Mortality and growth inhibition of γ-irradiated red scale *Aonidiella aurantii* (Hemiptera: Diaspdidae) on 'Kinnow' citrus (Sapindales: Rutaceae) fruits

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Abstract

Gamma-irradiation was assessed as a potential phytosanitary treatment to control the red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), a quarantine pest of citrus. Dose response tests were conducted with first and second stage nymphs and adult females—before they had given birth to 1st instars (crawlers)—with a series of radiation doses ranging from 100 to 300 Gy to determine the minimum dose required to inhibit normal adult emergence of the most radiation tolerant stage. All tests involved the use of the fruits of 'Kinnow', a mandarin hybrid (*Citrus reticulata* Blanco; Sapindales: Rutaceae). Irradiation affected every life stage of *A. aurantii*. The pattern of tolerance to irradiation in *A. aurantii* was eggs < 1st instars < 2nd instars < adult females with eggs but not crawlers. Adult females with eggs irradiated with 220 Gy did not produce a F_1 generation. Validation tests showed that a minimum dose of 222 Gy would serve as a phytosanitary treatment against *A. aurantii*.

Key Words: citrus; phytosanitary; export; quarantine pests; 'Kinnow'; Pakistan

Resumen

Se evaluó la irradiación-gamma como tratamiento fitosanitario potencial para controlar la escamaroja, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), una plaga cuarentenaria de los cítricos. Se realize una prueba de respuesta de dosis sobre ninfas del primer y segundo estadio y de hembras de tercer estadio—antes de que hubieran dado a luz a 1er estadios (rastreadores)—con una serie de dosis de radiación de 100 a 300 Gy para determinar la dosis minima requerida para inhibir la emergencia de adultos normales del estadio mas tolerante de la radiación. La irradiación afectó todos los estadios de vida de *A. aurantii.* Todas las pruebas incluyeron el uso de los frutos de 'Kinnow', un híbrido de mandarina (*Citrus reticulata* Blanco; Sapindales: Rutaceae). El patrón de la tolerancia a la radiación en *A. aurantii* fue huevos < 1ª estadio < 2º estadio < hembras pre-oviposición. Las hembras adultas irradiadas con 220 Gy no produjeron una generación F_1 grávido. Las pruebas de validación mostraron que una dosis mínima de 220 Gy serviría como tratamiento fitosanitario contra *A. aurantii*.

Palabras Clave: cítricos; fitosanitaria; exportación; plagas de cuarentena; 'Kinnow', Pakistán

A broad variety of fruits are grown in Pakistan thanks to its wide range of tropical, sub-tropical and temperate climates. Pakistan ranks among the 10 leading citrus growing countries and exports citrus to Russia, Iran, Afghanistan and the Middle East. Domestic and international demand for Pakistani 'Kinnow', a mandarin hybrid (*Citrus reticulata* Blanco; Sapindales: Rutaceae), is strong. Foreign fruit vendors generally prefer the 'Kinnow' mandarin hybrid from Pakistan due to its good taste and easy peeling. However, 'Kinnow' like other Pakistani citrus fruits is often infested by citrus scale insects, which are regulated pests in many parts of the world where the eggs and nymphs can be transmitted to fresh fruits and nursery plants (Hennessey et al. 2014). For this reason entry into such countries of Pakistan-grown citrus is prohibited. Recently phytosanitary irradiation has been adopted as a safe measure against quarantine pests (Hallman 2012) and its use has gained importance in the export of fresh agricultural commodities (Heather & Hallman 2008; Hallman 2011).

Radiation of fresh commodities has advantages over other post-harvest treatments, such as cold and hot water treatment, pressure washing and air tight treatments. Heat and cold treatments require a balance between killing the pest and commodity quality, while irradiation treatment can be developed and applied for a pest species irrespective of the host. Furthermore, the quality of most commodities is not affected at radiation doses that the required phytosanitary level of control the pests (Hallman 2011)

Research data on the quarantine treatments for scale insects using irradiation are limited. Pakistan Radiation Services (PARAS)—a joint venture of Pakistan Horticulture Development and Export Board (PHDEB) and Pakistan Atomic Energy Commission (PAEC)—has adopted a dose of 400 Gy for disinfestation of insect pests of citrus and mango. The 400 Gy dose is probably excessive for many pests. A reduction in the dose will reduce the risk of damage to fresh commodities as well as the cost and duration of treatment (Hallman 2012). Specific doses developed for individual pest species would increase the possibility of establishing new generic doses. This is especially true for scale insects since relevant data are limited. The objective of the present study was to determine the minimum effective irradiation dose to inhibit development and emergence of *Aonidiella* spp. (Hemiptera: Diaspididae) armored scale insects.

