blight. Blossoms were collected at white bud, allowed to open in the laboratory, and treated using a hand sprayer. After 12 h, blossoms were inoculated with a spore suspension of *M. fructicola* or *B. cinerea* (30,000 conidia/ml) until water droplets formed on anther filaments. Blossoms were evaluated for stamen infection after 4-5 days of incubation at 20 C, >95% relative humidity. Disease incidence was evaluated as the number of stamens infected divided by the total number of stamens per blossom. Three replications of 8 blossoms were used for each treatment and data were analyzed using analysis of variance and mean separation procedures (SAS 9.4).

Table 1: Fungicides, bactericides, and biologicals used in 2017 studies*.

Pesticide	FRAC group	Trade name	Active ingredient
Fungicides	***Single active ingredients***		
	3	Indar	fenfuconazole
	3	Quash	metconazole
	3	Rally	myclobutanil
	3	Tebucon/Toledo	tebuconazole
	3	Rhyme	flutriafol
	3	Fontelis	penthiopyrad
	7	Kenja	isofetamid
	7	Miravis (Adepidyn)	pydiflumetofen
	7	Procure	triflumizole
	7	Pyraziflumid	pyraziflumid
	12	Scholar	fludioxonil
	13	Quintec	quinoxyfen
	17	Elevate	fenhexamid
	19	Ph-D	polyoxin-D
	49	BioSpectra	natamycin
	Experimentals	EXP-AD	not disclosed
		EXP-AF	not disclosed
		UC-1	DMI
		UC-2	not disclosed
		IL-5412 (-54111)	not disclosed
	Double (Premixtures)		
	7 + 11	Luna Sensation	fluopyram + trifloxystrobin
	7 + 3	Luna Experience	fluopyram + tebuconazole
	7 + 11	Merivon	fluxapyroxad + pyraclostrobin
	3 + 11	Quadris Top	difenoconazole + azoxystrobin
	12 + 3	Chairman	fludioxonil + propiconazole
Bactericides	M1	ChampION^{++}	copper hydroxide
	24	Kasumin	kasugamycin
	---	ZTD	zinc thiadiazole
	---	DAS1	copper activity enhancer
Biologicals	Bacterium	Serenade Opti	*Bacillus subtilis* QST713
	Plant extract	Fracture	protein from *Lupinus* sp.
	Plant extract	Terraneem	*Azadirachta indica*
	---	MBI-110AF5	not disclosed
	---	MBI-106125	not disclosed

* - Alphabetical by trade name for each Fungicide Resistance Action Committee (FRAC) group or mode of action. Some fungicides were used with adjuvants such as Breakthru or DyneAmic.

To evaluate preharvest fungicide applications for control of fruit decay, an experimental orchard was used at UC Davis and a commercial orchard was used in San Joaquin Co. Treatments were applied 4 days (UC Davis) or 6 days (commercial orchard) PHI using a back-pack sprayer calibrated to deliver 100 gal/A. Fruit (8 fruit from each of three single-tree replication) were harvested, wounded with a glass rod (1 x 1 x 0.5 mm), and inoculated with 20 µl of a conidial suspension of *M. fructicola* (30,000 conidia/ml). For gray mold, 16 fruit from each replication at UC Davis were non-wound drop-inoculated with a spore suspension (200,000 spores/ml prepared in cherry juice). In the San Joaquin trial, approximately 50 to 60 fruit from each replication were non-wound inoculated by spraying with conidia of *M. fructicola* and *B.*

cinerea (25,000 spores/ml each). All fruit were incubated for 5-7 days at 20 C, >95% RH. Percent incidence of infection was determined as the number of fruit infected of the total number of fruit evaluated. Data were analyzed as described above.

To evaluate preharvest fruit treatments for postharvest decay management and the persistence of the fungicides on the fruit that were treated in San Joaquin orchard, fruit were washed by spraying with high-volumes of water for 3 minutes prior to wound- and non-wound inoculations with *M. fructicola* or *B. cinerea* as described above.

Efficacy of new and registered postharvest treatments for managing brown rot, gray mold, Rhizopus rot, and Alternaria/Fusicladium fruit rots of sweet cherry. Three commercial packingline studies and one laboratory study were done with emphasis on the recently registered bio-fungicide BioSpectra (natamycin), Chairman (a pre-mixture of fludioxonil and propiconazole), and Scholar (or other formulations of fludioxonil). In the commercial packingline studies applications were done by flooder or by two sequential T-Jets that are separated by a step on the belt so that fruit slightly tumbled and turned. These treatments were sometimes compared to dip applications. Treatment solutions for the flooder applications contained citric acid (1000 ppm) and a wetting agent. Fruit were wound-inoculated with 20 µl of a spore suspension of *M. fructicola, B. cinerea,* or *R. stolonifer* (30,000 spores/ml each). Additional treated fruit were spray-inoculated with *B. cinerea* or incubated for the development of natural incidence of decay. In the laboratory, fungicides were applied as aqueous solutions using an air-nozzle sprayer or as drenches 11-14 h after (Inoculated-Treated) inoculation with the fungal decay pathogens. After treatment, fruit were incubated for 4-7 days at 20 C, >95% RH. Incidence of decay was determined as the number of fruit infected of the total fruit evaluated. Data were analyzed using analysis of variance procedures of SAS 9.4.

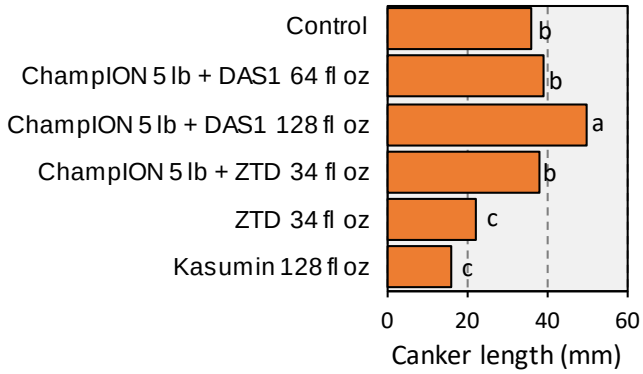
RESULTS AND DISCUSSION

Evaluation of treatments for control of bacterial canker. Treated injured branches inoculated with a copper-resistant strain of the pathogen develop canker at high incidence and treatment efficacy could be statistically separated by measuring canker length. Only Kasumin and ZTD significantly reduced canker formation. Copper by itself was not effective in previous studies, and in the current study, potential enhancers of copper activity (i.e., ZTD and DAS1) did not improve performance of copper.

Based on recent reviews by EPA specifically requested by us to find alternatives chemical for managing bacterial diseases of plants, ZTD is unlikely to be registered in the US, and thus, Kasumin is one of the few chemicals that have potential for bacterial canker and blast management. With widespread copper resistance in the bacterial pathogen *Pseudomonas syringae* pv. *syringae*, new effective treatments are needed to manage bacterial canker and blast. These are important diseases of sweet cherry that can impact cherry production in seasons with favorable environmental conditions and can also have long-term effects on tree health. In our studies over the years, Kasumin was the most effective and consistent treatment against both phases of the disease. Oxytetracycline was evaluated previously and also was identified as a promising bactericide against *P. syringae*. Registrants of both of these antibiotics are supportive of a registration on sweet cherry and this is currently pursued. Oxytetracycline (i.e., Mycoshield) and kasugamycin (i.e., Kasumin) have been submitted to EPA by the IR-4 program and are being reviewed both federally and by the state for use on sweet cherry. Kasumin is set for full registration on cherry in January 2018 based on the US EPA PRIA date. The antibiotic is federally registered on pome fruit since 2014 for management of fire blight. Over the years of our evaluations, Actinovate also showed good efficacy in reducing blossom blast (but was less effective against canker), and Blossom Protect/Botector also reduced the disease.

Evaluation of new fungicides for control of powdery mildew of sweet cherry. Our epidemiological studies have shown that mildew sequentially develops on: 1) leaves of inside shoots (water sprouts); 2) leaves of outer shoots; 3) green stems of fruit; and 4) on ripening fruit (fruit with color). The disease has not been found on green fruit mesocarp tissue. We have shown that young leaves are more susceptible than old leaves. The efficacy of new fungicides and new pre-mixtures was evaluated in a trial in San

Fig. 1. Evaluation of antibacterial treatments for protection of inoculated cv. Coral cherry branches from bacterial canker – Field studies in 2017



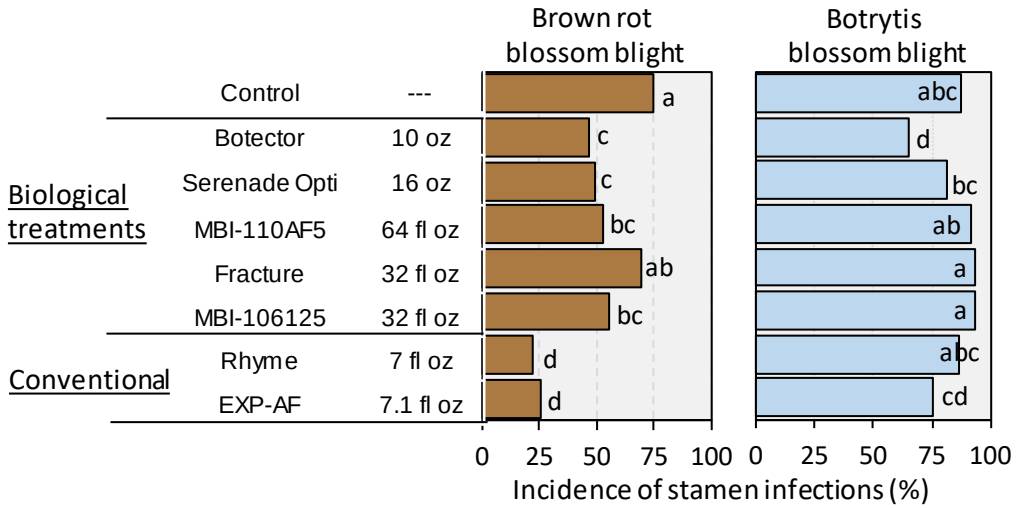
Branches were puncture-wounded laterally in winter 2017 using a nail. Wounds were treated with bactericides, allowed to air-dry, and were then spray-inoculated with *P. syringae* (approximately 2 x 10^7 cfu/ml). Branches were evaluated for canker formation in May 2017.

Fig. 2. Evaluation of preharvest fungicide treatments for management of powdery mildew of Bing cherries in San Joaquin Co. 2017

		Application dates	3-16	4-5	4-26	Inside shoots	Outside shoots
	Control	---	---		---	a	abc
Single	Terraneem EC	160 fl oz	@	@	@	ab	a
	Rhyme + Breakthru	7 + 7 fl oz	@	@	@	f	abcd
	Procure	12/16 fl oz**	@	@	@	def	cde
	Kenja	12.5 fl oz	@	@	@	abcde	de
	Pyraziflumid + Breakthru	3.38 + 8 fl oz	@	@	@	def	e
	UC-1 + Breakthru	4 + 8 fl oz	@	@	@	abcd	ab
	Pydiflumetofen	5.3 fl oz	@	@	@	abcde	de
Mixtures	Ph-D + Procure	6.2 oz + 12 fl oz	@	@	@	cdef	de
	Fontelis + Quintec	14 +7 fl oz	@	@	@	def	cde
	Indar + Quintec	8 + 7 fl oz	@	@	@	abc	abcde
Pre-mixtures	Luna Experience	6 fl oz	@	@	@	cdef	bcde
	Luna Sensation	6 fl oz	@	@	@	ef	e
	Merivon	6.5 fl oz	@	@	@	def	de
	UC-2 + Breakthru	6 + 8 fl oz	@	@	@	f	e
	EXP-AD	14.1 fl oz	@	@	@	def	e
	EXP-AF	7.1 fl oz	@	@	@	def	abcde
	IL-5412	15 fl oz	@	@	@	bcdef	de
Rotation	Fontelis	20 fl oz	@	---	---	abcde	bcde
	Rally + Quintec	6 oz + 7 fl oz	---	@	---		
	Fontelis + Quintec	14 + 7 fl oz	---	---	@		

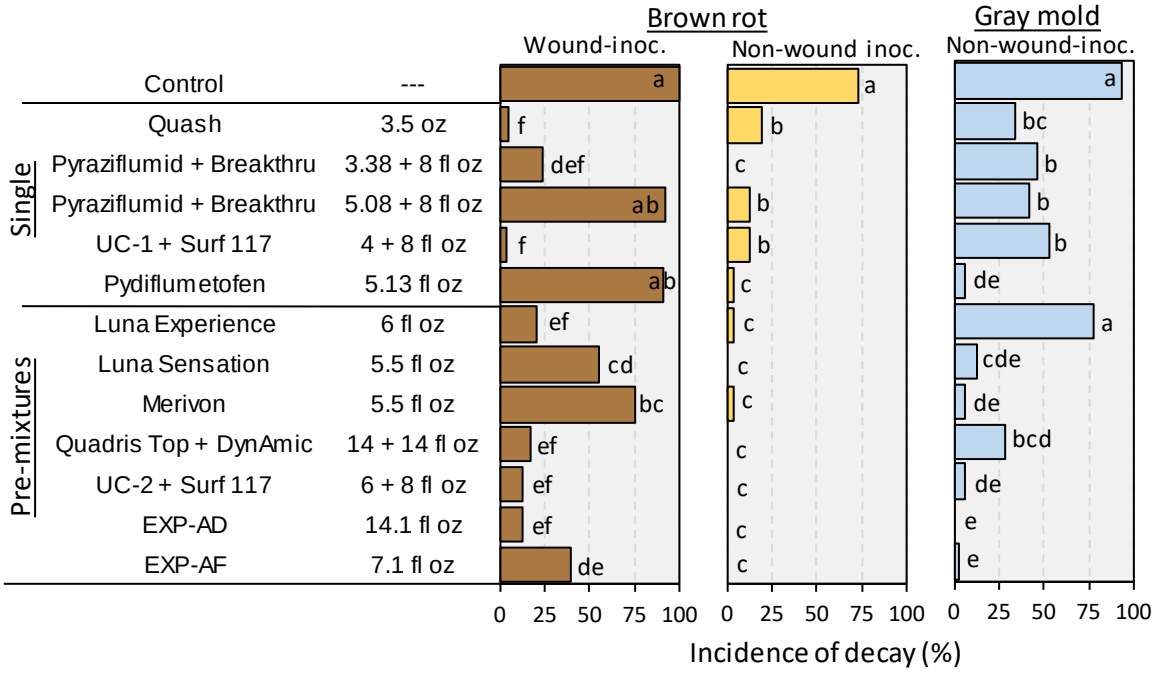
Applications were done using an airblast sprayer at 100 gal/A. For evaluation on 6-6-17, 20 leaves from 4 shoots each from inside or outside of the tree were sampled. The rating scale was: 0=healthy, 1=1-3 lesions/leaf, 2=<25%, 3=26-50%, 4 = >50% of leaf area diseased.

Fig. 3. Efficacy of pre-infection treatments for control of brown rot and Botrytis blossom blight of Bing cherry – Laboratory studies 2017



Pre-infection activity: closed blossoms were collected in the field, allowed to open, and treated in the laboratory using a hand sprayer. After 12 h blossoms were inoculated with a spore suspension of *M. fructicola* or *B. cinerea* (30 K/ml). Blossoms were evaluated for stamen infections after 4-5 days of incubation at 20 C.

Fig. 4. Efficacy of 4-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - Orchard 1 UC Davis - 2017



Treatments were applied on 5-19-17 using an air-blast sprayer at a rate of 100 gal/A. Non-washed fruit were wound-inoculated after harvest with *M. fructicola* (30,000 spores/ml) or non-wound-inoculated with *M. fructicola* or *B. cinerea* (200,000 spores/ml) and incubated at 20C for 6 days.

Joaquin Co. Three applications were done in ca. three-week intervals over a 6-week period starting at full bloom with fungicide applications for brown rot blossom blight. At evaluation time in early June, a high incidence of disease (90.7%) was present on leaves of inside shoots, but disease on the outer shoots was low (14% incidence) (Fig. 2). Thus, environmental conditions were not highly favorable at our trial site in the spring of 2017 possibly because of the late spring rainfall.

The most effective treatments included SDHI (FG 7)-containing fungicides including Fontelis, pyraziflumid, Luna Sensation, and Merivon, selected DMI (FG 3)-containing fungicides such as Rhyme and Procure, as well as the experimental fungicides UC-2, EXP-AD, and –AF (Fig. 2). Numerically, the lowest incidence on inside shoots was observed after treatments with the experimental UC-2 (6.3%). Quintec (FG 13) that was highly effective in the first years after its registration on cherry continued to show reduced performance and was only effective in mixture with other FRAC Groups such as FG 7 (i.e., Fontelis), but not in a rotation of Fontelis – Rally + Quintec – Fontelis + Quintec. The natural product Terraneem was not effective in these studies.

Thus, this research demonstrated excellent activity of several newly registered, as well as of numbered compounds against powdery mildew. We show that the disease can be reduced to acceptable levels by properly timed applications. Because of the potential of resistance to single-site mode of action fungicides, pre-mixtures or tank mixtures of FG 3, FG 7, FG 11, and FG 19 fungicides will be most sustainable. This limits the use of any single-site mode of action fungicide (i.e., single FG number) and reduces the selection pressure for selecting for fungicide resistance. Limiting the number of applications of any one mode of action (i.e., FG) will also reduce the residue and ensure that MRLs are not exceeded with any of the trade partners of the cherry industry.

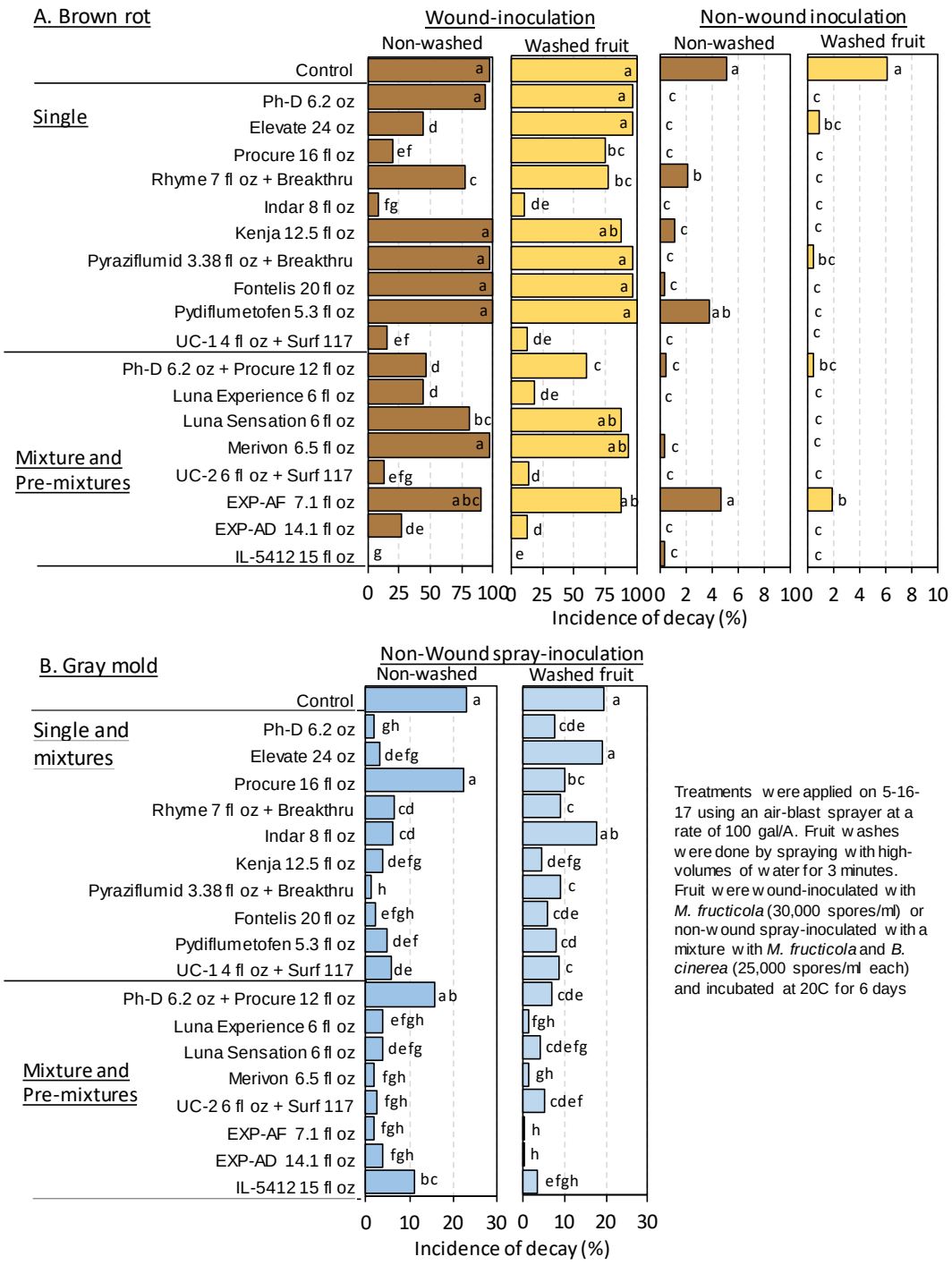
Under conditions where fungicides have to be used as post-infection treatments when visible symptoms are already present on fruit, we showed previously that Ph-D can be used with a multi-site fungicide like Kaligreen or with DMI fungicides like Procure for effective suppression of the disease.

Efficacy of new fungicides for control of brown rot and Botrytis blossom blight. Selected biological treatments were evaluated in comparison to two conventional fungicides for their pre-infection activity on detached opened blossoms in laboratory studies. For brown rot blossom blight, Rhyme and the experimental EXP-AF were highly effective, whereas the biological treatments Botector, Serenade Opti, and two MB compounds were less effective, but still significantly reduced the disease from the control (Fig. 3). For gray mold blossom blight, none of the evaluated compounds was very effective, but Botector, Serenade Opti, and EXP-AF showed some reduction in disease.

Due to the pre- and post-infection activity of most of the conventional fungicides that was shown previously, the practice of a single delayed-bloom application when environmental conditions are not favorable for disease is an excellent strategy for obtaining highly effective blossom disease management and result in a minimal number of blossom treatments on sweet cherry. Although having reduced efficacy, selected biological treatment can benefit disease management in organic production systems.

Evaluation of preharvest treatments for fruit decay control without postharvest washes and for postharvest decay control after postharvest washes. Two preharvest efficacy trials with 4- or 7-day PHI applications were done in 2017 (Figs. 4, 5). In wound inoculation studies on non-washed fruit, several fungicides applied 4 days before harvest provided excellent protection against brown rot and these included the registered Quash and the pre-mixtures Quadris Top and Luna Experience, as well as the experimental compounds UC-1, UC-2, and EXP-AD (Fig. 4). In the second trial with 7-day PHI applications, Procure, Indar, UC-1, UC-2, EXP-AD, and IL-5412 were all highly effective against brown rot on wound-inoculated fruit (Fig. 5A). When harvested fruit were washed and then inoculated, most treatments containing DMI fungicides were still very effective (Fig. 5A). When harvested fruit were non-wound inoculated, most treatments were highly effective against brown rot fruit decay on non-washed and washed fruit (Figs. 4, 5A). This emphasizes the importance of care in handling fruit to prevent injuries that by-pass the protective fungicides.

