The postharvest treatments of California blueberries to eliminate insects with potential to serve as export trade barriers: 2015 research

Postharvest treatment of blueberries with phosphine for control of spotted wing drosophila, *Drosophila suzukii*

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Executive summary:

A new postharvest treatment option to control spotted wing drosophila (SWD), *Drosophila suzukii*, and brown marmorated stink bug (BMSB), *Halyomorpha halys,* has been developed for US blueberry growers/packers. Packed-boxes were fumigated at cold-storage temperature for 48 h. A report can be drafted and presented to Industry (and thereafter APHIS) for consideration. Currently, market options include those countries willing to fumigate with phosphine on arrival (e.g., Chile). ARS is working with industry and USEPA to gain registration for PH3 so that fumigations can be done at the packinghouse.

PH3 chamber fumigations at 1.4 ± 0.5 ºC ($\bar{x} \pm s$)(~34.5°F) were evaluated for postharvest control of SWD and BMSB in fresh blueberry exports from Western USA. The most PH3-tolerant age of SWD (0 – to 24-h old, ~92% eggs) as well as life stage of BMSB (2^{nd} & 3^{rd} instars) was established via a series of exploratory fumigations. In confirmatory fumigations, which simulated the commercial scenario, complete mortality of 31,428 ± 1,129 (*n* ± *SE*) SWD eggs as well as 3,7008 BMSB, was achieved with an applied dose of 1000 ppmv, a load factor of ~ 55.3 ± 0.7% ($\bar{x} \pm s$), and a treatment time of 48 h at 1.4 ± 0.5 ºC ($\bar{x} \pm s$)(~34.5°F).

Sorption, off-gassing (i.e., depuration), and residue data were obtained. Results can be used by industry in the context of quantifying fumigant inputs to ingestion exposure and worker inhalation exposure that are respectively derived from the consumption of fruit residues and off-gassing of palletized fruit in cold-storage. Relative to methyl bromide, ~10-fold less mass of phosphine is sorbed by palletized loads of fruit during fumigation, phosphine respectively off-gasses ~15-fold faster from loads in cold-storage, and ~15-fold shorter amount of time is required for phosphine residues in blueberries to reach a generic USEPA food tolerance (for citrus) of 0.01ppm.

Materials and Methods.

Insects

SWD pupae were obtained from the laboratory colonies of Drs. Arytom Kopp (University of California at Davis) and Robert Van Steenwyk (University of California at Berkeley; both colonies originated from wild

specimens captured in cherry orchards of coastal California USA. SWD pupae were also obtained from a laboratory colony of Dr. Jana Lee (USDA-ARS), which originated from wild specimens captured in raspberry fields of Marion County, Oregon USA. Pupae from these three sources were integrated into a single colony that was maintained in several (6-8 ct.) nylon mesh enclosures (Bug Dorm-2®, BioQuip Products, Rancho Dominguez, CA, US) housed in an 22.65-m^3 incubation unit (24-27 ℃, 80% RH, 16:8 [L:D] h) at the USDA-ARS-SJVASC (Parlier, California USA). Approximately twice a year, SWD adults were captured in raspberry fields located in the Salinas Valley of California and introduced into the SJVASC colony along with new pupae from each of the original sources. Plastic vials (20-dram) containing saturated aqueous solutions of sucrose were capped with cotton wicks to serve as a food and water source for adults. As described in Walse et al. (2012), larvae were reared on standard cornmeal-(dextrose or sucrose)-agar-yeast medium layered to ($\bar{x} \pm s$, AVE. ± STDEV) 4.0 ± 0.6 mm on the bottom of 8.7 ± 0.1-cm diameter Petri dishes, which also served as ovipositional substrate. Formalin ® (2 mL), a fungistat, was added to each 4-L batch of diet. Four diet-containing Petri dishes were placed in each enclosure, replaced after 1-d ovipositional periods, and transferred to a separate communal rearing enclosure for the duration of development. When adults began to emerge from a particular dish, it was transferred back into a community of reproductively-active adults maintained at ~ 2000 individuals per enclosure.

