Ionizing Radiation as a Phytosanitary Treatment Against Fruit Flies (Diptera: Tephritidae): Efficacy in Naturally Versus Artificially Infested Fruit

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ABSTRACT Some phytosanitary irradiation treatment research against tephritid fruit flies (Diptera: Tephritidae) has used artificially infested fruit with the unstated and untested assumption that the method adequately simulated a natural situation. We compare grapefruit, *Citrus paradisi* Macfayden, naturally infested by Mexican fruit fly, *Anastrepha ludens* (Loew), via oviposition until larvae reached the late third instar versus insertion of diet-reared third instars into holes made in grapefruits 24 h before irradiation; the latter technique has been used in other studies. Both infestation techniques resulted in statistically indistinguishable results, indicating that insertion of diet-reared third instar Mexican fruit fly into holes bored into grapefruit and subsequently sealed 24 h before irradiation would adequately represent natural infestation and could be used to develop a radiation phytosanitary treatment of the insect in grapefruit when prevention of adult emergence is used as the measure of efficacy. Nevertheless, it may not be advisable to extend this conclusion to other fruit fly/fruit combinations without doing appropriate comparison studies. Dissection of puparia from nonirradiated control insects that failed to emerge as adults showed a relatively even distribution of mortality among the developmental stages within the puparium. In contrast, dissection of puparia from irradiated third instars that did not emerge as adults revealed a sharp attenuation in development from cryptocephalic to phanerocephalic pupae demonstrating this transition to be the developmental step most affected by radiation.

KEY WORDS *Anastrepha ludens*, quarantine, commodity treatment, radiation, disinfestation

Ionizing radiation is used as a phytosanitary treatment in Australia, India, Mexico, the United States, India, Thailand, and Vietnam at present. Interest in the treatment is increasing because it possesses some advantages compared with other treatments. In general, irradiation is tolerated by commodities better than fumigation or temperature treatments (Heather and Hallman 2008). A key technical disadvantage is that it is the only commercially applied treatment that does not result in death of the pest soon after the treatment is applied, providing no direct verification of treatment efficacy. The measure of efficacy of irradiation is prevention of development and/or reproduction, rather than acute mortality. Consequently, the discovery of live quarantine pests on inspection of the commodity for which a certified irradiation treatment is applied does not indicate treatment failure. For virtually every other treatment, when live quarantine pests are found at inspection the consignment is rejected or retreated regardless of treatment certification. In such cases it is assumed that the treatment was not properly done or did not work or that the shipment was contaminated with nontreated, infested commodity or reinfested after treatment. Because there is no independent confirmation of efficacy, greater validation of the research process may be advisable for irradiation treatments compared with those treatments where acute mortality is the endpoint.

Fruit flies (Diptera: Tephritidae) comprise the chief group of quarantine pests for which phytosanitary treatments are targeted (Hallman 1999). The measure of efficacy of fruit irradiation against tephritid fruit flies is typically the prevention of adults capable of flight regardless of the stage (eggs or larvae) infesting the fruit and efficacious doses range from 50 to 150 Gy (Heather and Hallman 2008). Research typically involves infesting fruit and irradiating it when the insect is in the third instar, the most radiotolerant developmental stage that may be present in fruit (Hallman 1999). Irradiation of fruit fly immatures in diet or in vitro may result in a greater effect than the same dose in fruit. For example, the estimated dose to prevent adult emergence of irradiated third instar Mexican fruit fly, *Anastrepha ludens* (Loew), in grapefruit, *Citrus paradisi* Macfayden, was >5× the dose needed in vitro (Hallman and Worley 1999).

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Testing procedures, in particular the method of infestation may significantly alter the results of phytosanitary treatments (Heather and Hallman 2008). The most accurate method is arguably the method closest to the natural situation. However, such methods may have disadvantages, such as difficulty in controlling the infestation rate or achieving uniformity of stage and pest distribution. For purposes of developing an effective quarantine treatment, a more manageable, artificial method may be used if it results in insects that are not less susceptible to the treatment in comparison with insects present in a commodity infested using a more natural method.