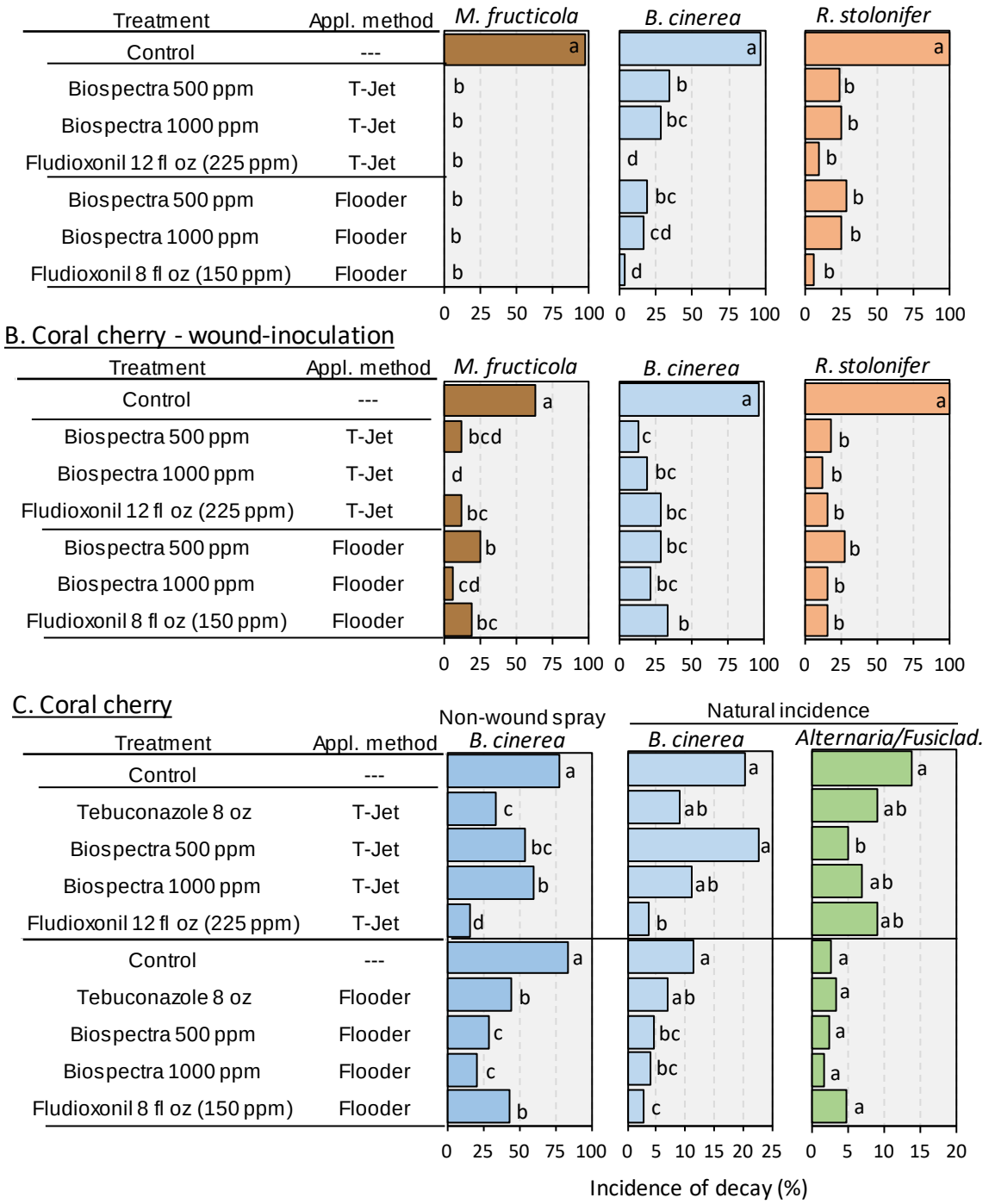
Fig. 5. Efficacy of 7-day preharvest fungicide treatments for management of postharvest brown rot and gray mold of Bing cherries - Orchard 2 San Joaquin Co. - 2017



Treatments were applied on 5-16-17 using an air-blast sprayer at a rate of 100 gal/A. Fruit washes were done by spraying with high-volumes of water for 3 minutes. Fruit were wound-inoculated with *M. fructicola* (30,000 spores/ml) or non-wound spray-inoculated with a mixture with *M. fructicola* and *B. cinerea* (25,000 spores/ml each) and incubated at 20C for 6 days

For gray mold, Luna Sensation, Merivon, and the experimentals pydiflumetofen, UC-2, EXP-AD, and EXP-AF were most effective after non-wound inoculation in the first study (Fig. 4), whereas Ph-D, Elevate, Kenja, Fontelis, pyraziflumid, pydiflumetofen, and most of the premixtures had the lowest incidence of gray mold in the second study on non-washed fruit (Fig. 5B). On washed fruit, performance of the preharvest fungicide treatments was reduced for managing gray mold. The most effective treatments were Luna Experience, Merivon, EXP-AD, and EXP-AF (Fig. 5B). These results demonstrate that preharvest treatments with SDHI (FG 7), hydroxyanilide (FG 17), or polyoxin (FG 19) fungicides are

Fig. 6. Postharvest treatments of inoculated sweet cherry fruit with fludioxonil and BioSpectra in a commercial packingline study – Packinghouse 1, 2017



Fruit were wound-inoculated with spores of *M. fructicola, B. cinerea*, or *R. stolonifer* (30,000 spores/ml) and incubated for 14 h at 18C. Flooder treatments (except tebuconazole) were done in combination with 1000 ppm citric acid + 50 ppm SDBS (sodium dodecyl benzene sulfonate). T-Jet treatments were done by 2 sequential spray bars separated by a step on the belt to promote turning of the fruit. Additional treated fruit were spray-inoculated with *B. cinerea* (25,000 spores/m) or were incubated for development of natural incidence of decay. Decay incidence is based on the number of decayed fruit of the total number of fruit treated. Statistical analyses for the non-wound spray inoculation and natural incidence were done separately for T-Jet and flooder applications.

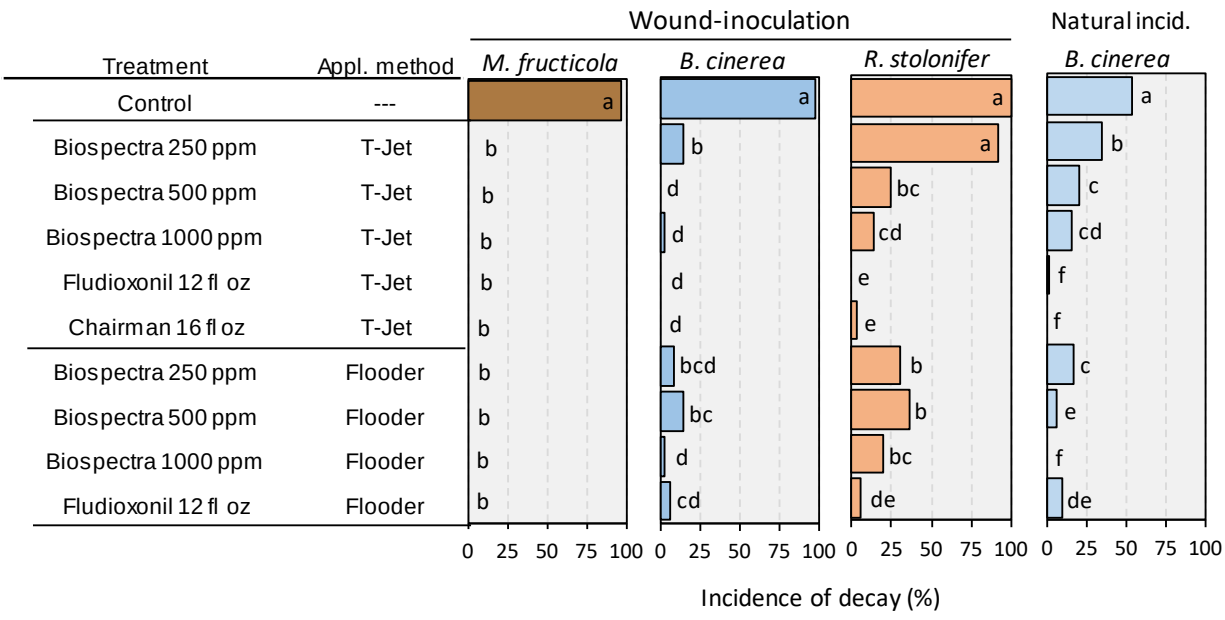
highly effective against gray mold. Thus, we are developing and identifying preharvest treatments that are highly against both brown rot and gray mold and that can protect fruit from infections before and during harvest. These fungicides will be evaluated again in 2018.

Postharvest decays, however, can still develop due to minor injuries that occur during the bulk handling of fruit resulting in stem punctures and epidermal abrasions. To obtain the highest level of decay control for shipping to distant markets, postharvest treatments are still needed to reduce crop losses and minimize claims against shippers, packinghouses, and ultimately growers.

Efficacy of new postharvest treatments for managing brown rot, gray mold, and Rhizopus rot of sweet cherry. Three commercial packingline studies with flooder and T-Jet applications were done for the evaluation of postharvest fungicides (Figs. 6-8). Emphasis was on the recently registered bio-fungicide BioSpectra (natamycin), the pre-mixture of fludioxonil and propiconazole Chairman, and Scholar (or other formulations of fludioxonil). Treatments were evaluated using fruit wound-inoculated before application or spray-inoculated after application, or treated fruit were incubated for development of natural incidence of decay. Fruit from an experimental orchard was used for the wound-inoculations, whereas commercial fruit was used for the two other evaluations. Because commercial fruit were likely treated with a preharvest fungicide in the orchard, no brown rot developed and only data for gray mold and Alternaria/Fusicladium rots were obtained.

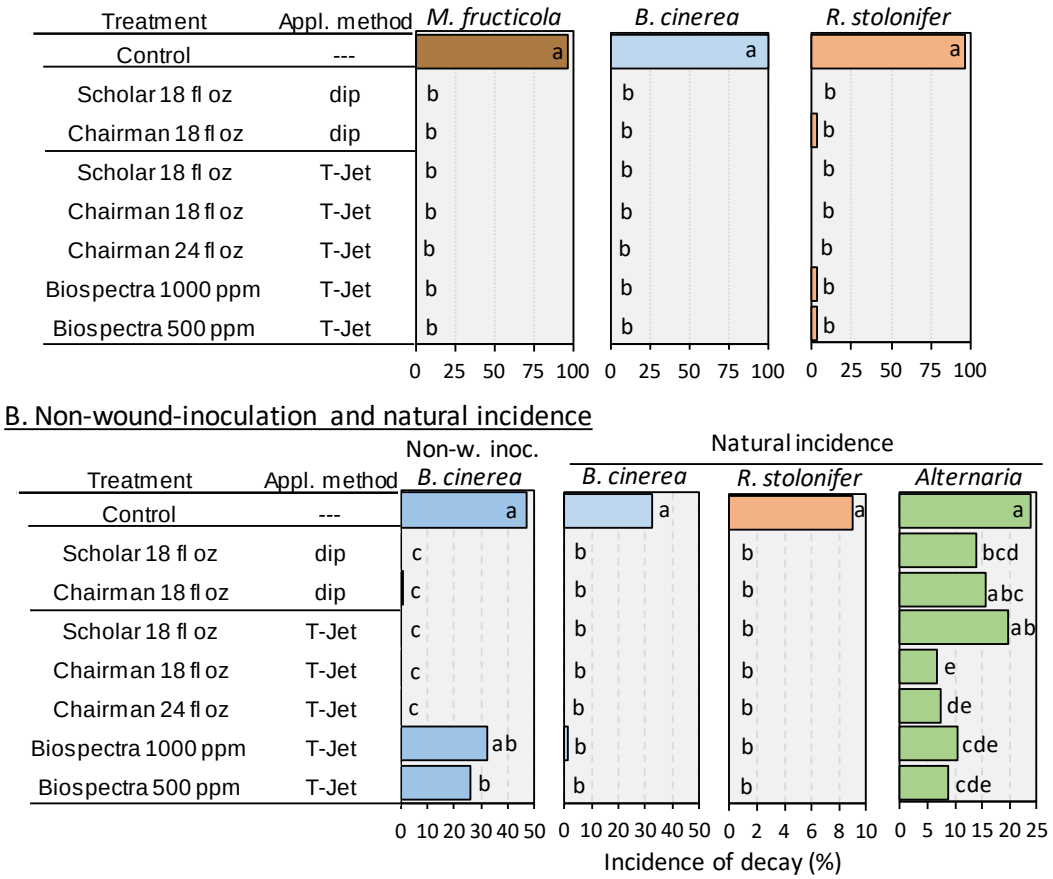
In the three trials, Scholar continued to perform very well against the major decays on inoculated fruit and natural incidence of gray mold and Rhizopus rot (Figs. 6-8). BioSpectra using flooder and T-Jet applications was in most cases highly effective against the major decays brown rot, gray mold, and Rhizopus rot when fruit were wound-inoculated before treatment (Figs. 6-8). Brown rot was reduced to zero levels in most cases, similar to Scholar, but for gray mold, Scholar was sometimes more effective than BioSpectra. These results are in agreement with our previous studies. BioSpectra was also very

Fig. 7. Postharvest treatments of inoculated Bing cherry fruit with Fludioxonil, Chairman, and BioSpectra in a commercial packingline study – Packinghouse 1, 2017



Fruit were wound-inoculated with spores of *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml) and incubated for 10 h at 20C. Flooder treatments were done in combination with 1000 ppm citric acid and wetting agent. T-Jet treatments were done by 2 sequential spray bars separated by a step on the belt to promote turning of the fruit. Additional treated fruit were incubated for development of natural incidence of decay. Decay incidence is based on the number of decayed fruit of the total number of fruit treated.

Fig. 8. Postharvest treatments of inoculated Bing cherry fruit with Scholar, Chairman, and BioSpectra in a commercial packingline study – Packinghouse 2, 2017



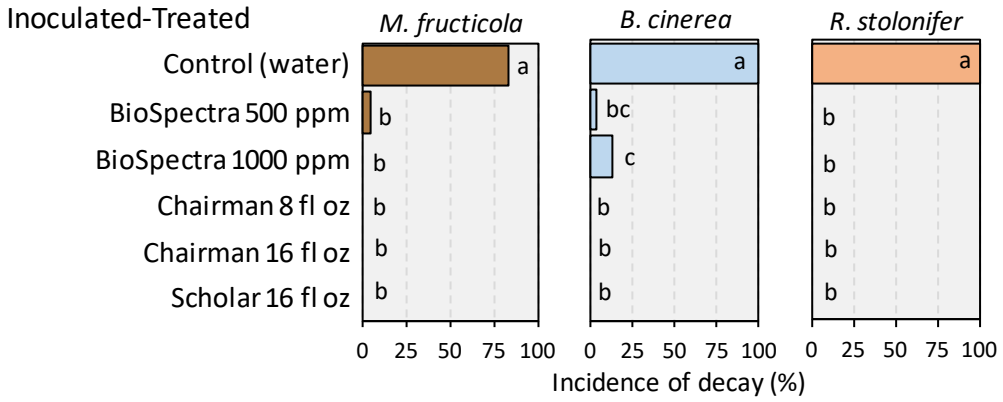
Fruit were wound-inoculated with spores of *M. fructicola*, *B. cinerea*, or *R. stolonifer* (30,000 spores/ml). T-Jet treatments were done by 2 sequential spray bars separated by a step on the belt to promote turning of the fruit. Dips were done for 15 sec. Additional treated fruit were spray-inoculated with *B. cinerea* (25,000 spores/m) or were incubated for development of natural incidence of decay. Decay incidence is based on the number of decayed fruit of the total number of fruit treated.

effective in reducing the natural incidence of gray mold and Rhizopus rot, especially in flooder applications, and sometimes reduced the natural incidence of Alternaria/Fusicladium rots. But again, Scholar was sometimes more effective. Gray mold was generally not well controlled by BioSpectra when fruit were spray-inoculated after treatment, and this can currently not be explained. In comparing the 500-ppm and 1000-ppm rates of BioSpectra, there were often no significant differences in efficacy. Thus, when properly applied, the 500-ppm rate should provide adequate decay control, but the 250-ppm rate showed reduced efficacy (Fig. 7). Chairman was highly effective against the three major decays after wound-inoculation, and reduced natural incidence of gray mold and Rhizopus rot to zero levels. BioSpectra, Scholar, and Chairman also demonstrated high decay control efficacy in a laboratory study using fruit inoculated 12-14 h before treatment (Fig. 9).

Thus, in our postharvest studies, we identified, optimized, and helped registered the new postharvest treatment BioSpectra for sweet cherry. Excitingly, resistance has never been reported to the active ingredient natamycin. Still, combination treatments of BioSpectra with other postharvest fungicides such as Scholar or Tebucon will be most beneficial in providing consistent, high efficacy. This strategy will also reduce the risk of selecting resistant sub-populations of the decay pathogens to other registered

postharvest fungicides. At this time, MRLs have not been established in many countries and use is suggested only for domestic markets (including Canada). A FAT in Japan is expected for propiconazole in Jan. 2019. FATs for natamycin are already established in Japan for other food products, and the registrant expects an expedited review for use on cherry and other fruit crops. With increasing emphasis on food safety and consumer concerns, natamycin with 'exempt from tolerance status' will likely become an important component of postharvest decay management in the future. We will continue our evaluations of these treatments in 2018 in cooperation with commercial packinghouses.

Fig. 9. Postharvest treatments with Scholar, Chairman, and BioSpectra for decay control of inoculated Bing cherry fruit in a laboratory study - 2017



Fruit were wound-inoculated (30,000 spores/ml) and incubated for 12-14 h at 20C. Aqueous treatments were applied using an air-nozzle sprayer and fruit were incubated at 20C for 6-8 days.

Annual report 2017

Project Title: INVESTIGATING THE CAUSE OF SUDDEN DECLINE OF SWEET CHERRY IN CALIFORNIA

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Interpretive summary
During the springs and summers of the past two growing seasons, multiple cherry orchards expressing symptoms of sudden decline were detected in the main sweet cherry producing counties in California. Symptoms included wilting of the entire tree crown followed by tree death. Cherry orchards with sudden decline were observed in Contra Costa, San Joaquin, Fresno, Kern and Kings Counties. Comprehensive examinations of affected trees revealed lesions and rot symptoms in the root systems, suggesting that the causal pathogens are soil-borne. Isolations from diseased roots revealed the occurrence of common soil-borne fungal pathogens in the genera *Fusarium*, *Cylindrocarpon*, and *Macrophomina*. Morphological and molecular identifications refined these pathogens to the species *Fusarium oxysporum*, *Fusarium solani*, *Cylindrocarpon liriodendri* and *Macrophomina phaseolina*. These fungi are known soil-borne pathogens of other crops in California, including Charcoal rot of strawberry and Black foot disease of grapevines. This is, however, the first report of *M. phaseolina*, *C. liriodendri* and *Fusarium* spp. affecting cherry in California. We are currently determining the pathogenicity of these fungal species in three important cherry rootstocks, Mahaleb, Colt and Krimsk5 to establish a causal relationship between the various fungi and the sudden decline of sweet cherry and eventyally reveal differences in rootstock tolerance to this emerging problem of sweet cherry in California.

Objective 1: Surveys (completed)
Surveys and sampling of sweet cherry orchards with symptoms of sudden decline were conducted in 2016 and 2017. Farm advisors and PCA collaborators identified orchards expressing sudden decline. Cherry trees expressing sudden decline were observed in orchards in Contra Costa, San Joaquin, Fresno, Kern and Kings Counties. Symptoms included rapid wilting of the entire tree crown, yellowing of leaves, leaf scorch, leaf drop, and tree death (**Figure 1**). Trees were backhoed to examine and sample roots showing symptoms of root rot and necrosis. Following surveys, diseased root samples were taken to the laboratory to determine the pathogens present. Pieces of roots were surface sterilized by immersion for 2 mins in a 1.5 % sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces from the margin between healthy and necrotic tissues were placed onto

petri dishes filled with potato dextrose agar (PDA) amended with 100 ppm tetracycline (PDA-tet) for isolation of fungi.

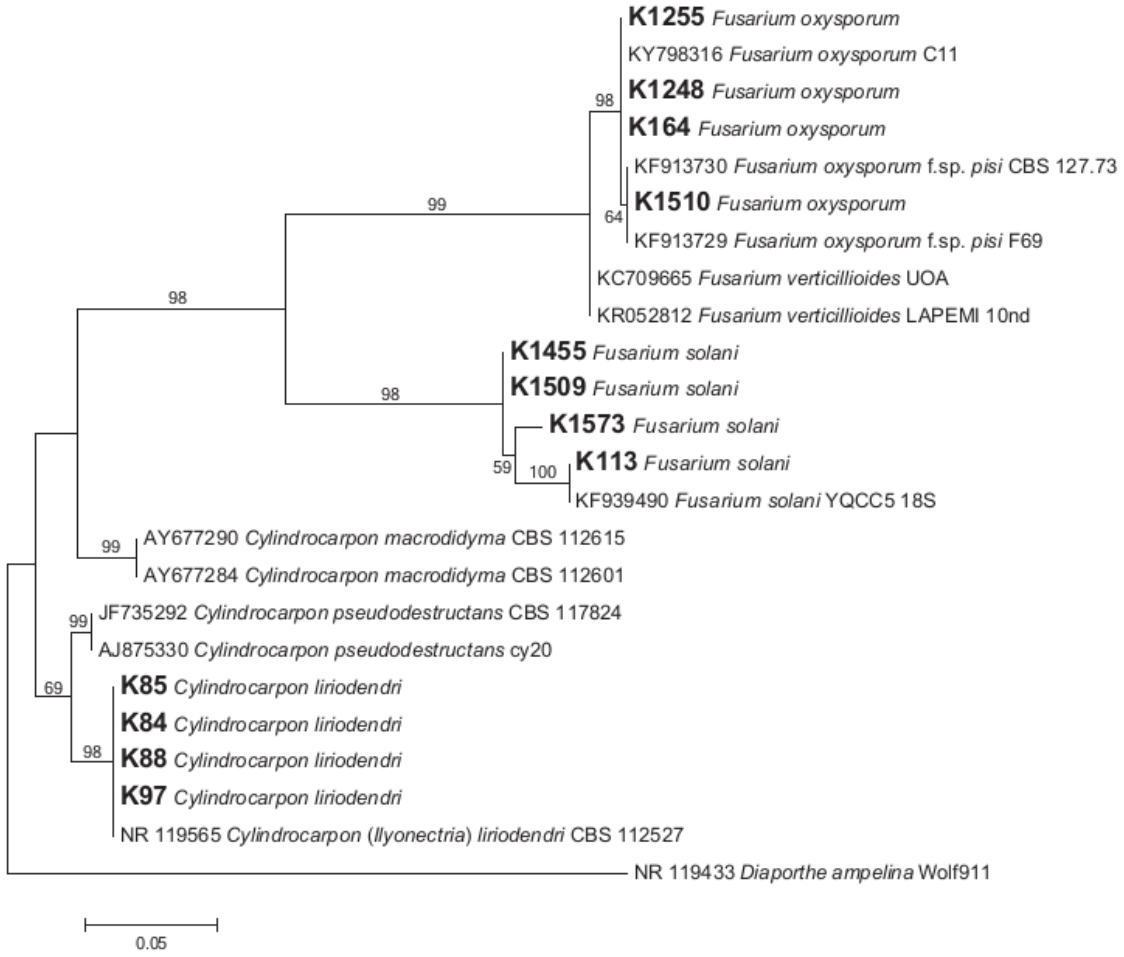
Figure 1. Symptoms of sudden decline of sweet cherry showing wilting of leaves of the entire tree crown. Complete wilting of the entire canopy of trees often indicates a problem in the root system.

Objective 2: Identify and characterize the disease causal agents (completed)

Isolation from diseased roots yielded consistently fungal colonies with morphological characteristics of the genera *Fusarium*, *Cylindrocarpon* and *Macrophomina*. These colonies were isolated into pure cultures and their DNA was extracted. Molecular refinement of pathogen identification was performed through PCR amplification and sequencing of the internal transcribed spacer region (ITS) of the ribosomal DNA using primers ITS1 and ITS4. The ITS sequences generated were compared to DNA sequences present in public databases using the nucleotide query algorithms BLAST in GenBank. Phylogenetic analyses were further used for unequivocal taxonomic placement among fungal members of related species.

Phylogenetic placement of representative isolates revealed that a large proportion of fungal pathogens belonged to the species *Fusarium oxysporum*, *Fusarium solani*, and *Cylindrocarpon liriodendri* (**Figure 2**).