BMSB (*Halyomorpha halys*) eggs were obtained from the laboratory colony of Dr. Tracey Leskey (USDA-ARS-Kearneysville, WV) that originated from wild specimens captured in small fruit and orchard crops in West Virginia, USA. Upon receipt of the BMSB eggs in Oct. 2012 at the Contained Research Facility at UC Davis (Davis, CA), a BSL-III agricultural quarantine facility, all specimens were transferred to an environmentally-controlled chamber set at 26 °C, 65% RH and a 16:8 diurnal light cycle until a strong colony of mixed life stages could be established. A second shipment of eggs was received from Dr. T. Leskey in March 2013 to supplement the CA colony. All non-egg life stages are reared concurrently inside 0.34-m^3 aluminum mesh cages on live bush bean and cowpea plants, supplemented with organic raw almonds, pumpkin seeds, sunflower seeds, and walnuts. Cages are maintained inside a greenhouse at 26 °C, 65% RH and a 16:8 diurnal light cycle. Eggs are collected every 48 h from rearing cages and allowed to hatch on green bean plants in an isolated cage to prevent egg predation. 1^{st} instar nymphs are then transferred via a #4 (3/32”) round brush back into the rearing cages 4 d after hatching to complete their life cycle and to maintain reproductive populations at ~500/cage.

Fruit infestation

To simulate a naturally occurring infestation scenario for SWD, ovipositional/diet substrate was removed from an enclosure and replaced with stainless-steel trays (30 × 30 × 2 cm) that were filled with a monolayer of fresh blueberries. The stainless-steel trays containing infested blueberries were removed after ovipositional periods that varied by test type, infested blueberries were transferred to a pull-string cloth bag (~25 per bag) (8” x 12”; ULine, Waukegan IL), and used in laboratory-scale exploratory fumigations or buried throughout the load of commercial fruit boxes in confirmatory-scale fumigations. Alternatively, cloth bags were not fumigated and held as untreated controls to estimate the number of individuals treated during a respective fumigation.

All seven life stages (eggs, 1^{st} – 5^{th} instar nymphs, and adults) of BMSB were evaluated in the exploratory fumigations. Egg samples were cut from oviposition substrate (usually a dried bean leaf) and counted under a dissecting microscope under low (10x) power. Nymphs (1^{st} – 5^{th} instars) were randomly collected from the rearing cages described above. Adults were aspirated from the walls and top of the rearing cages. Samples

from each life stage were then isolated in vials with mesh-screen covered openings on the top, bottom and sides (2 locations) and placed into cloth bags (8" x 12"; ULine, Waukegan IL). Several pumpkin seeds and a wetted cotton wick were placed in to the cages to serve as food and water sources, respectively. Cloth bags containing vials of the life stages to be treated, were placed inside the environmental room, housing the fumigation chambers, for tempering.

For the exploratory fumigations, SWD-infested fruit were incubated for 0, 24, 72, 144, or 192 h to yield ages, respectively, of 0- to 24-h (92% eggs), 24- to 48-h (83% 1st instar), 72- to 96-h (78% 2nd instar), 144- to 168-h (74% 3rd instar), and 192- to 216-h old (40% pupae, 57% adults) specimens at the start of a 12-h pre-fumigation period of temperature equilibration (i.e., tempering). Developmental times and life stage assignments were based on literature (Ashbruner, 1989; Bellamy et al. 2013; Kanzawa, 1936; Kanzawa, 1939; USDA, 2014). Infestations and subsequent incubations were synchronized so that all BMSB life stages and all SWD ages concurrently entered the tempering period that preceded fumigation. For the confirmatory fumigations, infestation and incubation was planned to yield 2nd and 3rd instar BMSB as well as 12- to 36- h old SWD (age at fumigation), only the most PH3-tolerant forms (*vide infra, Figure 1*).

