Postharvest Irradiation Treatment for Quarantine Control of *Drosophila suzukii* (Diptera: Drosophilidae) in Fresh Commodities

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ABSTRACT Irradiation is a postharvest quarantine treatment option for exported commodities such as stone fruits and small fruits to prevent movement of the new invasive pest spotted wing drosophila, *Drosophila suzukii* (Walker) (Diptera: Drosophilidae). The effects of irradiation on larval and pupal development and adult reproduction in *D. suzukii* were examined. Larvae (first, second, and third instars) and pupae (1–2-d-old, 3–5-d-old, and 7–8-d-old) on diet were irradiated at target doses of 20, 30, 40, and 50 Gy in replicated factorial experiments and survival to the adult stage was recorded. Tolerance to radiation increased with increasing age and developmental stage. Males and females were equally susceptible. A radiation dose of 40 Gy applied to first- and second-instar larvae prevented adult emergence. The late-stage pupa was the most radiation-tolerant stage that occurs in fruit, and individuals irradiated at this stage readily emerged as adults; therefore, prevention of F_1 adults was the desired treatment response for large-scale validation tests with naturally infested fruit. In large-scale tests, a radiation dose of 80 Gy applied to late-stage pupae in sweet cherries or grapes resulted in no production of F_1 adults in >33,000 treated individuals, which meets the zero tolerance requirement for market access. A minimum absorbed dose of 80 Gy is recommended for quarantine control of *D. suzukii*.

KEY WORDS X-ray radiation, radio-tolerance, invasive species, regulatory pest, phytosanitary treatment

Spotted wing drosophila, *Drosophila suzukii* (Walker) (Diptera: Drosophildae), is native to Asia and has been recorded from China, Japan, Korea, and Thailand. In 2008, *D. suzukii* was trapped for the first time in California, and has since spread to many other states in the United States (Lee et al. 2011a) and to Europe (Cini et al. 2012). *D. suzukii* mainly infests stone fruits and small fruits, and damage is caused by larvae feeding internally on the fruit pulp, and by the introduction of rot-type pathogens at the site of oviposition. Australia, New Zealand, and Mexico have imposed restrictions on the importation of strawberries, cherries, stone fruit, and table grapes from states with infested areas in the United States (Lee et al. 2011b, Cini et al. 2012).

Irradiation is a postharvest quarantine treatment option for exported commodities such as fruits and vegetables to prevent movement of viable *D. suzukii.* Irradiation treatment is effective against insect pests at doses that typically do not harm the quality of fresh commodities (Wall 2008, Follett 2009). In 2006, the U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA–APHIS) approved generic radiation doses of 150 Gy for any tephritid fruit fly and 400 Gy for all other insects, except the pupa and adult stages of Lepidoptera (which may require higher doses) (USDA–APHIS 2006). *D. suzukii* is a drosophilid, not a tephritid fruit fly, and therefore the 400-Gy radiation treatment would apply. During commercial irradiation, the actual dose received by the commodity varies because the ionizing radiation must penetrate to the center of a three-dimensional load of boxes (Follett and Weinert 2009). The dose-uniformity ratio (DUR = maximum or minimum dose) for commercial irradiators can range from 1.5 to 3.0 (Mehta and O'Hara 2006). Ensuring that a minimum radiation dose of 400 Gy is applied to a commodity against *D. suzukii* life stages, therefore, may result in part of the load receiving doses between 600 and 1,200 Gy. Irradiation treatment at this level may negatively affect commodity quality (Wall 2008), and the treatment may exceed the limit of 1,000 Gy (1 kGy) established by the Food and Drug Administration for fresh commodities. Safely lowering the dose from 400 Gy for *D. suzukii* will reduce any deleterious effects on commodity quality, ensure the applied treatment dose is below the 1-kGy limit, and lower the cost of treatment owing to shorter treatment time (Follett 2009).

The objective of this study with *D. suzukii* was to examine the effects of irradiation on larval and pupal development and adult reproduction, and to conduct validation tests with a radiation dose predicted to

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control *D. suzukii* to demonstrate it provides quarantine security.