Infestation involving placement of insects in sealed containers inside of fruit (Raga 1990, 1996) is essentially an in vitro situation as there is no biological interaction between the fruit and insect; thus, no direct effect of the fruit on radiation response of the insect. Although fruit flies treated in vitro are often controlled with lower doses than those treated in fruit (Hallman and Worley 1999, Follett and Armstrong 2004), the use of sealed containers could result in a low oxygen environment that might increase radiotolerance (Hallman and Phillips 2008). Regardless, insertion of fruit flies in sealed containers in fruit seems excessively artificial and of little practical merit.

Another technique used in tephritid research involves sealing a uniform number of diet-reared third instars inside a fruit, followed by application of the irradiation treatment (Mansour and Franz 1996, Follett and Armstrong 2004, Palou et al. 2007). A more natural technique involves exposing fruit to oviposition in cages and holding the fruit until the resulting infestation reaches the desired stage of development before irradiation (Arthur et al. 1993b, Bustos et al. 2004). This infestation method is close to the natural situation provided that care is taken to avoid overinfestation, which might cause decomposition before treatment. This may be difficult to avoid for some fruits, but has been accomplished adequately for many fruit, such as plum (*Prunus* spp.), mango (*Mangifera indica* L.), apple (*Malus* spp.), and citrus (*Citrus* spp.) (Hallman 1999).

The objective of this research was to compare two infestation techniques: 1) simulation of the natural situation by exposing fruit to oviposition and holding the fruit until third instars develop; and 2) rearing third instars in diet and inserting them into fruit before irradiation, for radiotolerance of a tephritid in a fruit. This objective includes examination of the literature where applicable because the intent of this research is to address this issue for tephritids in general, not simply the one species studied here.

Materials and Methods

Source of Insects. Mexican fruit flies were from the USDA–APHIS Mexican Fruit Fly Rearing Facility at Mission, TX, and originated with flies collected in Morelos, Mexico (Moreno et al. 1991). This strain is used in the Mexican fruit fly sterile release programs in Texas, California, and Mexico.

Irradiation Source and Dosimetry. The irradiation machine (Husman model 521A, Isomedix, Inc., Whippany, NJ) uses gamma rays from ^{137}Cs in a sealed environment and is located at the USDA–APHIS Mexican Fruit Fly Rearing Facility. It emitted a dose rate of ≈40 Gy/min. Reference standard dosimetry was done in 1996 with the Fricke system and routine dosimetry was performed at the time of the treatment with radiochromic film (Gafchromic MD-55, ISP Technologies, Inc., Wayne, NJ) placed in areas of the load (center and edges) with the most extreme dose readings. Dosimeters were read with a spectrophotometer (Milton Roy Spectronic 401, Ivyland, PA) at 600 nm.

Fruit Infestation and Irradiation. Two infestation techniques were compared for response of Mexican fruit fly to irradiation. For one technique, lots of ≈50 'Rio Red' grapefruit harvested near Weslaco, TX, were placed in a screen cage (1.2 by 0.8 by 0.5 m) with ≈20,000 Mexican fruit fly adults for 1.5 h. Fruit were subsequently held at 26.5 ± 0.5°C for sufficient time (14–19 d) to allow all or most of the larvae to become late third instars but were treated before mature larvae began emerging. Fruit were periodically opened to determine stage. When late third instars were the predominant stage the grapefruits were irradiated with 15, 20, 25, or 30 Gy; total numbers of third instar irradiated at each dose were 1,804, 689, 2,300, and 589, respectively. In addition, there was a nonirradiated control with a total of 436 larvae. The mean number of third instars per grapefruit over the entire experiment was 14.5 ± 3.2, and there were three to five replicates at each dose. Decomposing fruit or those with emergence holes were removed from the experiment.

For the second infestation technique, 25 third instars reared on diet were placed in 10-mm-diameter holes bored to the center of 10–20 grapefruits, and the grapefruit plug was then replaced and sealed with hot-melt glue. After 24 h at 26.5 ± 0.5°C, the grapefruits were irradiated with 15, 20, 25, or 30 Gy. A nonirradiated control with a total of 806 larvae was included. There were three to five replicates of each dose with total numbers of third instars tested ranging from 740 to 2,390 per dose; higher doses used a greater number of larvae to detect survivors. Fruit from both infestation techniques were irradiated together.