Exploratory fumigations

Exploratory fumigations were performed in modified Labonco® 28.32-L vacuum chambers housed in a walk-in environmental incubator with programmable temperature and humidity (USDA, 2009). A series of experiments was conducted determine the treatment duration, ranging from 6 to 72 h, required to control ages comprising the egg through pupal life stages of SWD as well as all life stages of BMSB with phosphine (PH3) at headspace concentrations ≥ 1.5 mgL^{-1} (1000ppmv) at 1.4 ± 0.5°C ($\bar{x} \pm s$). Chambers loaded with SWD-infested fruit, fruit infested with control specimens of SWD, BMSB cages (treated and untreated control specimens), source-gas cylinders, and gas-tight syringes were tempered for at least 12 h to treatment temperatures prior to fumigation. Chamber temperature was confirmed prior to fumigation by a HOBO data logger (HOBOware version 2.7). Chamber lids were then clamp-sealed in preparation for treatment. SWD and BMSB were fumigated concomitantly in a chamber.

A pressure of approximately 70 mmHg was established in each chamber. Gas-tight super-syringes (Hamilton ® 500, 1000, or 1500 mL) were filled with a volume of fumigant from a cylinder of 1.6 % (v/v) PH3 balanced with nitrogen (Cytec Canada, Inc., Niagara Falls, Ontario, Canada) to achieve the requisite applied dose of ~ 1.65 mgL^{-1} (1100ppmv) as predetermined in preliminary calibration studies. A syringe was fitted to a LuerLok ® sampling valve, which was subsequently opened so that fumigant was steadily drawn into the chamber. The syringe was then removed and normal atmospheric pressure was established in each chamber before the valve was closed; this marked the beginning of the exposure period. Gas samples (40 mL) were taken temporally at standard intervals from the chamber headspace through a LuerLok® valve using a B-D® 100 mL gas-tight syringe and quantitatively analyzed for PH3 with GC-PFPD. Fumigant concentrations were measured and exposures, expressed as concentration × time product ("CT"s) calculated by the method of Monro (1969), were tracked.

Following the final sampling to determine headspace concentration, chamber valves were opened to atmosphere, a 1-h aeration period was initiated, and chamber lids were then opened. Treated and non-treated SWD-infested blueerries were retrieved from the bags, transferred in pairs into a stainless-steel mesh ball cage (5.1-cm diameter), and the mesh cage was placed back into the respective bag. SWD-infested blueberries as well as caged-BMSB were transferred into a rearing incubator at 27.0 ± 1.0 °C and 80 ± 2% RH ($\bar{x} \pm s$), and placed into treatment-respective 0.03-m^3 nylon-mesh rearing cubicle.

Confirmatory export fumigations

To simulate a commercial scenario, fumigations were conducted using 241.9-L steel chambers housed in a walk-in environmental incubator with programmable temperature and humidity (USDA, 2010). On the same day that they were packaged, blueberries were obtained from a commercial wholesale source in California. Each chamber contained sixteen cardboard trays (25.40 x 39.37 x 8.26 cm) stacked in two eight -tray columns with tray consisting of two layers of six plastic clamshell containers (~ 170 g of fruit each). In fifteen of the trays, five clamshells in the top layer were removed and the blueberries were emptied. The content of each clamshell was replaced with an equivalent volume of SWD-infested blueberries or caged-BMSB. The chamber load was estimated as a fractional percentage, 55.3 ± 0.7% ($\bar{x} \pm s$), of the volume occupied by the load relative to the chamber volume (i.e., V_L $(V_{chamber})^{-1}$ x 100) (Monro, 1969).