Materials and Methods

Insect Rearing. A laboratory colony of *D. suzukii* was started from flies (≈100) trapped in a stand of strawberry guava, *Psidium cattleianum* Sabine, in Volcano, HI. Adult flies were held in 30- by 30- by 30-cm screen cages (Bioquip, Rancho Dominguez, CA) and maintained at 500–1,000 flies per cage. Immature stages were reared in 275-ml plastic containers with ventilated lids on an agar-based cornmeal–sugar–yeast diet using methods similar to those proposed by Walse et al. (2012). The diet recipe consisted of agar (13.5 g), water (1,500 ml), cornmeal (37.5 g), sugar (60 g), nutritional yeast (21 g), methyl paraben (1 g, dissolved in 10 ml of 90% ethanol), and propionic acid (12 ml). To obtain experimental insects, plastic containers without lids containing fresh diet were placed in adult cages for 24 h for oviposition, then removed, covered, and held in controlled environment rooms for development to the target life stage. Rearing conditions were 24°C (±2°C) and a photoperiod of 12:12 (L:D) h, and the time from egg to adult under these conditions was ≈14 d. Adults newly emerged from diet were transferred to adult cages as needed to maintain a relatively constant adult density. Larvae are difficult to count in diet, as they may feed at various depths, but pupation occurs at the surface of the diet and pupae are easily counted. The time from egg to pupation was ≈7 d and duration of the pupal stage was 7–8 d under our rearing conditions (24 ± 2°C). The colony had been in the laboratory for five generations at the time the irradiation experiments were initiated.

Dose–Response Tests. Larvae (first, second, and third instars) and pupae (1–2-d-old, 3–5-d-old, and 8–9-d-old) on diet were irradiated at target doses of 20, 30, 40, and 50 Gy in replicated factorial experiments. The egg is invariably the least radiation-tolerant immature stage when prevention of adult emergence or adult sterility is the desired response (Follett and Griffin 2006, Follett 2009), and therefore this stage was not tested. For each test, 275-ml plastic containers containing 45 ml of diet were exposed to reproductive adults for 24 h, and then removed and covered with ventilated lids to rear cohorts to the target life stage. Thirty larvae or pupae of the appropriate age were transferred from rearing containers using soft forceps to 59-ml plastic cups with lids containing 10 ml of diet and irradiated. After treatment, larvae and pupae were held in the cups and followed until pupation (larvae only) and adult emergence under normal rearing conditions. Emerged adults were frozen, counted, and sexed. For each larval stage or pupal age, six to eight replicates were irradiated at each dose, and an equal number of replicates were left untreated as a control group and held under the same conditions.

Large-Scale Validation Tests. *D. suzukii* eggs, larvae, and pupae can occur with exported fresh fruits and vegetables, and these life stages occur inside the commodity. Dose–response tests indicated that the late (5–9-d-old) pupa is the most radiation-tolerant stage and age. Large-scale validation testing was done with late pupae in sweet cherries or grapes at radiation doses of 60 and 80 Gy to identify a potential quarantine treatment.

Approximately 2.5 kg of fresh harvest-mature sweet cherries ('Bing') or grapes ('Thompson') were placed in an open-top clear plastic box (26.5 liter, 58 by 41 by 15 cm, Sterilite, Lima, OH) containing a 3-cm layer of sand to absorb any juice. The plastic box with fruit was placed inside a flexible screen cage (36 by 36 by 61 cm, Bioquip, Rancho Dominguez, CA) with ≈2,000 breeding *D. suzukii* for 72 h. After 72 h, the cage and plastic box with fruit and flies were placed in a 2°C room for 20 min (to arrest flight), and flies were brushed off the fruit with a soft bristled paint brush. Infested cherries or grapes were placed in a monolayer fashion in a new clear plastic box with sand and a ventilated lid, and the lid was taped to seal the edges and prevent escape. A custom-fit piece of fine mesh screen was put between the sand and fruit to prevent *D. suzukii* larvae from pupating in the sand. Sweet cherries or grapes in sealed plastic boxes were held at 24°C for up to 11 d to allow development to the late pupal stage and then irradiated. The holding time for development to the late pupa stage in fruit was determined by dissections before initiating the experiment. For each replicate, a subsample (10%) of infested sweet cherries or grapes was processed using a modified fruit screening (Liquido 1992) and dunking (Dreves and Langellotto-Rhodaback 2011) method to estimate the number of pupae and the percentage adult emergence in the irradiation and control treatments. After irradiation treatment, plastic boxes with infested fruit were opened and placed into new flexible screen cages for adult emergence. Emergence cages were placed inside larger flexible screen cages (61 by 61 by 91 cm, Bioquip, Rancho Dominguez, CA) to prevent contamination by other *Drosophila* fruit flies. Adults (parentals) emerging during 5 d were transferred in a 2°C cold room (where movement was arrested) to new screen cages, and then allowed to breed for 14–21 d at 24°C. Two 830-ml plastic food service trays (TriPak, Toronto, Canada) containing artificial diet were placed in the breeding cages to allow for oviposition and replaced every 3–4 d during the 14–21-d period. Diet was used instead of fruit to maximize survival and reproduction in adults surviving irradiation, and to observe any oviposition and immature development. After exposure to breeding adults, the trays with diet were transferred to separate screen cages and held for emergence of F_1 adults. After 14 d, any F_1 adults emerging from diet in the control and irradiated treatments were frozen and counted, and a subsample was sexed. Prevention of F_1 adult emergence was the desired response, as it indicated radiation-induced mortality or sterility in the parental generation and the absence of successful reproduction.