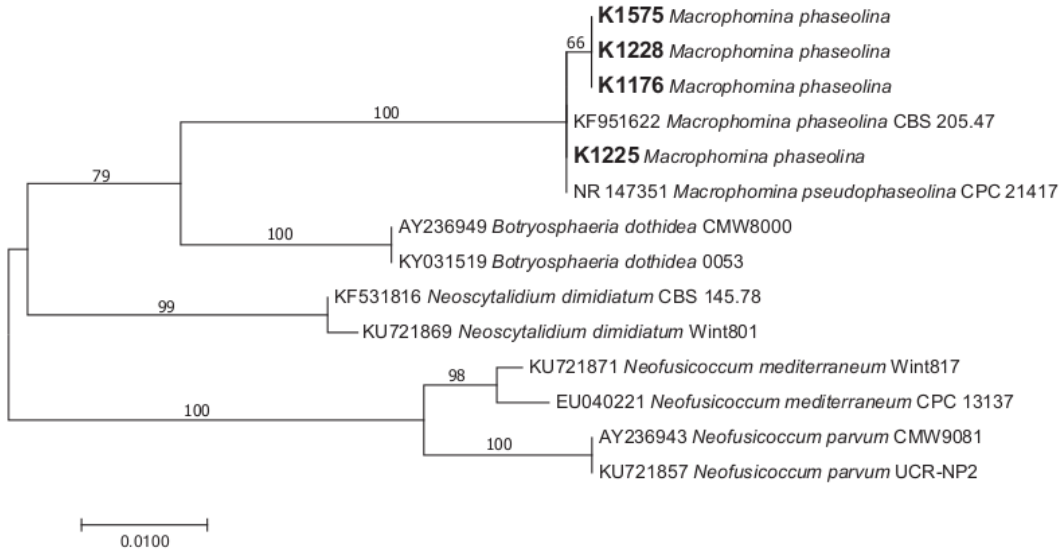
Figure 2. Phylogenetic analysis of ITS sequences of representative isolates (in bold) of the genera *Fusarium* and *Cylindrocarpon* recovered from diseased cherry roots. Isolates belong to the species *Fusarium oxysporum*, *Fusarium solani*, and *Cylindrocarpon liriodendri*.



Fusarium oxysporum was recently shown to be responsible for a root and crown rot disease of sweet cherry in British Columbia (Urbez-Torres et al. 2016); it is very likely that this species and the closely-related species *Fusarium solani* are also causing root damages to cherry trees in California. *Cylindrocarpon* species have been associated with Black foot disease of grapevine in California, with roots of symptomatic grapevines showing black, sunken, necrotic lesions (Petit and Gubler 2005). Grape leaves appear scorched and the stunting and decline of the entire vine frequently follows. *Cylindrocarpon liriodendri* role in sudden cherry decline is plausible and under investigation.

Another large proportion of fungal isolates belonged to the genus *Macrophomina* from the family Botryosphaeriaceae which is known to include aggressive pathogens of numerous perennial plants. Phylogenetic analyses revealed that these isolates belonged to the species *Macrophomina phaseolina* (**Figure 3**).

Figure 3. Phylogenetic analysis of ITS sequences of representative isolates (in bold) of the genera *Macrophomina* recovered from diseased cherry roots. Isolates belong to the species *Macrophomina phaseolina*.



Macrophomina phaseolina is the causal agent of Charcoal rot of strawberry roots in California (Koike 2008). It is also known to cause root rot in other crops such as corn, cotton, sunflower, potato and sorghum. In California, charcoal rot appears to be the most important current concern for the strawberry industry due to its steady increase over the past 10 years (Koike 2008). Each year finds additional new fields infested, and the disease has now been found in all the major strawberry producing counties in the state. The fungus is known to produce microsclerotia, below ground survival structures, which production increases under low water potentials that occurs during drought. Despite little reports of *Macrophomina phaseolina* affecting perennial woody crops, we have isolated it recently also from declining table grapes and pistachio trees in California. The role of *Macrophomina phaseolina* in root diseases of perennial crops is under further investigation.

Objective 3: Determine the pathogenicity and aggressiveness of the various fungi in the main cherry rootstocks (on-going)

Pathogenicity studies using the putative pathogens have been initiated during fall 2017. We selected three commercially-important cherry rootstocks: Performer Mahaleb, Colt and Krimsk5. Some of the surveyed orchards with sudden decline symptoms were grafted onto these rootstocks. Pathogenicity tests have been initiated to perform Koch's postulates and

to establish a causal relationship between the various fungi and the sudden decline of sweet cherry.

Four fungal species and a total of 16 fungal isolates recovered from the roots of diseased cherry trees were used for pathogenicity assays (**Table 1**).

Table 1. Isolates used in pathogenicity studies.

Isolate	Genus	species	Host	Rootstock	Isolation date	Location
K 84	Ilyonectria	liriodendri	Cherry	Colt	4/1/2015	Reedley
K 85	Ilyonectria	liriodendri	Cherry	Colt	4/1/2015	Reedley
K 88	Ilyonectria	liriodendri	Cherry	Colt	4/1/2015	Reedley
K 97	Ilyonectria	liriodendri	Cherry	Colt	4/1/2015	Reedley
K 164	Fusarium	oxysporum	Cherry	Colt	4/1/2015	Reedley
K1248	Fusarium	oxysporum	Cherry	Mahaleb	6/17/2016	San Joaquin County
K1255	Fusarium	oxysporum	Cherry	Krymsk5	6/17/2016	San Joaquin County
K1510	Fusarium	oxysporum	Cherry	Mahaleb	8/12/2016	Brentwood
K 113	Fusarium	solani	Cherry	NA	4/10/2015	Reedley
K1455	Fusarium	solani	Cherry	Mahaleb	6/17/2016	San Joaquin County
K1509	Fusarium	solani	Cherry	Mahaleb	8/12/2016	Brentwood
K1573	Fusarium	solani	Cherry	Maxma14	10/6/2016	Fresno
K1176	Macrophomina	phaseolina	Cherry	Mahaleb	6/17/2016	San Joaquin County
K1225	Macrophomina	phaseolina	Cherry	Krymsk5	6/17/2016	San Joaquin County
K1228	Macrophomina	phaseolina	Cherry	Mahaleb	6/17/2016	San Joaquin County
K1575	Macrophomina	phaseolina	Cherry	Maxma14	10/6/2026	Fresno

For each species, a mixture of four isolates was used as inoculum. In addition, a known and aggressive pathogen of cherry trees (*Phytopthora cambivora*) was used to serve as a positive control. The plant growth media (UC potting mix) was inoculated either using millet seeds (ascomycetes) or sand-bran (*P. cambivora*). Sterile water (125mL) were added to millet seeds (250g) in 1 L flasks and steeped for 12 hours. Flasks were autoclaved at 120C for 20 min 24 h apart. Autoclaved millet seeds were inoculated with 10 five mm diameter mycelial plugs from 10 days-old PDA cultures of each isolate (2 flasks per isolate, 8 flasks per ascomycete species) and incubated for 14 days in the dark at 25C and shaken every other day (**Figure 4**). Control bottles were inoculated with plugs of PDA only. The sand-bran inoculum of *P. cambivora* consisted of 200g of sterile river sand, 20 g of wheat bran and 30 mL of sterile water in Schott bottles autoclaved at 120C for 20 min 24 h apart. Each bottle was inoculated with ten 5-mm mycelial plugs from a 10 days-old *P. cambivora* CMA (Corn Meal Agar) whereas control bottles were inoculated with ten 5-mm uncolonized CMA plugs. Bottles were incubated for 14 days in the dark at 25C and shaken every other day. Millet seed and sand-bran inoculum were added at a rate of 10% (v/v) in the plant growth media, using pots of 880 ml (MT38BT; Stuewe and Sons, Tangent, OR) held in trays.

Figure 4. Millet seeds colonized with the selected fungal isolates are used as inoculum when amended into the potting soil of cherry rootstock plants.

One-year-old cherry rootstocks plants (**Figure 5**) were obtained from a commercial nursery in northern California and grown in the greenhouse for eight weeks before inoculations. The pathogenicity of each fungal species is being evaluated using nine replicates, each replicate corresponding to a tree in a single pot. The experiment was set up as a complete random block design, and the experiment was repeated once a month apart. Inoculated trees are grown at temperatures ranging from 20C to 25C and will be harvested after 8-10 months (summer 2018).

Figure 5. Cherry rootstocks Mahaleb, Colt and Krimsk5 were grown in the greenhouse before inoculations.

After 8-10 months (summer 2018) plants will be rated. Root system will be washed and we will perform the following assessments: root mass fresh weight, root length, plant height (at inoculation and at harvest), trunk diameter (at inoculation and at harvest), presence or absence of root/crown rots, rating of root rot on a notation scale to estimate percent root rot. Feeder roots will be plated onto Potato Dextrose Agar for isolation of fungi and the incidence of root colonization will be assessed. Data will be analyzed using analysis of variance and multiple mean separation methods using SAS ver. 9.4.

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E. Petit and W.D. Gubler. 2005. Characterization of *Cylindrocarpon* Species, the Cause of Black Foot Disease of Grapevine in California. Plant Disease 89:1051-1059.

J.R. Urbez-Torres et al. 2016. First Report of Root and Crown Rot Caused by *Fusarium oxysporum* on Sweet Cherry (*Prunus avium*) in British Columbia. Plant Disease 100:855.

Investigating Biological Controls to Suppress Spotted Wing Drosophila Populations

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Summary. The spotted wing drosophila (SWD), *Drosophila suzukii* has become a major cherry pest in California. To develop sustainable management options for this highly mobile pest, we evaluated the potential of biological control using parasitoids. Here, we report on our continual evaluations of imported parasitoids that were discovered during our pervious explorations in Asia in the quarantine in 2017. We focused on the evaluations of two larval parasitoids that have been selected for the approval of field release based on previous evaluations on their efficiency and relative host specificity. We conducted additional collections for these native parasitoids in South Korea in 2017 to increase the vigor of the quarantine colonies for the rearing and studies. While we are preparing for release permit of the two selected larval parasitoids, we also evaluated the potential of two indigenous pupal parasitoids for the control of SWD, developed mass-rearing and storage methods, and prepared for augmentative field release.

Introduction

Spotted wing drosophila (SWD), *Drosophila suzukii* is native to East Asia, but has invaded North America and become a key pest of soft and thin skin fruits such as blueberries, cherries, raspberries, and strawberries in California and other invaded regions in North America. Currently, control efforts rely on the use of insecticides that target adult SWD. However, insecticide-based programs can be limited by the fact that many host fruits in non-crop habitats act as reservoirs for SWD and support its reinvasion into commercial fields.

Our work focuses on improving biological controls. Our surveys in California showed a lack of specialized parasitoids that can specifically attack SWD larvae. Only two generalist indigenous pupal parasitoids *Trichopria drosophilae* (Diapriidae) and *Pachycrepoideus vindemiae* (Pteromalidae) were found to attack SWD in California. The lack of effective biological controls in California (and other North American regions) led to the initiation of a classical biological control program. Our goal is to discover, import and select the most effective but also safest parasitoids for field release to control SWD in North America. This will be accomplished through foreign exploration to find novel parasitoids and then quarantine evaluations of the parasitoids efficiency, specificity (no costly non-target impacts), and temperature tolerances (where can they be released and establish).

During explorations in South Korea and China, we discovered three major larval parasitoids, *Asobara japonica* (Braconidae), *Ganaspis brasiliensis* and *Leptopilina japonica* (Figitidae) (there are no 'common names"). These parasitoids have been imported into our quarantine at UC Berkeley. We completed part evaluations of the three larval parasitoids (*A. japonica*, *G. brasiliensis* and *L. japonica*) on their biology and efficiency (e.g. egg maturation dynamics, functional responses, host age preference and suitability and life-time fecundity) as well as potential non-target impacts (host range test). This information is needed to develop effective rearing methods for these parasitoids, and more importantly for obtaining the release permits. The two dominant parasitoids (*G. brasiliensis* and *L. japonica*) (Fig. 1) both in South Korea and China, were shown to be the most promising agents in terms of their low potential risk to non-target species and their effectiveness against SWD. Petitions to release both parasitoids were submitted and reviewed, and while 5 of 8 reviewers approved the petition, the USDA APHIS regulators have requested

that we complete more studies on a) temperature development, b) non-target impact, and c) parasitoid taxonomy. We will complete this work in 2018.

In 2017, we (1) continued our studies to improve SWD biological control, especially on these two selected parasitoids; (2) conducted more collections of these exotic parasitoids in South Korea, and (3) evaluated the potential of the two North American pupal parasitoids (*T. drosophilae* and *P. vindemiae*) (Fig. 1) for use in an augmentative field release.

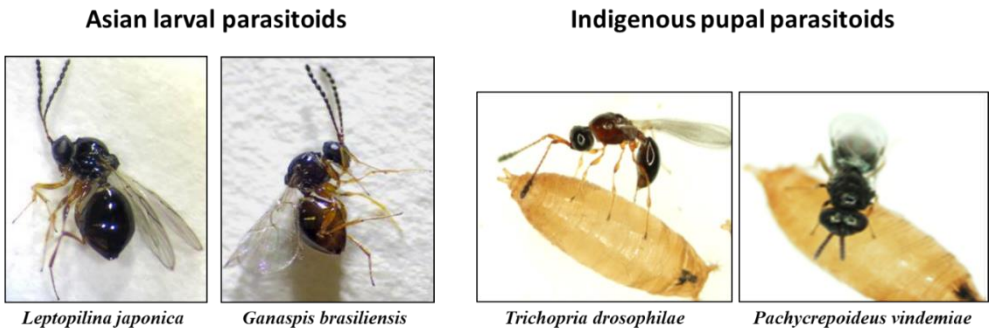

Fig. 1. Major Asian larval parasitoids and indigenous pupal parasitoids

1. Evaluations of Asian larval parasitoids

1.1. Host age preference

Last year, we reported that all three Asian larval parasitoids tested (*A. japonica*, *G. brasiliensis*, and *L. japonica*) performed better on younger SWD larvae in no choice test with four different host ages (1, 2, 3 or 4 days old larvae). However, there was no difference in parasitism rates between 1- or 2-day-old hosts or between 3- or 4-day-old hosts for either *G. brasiliensis* or *L. japonica*.

In 2017 we continued this research using a "choice test" – in other words, each parasitoid could choose to attack a younger (1-day-old) or older (4-day-old) host larvae. For this test, 5 SWD larvae of each age group (1-day and 4-day) were provided simultaneously to a single female parasitoid for 24 h exposure period (at room temperature (23 ± 2 °C), with 18-20 replicates of each age group). Following the exposure, all host larvae were dissected to determine the percentage of hosts parasitized. Results showed that both parasitoids preferentially attacked the younger hosts (*G. brasiliensis*: $F_{1,37} = 5.3$, $P = 0.029$; *L. japonica*: $F_{1,39} = 4.9$, $P = 0.045$) (Fig. 2).

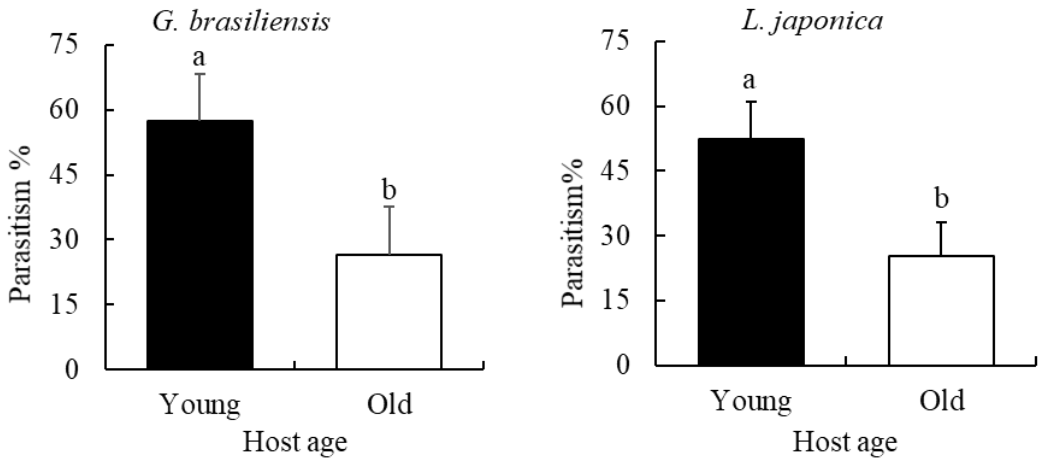

Fig. 2. Host age preference by *L. japonica* or *G. brasiliensis* in a choice test. Values are mean ± SE and bars bearing different letters are significantly different ($P < 0.05$).

1.2. Life-time fecundity

We compared the reproductive potential of *G. brasiliensis* and *L. japonica* by offering them unlimited access to SWD larvae. For each test, a pair of newly emerged (<12 h old) adult female and male parasitoids were daily provided 10 larvae (in artificial diet) until the female died (e.g., life time reproductive potential). We recorded adult female longevity, the number of offspring produced, offspring sex ratio and survival rate, and juvenile developmental time. From these data, life table fertility parameters – which are used to better compare animals – were estimated for each parasitoid species.

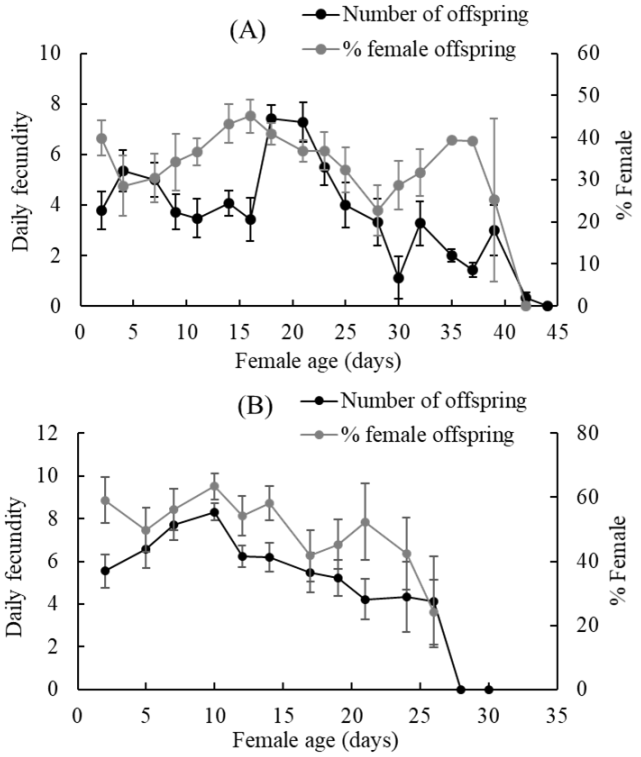

Fig. 3. Life-time fecundity and offspring sex ratio (% female offspring) of *G. brasiliensis* (A) and *L. japonica* (B) when parasitizing SWD larvae at 23 ± 2°C. Values are mean ± SE and dark and gray lines refer to daily fecundity and sex ratio, respectively.

Our studies showed that female parasitoids started oviposition within 2 days after emergence and the number of offspring produced generally decreased with the adults' increasing age when provided the hosts (Fig. 3). When the adults were provided with food (honey-water) and host SWD larvae, *G. brasiliensis* adult females survived 27.6 ± 1.9 days (range: 14–40 days) and produced 111.1 ± 7.5 offspring, while *L. japonica* survived 18.7 ± 1.1 days (range: 7–30 days) and produced 107.2 ± 9.9 offspring. The percentage of female progeny produced decreased as the "mother's" age increased, although this trend was more apparent for *L. japonica* (Fig. 3). The estimated life table fertility parameters were lower for *G. brasiliensis* than *L. japonica*: *Net reproduction rate* was 34.9 and 47.3; *intrinsic rate of increase* was 0.138 and 0.108; *mean generation time* was 33.5 and 28.1 days; and *doubling time* was 6.4 and 5.0 days for *G. brasiliensis* and *L. japonica*, respectively. Basically, these parameters indicate that under our laboratory conditions *L. japonica* will kill more SWD than *G. brasiliensis* – but the values are very close.

1.3. Host species preference

Our previous studies suggest that all three larval parasitoids (*A. japonica*, *G. brasiliensis* and *L. japonica*) can also attack *D. melanogaster* (DM), a species 'phylogenetically' related to SWD. For

each parasitoid, we (1) compared its performance on SWD and DM in cherries or artificial diet medium in no choice test (20 SWD or 20 DM only); (2) tested its preference when the two hosts were presented at equal proportion ratio (10 SWD + 10 DM) in choice test; and (3) determined its response to relative host abundance when the two hosts were presented at different proportion ratios (i.e. 15 SWD + 5 DM or 5 SWD + 15 DM). All tests conducted at the room temperature (23 ± 2°C) and with a 24 h exposure period, and 20-30 replicates for each treatment. Tests with artificial diet consisted of exposure of one female wasp to a total 20 host larvae (in drosophila vials filled with 20 mm standard cornmeal-based artificial diet. Tests with cherries consisted of exposure of infested cherries (15-20 fruit fly eggs per cherry per replicate) to one female wasp in small containers. All exposed hosts were reared until the emergence of flies or parasitoids. As a control, 10 unexposed replicates were simultaneously tested. We estimated the 'Degree of Infestation (DI)' to measure the proportion of hosts that were successfully parasitized. The DI was estimated as (T- di)/T where T= the number of emerging flies in the absence of the parasitoids and di = the number of emerged flies in the presence of parasitoids.

The results from the no choice test show that parasitism was significant different among different parasitoids (**stats 1**… if interested, the statistics are in the figure caption), but was not affected by host species (**stats 2**) (Fig. 4A-B). Overall, parasitism by *A. japonica* was higher than that of *G. brasiliensis* or *L. japonica* while the two figitids performed similarly in both cherry (**stats 3**) (Fig.4A-B). In the choice test with an equal proportion of SWD and DM, none of the parasitoid species showed a preference between the two host species in cherry (**stats 4**) or artificial diet (**stats 5**) (Fig. 4C-D).

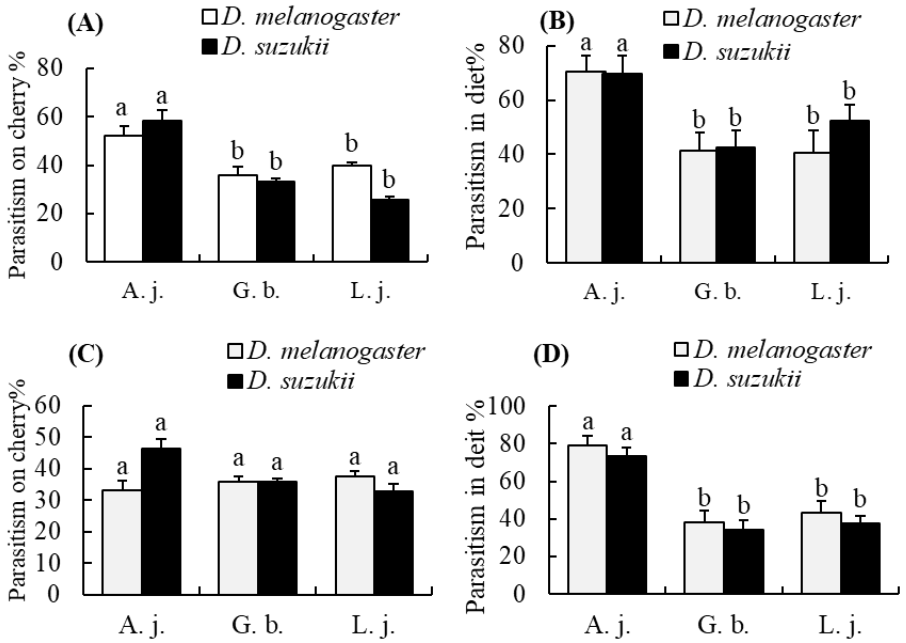

Fig. 4. Parasitism of both host species by *A. japonica* (A. j.), *G. brasiliensis* (G. b.) or *L. japonica* (L. j.) in no choice test in cherry (A) or artificial diet (B) and choice test in cherry (C) or artificial diet (D). Bars refer to mean and SE and different letters above the standard error bars indicate significant differences ($P < 0.05$). (**stats 1** cherry: $\chi^2 = 8.72$, df = 2, $P = 0.013$; diet: $\chi^2 = 7.59$, df = 2, $P = 0.022$; **stats 2** cherry: $\chi^2 = 0.09$, df = 1, $P = 0.755$; diet: $\chi^2 = 0.21$, df = 1, $P = 0.646$; **stats 3** cherry: $\chi^2 = 8.63$, df = 2, $P = 0.013$ or diet: $\chi^2 = 7.55$, df = 1, $P = 0.029$; **stats 4** *A. japonica*: $\chi^2 = 1.14$, df = 1, $P = 0.289$; *G. brasiliensis*: $\chi^2 = 0.01$, df = 1, $P = 0.989$; or *L. japonica*: $\chi^2 = 0.14$, df = 1, $P = 0.727$; **stats 5** *A. japonica*: $\chi^2 = 0.24$, df = 1, $P = 0.626$;*G. brasiliensis*: $\chi^2 = 0.07$, df = 1, $P = 0.785$; or *L. japonica*: $\chi^2 = 0.13$, df = 1, $P = 0.718$).