Chambers loaded with test specimens and uninfested fruit as well as control specimens were acclimated to fumigation temperature of ~1.4 °C (~34.3°F) for 12 h prior to treatment (i.e., tempered) within a temperature-controlled storage unit (USDA, 2009). Fruit pulp temperature was confirmed prior to fumigation by each of three probes (YSI scanning tele-thermometer) that recorded the respective pulp temperature in three uninfested fruit distributed at different locations within the load of the fruit undergoing treatment. Temperature probes were then removed and chamber lids clamp-sealed in preparation for treatment. The chamber ventilation valve was opened and chambers were filled with a volume of fumigant from a cylinder of 1.6 % (v/v) PH3 balanced with nitrogen (Cytec Canada, Inc., Niagara Falls, Ontario, Canada) to achieve the requisite dose as predetermined in preliminary calibration studies. The valve was then closed which marked the beginning of the exposure period. Gas samples (40 mL) were taken from the chamber headspace through a LuerLok® valve using a B-D® 100 mL gas-tight syringe and quantitatively analyzed for PH3 with GC-PFPD at standard intervals corresponding to 5 (initial), 60, 480, 1440 (1-d end), or 2880 (2-d end) min. Fumigant exposures were expressed as concentration × time cross products, "CTs", and calculated by the method of Monro (1969).

After completion of the exposure, chamber valves were opened to atmosphere and vacuum was pulled to aerate the chamber until headspace concentration of the fumigant was below the mandated ventilation requirements of 0.3 ppm (0.45μg/L) phosphine. Chamber lids were opened and the treated and non-treated specimens were collected, SWD-infested blueberries were transferred to mesh cages as described above, and all specimens were placed into separate 0.03-m^3 nylon-mesh rearing cubicles maintained in an incubator at 27.0 ± 1.0 °C and 80 ± 2% RH ($\bar{x} \pm s$) as described above. Noninfested fruit was retrieved and used for residue determination and fruit quality evaluation. Samples of noninfested fumigated fruit (75 g each), selected from 3 different locations within the load, were placed into a cooler filled with dry ice within 5 minutes of the end of aeration and were used to estimate initial residue levels. The remaining noninfested fumigated fruit transferred into cold storage at 1.1 ± 0.6 °C ($\bar{x} \pm s$) (~34.0°F) and temporally retrieved from storage and used for residue determination(s)(*methods and results available upon request*).

Mortality evaluation

SWD mortality was assessed at 1-d intervals post-fumigation for 21 d; rearing cubicles were examined and live adult specimens were tallied and discarded before cubicles were resealed for further incubation and evaluation. The cumulative number of adults, which emerged from each piece of fruit designated as an untreated control for paired fumigation trials, was counted. An average ($\bar{x}$) emergence from each infested fruit left untreated was calculated along with a standard deviation ($\pm s$). The number of SWD specimens ($n \pm s$) that were treated was estimated by multiplying the number of infested fruit treated in each trial by the average emergence from each fruit that was infested and untreated ($\bar{x} \pm s$). The total number of specimens that were treated across all

exploratory- or confirmatory-trials was estimated by summing the number from each respective trial and propagating the respective standard deviation.

Two days following treatment, BMSB specimens were retrieved from all cages for evaluation. Egg mortality was diagnosed visually by discoloration, while survivability of other life stages was diagnosed by locomotion or by prodding-induced motion. Post-embryonic BMSB were categorized as moribund if the survivability was inconclusive. Moribund BMSB were placed inside a labeled plastic snap-cap cage with food and water source as described above prior to further incubation until an additional evaluation the following day. Mortality of control BMSB specimens was included as a natural response in the efficacy modeling for exploratory fumigations. For the confirmatory trials, Abbott's method (Finney, 1971) was used to estimate the percentage mortality of BMSB used in Probit calculations. The total number of specimens that were treated for each exploratory- or confirmatory-trial was estimated by summing the numbers treated, while the total number of specimens treated across exploratory- or confirmatory-trials was estimated by summing the numbers from each respective trial.