Irradiation Treatment. Irradiation treatment was conducted at a nearby commercial X-ray facility (CW Hawaii Pride LLC, Keaau, HI) using an electron linear accelerator (5 MeV, model TB-5/15, L-3 Communi-

Table 1. Maturation of *D. suzukii* larvae and pupae treated by irradiation

Stage	Dose (Gy)	Reps	No. of insects treated	No. of pupae	No. of adults			% adult emergence (±SE)
					Total	Males	Females	
First instar	0	8	240	169	156	85	71	65.0 (4.5)
	10	8	240	185	11	4	7	4.6 (1.8)
	20	8	240	187	3	2	1	1.3 (0.9)
	30	8	240	165	0	0	0	0
	40	8	240	165	0	0	0	0
Second instar	0	8	240	205	152	63	85	72.9 (5.9)
	10	8	240	214	112	59	53	46.7 (3.0)
	20	8	240	214	120	67	53	50.0 (7.7)
	30	8	240	211	77	36	41	32.1 (9.0)
	40	8	240	186	0	0	0	0
Third instar	0	8	250	243	213	93	120	84.9 (4.1)
	10	8	240	223	196	96	100	81.7 (3.5)
	20	8	240	224	177	80	97	73.8 (3.6)
	30	8	240	211	97	27	49	40.4 (8.4)
	40	8	240	217	1	0	1	0.4 (0.4)
Pupa (1–2 d)	0	8	257	–	216	103	113	84.5 (3.5)
	10	8	239	–	155	49	97	64.4 (6.9)
	20	8	235	–	141	46	95	59.8 (3.2)
	30	8	230	–	34	10	24	15.3 (5.2)
	40	8	232	–	2	0	2	0.9 (0.6)
Pupa (3–5 d)	0	9	270	–	226	104	122	83.7 (2.9)
	10	4	120	–	112	42	70	93.3 (1.9)
	20	4	120	–	105	52	53	87.5 (2.5)
	30	14	420	–	310	132	178	73.8 (4.2)
	40	14	420	–	317	147	170	75.5 (4.4)
	50	10	300	–	198	103	95	66.0 (5.6)
Pupa (8–9 d)	0	6	179	–	143	58	85	79.7 (5.4)
	10	6	181	–	137	49	88	75.7 (7.3)
	20	6	172	–	148	66	82	85.4 (4.3)
	30	6	179	–	140	67	73	78.1 (4.8)
	40	5	150	–	118	57	61	78.7 (5.2)

cations Titan Corp., San Diego, CA) at ambient temperature and atmosphere. Dosimeters (Opti-chromic detectors, FWT-70–40M, Far West Technology, Goleta, CA) were attached to containers containing larvae or pupae at each dose in each replicate. The dosimeters were read with a FWT-200 reader (Far West Technology, Goleta, CA) at 600-nm absorbance to measure dose variation in each treatment replicate. To minimize the DUR, plastic containers with *D. suzukii* life stages were placed in a single row perpendicular to the X-ray beam and elevated by placement on a cardboard box positioned in the center of the carrier. Each carrier passed in front of the beam in a forward and then a reverse orientation. The DUR during the *D. suzukii* research was <1.1. After irradiation treatment, *D. suzukii* life stages were returned to the laboratory and held under standard rearing conditions.