Parasitism was also not different between the two host species when the hosts were present at a ratio of 5 SWD and 15 DM (**stats 6)** or at a ratio of 15 SWD and 5 DM in artificial diet medium (**stats 7**) (Fig. 5A-B). The observed and expected parasitism were similar by each parasitoid species at the ratio of 5 SWD and 15 DM (**stats 8**) or at the ratio of 15 SWD and 5 DM (**stats 9**) (Fig. 5 C-D). What do these studies show? Basically, there was no host preference or host switching by these parasitoids for either the common fruit fly (*D. melanogaster*) or spotted wing drosophila (*D. suzukii*).

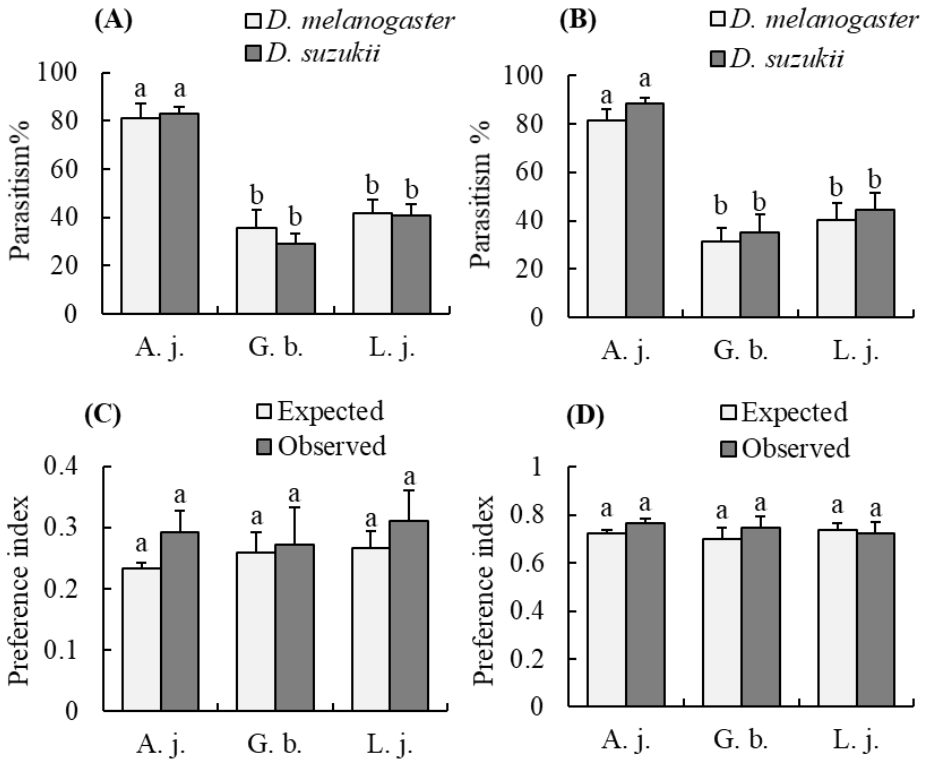

Fig. 5. Parasitism of both host species by *A. japonica* (A. j.), (B) *G. brasiliensis* (G. b.) or *L. japonica* (L. j.) when both hosts were present at a ratio of 5SWD: 15DM (A) or 15DM: 5SWD (B) in a choice test and the preference index at the two different ratios (C-D) based on the observed and expected parasitism. Bars refer to mean and SE and different letters above the standard error bars indicate significant differences ($P < 0.05$). (**stats 6**: *A. japonica*: $\chi^2 = 0.39$, df = 1, $P = 0.533$; *G. brasiliensis*: $\chi^2 = 0.06$, df = 1, $P = 0.813$; or *L. japonica*: $\chi^2 = 0.07$, df = 1, $P = 0.795$; **stats 7**: *A. japonica*: $\chi^2 = 0.03$, df = 1, $P = 0.864$; *G. brasiliensis*: $\chi^2 = 0.18$, df = 1, $P = 0.673$; or *L. japonica*: $\chi^2 = 0.01$, df = 1, $P = 0.961$; **stats 8:** *A. japonica*: $\chi^2 = 5.85$, df = 1, $P = 0.016$; *G. brasiliensis*: $\chi^2 = 0.52$, df = 1, $P = 0.472$; or *L. japonica*: $\chi^2 = 0.13$, df = 1, $P = 0.715$**stats 9**: *A. japonica*: $\chi^2 = 0.68$, df = 1, $P = 0.410$; *G. brasiliensis*: $\chi^2 = 0.26$, df = 1, $P = 0.609$; or *L. japonica*: $\chi^2 = 0.12$, df = 1, $P = 0.914$).

We also assessed each parasitoid's response to 'volatile cues' emitted by either the fruit or fruit fly host using dual-choice bioassays with a Y-tube olfactometer, basically a glass tune that forms a "Y" and the tested gasses are passed down either of -y-tube ends and the tested parasitoid selects which path to take (Fig. 6). Our tested female parasitoids were attracted by the volatiles from SWD-infested fresh cherries more than clean air or healthy cherries, or were attracted by the volatiles from DM-infested rotten cherries vs. clean air (Fig. 6). However, there was no difference in the parasitoid's response to the volatiles from DM-infested rotten cherries vs. uninfested rotten cherries or to the volatiles of SWD-infested fresh cherries vs. DM-infested

rotten cherries (Fig. 6). In other words, these parasitoid species were attracted to fruit fly infested fruit, but it didn't matter if the fruit was infested by the common vinegar fly or SWD. The morphological evolution of SWD's ovipositor confers an adaptive advantage by allowing it to exploit a new ecological niche: young, undamaged fruit that is inaccessible to the larvae of other Drosophila species such as DM. Depsite the lack of prefernce by these parasitoids on these two hosts, SWD would mostly likely become available for these parasitoids in the fruit ripe or ripening stages, before they are available to DM. DM may dominate the Drosophila fauna in decaying fruit medium and serve as an alternative host for these parasitoids if they were released in North America. This could help these parasitoids to persist, increase the wild population sizes or enhance their activity in crops systems, and may eventually lead to an increased impact on SWD populations.

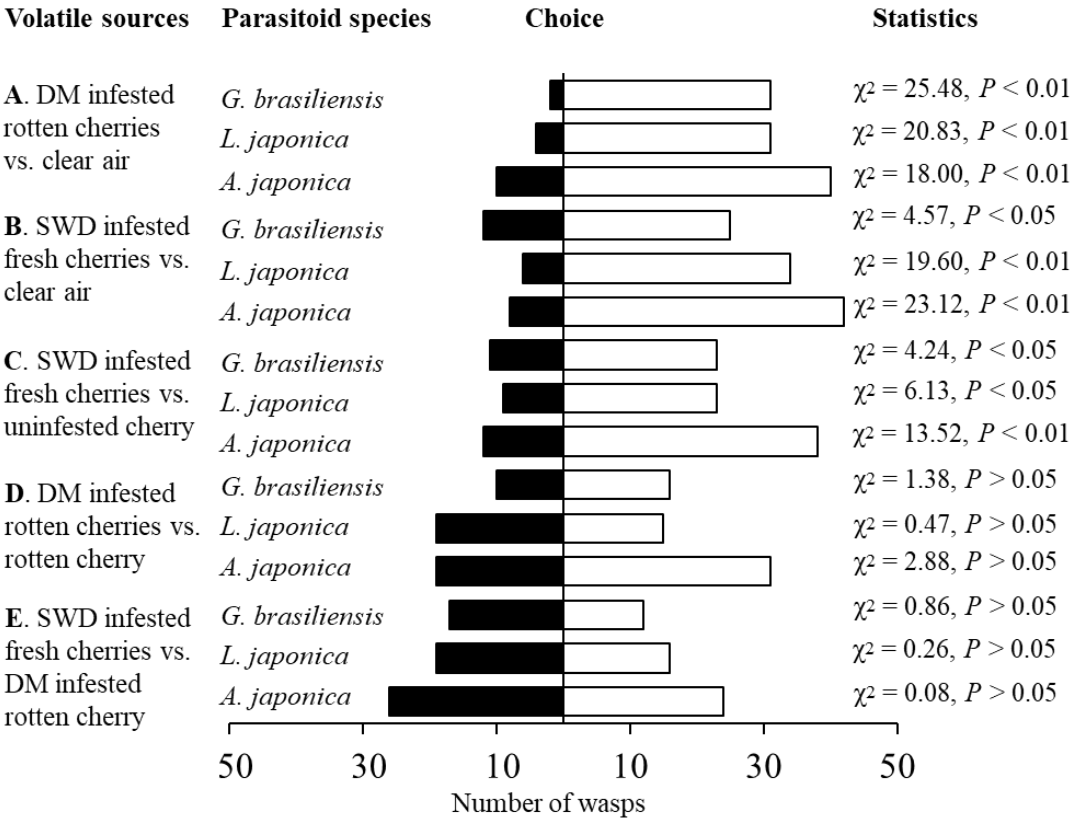

Fig. 6. Number of female *G. brasiliensis*, *L. japonica* or *A. japonica* wasps choosing odor sources in a Y-tube in five different choice tests: (A) 50g of SWD-infested fresh cherries vs. Clear air, (B) 50g of SWD-infested fresh cherries vs. 50g uninfested fresh cherries, (C) 50g of DM-infested rotten cherries vs. Clear air; (D) 50g of DM-infested rotten cherries vs. 50g of uninfested rotten cherries, and (E) 50g of SWD-infested fresh cherries vs. 50g of DM-infested rotten cherries.

2. Evaluations of indigenous pupal parasitoids

2.1. Functional response

To predict the host suppression potential by the two pupal parasitoids (*P. vindemiae* and *T. drosophilae*), the functional response of each parasitoid to eight different host densities (3, 6, 9, 12, 15, 18, 21 or 24) of SWD or DM pupae was tested. For each replicate, fly pupae were exposed to a female parasitoid for 24 h. All exposed pupae were reared until the emergence of adult wasps or flies. Each tested combination had 30–35 replicates. As a control, 10 replicates (each with 10 pupae) of each host species were held under the same conditions to estimate the natural

mortality of fly pupae that were not exposed to parasitoids. The proportion of hosts parasitized at each density was also estimated based on the number of emerged flies in the presence or absence of the parasitoid using the 'Degree of Infestation (DI) as described previously.

The number of hosts parasitized increased with increasing host density to upper limits of 18 hosts for *P. vindemiae* and 24 hosts for *T. drosophilae*. The functional response curves generated were similar between the two drosophilid species (Fig. 7). Using the data sets that were restricted to 3–18 hosts for *P. vindemiae* and 3–24 hosts for *T. drosophilae* the functional response by both parasitoids on both host species are type I. The 'area searched' was the slope of the linear cure (Fig. 7) and was 0.754 and 0.777 for *P. vindemiae* on SWD and DM, respectively, and 0.857 and 0.774 for *T. drosophilae* on SWD and DM, respectively. The number of hosts parasitized (within the density range of 3–24) was influenced by host density (χ^2 = 2285.6, df =1, $P < 0.001$), host species (χ^2 = 165.3, df = 1, $P < 0.001$) and parasitoid species (χ^2 = 10.2, $P = 0.001$). Overall, *P. vindemiae* parasitized fewer hosts than *T. drosophilae*, and more SWD were parasitized than DM by each parasitoid species (Fig. 7).

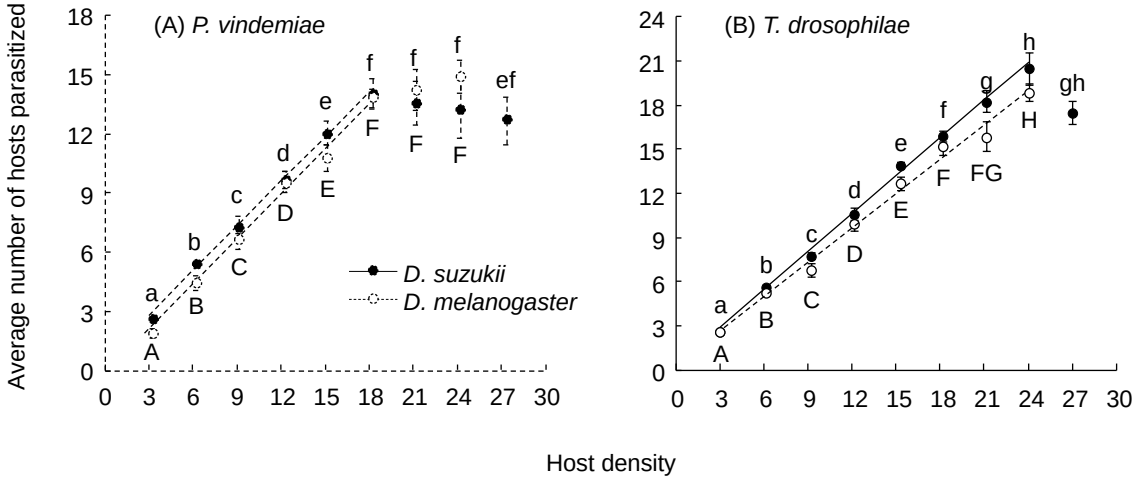

Fig. 7. Functional response of (A) *P. vindemiae* and (B) *T. drosophilae* to the host pupal density of SWD or DM. Data within the host density range from 3 to 18 (*P. vindemiae*) and 3 to 24 (*T. drosophilae*) were fitted to a linear model and are: $y = 0.594 + 0.754\ x$, $R^2 = 0.998$; $y = -0.293 + 0.777\ x$, $R^2 = 0.992$; $y = 0.311 + 0.857\ x$, $R^2 = 0.997$; $y = 0.431 + 0.774\ x$, $R^2 = 0.989$ for *P. vindemiae* on SWD and DM and for *T. drosophilae* on SWD and DM, respectively. Data (mean ± SE) were analyzed for each host-parasitoid combination separately and different letters indicate significant difference among different host densities of SWD (lower case) or DM (upper case) ($P < 0.05$).

2.2. Thermal performance

Temperature is a crucial environmental factor that affects the distribution, physiology and fitness of most animals, including insect parasitoids. How parasitoids adapt to different temperature ranges are particularly important for biological control as it can help to predict their potential geographical ranges and determine whether they can establish and proliferate if introduced in newly regions. Among-population variation in parasitoid thermal performance may occur and is also important for predicting parasitoid responses to altered climate regimes in future or novel environments. For these reasons, we compared thermal performance profiles (developmental time, survival and reproduction) between *P. vindemiae* and *T. drosophilae* and between a Californian and an Oregon population of *P. vindemiae*.

To determine each parasitoid's immature developmental time and survival, 10 host pupae were exposed to a single female wasp for 24 h in per dish at suitable room temperature (23°C),

and exposed pupae were assigned to different temperature incubators (12, 16, 20, 24, 29, 30, 31, 32 °C, 14L:10D). Relative fecundity (offspring produced over a 2-day exposure) were estimated by exposing 20 host pupae to a single female wasp at the different temperatures, and exposed host pupae were reared at the room temperature (23°C). Each temperature treatment had 30-50 replicates. The number, sex and date of emerged wasps were recorded. Immature survival was based on the initial parasitism and emerged wasps. Initial parasitism was estimated based on the rearing part of exposed pupae and unexposed pupae at the room temperature. A nonlinear developmental model was used to describe the relationship between developmental rate and temperature (see figure caption for details).

Results predict that *P. vindemiae* has a wider temperature range than *T. drosophilae* for development, but the acceptable temperature range for development is similar between the California and Oregon *P. vindemiae* populations (Fig. 8). *T. drosophilae* failed to develop above 29°C whereas *P. vindemiae* could develop at 32°C. The lower temperature threshold was similar for both species and populations (Table 1).

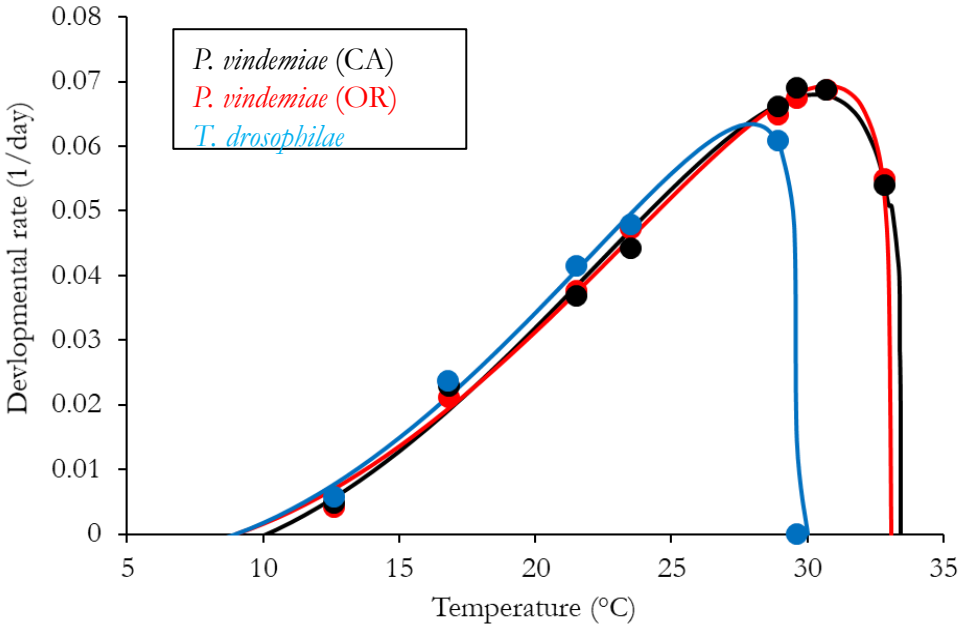

Fig. 8. Relationship between temperature and development rate (1/day) for the California (CA) and Oregon (OR) population of *P. vindemiae* and *T. drosophilae*, fitted to a nonlinear model. The model used was $D(T) = nT(T - T_b)(T_L - T)^{\frac{1}{m}}$ where $D(T)$ is the developmental rate at temperature T, with T_b, and T_L being the lower and upper thermal threshold of development, and n and m are empirical constants. The operative temperature range, defined as the difference between T_b and T_L, and the optimum temperature, defined as the temperature at which the insect develops at its maximal rate was determined. Data in mid-range of the nonlinear developmental rate model were selected to determine the best-fit by linear regression analysis.

Table 1. Estimates of base (T_b), optimal (T_{opt}) and upper (T_L) threshold temperature and thermal requirements (DD) for the development of the California (CA) and Oregon (OR) population of *P. vindemiae* and *T. drosophilae* using nonlinear and linear models

Species	Linear Model			Nonlinear Briere Model			
	Tb (°C)	DD	r^2	T_b (°C)	T_{opt} (°C)	T_L (°C)	r^2
P. vindemiae (CA)	11.1	276.1	0.997	10.1	30.3	33.4	0.998
P. vindemiae (OR)	11.0	276.3	0.995	8.9	30.7	33.0	0.995
T. drosophilae	10.1	292.7	0.983	9.0	27.9	29.6	0.925

At the lower temperatures tested, *T. drosophilae* survival rate was lower than *P. vindemiae*, but at the middle temperatures tested *T. drosophilae* survival rate was higher. than that of *P. vindemiae*. (Fig. 9A). At the highest temperature tested, the Californian *P. vindemiae* population survived better than the Oregon population. (Fig. 9A). Both parasitoid species reproduced at all temperatures, but *T. drosophilae* and the Oregon *P. vindemiae* population produced more offspring from low to middle temperatures than the Californian *P. vindemiae* population, whereas at the highest temperature the Californian *P. vindemiae* produced more offspring (Fig. 9B).

These results showed slight thermal profile differences between the two species and two *P. vindemiae* populations, with *T. drosophilae* being less cold tolerant whereas the Oregon *P. vindemiae* population was much cold tolerant than the Californian population. In addition, we also tested the effects of a varying diurnal temperature (15-32°C) that simulates the summer high temperatures in California Central Valley on the development and survival of different *T. drosophilae* stages.

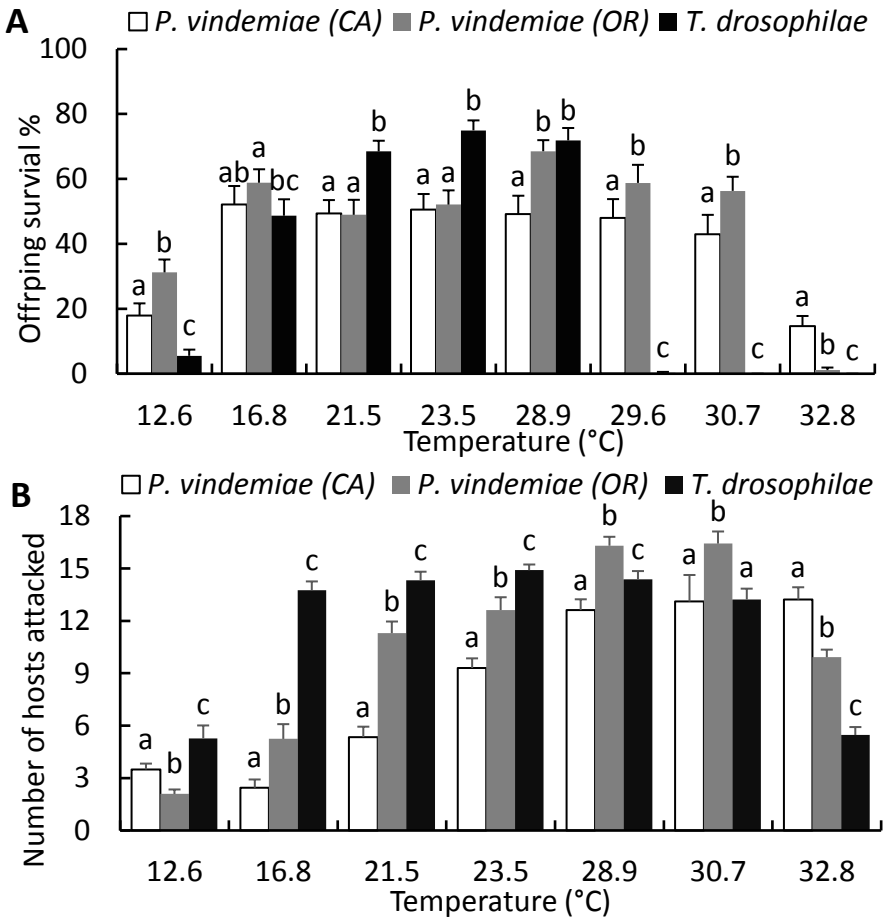

Fig. 9. Effects of temperature on the immature survival (A) and adult reproduction (B) of *P. vindemiae* (both California and Oregon populations) and *T. drosophilae*. Bars refer to mean and SE and different letters above the standard error bars indicate significant differences ($P < 0.05$).