Rearing and incubation conditions of 27.0 ± 1.0 °C, 80 ± 2% RH, and 16:8 [L:D] h photoperiod were fixed to maintain a consistent progression of development between trials and controls; resulting mortality in control specimens was assumed to be equal to that in fumigation trials. Insects were more likely to survive and there was greater certainty in diagnosing survivorship after the treatment if incubated under conditions described above rather than if refrigerated post-fumigation at < 5 ℃ under simulated commercial transport conditions, which confound the effect of a fumigation event on mortality. To be detailed in a forthcoming publication on the effect of refrigeration on SWD and BMSB development, for both species we generally observed increases in the mortality of all life stages, the length of the developmental periods of each life stage, and heterogeneity in the times required to complete development within each life stage.

Chemicals and Chemical analysis

A 300-lb cylinder of 1.6 % (v/v) PH3 balanced with nitrogen was obtained from Cytec Canada, Inc. (Niagara Falls, Ontario, Canada) and used as the source for gas chromatography calibrations as well as fumigations. PH3 levels in headspace of fumigation chambers were measured using gas chromatography; retention time (PH3, t_r = 3.2 ± 0.2min) was used for chemical verification and the integral of peak area, referenced relative to liner least-squares analysis of a concentration – detector response curve, was used to determine concentration. Detector response and retention indices were determined each day in calibration studies by diluting known volumes of gases into volumetric gas vessels. PH3 analyses were with a Varian 3800 and splitless injection (140 ℃) using a gas sampling port with a 10 μL-sample loop, a Teflon column (L = 2 m, OD = 2 mm) packed with Porpak N (80/100 mesh) held at 130 ℃ for 10 min, and a PFPD detector (13 mL/min H_2, 20 mL/min air, and 10.0 mL/min N_2 make-up) at 250 ℃ that received only 10% of the 15 ml He/min column flow.

Fruit quality

The effects of fumigation on fruit quality were quantified by methods reported in Obenland et al. (2011) and Mitcham et al (2003) by evaluating characteristics of non–fumigated cherries relative to those fumigated in confirmatory SWD fumigations with 1000 ppm PH3 and treatment durations of either 24 or 48 h. Quality parameters were evaluated after storage for 2 days at 1.1 ± 0.6 ℃ ($\bar{x} \mp s$) (~34.0°F) plus 16 hours at 22.2 ± 0.6 ℃ ($\bar{x} \pm s$) (~72.0°F) to simulate air shipment and marketing. Surface browning, stem browning, pitting,

cracking, shrivel, decay and overall acceptability were subjectively evaluated as listed in Table 1. Ratings that would likely be unacceptable to a consumer are indicated. Ratings are presented as calculated indices or in terms of acceptability. Skin color was evaluated using a Minolta colorimeter by measuring the same spot on the skin of 10 fruit for each replication before treatment and after storage and expressed in the L*C*h scale as amount of color difference (poststorage - pretreatment). Acidity was determined from the juice of 5 pooled fruit for each replication by titration with NaOH. Soluble solids were measured from the same juice using a digital refractometer as in Obenland et al. (2005). Firmness (g-1mm deflection) was measured with a Bioworks Firm Tech 2 instrument. (*Results were reported in the 20145 final report).*