Statistical Analysis. To make comparisons of radiotolerance between life stages, dose–response data on percentage adult emergence were arcsine-transformed and subjected to linear regression and analysis of covariance using the standard least squares model (SAS Institute 2002, Cary, NC). Linear regression was selected after probit and logit models showed poor fit to the data in four of the five life stages tested (Robertson et al. 2007). For each replicate, mortality values <100% were adjusted for control mortality using Abbott's formula (Abbott 1925). Residual plots were evaluated to ensure regression model assumptions were met for each treatment combination. Covariance analysis requires the slopes of the regression lines fitted to each group to be parallel, so the assumption of parallelism (nonsignificant life stage × dose interaction effect) was tested before evaluating intercepts (life stage effects; Sokal and Rohlf 1981). For the large-scale validation tests, the level of confidence associated with treating a number of insects with zero survivors is given by the following equation:

$$C = 1 - (1 - p_u)^n$$

where p_u is the acceptable level of survivorship (as a proportion) and n is the number of test insects (Couey and Chew 1986). Confidence levels were calculated for the number of *D. suzukii* irradiated in sweet cherries, assuming the required efficacy ($[1 - p_u] \times 100$) is 99.99% (Follett and Neven 2006, Follett and Hennessey 2007).

Results

Tolerance to radiation in *D. suzukii* increased with increasing age and developmental stage (Table 1). No first or second instars, and only one third instar and two 1–2-d-old pupae, developed to the adult stage at a radiation dose of 40 Gy. Late-stage pupae (3–5-d-old and 8–9-d-old) readily completed development and emerged as adults in all radiation treatments. Covariance analysis was performed to compare the relative tolerance of the *D. suzukii* life stages found in fruit. Dose–response data for percentage adult emergence were significant ($P < 0.01$) for the effect of life stage, irradiation dose, and the life stage by irradiation dose

Table 2. Linear regressions on prevention of development to adult when various life stages of *D. suzukii* were treated by irradiation

Stage	Observations	Y-intercept (±SE)	Slope (±SE)	R^2	Predicted doses (±95% CL)	
					LD_{50}	$LD_{99.99}$
First instar	32	92.0 ± 1.9	0.2 ± 0.07	0.27	–	34.7 (27.7–53.2)
Second instar	32	2.4 ± 11.0	2.2 ± 0.40	0.49	22.1 (16.8–26.4)	45.4 (38.6–59.1)
Third instar	32	−36.4 ± 8.1	3.2 ± 0.30	0.79	27.2 (25.1–29.5)	43.0 (39.5–47.8)
1–2 d pupa	32	−10.0 ± 7.7	2.7 ± 0.28	0.76	21.8 (19.2–24.2)	40.0 (36.7–44.8)
3–5 d pupa	46	−4.8 ± 7.2	0.5 ± 0.20	0.14	104.0 (73.7–308.4)	198.8 (128.4–681.8)
8–9 d pupa	23	8.4 ± 5.9	−0.1 ± 0.21ns	0.01	–	–

Regression analysis used adult survivorship data from Table 1 corrected for control mortality using Abbott's formula; ns, slope not significantly different from 0.

interaction. For development to the adult stage, first instars were less radiation-tolerant than third instars (t-ratio = 2.0; $P < 0.001$), third instars were less tolerant than 1–2-d-old pupae (t-ratio = 2.0; $P < 0.001$), and 1–2-d-old pupae were less tolerant than 3–5-d-old pupae (t-ratio = 1.9; $P < 0.001$). Three- to five-day-old pupae and 8–9-d-old pupae were equally tolerant to radiation treatment (t-ratio = 2.0; $P = 0.15$), and were designated as the most tolerant life stages. For larvae and 1–2-d-old pupae, the dose by sex ratio interaction was not significant ($F_{1,79} = 0.9$; $P = 0.34$), indicating that males and females were equally susceptible to irradiation.

Linear regression was used to test whether slopes were significantly different from 0 (significant effect of radiation dose) for each life stage. Slopes were positive and significant for first, second, and third instars, and for 1–2-d-old and 3–5-d-old pupae ($P < 0.001$), indicating that mortality generally increased with increasing dose. The slope for 8–9-d-old pupae was not significantly different from 0, indicating that radiation treatment at 10–40 Gy did not affect adult emergence when pupae were close to ecolsion (Table 2).