2.3. Cold storage

Low temperature storage of parasitoids is one possible solution to the production and timely availability of parasitoids for field release during critical stages of pest outbreaks. We compared the cold tolerance of both pupal parasitoids (*P. vindemiae* and *T. drosophilae*) after they were exposed to low temperatures (10 or 12°C) at different development stages (egg, larva, pupa, and pre-adult) for a period of 1, 2 or 3 months.

To obtain parasitized hosts, 10 pupae were first exposed to a single female for 24 h in petri dish at the room temperature (23°C). Exposed pupae were then moved into the incubators (10, 12 °C, 12L: 12D) immediately when parasitoids were at the egg stage, 6 days later (at larval stage), 15 days later (at pupal stage) or 18 days later (at pre-adult stage). Parasitized hosts are recognizable after the parasitoids have developed into early larval stage since flies would have already emerged from unparasitized pupae after 6 days or developed into late adult stage with visible red eyes under microscope. Therefore, all exposed pupae were examined under the microscope to remove unparasitized (i.e. emerged fly pupae) and dead (blacken) fly pupae for the storage of parasitoid larva, pupa or pre-adult. It is impossible to determine if a host is parasitized at the parasitoid or fly pupal early developmental stage. Therefore, for the storage parasitoid egg we estimated initial parasitism of the exposed host pupae based on the rearing of parts of exposed hosts and control of unexposed hosts at the room conditions as described previously. Host pupae were returned to the room conditions after the exposure periods, and the developmental time, sex and date of emerged wasps were recorded. A sub-sample (20-25 females) of emerged female wasps were mated with emerged males and tested for a 48h fecundity by providing each female with 20 host pupae in petri dish.

Results show that at 12°C, neither eggs or larvae developed into adults after a 3-month storage period (Table 2). In contrast, all *T. drosophilae* pupae emerged within 2 months and up to 69.4% *P. vindemiae* pupae had emerged within 3 months. Almost all pre-adult had emerged within one month for both parasitoids (Table 2). After the surviving cold-stored parasitoids were returned to room temperatures, the percentage of those that developed into adults decreased with increasing storage duration but increased with the advanced development stages (Fig. 10). Both cold-stored pupae and pre-adults had > 80 survival (Fig. 10).

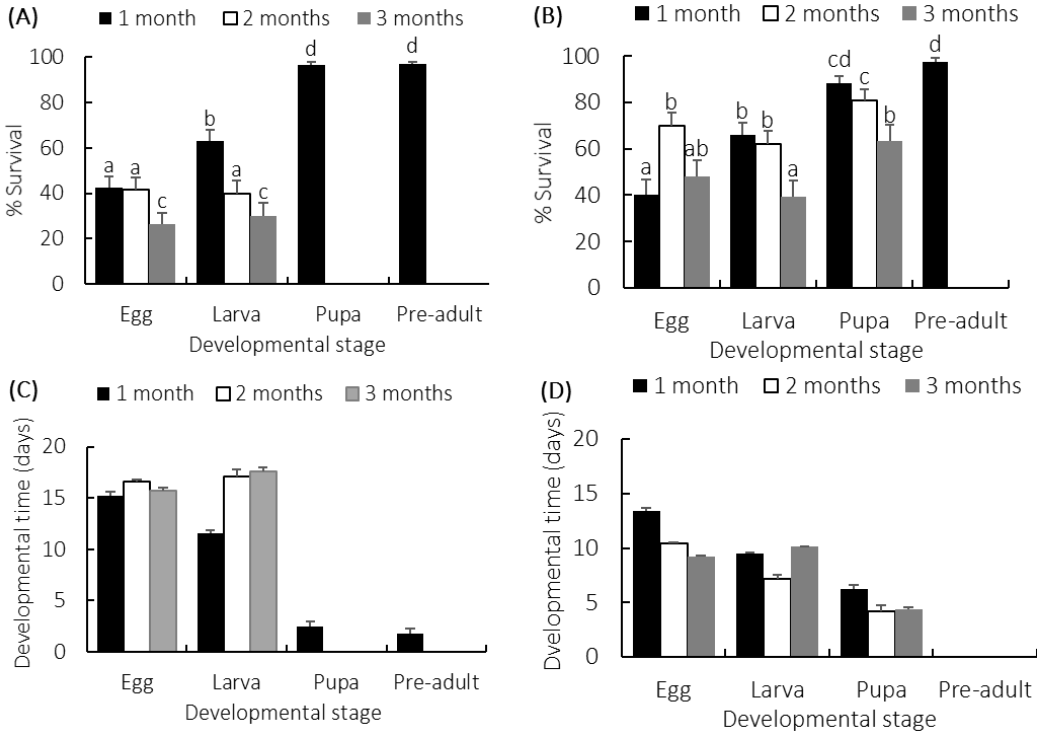

Fig. 10. Effects of cold exposure at 12°C for 1-3 months on the survival of *T. drosophilae* (A) and *P. vindemiae* (B) and post-storage developmental time of *T. drosophilae* (C) and *P. vindemiae* (D) when they parasitoids were exposed at different developmental stages. Bars refer to mean and SE and different letters above the standard error bars indicate significant differences ($P < 0.05$).

Table 2. Percentage (%) of *P. vindemiae* and *T. drosophilae* emerged during the storage at 12°C

Parasitoid	Storage duration (month)	Developmental stage			
		Egg	Larva	Pupa	Pre-adult
P. vindemiae	1	0	0	5.5	100
	2	0	0	38.2	100
	3	0	0	69.4	100
T. drosophilae	1	0	0	58.1	94.9
	2	0	0	100	100
	3	0	0	100	100

Most *T. drosophilae* pupae or pre-adult emerged within 1-3 days while developmental time for its egg or larva generally increased with storage duration. In contrast, developmental time for *P. vindemiae* generally decreased with increased storage for all developmental stages. Fecundity of emerged females after the cold exposure was not affected by the developmental stage but percentage of female offspring significantly decreased when the wasps were stored at pre-adult stage (Fig. 11). Many of the tested insects produced only male offspring after being stored at pre-adult stage (possibly because low temperature sterilizes males or females).

In conclusion, cold exposure has some sub-lethal effects on egg, larva or pre-adult stages, but the pupal stage handled cold temperature storage best and could be held at 10-12°C for 1 month with minimum lethal and sub-lethal effects. We are using these results now for our release studies in Salinas strawberries.

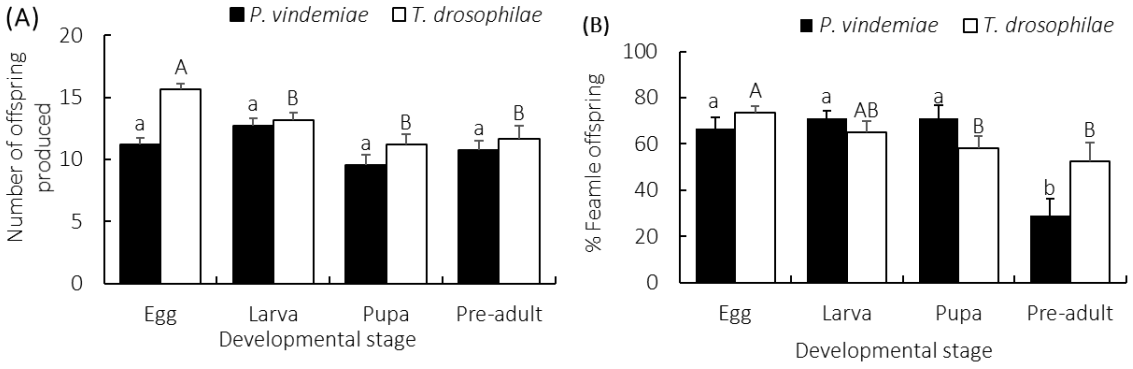

Fig. 11. Fecundity (A) and offspring sex ratio (B) of adult *P. vindemiae* and *T. drosophilae* after cold storage at different developmental stages for one month at 12 °C. Bars refer to mean and SE and different letters above the standard error bars indicate significant differences ($P < 0.05$).

3. Foreign exploration in South Korea

We conducted additional collections of native SWD parasitoids at 4 locations and two provinces in South Korea during July 2017. About 11,300 SWD pupae were collected from wild *Rubus* fruits and imported into the University of California Quarantined Facility. Of the collected pupae, about 500 wasps emerged including 440 figitids (88%), 41 braconids (8%, all *Asobara*) and 19 diapriids (8%) (all *Trichopria*). The figitids were *G. brasiliensis* (35%), *L. japonica* (13%) and one unidentified species (26%). These different parasitoids species co-occurred in all collection sites.

4. Ongoing studies

4.1. Quarantine evaluation of imported larval parasitoids

We are investigating abiotic factors (e.g. temperature) that could affect the performance and establishment potential of two selected larval parasitoids (*G. brasiliensis* and *L. japonica*). The

information will help us to predict the potential geographical ranges of these introduced parasitoids if released in North America.

4.2. Field monitoring of SWD and its associated parasitoids in California

We are also monitoring SWD population dynamics and resident parasitoid species that attack SWD in Sierra foothills using sentinel traps. Traps were baited with apple cedar vinegar or uninfested fruit and placed at different latitudes along the foothills to determine possible movement of the fly populations and host-parasitoid dynamics throughout the seasons.

4.3. Augmentative release of indigenous pupal parasitoids

Since many non-crop habitats could sever as refuges or overwintering sites for SWD in later seasons, providing source population for early generations, it is crucial to suppress source populations on non-crop hosts and post-harvest cash crops to reduce pest pressure in susceptible crops. In cooperation with Dr. Brian Hogg (USDA ARS Western Regional Research Center, Albany, CA), we are mass-producing the two pupal parasitoids (*P. vindemiae* and *T. drosophilae*) and will conduct augmentative release in the habitats surrounding crops in October 2017 (i.e. targeting overwintering populations but could potentially move into early fruit crops such as cherry).

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Spotted Wing Drosophila 2017 Progress Report –Akbari and Hay

Project Title Engineered transgenic *Drosophila suzukii* for wild population suppression and eradication: production, performance assessment, and effective wild releases.

Collaborating PIs
Bruce A. Hay, California Institute of Technology
Omar S. Akbari, University of California, San Diego.

October 11, 2017

Current Progress (2017) and Future work (2018) in *Drosophila suzukii*

Generation and testing of *D. suzukii Medea*

We have developed the first *Drosophila suzukii* functional replacement system termed *Medea*, have rigorously tested it in laboratory cage populations, and have performed crosses with different genetic backgrounds to determine effectiveness and fecundity. A manuscript detailing our findings is in review an PNAS, a portion of which is attached below. We plan to work with USDA-Aphis to begin field testing this approach, and are also developing a second-generation *Medea* system in *D. suzukii* that will be more robust in the face of genetic diversity and could be used to replace the original *Medea* in case a recall is necessary. To safeguard, reduce risk, and mitigate the spread of the *D. suzukii Medea* system into wild populations it will be important to develop a reversal *Medea* (RM) system and demonstrate that it can function as advertised. Reversing the drive of a *Medea* system has been theorized; however, it has never been experimentally demonstrated. We have engineered a reversal *Medea* construct, and will work on generating *D. suzukii* bearing this construct and testing its effectiveness.

Development of Y Drive

We have developed and tested several components that are needed to build a successful Y drive. We have engineered two separate germline-specific Cas9 lines, integrated them into *D. suzukii*, and shown that function efficiently when gRNAs are injected into said lines. We have also been able to generate targeted insertions on the *D. melanogaster* Y chromosome using CRISPR/Cas9 and are currently testing this approach in *D. suzukii*. We have also determined that the *D. melanogaster* U6 promoter needed to express gRNAs in transgenic flies does not function in *D. suzukii*, and are working on identifying *D. suzukii* specific U6 promoters and testing them in D. suzukii with gRNAs that we know work. Once we optimize the above three components, we will move on to designing and testing an X shredding system in *D. suzukii*.

Going forward with *D. suzukii Medea*

We will focus on:

A. Field testing the *D. suzukii Medea we generated*
B. Developing and optimizing a reversal *Medea*
C. Developing evolutionary stable *Medea*

Going forward with Y Drive/Homing based Suppression.

We will focus on:

A. Identifying functional U6 promoters to drive gRNA expression
B. Further characterize/Publish on developed Cas9 strains.
B. Using CRISPR/Cas9 to dock genes on the Y

Summary

In 2017, we have made considerable progress: we characterized a functional gene drive system in D. suzukii, we constructed a second-generation replacement system for this system, and we developed functional germline Cas9 expressing lines that can be used to develop Y-drive and other systems. In 2018, we will keep up this progress and if we are successful in these key lab-based experiments, our plan is still to continue with cage trials and ultimately wild releases, as outlined in our initial proposal.

Results

Generation of Synthetic *Medea* Element

To create a synthetic *Medea* element in *D. suzukii*, we engineered a *piggyBac* vector comprising a miRNA toxin coupled with a toxin-resistant antidote, inspired by the architectures used to generate previous *Medea* systems in *D. melanogaster* (37, 42). We designed synthetic miRNAs to target *D. suzukii* myd88, a highly conserved gene shown to be maternally deposited and required for dorsal-ventral patterning in the early embryo in *D. melanogaster* (43). We used the predicted *D. suzukii* female germline-specific bicoid (BicC) promoter to drive expression of a "toxin" consisting of a polycistronic set of four synthetic microRNAs (miRNAs) each designed to target the 5' untranslated region (UTR) of *D. suzukii* myd88 (Figure 1A). Importantly, to ensure these miRNAs could target the desired sequence, we performed genomic DNA sequencing of the myd88 5'UTR target region in our reference *D. suzukii* strain (collected from Corvallis, Oregon) and designed the miRNAs against this sequence (Supplementary Figure 1). This *Medea* element also contained an "antidote" consisting of the *D. suzukii* myd88 coding region, insensitive to the miRNAs as it does not contain the miRNA-targeted 5'UTR, driven by the predicted *D. suzukii* early embryo-specific bottleneck (bnk) promoter, and two separate transformation markers – eGFP driven by the eye-specific 3xP3 promoter (44), and dsRed driven by the ubiquitous hr5-IE1 promoter (45).

Characterization of *Medea* Genetic Behavior

Following microinjection of the *Medea* transgene into *D. suzukii* embryos, a single G_1 transformant male was recovered, as identified by ubiquitous hr5-IE1 driven expression of dsRed (Figure 1F), and weak eye-specific 3xP3-driven eGFP. When outcrossed to several wildtype (non-*Medea* bearing; +/+) females, this male produced roughly ~50% *Medea*-bearing and ~50% wildtype offspring, as would be expected from standard Mendelian segregation without dominance (Table 1). Resulting heterozygous G_2 *Medea*-bearing progeny were individually outcrossed to wildtype individuals of the opposite sex to determine inheritance patterns, and these individual outcrosses were continued for six generations (Table 1). Remarkably, until the G_5 generation, all heterozygous *Medea*/+ mothers (n = 91) produced 100% *Medea*-bearing progeny (n = 1028), while heterozygous *Medea*/+ fathers (n = 16) produced ~50% *Medea*-bearing progeny (n = 268). While the majority of heterozygous *Medea*/+ G_5 (23/31) and G_6 (16/25) generation females also produced 100% *Medea*-bearing progeny, some heterozygous G_5 (8/31), and G_6 (9/25) females unexpectedly produced a small yet notable number (52/1219) of wildtype offspring. Notwithstanding, individually these G_5 and G_6 heterozygous *Medea*/+ females displayed significantly biased biased inheritance rates ranging from 76%-96%, with an average rate of 86.4%. Overall, in six generations of individual female outcrosses, the percentage of *Medea*-bearing progeny borne by single heterozygous *Medea*/+ mothers (n = 147) was 97.7% (2195/2247; Table 1) as opposed to the 50% that

would be expected with standard Mendelian segregation without dominance, indicating that the *Medea* element is extremely functional at biasing inheritance.

D. Suzukii Medea Exhibits Maternal-Effect Lethality and Zygotic Rescue

To further characterize the genetics behind the highly biased inheritance patterns described above, additional crosses between individuals of various *Medea* genotypes were performed, and confirmed that *Medea* exhibits maternal-effect lethality and zygotic rescue (Table 2). For example, matings between heterozygous *Medea*/+ mothers and wildtype fathers resulted in 55.63±0.76% total embryo survival with 94.20±1.33% of the progeny being *Medea*-bearing, while matings between heterozygous *Medea*/+ mothers and heterozygous *Medea*/+ fathers yielded 79.11±3.95% total embryo survival with 94.12±0.67% of the progeny being *Medea*-bearing. The higher-than-expected embryo survival is consistent with the observation that not all heterozygous *Medea*/+ mothers give rise to 100% *Medea*-bearing progeny, indicating that not all wildtype progeny from a heterozygous *Medea*/+ mother perish.

Medea Functionality in Geographically Distinct Populations

To assess whether the *D. suzukii Medea* could function in geographically distinct populations that possibly harbor genetic variability in regions that canonically have less conservation such as the 5'UTR, heterozygous *Medea*/+ flies were tested in eight additional *D. suzukii* strain backgrounds. These strains were collected from various locations around the world, including: Mt. Hood, OR; Clayton, WA; Brentwood, CA; Tracy, CA; Watsonville, CA; Oahu, HI; Beltsville, MD; and Ehime, Japan. Interestingly, for 3/8 strains, the *Medea* inheritance rate from heterozygous *Medea*/+ mothers was 100%, while from 5/9 strains the inheritance rate ranged from 87.6% to 99.4%, with an overall transmission rate of 94.2% (Figure 2). These results strongly demonstrate that the *Medea* element described here can dominantly bias transmission in diverse *D. suzukii* populations.

Long Term Population Cage Experiments

The above observations suggested that *D. suzukii Medea* should be able to drive robust population replacement. To test this prediction, after mainiting this population for at least 8 generations, we mated *Medea*-bearing fathers to wildtype Corvallis, OR, strain mothers at three distinct introduction frequencies: low frequency (equal numbers of heterozygous *Medea*/+ and wildtype +/+ fathers mated to wildtype +/+ mothers); medium frequency (equal numbers of heterozygous *Medea*/+ fathers to wildtype +/+ mothers); and high frequency (equal numbers of homozygous *Medea*/*Medea* fathers to wildtype +/+ mothers). These experiments were conducted in separate bottles in biological triplicate for the low and medium threshold and quadruplicate for the high threshold drives, producing ten distinct populations with initial *Medea* allele frequencies ranging from ~12.5-50%. Altogether, these population cage experiments were followed for 9 generation (for lower allele frequency populations, as the *Medea* allele disappeared from the population by that time) or 19 generations (for higher allele frequency populations), counting the number of *Medea*-bearing adults each generation, as described previously (35, 37). Interestingly, the observed changes in *Medea* frequency over time indicated that, for release proportions of 50% or smaller, the *D. suzukii Medea* element was unable to drive into the wildtype population, likely because of selected drive resistance combined with high fitness costs outweighing the effect of drive. However, at higher introduction frequencies of >90%, similar to classical chromosomal rearrangement thresholds (46), the drive largely compensated for the fitness cost, allowing the construct to remain in the population at high frequencies for the duration of the experiment (19 generations; Figure 3).

Molecular Characterization of Resistance

To understand whether resistance of the target mRNA to the toxin played a role in observed *Medea* inheritance rates of <100%, we performed genomic DNA sequencing of the myd88 5'UTR miRNA target region from randomly selected *Medea*/+ and +/+ progeny from generation 19 of the highest-threshold drive experiments described above to determine whether the miRNA target sites

contained any mutations as compared to our reference strain (against which the miRNAs were designed). Genomic sequence analysis revealed that, out of 4 miRNA target sites, one to two sites were perfectly conserved in *Medea*/+ individuals (site #4 or sites #1 and #4, depending on the individual), while only one (site #4) was perfectly conserved in +/+ individuals (Supplementary Figure 1); additionally, for sites that had mutations, some of the mutations were found in both *Medea*/+ and +/+ flies (for sites #2 and #3), while others were only found in one type of fly or the other (for site #1). To further this analysis, we also sequenced +/+ individuals from all of the geographically distinct populations tested for *Medea* functionality (shown in Figure 2) , and discovered a similar trend - i.e., that only one of the four miRNA target sites was perfectly conserved (#4), two others (#2 and #3) had the same mutations in all strains (including the *Medea*/+ and +/+ individuals from the drives described above), and a third site (#1) had variable mutations that may correlate with *Medea* efficiency. Together, these observations indicate that the nature of mutations differed between backgrounds with different observed *Medea* inheritance rates, suggesting that the efficiency of the miRNA "toxin" is likely influenced by resistance alleles, which influence *Medea* transmission.

Mathematical Modeling

To characterize the population dynamics observed in the above cage experiments, we fitted a mathematical model to the observed data in which the *Medea* element had an associated fitness cost in heterozygotes and homozygotes and there was a *Medea*-resistant allele present in the population that reduced toxin efficiency. For the fitted model, the *Medea* element was estimated to have a toxin efficiency of 93% in individuals homozygous for the resistant allele (95% credible interval (CrI): 90-95%) and was assumed to have a toxin efficiency of 100% in individuals lacking the resistant allele. The *Medea* element was estimated to confer a large fitness cost on its host - 28% in heterozygotes (95% CrI: 27-30%) and 65% in homozygotes (95% CrI: 62-67%) - and the resistant allele was estimated to have an initial allele frequency of 78% in the population (95% CrI: 57-97%).

Predictive mathematical modeling based on these parameter estimates suggests that the *Medea* element would spread to fixation in the absence of toxin resistance if released above a threshold frequency of 79% (Figure 4A). Spread to fixation would also be expected if the fitness costs of the generated *Medea* element were halved (Figure 4C), even if all individuals in the population were homozygous for the *Medea*-resistant allele (Figure 4D), provided the element was released above a threshold frequency of ~25-27%. Consistent with the experimental results (Figure 3), a *Medea* element with a large fitness cost in a *Medea*-resistant population is expected to be maintained at high frequencies through its drive; however, its eventual elimination is inevitable unless supplemental releases are carried out. However, for high release frequencies (90-95%), the element may be maintained at high frequencies (>75%) for ~20 generations (Figure 4B), which likely exceeds the duration required for agricultural impact. Of note, the ability of the drive to counteract large fitness costs is significant, as demonstrated by comparison to non-driving alleles with analogous fitness costs that rapidly decline in frequency following a 95% release (black lines in Figures 4A and 4C).

Discussion

This study represents the first comprehensive characterization of a fully functional *Medea*-based gene drive element being challenged with pre-existing resistance in a long term multi-generational population cage experiment (19 generations). The synthetic *Medea* element described here showed maximal levels of dominance, up to 100% in some populations, but <100% inheritance bias in other populations. We hypothesized that this difference could be attributed to the presence of resistance arising from naturally occurring genetic variation that rendered certain embryos immune to the miRNA toxin. This hypothesis is supported by the sequencing data, as many of the sequenced miRNA target sites contained mutations that likely affected miRNA function and lowered toxin efficiency. Although we did not attempt to measure individual miRNA efficiency, it is possible that not all of the miRNAs are effective at target gene knockdown, and that particular target site mutations reduce toxin efficiency significantly enough to allow survival of some wildtype individuals. This is supported by sequencing data

collected from the eight distinct geographic populations, which suggests that certain target site mutations are correlated with incomplete *Medea* dominance patterns.

The above observations highlight the importance of resistance as a possible impediment to the use of gene drives, including toxin-antidote drive systems, in the field (18, 26, 28). Multiple recent studies have highlighted resistance as a major obstacle to gene drive, mostly in the context of homing-based CRISPR/Cas9 drives (47–51). Although a *Medea* system may be less prone to resistance-associated spread impediment because, unlike homing-based drive, its mechanism of action is not likely to generate resistant alleles (18, 48), it will face pre-existing resistant alleles given the natural genetic diversity found in wild populations. Furthermore, such mutations would be expected to face strong positive selection and increase in frequency over time, which would likely expand their effect. Therefore, any meaningful attempt at generating a *Medea*-based gene drive system capable of manipulating diverse wild populations must plan for, and mitigate the effects associated with, resistant alleles and fitness costs.