Twenty-four hours after irradiation, the grapefruits were opened and all larvae removed and placed in plastic containers (230 ml) with moist vermiculite for further development. Larvae less developed than the late third instar were discarded. After adult emergence the remaining puparia with dead insects inside were opened to determine how close to adult emergence the insects developed. Nomenclature for puparial stages follows Thomas and Hallman (2000).

Adult emergence in the nonirradiated controls of both infestation techniques ($n = 22$) was tested for normality ($P < 0.05$) before being tested for mean difference with a two-tailed t-test. Adult emergence between the two infestation techniques at 25 Gy

Table 1. Percentage nonemergence of adult *A. ludens* when irradiated as third instars in grapefruit infested by inserting diet-reared third instars in grapefruit 24 h before irradiation or by placing grapefruit in cages with adult *A. ludens* and irradiating when third instars developed

Dose (Gy)	Non emergence for fruit infestation technique (% ± SEM)[a]	
	Insertion of diet-reared third instar in fruit	Oviposition and larval development in fruit
0	19.8 ± 3.7	12.2 ± 3.5
15	86.9 ± 3.6	89.2 ± 2.6
20	95.5 ± 2.0	98.84 ± 0.73
25	98.46 ± 1.5	99.03 ± 0.65
30	100 ± 0	99.83 ± 0.07

[a] Percentages are based on adults fully emerged per larvae treated.

(which results in a high level of prevention of adult emergence) was subjected to the same test ($n = 10$).

Dose–response data were analyzed by probit analysis (SAS 9.1, SAS Institute, Cary, NC) using the Gompertz probability density function (PDF).

Results

Absorbed doses varied consistently from −3 to +28% around the target doses, with means ≈16% greater than target doses. Lower absorbed doses were near the middle of the load whereas higher doses were at the peripheries. Adult emergence in the controls was 87.8 ± 3.7 and 80.2 ± 3.5% for the cage-infested and inserted insects, respectively. The data did not depart from normality (KS distance = 0.23 and 0.22 for the cage-infested and inserted insects, respectively). There was no significant difference between the two infestation techniques regarding adult emergence in the controls ($t = 1.45$, df = 20, $P = 0.16$).

For third instars irradiated at 25 Gy, mean adult emergence was 1.0 ± 0.65 and 1.5 ± 0.55% for the cage-infested and inserted insects, respectively. The data did not depart from normality (KS distance = 0.35 and 0.21 for the cage-infested and inserted insects, respectively). The t-value was 0.57 (df = 8; $P = 0.58$), indicating no statistically significant difference between the two infestation techniques regarding adult emergence at 25 Gy.

Postirradiation adult emergence among the two infestation techniques is summarized in Table 1. Data from larval insertion fit the Gompertz PDF, whereas that from cage infestation did not (Table 2). Because data from cage infestation did not fit the model it is not advisable to do direct statistical comparisons of the two infestation techniques regarding efficacy of irradiation. However, qualitative comparisons can be made. Response of Mexican fruit fly third instars to radiation essentially did not differ between the two infestation techniques compared, although one third instar of 589 emerged as an adult in the cage infestation at 30 Gy, whereas none did for the insertion technique when 931 third instars were irradiated.