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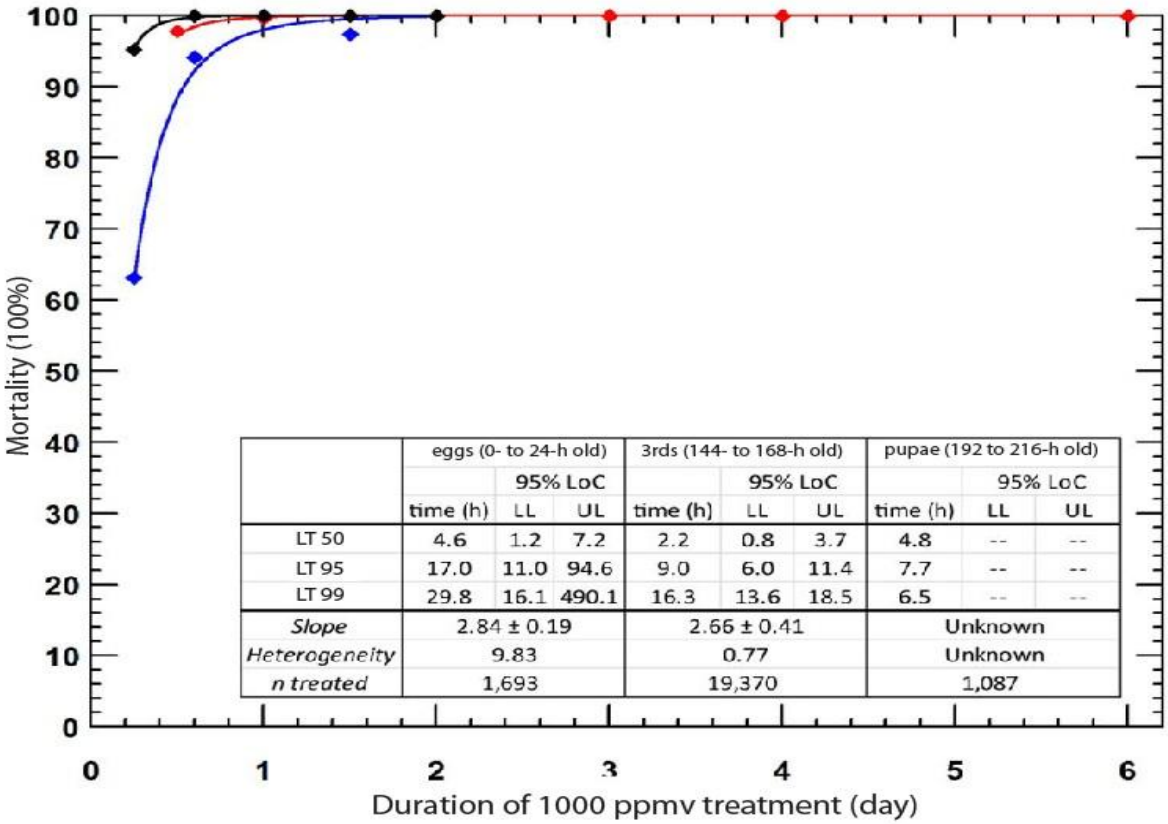

	eggs (0- to 24-h old)			3rds (144- to 168-h old)			pupae (192 to 216-h old)		
		95% LoC			95% LoC			95% LoC	
	time (h)	LL	UL	time (h)	LL	UL	time (h)	LL	UL
LT 50	4.6	1.2	7.2	2.2	0.8	3.7	4.8	--	--
LT 95	17.0	11.0	94.6	9.0	6.0	11.4	7.7	--	--
LT 99	29.8	16.1	490.1	16.3	13.6	18.5	6.5	--	--
Slope	2.84 ± 0.19			2.66 ± 0.41			Unknown		
Heterogeneity	9.83			0.77			Unknown		
n treated	1,693			19,370			1,087		

Figure 1. Mortality of 0- to 216-h old (pre-tempering age) spotted wing drosophila (SWD) following phosphine (PH3) fumigation at 1.4 ± 0.5 °C and probit regression analyses (Polo Plus, LeOra Software, 2002-2007) of the duration-mortality response respective to an applied doses of 1.65 mgL^{-1} (1000ppmv), showing the number of specimens treated, the regression heterogeneity (H), the predicted durations to cause 50, 95, and 99% mortality in the treated population (respectively LT_{50}, LT_{90}, and LT_{99}), and the corresponding estimates of the upper (UL) and lower limits (LL) at the 95% level of confidence (LOC) (Finney, 1971).