This may be achieved in several ways. Firstly, sequencing-based characterization of naturally occurring genetic variation in geographically distinct target populations can help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. Secondly, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5'UTR, which canonically have higher tolerance for sequence variation. Thirdly, the choice of multiple target sites that have been validated to achieve knockdown and the creation of a polycistronic "toxin" can ensure that toxin efficiency is maximally high. Fourthly, verifying that the "antidote" components (e.g., the recoded targeted gene and promoter used to express said gene) function efficiently enough to restore wildtype function can ensure that an imperfect "antidote" does not impose fitness costs. Finally, reducing the expression of the marker gene to a specific tissue type will likely reduce some of any possible fitness costs associated with high ubiquitous overexpression of an exogenous gene.

That said, modeling results suggest that a *Medea* element having a high fitness cost and high (though imperfect) toxin efficiency may be capable of maintaining itself in a population for a period of several years following a series of large-scale releases of homozygous males. Either decreasing the fitness cost of the element or minimizing resistance to the toxin are expected to enable the element to spread to fixation above a release threshold of ~25-79% (the lower bound corresponds to halved fitness costs). While the stated release thresholds are high, they may be achievable given multiple successive releases (releases associated with the classical sterile insect technique for the Mediterranean fruit fly were of an even higher magnitude (52)), and may be desirable for biosafety considerations related to novel genetic control strategies.

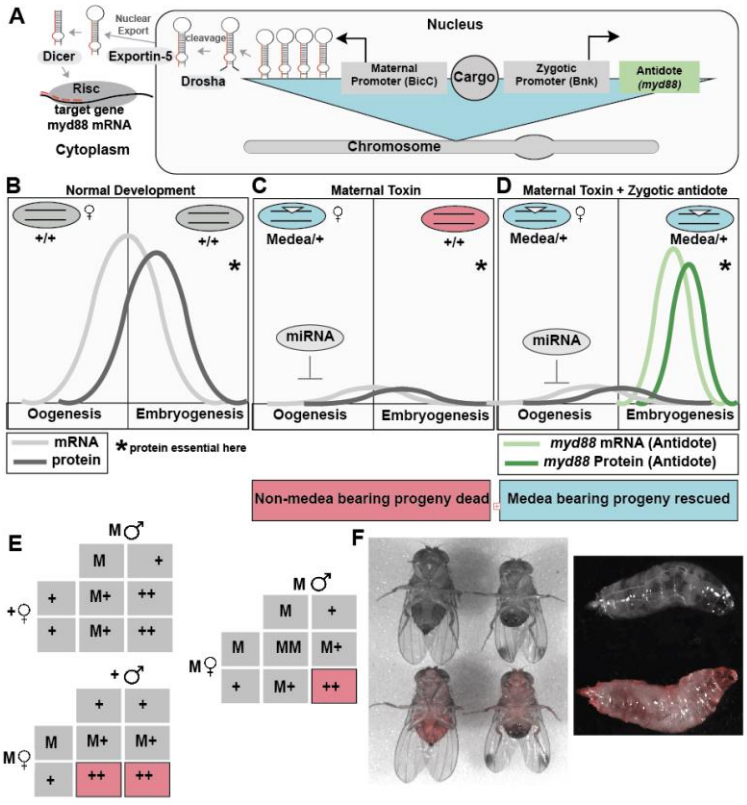

Figure 1. A synthetic *Medea* element in *D. suzukii*. *D. suzukii Medea* transgene was generated to comprise a miRNA "toxin" targeting the 5' UTR of *D. suzukii* myd88 expressed under the predicted *D. suzukii* female germ-line–specific bicoid (BicC) promoter, an "antidote" consisting of *D. suzukii* myd88 coding region driven by the predicted *D. suzukii* early embryo–specific bottleneck (bnk) promoter, and two separate transformation markers – eGFP under control of the eye-specific 3xP3 promoter, and dsRed under control of the ubiquitous hr5-IE1 promoter (A). During normal development maternal myd88 deposited into the embryo, where it is required for normal development (B). The *Medea* miRNA toxin targets myd88 mRNA during oogenesis, preventing proper deposition into the embryo and causing embryonic lethality in progeny that lack the *Medea* element (C). In embryos that possess a copy of the Medea element, a version of myd88 that is insensitive to the miRNA toxin is expressed during early embryogenesis, rescuing miRNA-induced lethality (D). When heterozygous Medea males are crossed out to wild type females, all progeny survive since the maternal toxin is not expressed; however, when heterozygous *Medea* females are crossed to wild type males, 50% of the progeny - the ones that fail to inherit *Medea* perish. When heterozygous females are crossed to heterozygous males, 75% of the progeny inherit *Medea*, either from the mother or the father, and survive, while those that fail to inherit a *Medea* element perish (E). The hr5-IE1 promotes robust expression of dsRed in both D. suzukii adults and larvae, allowing for facile identification of Medea-bearing individuals (F).

Figure 2. *Medea* functions in diverse populations of *D. suzukii*. Heterozygous *Medea*/+ individuals were crossed with eight geographically distinct *D. suzukii* populations and *Medea* inheritance was measured. Overall, *Medea* biased inheritance with rates ranging from 87.6-100%, suggesting that a *Medea* system generated in the laboratory could be utilized to manipulate some, but not all, diverse wild populations of *D. suzukii*. Green stars indicate the collection locations of the flies tested, green pie charts indicate the percentage *Medea* inheritance observed from heterozygous *Medea*/+ females, and shaded areas on the map indicate locations where *D. suzukii* populations have been confirmed. The Corvallis, OR, strain was our reference *D. suzukii* strain used to engineer the *Medea*.

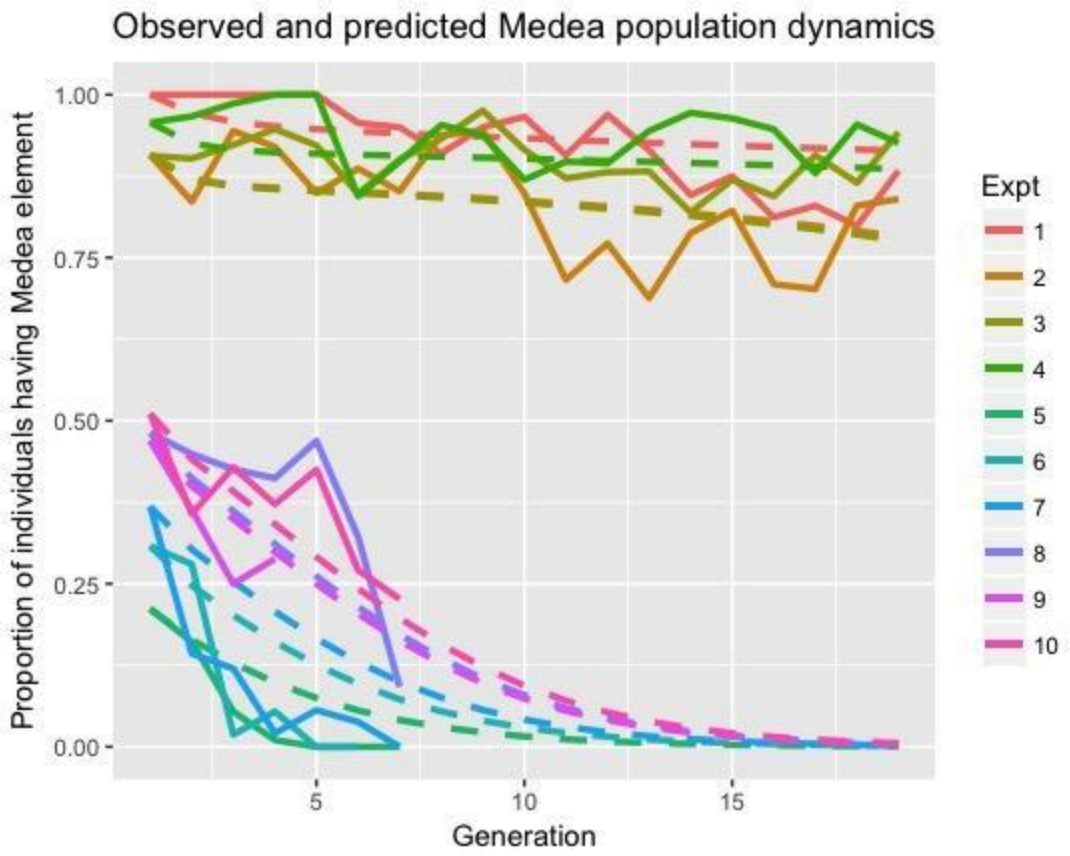

Figure 3. Observed and predicted dynamics of *D. suzukii Medea* element. Population cage experiments were set up by mating wild type (+/+) and homozygous *Medea* males (*Medea*/*Medea*) with wild type (+/+) females, producing a frequency of heterozygotes (*Medea*/+) in the first generation of 21-100%. Population counts were monitored over 19 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Observed data are consistent with a toxin efficiency of 100% in *Medea*-susceptible mothers, 93% in *Medea*-resistant mothers (95% CrI: 90-95%), a heterozygote fitness cost of 28% (95% CrI: 27-30%), a homozygote fitness cost of 65% (95% CrI: 62-67%), and an initial resistant allele population frequency of 78% (95% CrI: 57-97%). For high initial heterozygote frequencies (90-100%), the element is capable of manipulating inheritance in its favor in order to maintain its presence at high population frequencies, despite a fitness cost. For lower initial heterozygote frequencies (~50% or less), the element is eliminated from the population.

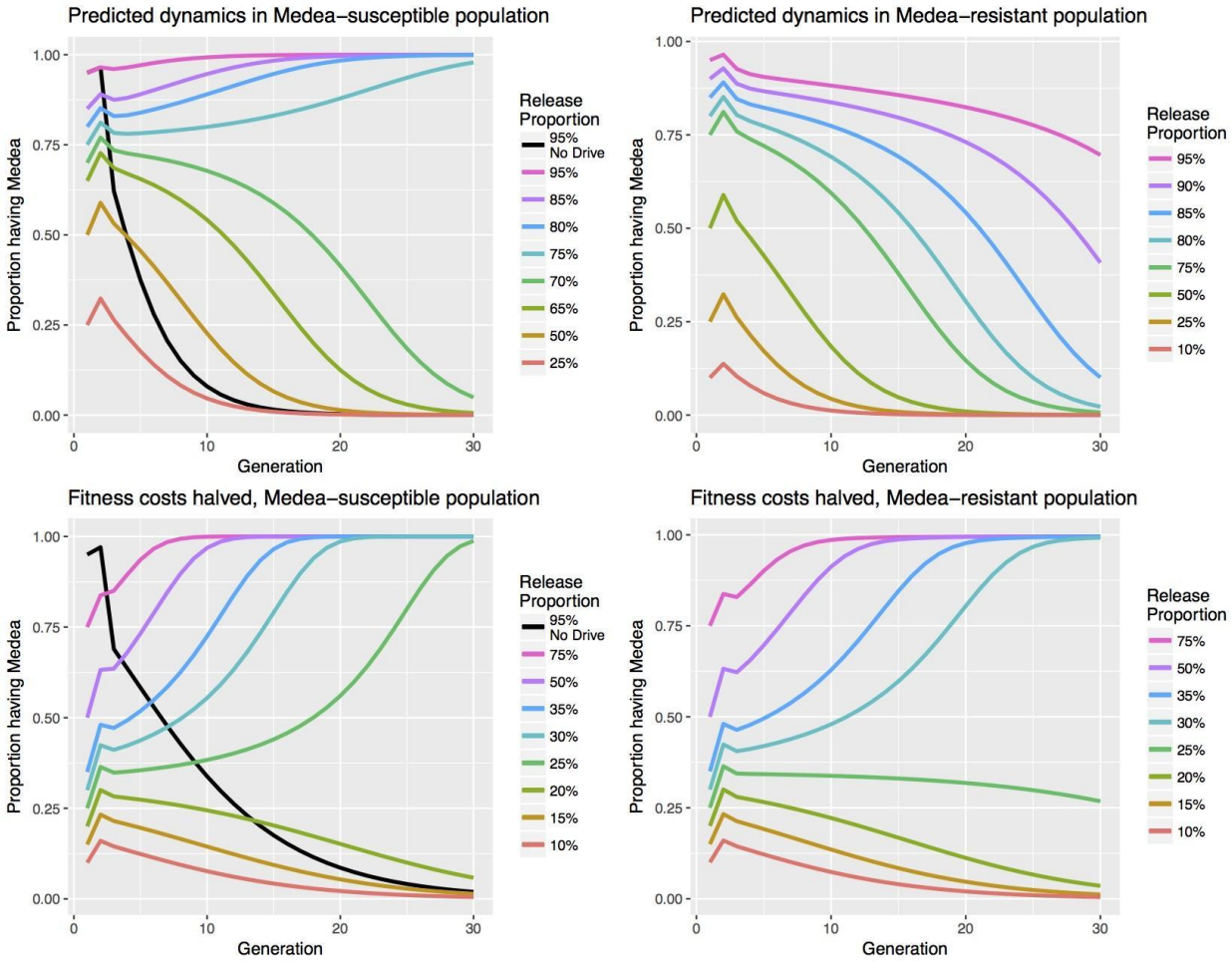

Figure 4. Predicted dynamics of *D. suzukii Medea* element. In all cases, releases of homozygous *Medea* males are assumed, except for black lines, which describe the dynamics of an equivalent release of non-*Medea* males. (A) In a *Medea*-susceptible population, the generated element (toxin efficiency = 100%, heterozygote fitness cost = 28%, homozygote fitness cost = 65%) displays threshold dynamics, spreading to fixation for release proportions of 73% or higher. (B) In a *Medea*-resistant population in which toxin efficiency is 93%, as inferred from the laboratory studies, the *Medea* element can be maintained at high frequencies following a high release proportion; however, its eventual elimination is inevitable unless supplemental releases are carried out. (C) In a *Medea*-susceptible population, if fitness costs are halved, the element displays threshold dynamics, spreading to fixation for release proportions of 23% or higher. (D) In a *Medea*-resistant population, if fitness costs are halved, the critical release threshold is raised slightly to 25%.

Generation	Sex (# crossed)	# of Progeny	Average % Medea
G_1	♂ (1)	22	54.5%
G_2	♀ (9)	126	100%
G_2	♂ (3)	45	43.3%
G_3	♀ (32)	299	100%
G_3	♂ (12)	201	48.8%
G_4	♀ (50)	603	100%
G_5	♀ (31)	785	96.8%
G_6	♀ (25)	434	93.8%
***Medea*+/total individuals from females (147)**		**2195/2247**	**97.7%**

Table 1. *D. suzukii Medea* shows predicted genetic behavior. Results of heterozygous *Medea D. suzukii* individual fly outcrosses to wild type *D. suzukii*. G_1 indicates the offspring from injected G_0 individuals, with subsequent numbers (G_2-G_6) indicating subsequent generations.

Parental Genotype			Embryo Survival %		Adult Transgenic %	
♀	♂	**Progeny Genotype (%)**	**Predicted**	**Observed**	**Predicted**	**Observed**
M/M	M/M	M/M (100%)	100	97.63±0.25	100	100
M/M	+/+	M/+ (100%)	100	95.67±1.87	100	100
+/+	M/M	M/+ (100%)	100	97.07±0.42	100	100
M/+	M/+	M/M (25%) M/+ (50%) +/+ (25%)	75	79.11±3.95	100	94.12±0.67
M/+	+/+	M/+ (50%) +/+ (50%)	50	55.63±0.76	100	94.20±1.33
+/+	+/M	M/+ (50%) +/+ (50%)	100	89.74±1.39	50	48.39±3.22

Table 2. *D. suzukii Medea* chromosomes show maternal-effect lethality and zygotic rescue. Crosses between parents of specific genotypes (indicated in the two leftmost columns) were carried out, and progeny survival to crawling first-instar larvae was quantified (third column from right). M indicates *Medea*, + indicates wild-type, red text indicates genotypes expected to be inviable. The percentage of transgenic adults resulting from each cross type, together with the standard deviation, was quantified (rightmost column).

Strain	Target Sequence	# of Flies Sequenced (n)
Corvallis, OR (reference strain)	Target 1 Target 2 Target 3 Target 4 ATCTGAAAAAAATTAAAAAAAATAGTAATA....TCCCGCGCTTCATCGTTTTATT....ACTGAAAAACGTCCCGTTGATAAATACATATATCATCG	4
Medea+ from drive (2 alleles)	ATCTGAAAAAAATTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG ATCTGAAAAAAATTTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	28
Medea- from drive (2 alleles)	ATCTGAAAAAAATTTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG ATCTGAAAAAAATTAAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	26
Clayton, WA	ATCTGAAAAAAATTAAAAAAA_TAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	16
Brentwood, CA	ATCTGAAAAAAATTAAAAAAA_TAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	10
Oahu, HI	ATCTGAAAAAAATTAAAAAAA_TAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	9
Enime, Japan	ATCTGAAAAAAATTTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	10
Beltsville, MD	ATCTGAAAAAAATTAAAAAAA_TAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	6
Mt. Hood, OR	ATCTGAAAAAAATTTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	14
Watsonville, CA	ATCTGAAAAAAATTTAAAAAAAATAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	21
Tracy, CA	ATCTGAAAAAAATTAAAAAAA_TAGTAATA....TCACGCGCTTCATCGTTTTATT....ACTGATAAACGTCCCGTTGATAAATACATATATCATCG	9

Supplementary Figure 1. Genomic DNA sequences of the myd88 5'UTR region of various strains/fly types targeted by the *Medea* toxin miRNAs. Green and black nucleotides represent sequence perfectly complementary to the miRNAs; red and other color nucleotides represent specific mutations and target sites that are not perfectly complementary to the miRNAs, respectively. Target site four is not highlighted/underlined as it is perfectly conserved among all sequenced flies.

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University of California

Agriculture and Natural Resources

PROJECT REPORT

Project Year 2016-17

Duration of Project: 2 year (no-cost extension of the project into the 2nd year)

Project Leader Jhalendra Rijal

Location UCCE-Stanislaus

Cooperating Personnel Joseph Grant, UCCE San Joaquin

Project Title Oviposition Deterrents and Insecticides for Spotted Wing Drosophila Control in Cherry

Problem and Previous Research Accomplishments:

Out of ~1500 *Drosophila* species worldwide, Spotted Wing Drosophila (SWD) is one of the two species capable of depositing eggs on healthy and ripening fruits because female SWD is equipped with a serrated (i.e., saw-like) ovipositor (i.e., egg laying apparatus) for depositing eggs inside fruits. One female is capable of laying more than 300 eggs during her lifetime and, in most instances, one fruit is infested with multiple larvae. The ovipositor is capable of incising the intact fruit skin rendering the cherry fruit with typical oviposition scars. Direct damage on fruits by internal-feeding larvae of SWD leads to fruit tissue damage and ultimately the fruit collapse. Fruits injured by oviposition and feeding become an easy target for several other pests such as vinegar flies and other secondary infections, which are otherwise not a threat to intact fruits. In addition to indirect damage associated with secondary pest and disease incidence, there is a high risk of fruit lots being rejected during the processing and/or exporting of fruits if SWD infestation is found on fruits. Thus the economic threshold for this pest in cherry is 'zero' in practical terms.

Because of its wide host range, unique egg-laying behavior, high fecundity, and a large number of generations per season, damage by SWD in susceptible fruits such as cherry become severe very quickly. Current management practices for SWD in California cherry production rely heavily on a limited number of insecticides, particularly of pyrethroid and spinosyn products. Frequent use of these insecticides can lead to pest resistance, adversely affect natural enemy populations, and lead to an outbreak of secondary pests such as scale insects. Also, use of the insecticide close to harvest can lead to unacceptably high

residue levels in fruits.Given this situation, exploring alternative measure(s) that can reduce the fruit damage by SWD, while minimizing insecticide-related problems is crucial. One option worth exploring to achieve this is to use oviposition deterrents activity of the commercial neem-based products. There are reports in pest management literature that this approach is working against some fruit fly and some *Drosophila* species in several crops. Several concentrations (0.2-4%) of neem seed kernel (NSK) extracts (in acetone) have reduced oviposition activity. The Oriental fruit fly, *Bactrocera dorsalis* egg deposition by 87.5-99.2% in guava fruit in choice tests (Chen et al. 1996). Similarly, acetone-based extract of deoiled NSK powder has significantly deterred oviposition by some tephritid fruit flies (*B. dorsalis* and *B. cucurbitae*) (Singh and Singh 1998). Some compounds derived from plant and microbes have shown repellent effects on vinegar fly (*D. melanogaster*) in laboratory bioassays (Devaud 2003, Inamdar et al. 2010). In addition to oviposition activity by neem-products, we have also looked at the efficacy of some commercial and/or experimental products against SWD mortality under laboratory condition.

Objectives and Anticipated Outcomes:

1. To evaluate oviposition deterrent activities of neem-based commercial products (containing either Azadirachtin or Clarified Hydrophobic Neem Oil Extract or both) in the laboratory using various combinations of choice and no-choice bioassays.
2. To test reduced-risk insecticides against SWD under laboratory condition.

Anticipated Outcomes. This project will ultimately help in improving current pest management practices targeting SWD in California cherry production by incorporating oviposition deterrent products in combination with other control methods. This approach may be beneficial in reducing the issues related to insecticide-focused pest management program of controlling spotted wing drosophila in cherries.

Plans and Procedures:

SWD rearing. SWD stock population was obtained from Dr. Chiu lab at UC Davis and establish colony at UCCE-Stanislaus using the Jazzmix-based fly diet. For diet preparation, 10 g Jazzmix was added to 70 ml of distilled water, boil it for about 2 min. The mix was poured into the Drosophila rearing vials and allow to cool overnight. Place roughly 10 flies (both males and females) were released into the vials for egg laying. The newly emerged adults were collected from the vials and used in studies.

Cherry fruit source. Cherry fruits with the same level of ripeness visually were collected from stores. Fruits were washed thoroughly to remove any external dirt, and other potential contamination, and

allowed to air dry for ~20 min. under room temperature. Cherries with peduncle were used for several no-choice and choice bioassays.

Effect of neem products on oviposition using no-choice studies. Cherry fruits treated singly with each of individual treatments were hung on the lid inside the small plastic cups (12 oz.) with a screened lid were used. The other sets were treated with distilled water (control) and use as the control. Several neem-based and other products were evaluated. 3 female and 2 male SWD flies (age: 7-10 days old) were released into the container to allow egg laying on fruits. Each set of the trial had ten replicates for each of treatment and control. The fruits were inspected for oviposition stings (oviposition scars on the fruit) at 24 hours. 2-3 repetitions of the trial were conducted for each product tested.

Effect of neem products on oviposition using choice studies. Single fruit treated with one of the neem and other products was hung in one quadrant inside the ventilated container (36 oz.) while an untreated fruit (i.e. control) was attached to the opposite quadrant of the container. 5 female and 3 male flies (age: 7-10 days old) were released into the containers to allow egg laying. Fruits were inspected and number of oviposition marks (i.e. stings) were counted using dissecting microscope after 24 hours

Effect of cyclaniliprole on SWD adult mortality. Laboratory bioassays were conducted by exposing SWD adults to a diamide insecticide, cyclaniliprole (rates: 12 oz/acre and 16 oz/acre). Cherries were treated with the insecticide and hung inside a ventilated cup (12 oz.). 10 adults of the same age (7-10 days old) were released into the container and closed the lid. Bioassay was conducted at room temperature condition. The mortality of the flies was recorded at 1, 4, 7 days after treatment (DAT).