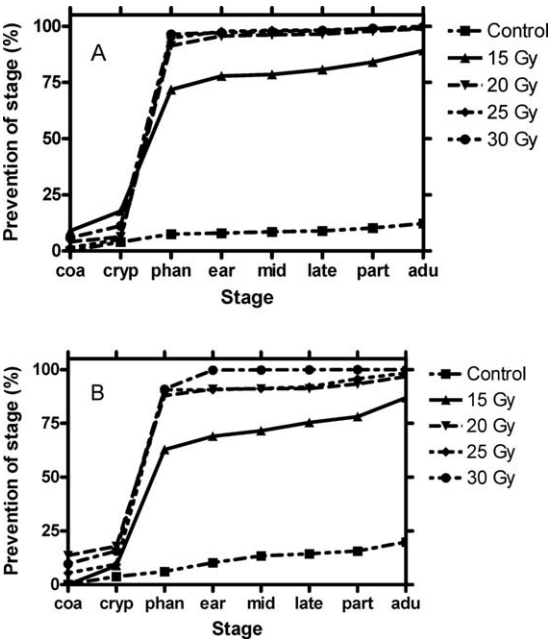

Fig. 1. Failure to achieve developmental stages when Mexican fruit flies are irradiated (15–30 Gy) as third instars in grapefruit by using two infestation techniques: oviposition in grapefruit (A) and insertion of third instars reared in diet into grapefruit 24 h before irradiation (B). Stages: coa, coarctate larva; cryp, cryptocephalic pupa; phan, phanerocephalic pupa; ear, early pharate adult; mid, middle (red-eye) pharate adult; late, late pharate adult; part, adult partially emerged from puparium; and adu, adult fully emerged from puparium.

Dissection of puparia revealed a relatively low lack of development among the developmental stages for nonirradiated third instars for both infestation techniques (Fig. 1). In contrast, dissection of irradiated third instars revealed a sharp cessation in development at the step from cryptocephalic to phanerocephalic pupa. Among previous and subsequent steps the distribution of lack of development is similar to that observed in the controls demonstrating that the most affected developmental step for irradiated Mex-

Table 2. Probit analysis (Gompertz probability density function)[a] of nonadult emergence of *A. ludens* adults from irradiated third instars infesting grapefruit by placing diet-reared third instars in grapefruit 24 h before irradiation (artificial) or by placing grapefruit in cages with adult *A. ludens* and irradiating when third instars developed (cage)

Infestation method	Slope ± SE	ED_{90} (95% CL) (Gy)	ED_{99} (95% CL) (Gy)	Probability > χ^2
Insertion of third instar	0.073 ± 0.0045	15.2 (6.6–18.2)	26.1 (25.2–27.3)	0.12
Oviposition in fruit	0.038 ± 0.012	15.1 (0.55–18.7)	24.2 (20.3–47.9)	0.001

[a] Degrees of freedom = 2.

ican fruit fly third instars was at the transition to the phanerocephalic pupal stage. The slopes of lack of development for irradiated third instars with both infestation techniques were similar, indicating no obvious difference in response among the puparial stages.

Unlike the transitions among the other stages of development, the transition to the phanerocephalic stage requires physical exertion. Eversion of the head and limbs is accomplished by means of vigorous muscular contractions (Zdarek and Friedman 1986). Hence, the radiation induced developmental arrest observed in the current study is the same as that reported previously in vitro (Thomas and Hallman 2000) and was induced to both treatment groups.

Discussion

Insertion of diet-reared Mexican fruit fly third instars into grapefruit 24 h before irradiation seems to be an acceptable substitute for more natural infestation of the fruit via oviposition. However, one anonymous reviewer pointed out the fact that one larvae (0.17%) emerged as an adult from cage-infested larvae irradiated with 30 Gy, whereas none emerged from inserted larvae at that same dose might cause some regulatory agencies to require that research be done using a natural infestation technique regardless of statistical significance. It must be noted that the regulatory agency of the importing country invariably has the last word regarding supporting data required and how the research is conducted to support a phytosanitary measure for export of commodities to their country. It would be safer in a situation like this to do the research in the way most reflective of the natural situation when there is any doubt.

The organism for which the most studies of phytosanitary irradiation have been done is arguably the most important arthropod from a plant quarantine standpoint, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Torres-Rivera and Hallman 2007). Because several studies have been done with *C. capitata* using different infestation techniques, together with our study on *A. ludens* these may shed light on any effect of infestation on the efficacy of phytosanitary irradiation. No other tephritid has been studied to an extent where the effect of infestation technique on efficacy can be examined. Those studies providing multiple dose–response points are of particular usefulness (Table 3) because they can be analyzed by appropriate statistical measures. The methods used for infestation of fruit with *C. capitata* can be divided into three categories: 1) placement of larvae within a small container inside a cavity bored into the fruit, 2) placement of larvae directly into a cavity bored into the fruit, and 3) oviposition into fruits allowing the larvae to range within the fruits in a close approximation to field conditions. These different infestation techniques for *C. capitata* have not been compared in the same study.