The late-stage pupa is the most radiation-tolerant stage that may occur in fruit. Because prevention of adult emergence is not easily achieved in late-stage pupae, the appropriate required response for radiation treatment in *D. suzukii* is sterility or prevention of F_1 adults. Mortality in immature life stages (eggs, larvae, and pupae) of *D. sukukii* is difficult to measure as individuals are hidden inside fruit. With diet, eggs are easily detected but emerging larvae burrow into the media and become cryptic. Therefore, the desired response criterion in large-scale validation tests was failure to produce F_1 adults in irradiated late-stage pupae. A radiation dose of 60 Gy applied to late-stage pupae in sweet cherries resulted in 51 F_1 adults after irradiation of 3,770 parental late pupae, whereas 15,696 F_1 adults were produced by 380 untreated control pupae (Table 3). A radiation dose of 80 Gy applied to 33,086 late-stage pupae in sweet cherries (25,046) and grapes (8,040) resulted in no production of F_1 adults (Table 3). No eggs or immature stages were observed in the diet that was exposed to irradiated flies, suggesting that the 80-Gy treatment caused a high level of sterility. Assuming a required efficacy of 99.99%, $C = 1 - (1 - 0.0001)^{33,086}$, and our confidence level is 96.3% and the true sterility of *D. suzukii* is <0.0001.

Discussion

Tolerance to radiation in *D. suzukii* increased with increasing age and developmental stage. The late-stage pupa was the most radiation-tolerant stage that may be found in fruit. Males and females were equally susceptible to radiation. Australia and Japan generally have required 99.99% efficacy as a basis for approving treatments as meeting quarantine security requirements, which can be demonstrated by treatment of 29,956 individuals with no survivors (Follett and Neven 2006). A radiation dose of 80 Gy applied to late-stage pupae prevented successful adult reproduction (no survivors to F_1 adult stage) in 33,086 treated individuals, which meets the zero tolerance requirements for market access.

Trade with international markets often requires the preshipment certification of a phytosanitary treatment that satisfies the efficacy requirements of the importing country. Several countries such as Australia regulate *D. suzukii* as a quarantine pest and require a postharvest fumigation with methyl bromide to mitigate the risk of introduction. Walse et al. (2012) developed a methyl bromide treatment schedule for control of *D. suzukii* in California strawberries to maintain market access to Australia. Cold treatment is another postharvest treatment option for tolerant

Table 3. Large-scale validation tests irradiating *D. suzukii* late-stage pupae in fruit

Fruit	Target dose (Gy)	Measured doses	No. of replicates	No. of pupae treated	No. of parental adults	No. of F_1 adults	F_1 adults	
							% males	% females
Sweet cherries	60	49	4	3,770	3,141	51	–	–
	Control	–	4	380	349	15,696	37	63
	80	70–78	35	25,046	10,572	0	–	–
	Control	–	15	1,361	1,248	17,590	50	50
Grapes	80	70–74	4	8,040	6,700	0	–	–
	Control	–	4	2,748	2,516	1,100	47	53

crops and may be applied before or during transit. Dalton et al. (2011) showed that exposure to 1°C for 17 d caused complete mortality in *D. suzukii,* suggesting a potential target for a cold treatment protocol. An advantage to irradiation is that a generic treatment is available for almost all pests irrespective of commodity (Follett 2009); use of the 400-Gy generic dose requires no specific data for the target pest or commodity, and therefore, this treatment can be used immediately to open or maintain an export market if the trading partner has approved phytosanitary uses of irradiation. Lowering the dose, as we have for *D. suzukii,* from 400 to 80 Gy will lower treatment costs, increase product throughput owing to shorter treatment time, and reduce or eliminate any adverse effects of irradiation on fruit quality.

Relatively few countries currently have regulatory approvals to accept irradiated fresh agricultural products. In 1989, USDA–APHIS published the first rule to allow the use of irradiation as a phytosanitary treatment (USDA–APHIS 1989, Follett and Griffin 2006). The earliest commodity approvals using irradiation provided treatment protocols for importation of several tropical fruits and sweet potato from Hawaii (Follett and Weinert 2012). For U.S. trade with a foreign country, a framework equivalency work plan is a prerequisite, bilateral agreement identifying the key components and steps for establishing cooperation in irradiation. The purpose of the agreement is to develop a common understanding of capabilities, capacities, intents, and expectations before both countries invest resources in this effort, and to establish that each country must accept each other's systems and irradiated products (Follett and Griffin 2006, Follett 2009). Currently, only India, Thailand, Vietnam, Mexico, Pakistan, the Philippines, Malaysia, South Africa, and Laos have signed a framework equivalency work plan. Australia, Jamaica, and Peru are on the horizon. The United States has not exported any irradiated fresh commodities partly because of the significant investment in a treatment facility, the limited number of countries with regulatory approvals, and the lack of approvals for many of our main trading partners for fresh agricultural products. Key trading partners in fresh agricultural products such as Canada, the European Union, Japan, Korea, and China must approve the use of phytosanitary irradiation before the availability and use of this technology can expand in the United States (Follett and Weinert 2012).

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