Table 1. Neem products used to conduct SWD oviposition deterrent studies-2016

Treatments	Active Ingredient	Rate
Bonide Neem Oil	Neem oil extract 0.90%	0.90%
Trilogy	Neem oil extract 70%	1%
Debug Turbo	67% (Neem oil extract + Azadirachtin)	1 quarts/100 gallon water
Triple Action Neem	Neem oil extract 70%	1 fl oz/gallon water
Neemix 4.5	Azadirachtin 4.5%	4 oz/acre
Neemix 4.5	Azadirachtin 4.5%	8 oz/acre
Azamax	Azadirachtin 1.2%	32 oz./gallon water

2017 studies. In 2017, the objective was to test some new and potential natural insecticides. Based on recent work, methyl benzoate, a natural volatile compound produced by several plant species such as petunia, snapdragon showed ovicidal activity against SWD eggs in blueberries (Feng and Zhang 2017;

Scientific Reports, 7:42168, DOI: 10.1038/srep42168). Following similar protocol, we conducted studies to look at the effect of methyl benzoate against SWD in cherries. We separated 40 cherries, put them in a small cage (13.5 x 13.5 x 24"), and 100 adult flies (male and female mixed) were released into the cage for 5 days to allow egg laying. After that, fruits were divided into two groups (20 in each). One group of fruits was dipped into 1% Methyl Benzoate solution (1:1::MB:Tween 20 plus Tween 80) while the other group of fruits was dipped into the solvent (i.e.Tween 20 plus Tween 80). Fruits were dried for ~30 min, and held separately in plastic containers in the lab and inspected for adult emergence after 7 days. The adult emergence was recorded.

In addition, we also evaluated some other insecticides such as Rimon (novaluron) and Dimilin (diflubenzuron) for their effectiveness not as the insecticide, but as the oviposition deterrence or ovicidal effect.

Results

Effect of neem products on oviposition using choice and no-choice studies-2016

Bonide neem oil. In choice tests, significantly less number of oviposition stings was recorded in Bonide neem oil treatment compared to the control (Fig 1).

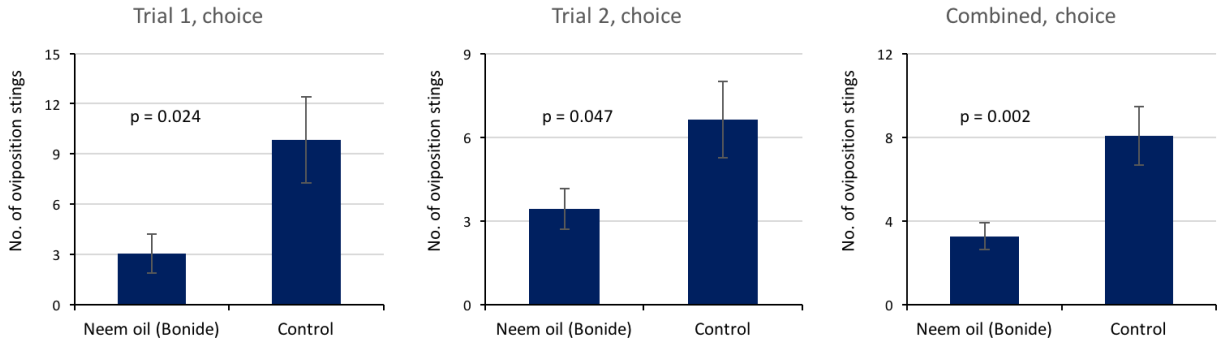


Fig. 1. Effect of Bonide neem oil on SWD oviposition in choice tests

In no-choice tests, significant oviposition activity was observed in Bonide neem oil treated cherries compared to the control (Fig 2).

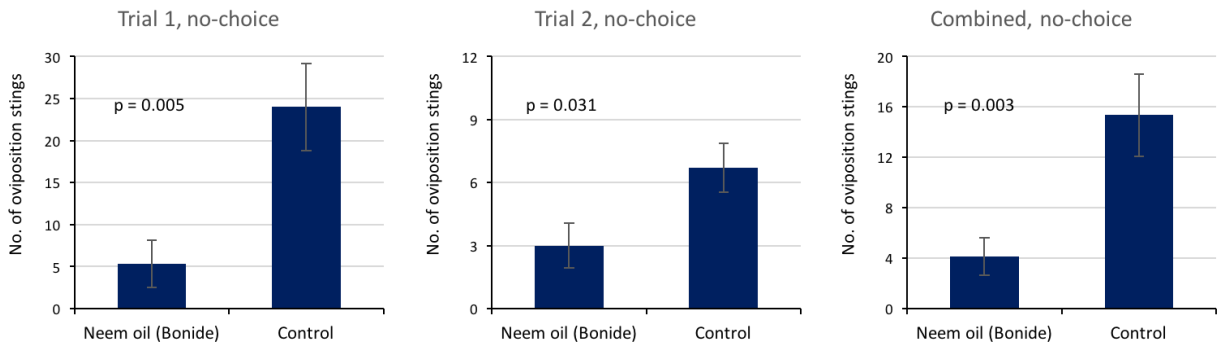


Fig. 2. Effect of Bonide neem oil on SWD oviposition in no-choice tests

Trilogy. Although oviposition sting counts were numerically higher in control compared to the Trilogy treatment in two sets of the choice tests conducted, no statistical difference was observed in no-choice studies. However, oviposition activity was significantly reduced in Trilogy when combined data from the two trials (Fig 3).

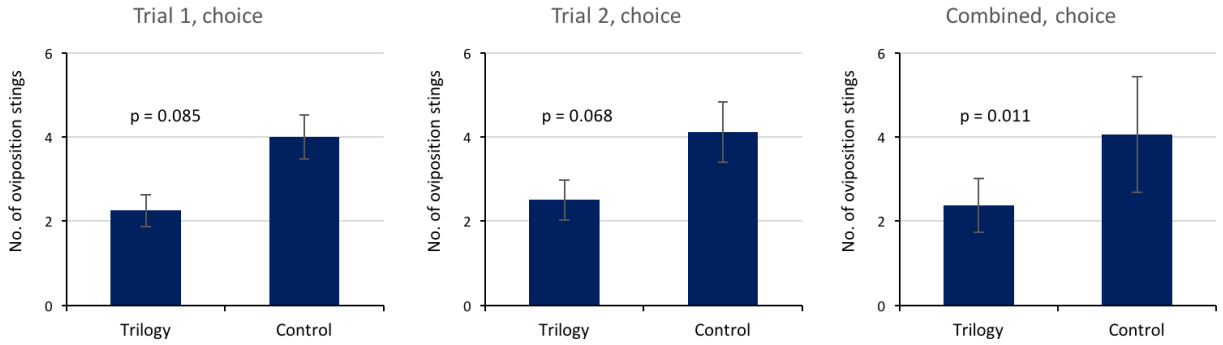


Fig. 3. Effect of Trilogy on SWD oviposition in choice tests

In no-choice tests, Trilogy did not perform well in reducing the oviposition activities (Fig 4)

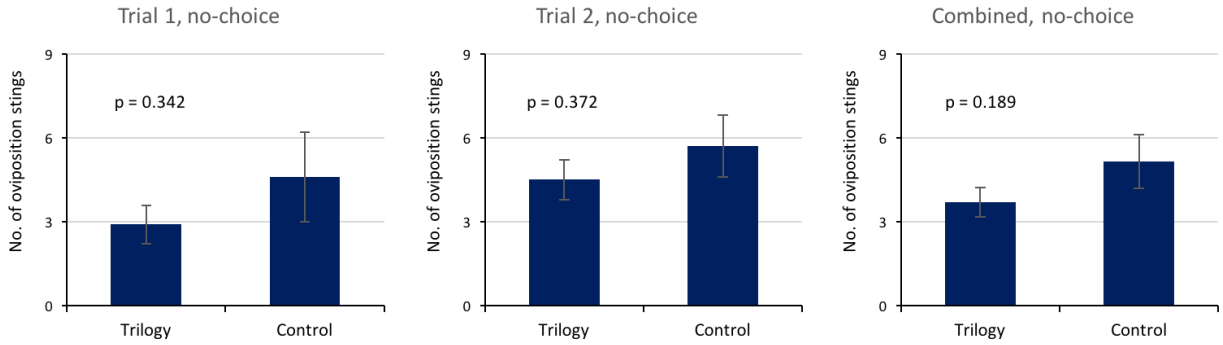


Fig. 4. Effect of Trilogy on SWD oviposition in no-choice tests

Debug Turbo. No significant effect of Debug Turbo was observed in no-choice test and two separate sets of choice bioassays. The effect was statistically significant when combined data from two sets of the choice tests (Table 2).

Table 2. Effect of Debug Turbo on SWD oviposition							
		Average no. of oviposition stings	SE	N	P-value	t	df
No-choice test							
	Treated	6.7	0.790569	10	0.325006	2.100922	18
Trial 1	Control	9.3	1.498888				
Choice test							
	Treated	2.53	0.567926	15	0.192368	2.048407	28
Trial 1	Control	3.93	0.880837				
	Treated	3.125	0.790569	16	0.123185	2.042272	30
Trial 2	Control	5.25	1.498888				
	Treated	2.84	0.420181	31	0.041938	2.000298	60
Combined	Control	4.614	0.742963				

SE=standard error, t = t-test value, df = degree of freedom

Triple Action Neem. We were able to conduct one set of choice test using this product, and there was a statistical difference between the treated and control (Table 3).

Table 3. Effect of Triple Action Neem on SWD oviposition						
Choice test	Average no. of oviposition stings	SE	N	P-value	t	df
Treated	8.07	1.64	15	0.003394	2.048407	28
Control	17.47	2.43				

Azamax. Azamax did not reduce the oviposition activities of the flies effectively (Table 4).

Table 4. Effect of Azamax on SWD oviposition						
	Average no. of oviposition stings	SE	N	P-value	t	df
No-choice test						
Treated	1.5	0.428174	10	0.073471	2.100922	18
Control	2.8	0.533333				
Choice test						
Treated	0.6875	0.222673	10	0.512312	2.042272	18
Control	0.875	0.279881				

Neemix. There was no statistical difference between Neemix and control treatments when used at the rate of 4 oz/acre. However, increased rate Neemix (8 oz/acre) showed a significant reduction in oviposition

activities (Fig. 5). Only single set of choice bioassays was conducted for the Neemix evaluation due to time constraint.

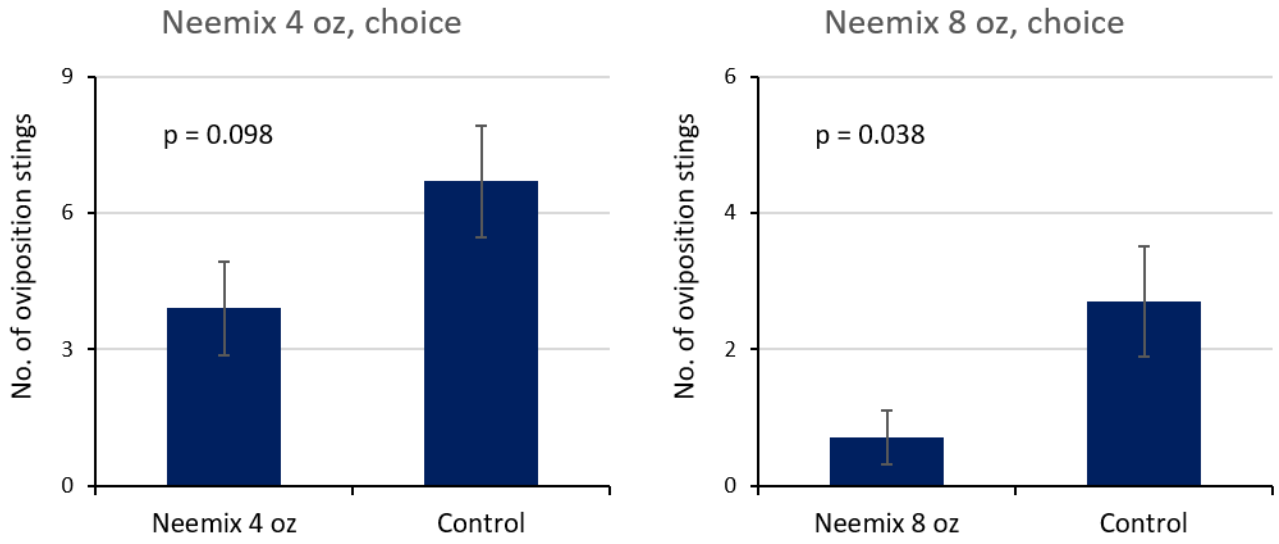
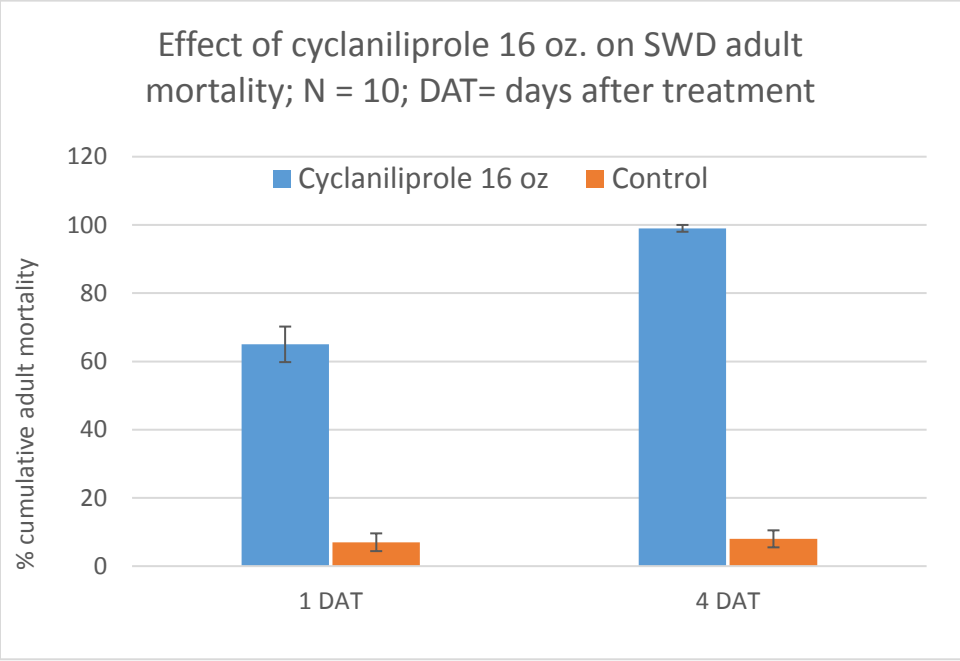
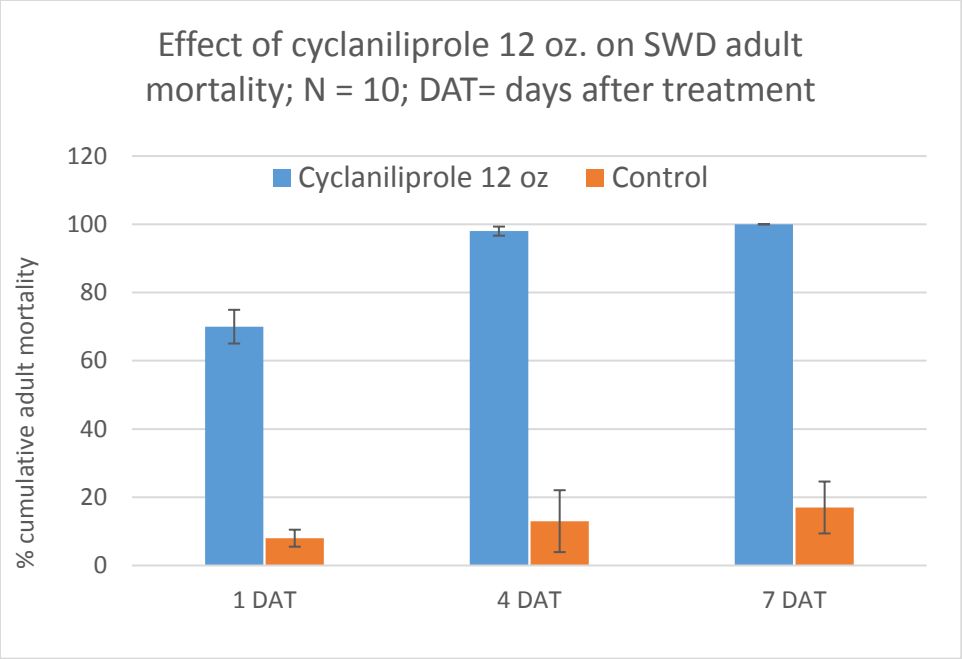


Fig. 5. Effect of Neemix on SWD oviposition in choice tests

Effect of cyclaniliprole on SWD adult mortality. Results showed that IKI-3106 (cyclaniliprole) at the rate of 12 fl oz/acre caused 70% mortality after 24 hours. The mortality was increased to 98% after 4 days and 100% at 7 DAT. Increasing the rate to 16 fl oz/acre did not improve the mortality significantly (i.e. 65% at 1 DAT; 99% at 4 DAT) compared to the 12 oz/acre rate used. Control mortalities were under 8% and 13% at 4 DAT for 16 oz. and 12 oz. rates, respectively.





Effect of methyl benzoate on SWD eggs and adult emergence -2017. Two sets of trial conducted using 1% methyl benzoate, and there was no statistical difference ($p<0.05$) on number of adult emergence between treated and control in both trials (Trial 1, t = 1.034; df =38, P =0.307; Trial 2, t = -0.152; df =38, P =0.135). Pooled data from two trials was not statistically significant (combined, t = -1.794; df =78, P =0.076). However, the numerical difference between two treatments was consistently showing the effect of methyl benzoate on adult emergence across the trial (Fig. 6).

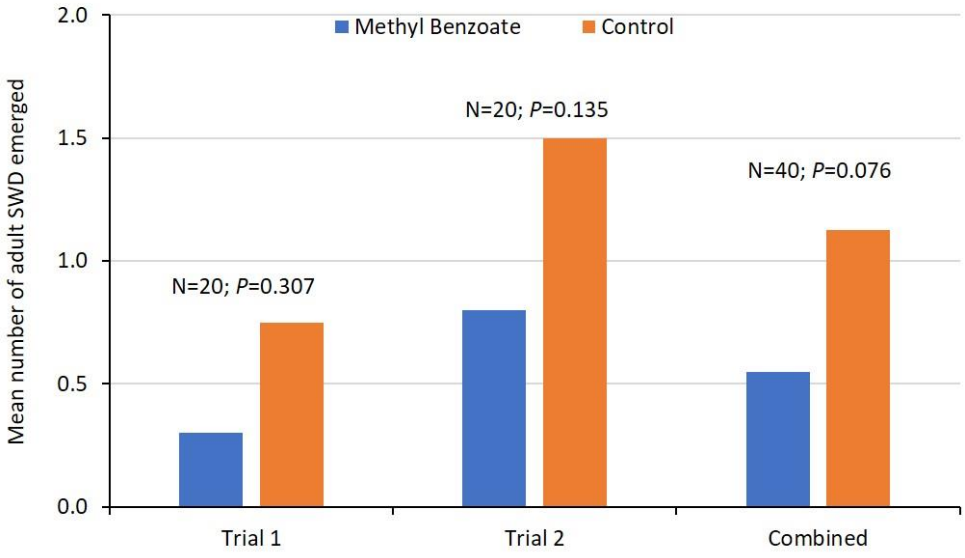


Fig. 6. Effect of Methyl Benzoate on mean number of SWD adult emergence

Also, we evaluated some other insecticides 1) Rimon (novaluron) and 2) Dimilin (diflubenzuron) for their the oviposition deterrent property using several sets of no-choice and choice based studies (details described above). No significant difference was observed between treated and control fruits on the number of oviposition marks/stings.

Conclusion and plan for further studies

Neem products used in the bioassay showed effectiveness in reducing spotted wing drosophila oviposition activity. The most effective product was Bonide Neem Oil which contains 0.90% Clarified Hydrophobic Extract of Neem Oil. When neem oil is mixed with alcohol, azadirachtin is separated from the oil, and the remaining oil without azadirachtin is called clarified hydrophobic extract of neem oil. Another promising product is Neemix 4.5 that contains 4.5% azadirachtin. Triple Action Neem, which is, also containing hydrophobic extract of the neem oil showed a good promise for future potential use in oviposition reduction.

The naturally occurring volatile compound, methyl benzoate showed a promise due to its potential ovicidal effect on spotted with drosophila eggs in cherries based on our studies. This compound is already proven to have an ovicidal effect on eggs of brown marmorated stink bug eggs, spotted wing drosophila eggs (in blueberries) and other insects, and toxic to other life stages such as nymphs of brown marmorated stink bug (Feng and Zhang 2017, Scientific Reports, 7:42168, DOI: 10.1038/srep42168). Although there is promise based on the lab studies across different insects, methyl benzoate has not been registered as an insecticide in the US. The field-trial using methyl benzoate needs to be done in the future.

Based on this year results, what we found was neem products might be helpful in reducing the oviposition activities. The further research question is, can we combine currently used insecticides with the neem products improve the effectiveness in reducing the oviposition in cherries? In addition to the continuation of the screening of newer and reduced-risk insecticides, we will conduct studies by combining the currently used or new insecticide(s) with the neem-based insecticide in the laboratory and/or in the field in coming season.

Additional objectives planned for 2018 field season are:

1. Evaluate the efficacy of experimental and registered insecticides against spotted wing drosophila
2. Compare the alternate-row spray method with the standard practice (spraying every row)to control spotted wing drosophila.

Evolution of sexual codes: Pheromone signatures and mate discrimination in related drosophilids

Nicole H Scheidler, Cheng Liu, Maria Fernandez, Cole M Johnson, Joseph Kinsella, Zainulabeuddin Syed

Abstract

Insects have been shown to use pheromone communication in many life history traits such as aggregation and host selection, oviposition, aggression, mate selection and courtship. Pheromones are often species specific among insects in either compound identity or compound ratios, creating a unique chemical code. This indicates the prominent role of pheromone communication within a species, as well as a role in reproductive isolation through pheromonal differences between species. In *Drosophlia*, hydrocarbons present on the cuticle (cuticular hydocarbons, CH) act as pheromones, and several CH compounds in *D. melanogaster* have been identified as important for mate selection and influence male courtship. While *D. melanogaster* pheromone communication has been relatively well studied, CH cues of other drosophilids and how they differ among related species are much less understood. We performed a comprehensive investigation of the CH profiles of three related drosophilids in the *D. melanogaster* species group. *D. suzukii*, a unique pestiferous drosophilid of economic importance, and it close relatives *D. biarmipese* and *D. takahashii* and were investigated with *D. melanogaster* as a reference. We found that hydrocarbon profiles do not reflect relatedness, however only a few compounds drive the major differences between species. Further investigation will elucidate if the compounds driving these differences play an important role in male courtship induction.