Studies with three host species used larvae in vials inserted into the fruit: Raga (1990) placed 20–25 *C.*

Table 3. Percentage emergence[a] of adult *C. capitata* when irradiated as third instars in fruit infested in various ways; see text for details of infestation

Dose (Gy)	Adult emergence (%) when infesting various fruits via three techniques										
	Infestation via insertion of third instars in vials in fruit			Infestation via insertion of third instars in fruit					Infestation via oviposition		
	Mango (Raga 1990)	Orange (Raga 1996)	Grapefruit (Raga 1996)	Papaya (Faria 1989)	Orange (Mansour and Franz 1996)	Peach (Mansour and Franz 1996)	Papaya (Follett and Armstrong 2004)	Mandarin (Palou et al. 2007)	Plum (Arthur et al. 1993a)	Plum (Arthur et al. 1993b)	Mango (Bustos et al. 2004)
0	92.5	95.5	98.0	87.3	68.2	48.9	75.1	≈44	24.4	82.9	90.4
10	87.5	86.5	92.5	77.5							75.1
15		86.5	59.5	62.8							
20	9.5	73.0	7.5	11.5	2.4	2.5	6.4				37.7
25		46.0	3.0	0.84			4.0		1.3	5.1	
30	0		0	0.67	0.8	0.7	1.5	≈1.5			4.5
35				0			0.57				
40					0	0	0.47				2.1
45							0.25				
50							0.07	0	0	0	
60							0				0.3

[a] Percentages are based on adults emerged per larvae treated except for Arthur et al. (1993b), which is based on adults emerged per puparia formed because no. of larvae were not given.

Table 4. Probit analysis (Gompertz probability density function) of nonadult emergence of *C. capitata* adults from irradiated third instars infesting fruit by placing diet-reared third instars in fruit before irradiation (artificial) or by placing grapefruit in cages with adults and irradiating when third instars developed (cage)

Infestation method	Fruit	Slope ± SE	ED_{90} (95% CL) (Gy)	ED_{99} (95% CL) (Gy)	Probability > χ^2	df	Reference
Insertion of third instar	Papaya	0.18 ± 0.034	22.7 (19.2–30.9)	28.6 (23.6–41.9)	<0.0001	5	Faria (1989)
Insertion of third instar	Orange	0.036 ± 0.0066	10.3 (2.1–14.5)	29.4 (27.0–33.1)	0.14	1	Mansour and Franz (1996)
Insertion of third instar	Peach	0.043 ± 0.0064	14.3 (9.7–17.1)	30.6 (28.5–34.0)	0.17	1	Mansour and Franz (1996)
Insertion of third instar	Papaya	0.039 ± 0.0026	18.0 (16.1–19.4)	36.0 (34.6–37.7)	0.41	6	Follett and Armstrong (2004)
Oviposition in fruit	Mango	0.10 ± 0.012	31.1 (27.2–36.9)	38.0 (33.1–46.0)	<0.0001	3	Bustos et al. (2004)

capitata larvae in polyethylene tubes (7 mm in diameter by 130 mm in length) with diet and inserted the tubes into 8-mm-diameter holes in papaya. The ends of the tubes were sealed with wax sheeting. The time intervals between infestation and irradiation or irradiation and removal were not given. Raga (1996) placed 20 diet-reared third-instar *C. capitata* into diet-filled polyethylene tubes (7 mm in diameter by 100 mm in length) with the ends sealed with wax sheeting or diet-filled glass vials (16 mm in diameter by 100 mm in length) with lids inside oranges and grapefruits, respectively, to be irradiated.

In a total of five studies (two reported in the same reference) larvae were inserted directly into the fruit. Faria (1989) put 15 diet-reared third instars into each of two 1- by 1.5-cm holes bored into papaya, sealing the hole with the same plug of papaya and some tape. The time intervals between infestation and irradiation or irradiation and removal were not given. A dose of 35 Gy prevented adult emergence. Mansour and Franz (1996) placed diet-reared *C. capitata* third instars into holes made with a dissecting needle in oranges and peaches. Larvae were left in the fruit for ≈30 h before irradiation. At 30 Gy, adult emergence (0.7–0.8%) was very similar to Faria (1989). Follett and Armstrong (2004) reared third-instar *C. capitata* in diet and placed them in the cavity of papayas, *Carica papaya* L., accessed through a hole made with a 12-mm-diameter cork borer 24 h before irradiation. A higher dose (60 Gy) than Faria (1989) and Mansour and Franz (1996) was required to prevent adult emergence. Palou et al. (2007) placed diet-reared third-instar *C. capitata* into 10-mm-diameter holes bored into mandarin, *Citrus reticulata* Blanco, fruit and sealed by replacing the fruit plug and covering it with warm paraffin. It is not clear how long they were left in the fruit before irradiation, but it was at least 36 h. Control emergence was low (≈44%), but other than that the two data points reported (30 and 50 Gy) were similar to Follett and Armstrong (2004).

Three studies with multiple doses used wild or cage infested *C. capitata.* Arthur et al. (1993a,b) used plums naturally infested from the field and also placed non-infested plums in a cage with adult *C. capitata* and held the fruit until third instars developed. Only two data points were reported; adult emergence was 1.3–5.1% at 25 Gy and 0% at 50 Gy. Bustos et al. (2004) infested mangoes by placing them in cages with adult *C. capitata* and holding the infested fruit until third instars had developed. The highest data point reported (60 Gy) resulted in 0.3% adult emergence, greater than all of the other studies using larval insertion or infestation by oviposition (Table 3). When corrected for failure of adult emergence in the controls (Abbott 1925) all of the studies using infestation via oviposition resulted in greater numerical radiotolerance than all of the studies using infestation by insertion of larvae directly into fruit.

Other studies using infestation via oviposition but without multiple doses yielding varying levels of efficacy also indicate that higher doses are required to prevent adult emergence of *C. capitata* although Hallman (1999) argues that some of these studies suffered from other problems, such as inadequate dose measurement or accidental posttreatment reinfestation.

Greater radiotolerance was noted in the study using cage infestation compared with the studies using inserted diet-reared third instars except for the study in oranges using third instars placed in polyethylene tubes, which reported 46% adult emergence at 25 Gy, the highest dose used (Raga 1996). This level of adult emergence was >15× the level reported in the same study using third instars in glass vials inserted in grapefruits. The author does not comment on this remarkable difference and we are at a loss to speculate how it might be valid.

Four of the five studies using infestation via insertion of third instars in fruit and one of the three studies using infestation via oviposition had enough data points (at least three plus a control) to do probit analysis, which we did using the Gompertz probability density function (SAS 9.1, SAS Institute). Unfortunately, the lone study using infestation via oviposition (Bustos et al. 2004) did not fit the model used (Table 4), so direct comparisons to insertion infestation techniques (where three of four studies fit the model) cannot be made. Nevertheless, it seems that *C. capitata* radiotolerance could be greater in the more natural infestation technique of subjecting fruit to oviposition compared with insertion of diet-reared third instars into fruit. If this is indeed the case, it would behoove researchers to use the more natural technique when developing radiation phytosanitary treatments. Even if the greater radiotolerance in more natural infestation techniques was minimal or not statistically significant the fact that there is evidence that insects

require a larger dose to control in a more natural setting compared with an artificial setting would necessitate that the more natural situation be used in research defining phytosanitary treatments. A more liberal stance in rejecting a null hypothesis than the 95% confidence level typical of statistical studies should be taken when deciding whether to use an artificial infestation system for supporting a phytosanitary treatment if there is indication that quarantine pests in the artificial system may be controlled with lower doses than in a more natural setting. The opposite would not be true, i.e., if pests in the artificial system required higher doses, the resulting treatment might be excessive, which would, of course, make it efficacious.

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