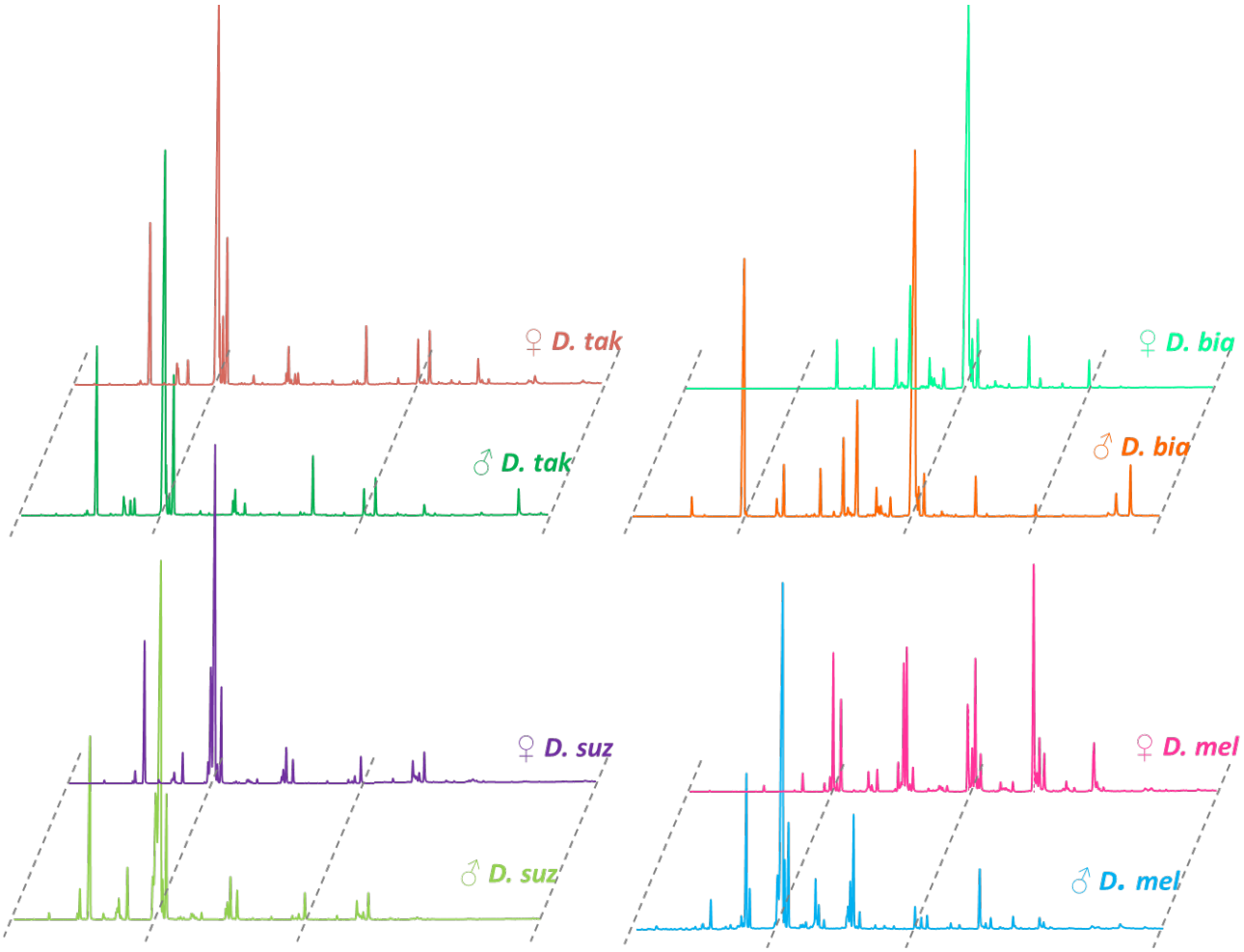


Figure 1. Fly species produce distinct cuticular hydrocarbon profiles. Representative CH profiles from four drosophilids for both sexes analyzed by gas chromatogram-mass spectrometry (GC-MS).

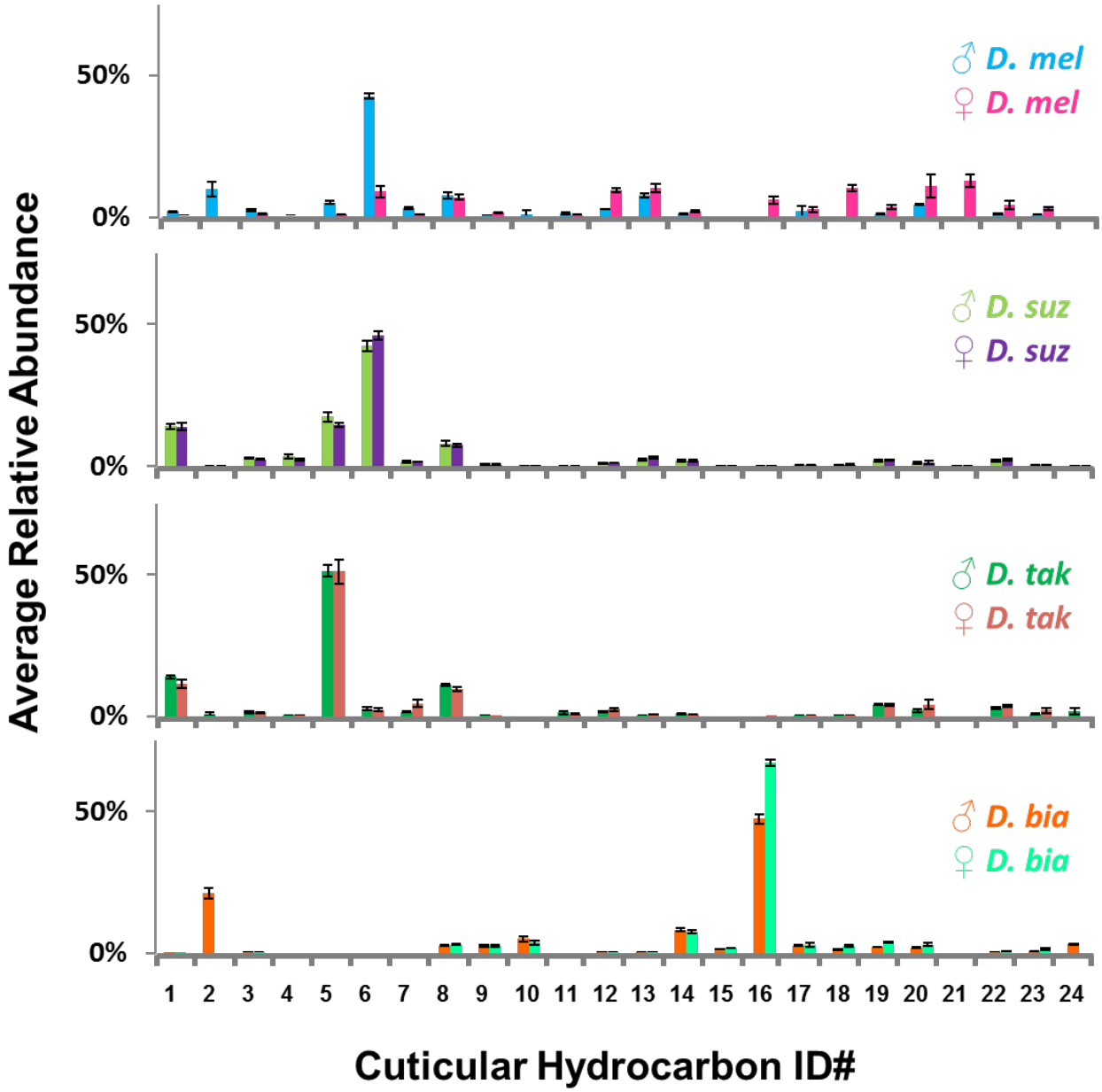


Figure 2. Relative abundance of 24 Cuticular Hydrocarbons of four drosophilids indicate differences in major constituents across species. The average relative abundance for males and females of a given species are graphed together for comparison. The X axis represents the 24 CHs of interest while the Y axis represents relative abundance

ID#	Compound	KI	♂ *D. m*	♀ *D. m*	♂ *D. s*	♀ *D. s*	♂ *D. t*	♀ *D. t*	♂ *D. bi*	♀ *D. b*
1	n-Heneicosane	2102	x	x	x	x	x	x	x	x
2	(Z)-11-Vaccenyl Acetate	2192	x				x		x	
3	n-Docosane	2202	x	x	x	x	x	x	x	x
4	2-Methyldocosane	2274	x		x	x	x	x		
5	(Z)-9-Tricosene	2278	x	x	x	x	x	x		
6	(Z)-7-Tricosene	2288	x	x	x	x	x	x		
7	(Z)-5-Tricosene	2298	x	x	x	x	x	x		
8	n-Tricosane	2305	x	x	x	x	x	x	x	x
9	NI	2402	x	x	x	x	x	x	x	x
10	2-Methyltetracosane	2469	x	x	x	x			x	x
11	NI	2472	x	x			x	x		
12	(Z)-9-Pentacosene	2479	x	x	x	x	x	x	x	x
13	(Z)-7-Pentacosene	2486	x	x	x	x	x	x	x	x
14	n-Pentacosane	2504	x	x	x	x	x	x	x	x
15	7,11-hexacosadiene	2565		x					x	x
16	7,11-heptacosadiene	2661		x				x	x	x
17	2-Methylhexacosane	2671	x	x	x	x	x	x	x	x
18	7-heptacosene	2687		x	x	x	x	x	x	x
19	n-Heptacosane	2702	x	x	x	x	x	x	x	x
20	2-Methyloctacosane	2867	x	x	x	x	x	x	x	x
21	7,11-Nonadosadiene	2877		x						
22	n-Nonacosane	2904	x	x	x	x	x	x	x	x
23	2-Methyltriacontane	3070	x	x	x	x	x	x	x	x
24	NI	ND					x		x	

Table 1. Fly species 10 most abundant cuticular hydrocarbon. An 'x' marks the presence of the compound while the colored fields indicate the compound is among the 10 most abundant compounds in the cuticular hydrocarbon profile for any given species. The KI is the calculated average across species.

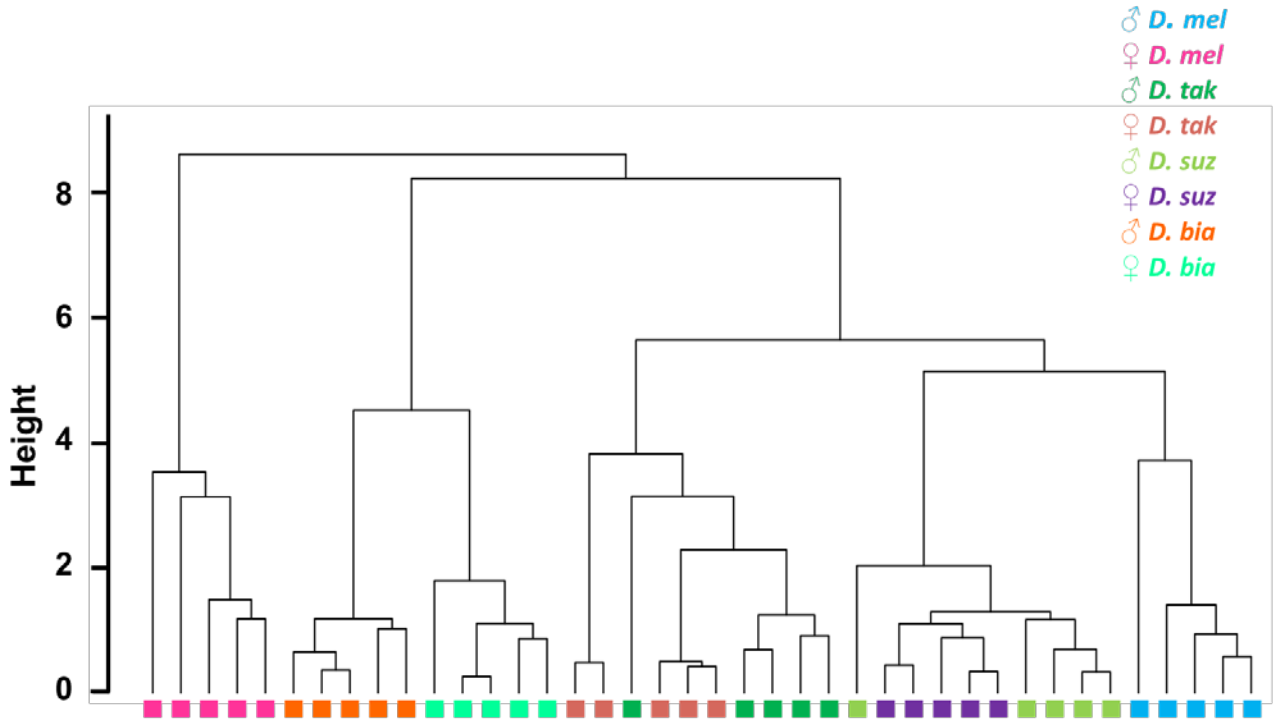


Figure 3. Relationship based on CH profile in hierarchical analysis for four drosophilids. Hierarchical cluster analysis was performed based on relative abundance of 24 CH for each fly. Branches were considered to be a cluster beneath a threshold height of 4 (indicated by the dashed line). Each leaf is identified to a single fly by a color and label

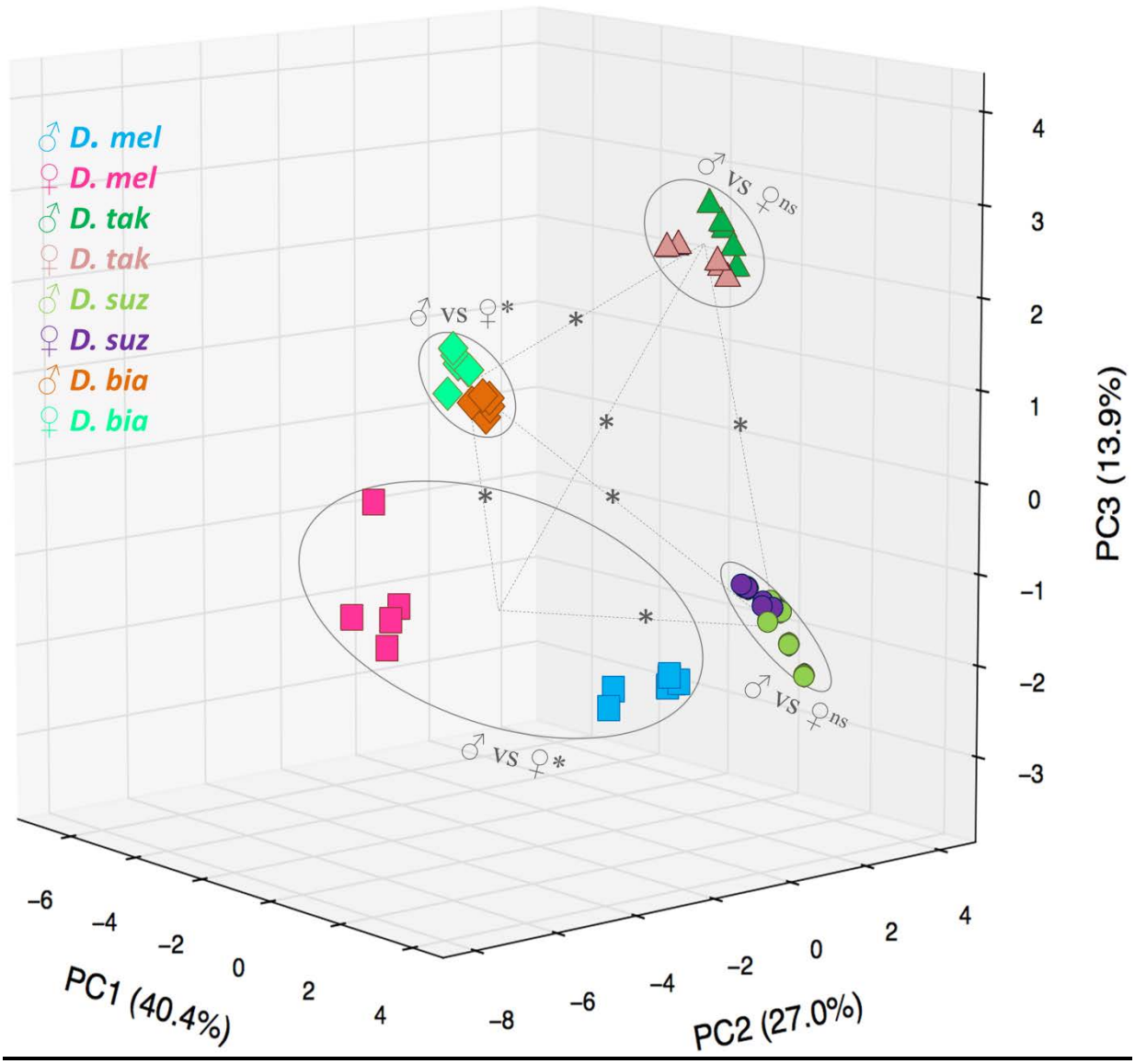


Figure 4. Fly species produce distinct cuticular hydrocarbon profiles. Principal Component Analysis (PCA) significantly resolved the cuticular hydrocarbon profiles into discreet species clusters (circled). Lines marked with an asterisk (*) between circled clusters indicates the significance ($*p \leq 10^{-3}$) relationship between species. Inraspecies-sex separation ($*p \leq 10^{-6}$) was detected in both *D. melanogaster* and *D. biarmipese.* Significance relationships between sexes of a single species is indicated by an asterisks (*) or marked not significant (ns) beside the ♂ vs ♀ notation.

	PC1			PC2			PC3		
	Impact	Compound Name	ID#	Impact	Compound Name	ID#	Impact	Compound Name	ID#
1	0.292	n-Tricosane	8	0.197	2-Methyldocosane	4	0.445	n-Heptacosane	19
2	0.260	n-Docosane	3	0.190	n-Heneicosane	1	**0.391**	**(Z)-9-Tricosene**	**5**
3	0.247	n-Heneicosane	1	**0.129**	**(Z)-7-Tricosene**	**6**	0.193	NI	24
4	0.221	(Z)-5-Tricosene	7	0.096	NI	24	0.156	n-Nonacosane	22
-4	**-0.304**	**7,11-heptacosadiene**	**16**	-0.356	2-Methyltriacontane	23	-0.267	2-Methyldocosane	4
-3	-0.304	2-Methyltetracosane	10	-0.358	2-Methyloctacosane	20	-0.278	n-Docosane	3
-2	-0.306	7,11-hexacosadiene	15	**-0.360**	**7,11-Nonacosadiene**	**21**	-0.334	(Z)-7-Pentacosene	13
-1	-0.313	n-Pentacosane	14	-0.366	(Z)-9-Pentacosene	12	**-0.428**	**(Z)-7-Tricosene**	**6**

Table 2. The top four positively and negatively weighted constituents in the first three PCs. For each PC, the factor impact, compound name, and compound ID# is listed for the top four positively and negatively impacting factors. Positive factors are in order of decreasing magnitude of impact [(1)-(4)] while negative factors are in order of increasing magnitude [(-1)-(-4)]. Major cuticular constituents within the four species of interest and appear in the top influencing factors are emboldened.

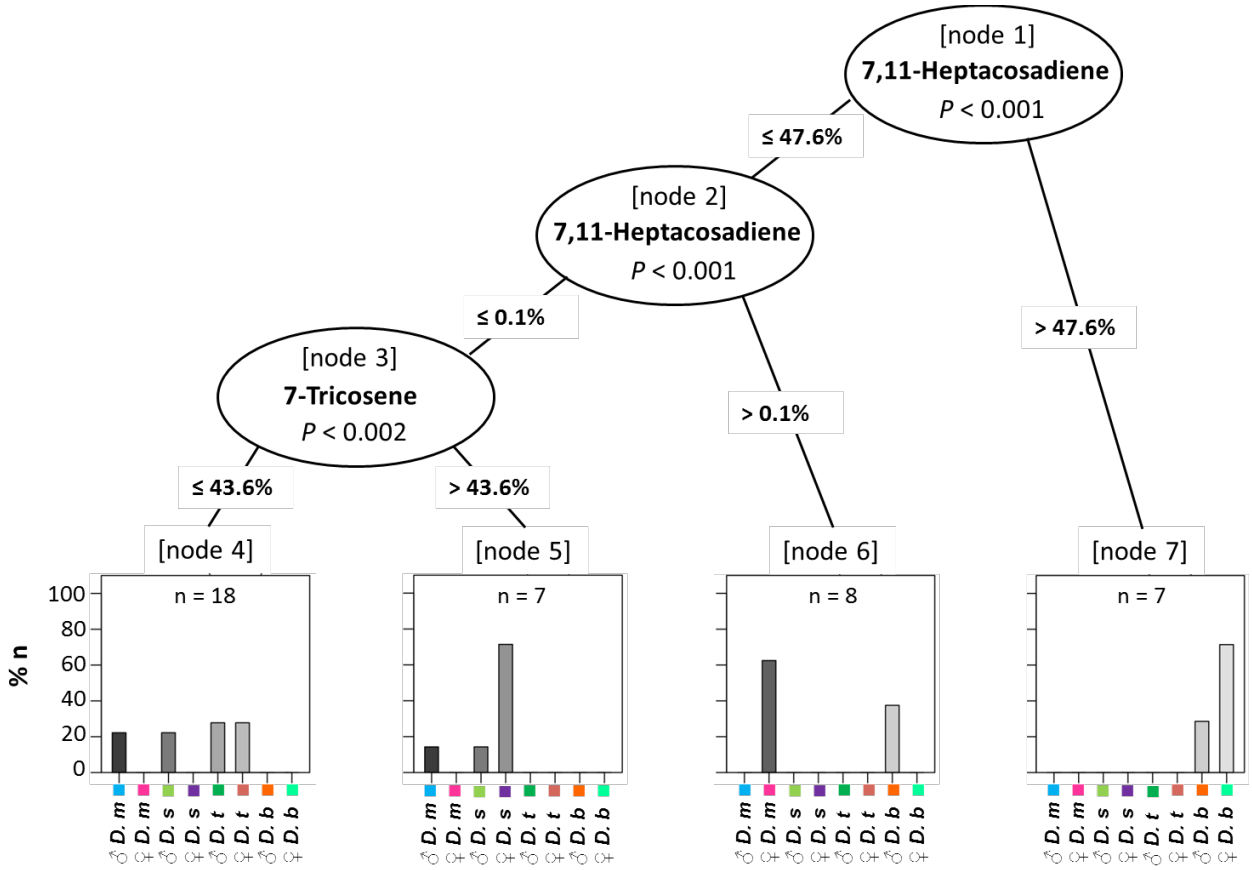


Figure 5. Two compounds figure prominently in sorting flies by conditional inference tree: 7,11-heptacosadiene and 7-tricosene. The tree has three branching nodes, circled and labeled as 1, 2, or 3. Within each branching node, the compound ID# is given along with a *p*-value indicating the significance of that constituent in sorting the CH profiles. 7,11-Heptacosadiene (compound 16) is significant for separation at nodes 1 and 2 (*p*-value ≤ 0.001); and node 3 significantly separates flies based on 7-Tricosene (compound 6) (*p*-value ≤ 0.002). The CH profiles were separated into four terminal nodes (4, 5, 6, or 7). The 'n' at each terminal node is the total number of CH profiles successfully sorted by the conditions leading to that terminal node. Each branch has a threshold relative abundance (normalized to 1) of the cuticular constituent separating at that node. The graphs at each terminal node indicate the relative number of sorted CH profiles corresponding to the eight categories of flies (fly species and sex). The Y-axis represents the percent of 'n' for any given node; and the X-axis represents the fly categories. The tree was generated with an N=5 of CH profile for each category of fly.

Technology Innovation Group

October 16, 2017

Mr. Nick Matteis
Ag Association Management Services, Inc.
1521 "I" Street
Sacramento, CA 95814

Dear Nick,

We would like to provide a summary of our recent and near term activities for creating a business to advance technologies to mitigate the damage caused by Spotted Wing Drosophila in cherry and other crops.

Dr. Tom Turpen attended a series of meetings at the Food Crunch conference in Montana in June. This conference is an invitation-only event comprised of agriculture experts, investors, and non-profit leaders devoted to exploring innovations in food systems. Tom was able to discuss gene drive technology and the concept of establishing a B Corporation to bridge the application of modified SWD from the laboratory to release in orchards. One of the emerging themes at the conference was the importance of building trust with the public, given that the pace of changes in technology is outpacing regulatory guidance.

One of the conference attendees works for the Lift Economy, a consultancy that provides advice and training to businesses that pursue dual mission objectives, such as B Corporations. We have had follow up contact with two principals with this group on the establishment of the B Corporation and will have further discussion on a few key organizational issues with the B Corporation just prior to when it is legally formed.

We will have additional discussions with a founder of a crowd source platform that focuses on agricultural related companies. This company has a fund and offers a method for individual investors to participate in a crowd funding round. The company has been active in California and has helped sponsor several events to match companies with investors.

We have identified a candidate law firm in Oakland, California to assist with the legal organization of the B Corporation and the drafting of the proposed investment document. The law firm is itself a B Corporation, which indicates their commitment to this type of legal entity. Upon the financial commitment of from California Cherry Growers and Industries Foundation, we will be in a position to initiate detailed discussions with the law firm and begin drafting the security agreement. Should the financial commitment from the Foundation not materialize, we will need to discuss other alternatives with the Research Committee of the Cherry Board.

Regards,

Dan Hanson, Principal
Technology Innovation Group