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Materials and Methods

COLONIZATION OF CITRUS RED SCALES AND SAMPLE PREPARATION

A colony of citrus red scales *Aonidiella aurantii* Maskell was started from newly hatched crawlers collected from 'Kinnow' citrus fruits at the Agricultural Research Institute (ARI), Tarnab, Peshawar, Pakistan. The infested fruit were placed at the bottom of a box on a wooden rack (60 × 30 cm). A sliding cover fitting the wooden rack was placed over the fruit. Each cover had 3 cm-diam holes spaced at a distance of 4 cm (total 21 holes). Connecting tubes (6 cm long × 2 cm diam) were inserted in the holes of the sliding cover in such a way that the lower end of each tube was touching an infested fruit at the bottom of the wooden rack and the upper end was touching a non-infested 'Kinnow' fruit on an upper rack. This whole apparatus was placed in an environmentally controlled room maintained at 28 ± 2 °C, 65 ± 5% RH. A 23-watt fluorescent lamp was used to provide light at the top of the apparatus. Crawlers moved to the light and settled on the non-infested fruits. Infested fruits at intervals of 3, 9, and 17 d after settlement of the crawlers presented 1st, 2nd, and 3rd instar scales that were used in irradiation experiments.

Aonidiella aurantii females have 4 major life stages: (i) the egg stage within the viviparous mother, (ii) the 1st stage, i.e., the crawler with legs (born alive)—which is the 1st instar, (iii) the 2nd stage, which is the legless 2nd instar and the last pre-imaginal feeding instar, and (iv) the 3rd stage, which is not a pre-imaginal instar, rather it is the female adult that initially is miniature but fertile. According to CABI (2016), the various adult growth and development stages are as follows:

- *Gray adult female or early 3rd stage female*. The female and its waxy covering enlarge considerably during this stage of the adult. The wax cover is very thin with a distinct gray color and the initially pear-shaped female body shows through it. The adult male fertilizes the female during the beginning of this stage. CABI (2016) asserts: "It is therefore considered incorrect to refer to the entire third stage (third instar of some authors) as the virgin female stage." The female does not grow and develop into a mature female unless mated. The gray adult stage terminates when the body of the female and the waxy covering reach their final size and become fused together. This growth and development stage is conveniently divided into 3 sizes: (a) small, up to 1/3 full size; (b) medium, > 1/3 and ≤ 2/3 of full size; and (c) large, > 2/3 to full size.

- *Fully grown adult stage*. The fully grown adult stage can be subdivided into an A− stage consisting of gravid females before crawlers hatch from the eggs, and an A+ stage consisting of actively crawler-producing females.

On the other hand for research on phytosanitary irradiation, we found it essential to designate the various growth and development stages of the female adult as follows:

A1, the early 3rd stage gray adult female before she has mated,
A2, the mated adult female still without any eggs, and usually ≤35 d-old, i.e., d measured since the egg stage,
A3, the mated adult female with eggs, but not with crawlers, and usually the female is 36–40 d-old, and
A4, the adult female with eggs actively hatching into crawlers, and usually a crawler-producing female is 45–50 d-old.

At 28 ± 2 °C the approximate development times for these stages are 4, 4, 6 and 12 or more d for the egg, 1st stage, 2nd stage and 3rd stage, respectively (Foster et al. 2007). Male and female scales can be distinguished by their shapes. Male are oblong while females are round. Each adult female lives under the scale cover formed from secreted wax material. Since the 3rd stage female does not become a mature female unless mated, the developmental period (d) of the 3rd stage female depends on when she mated. Crawlers appear at approximately 12 d after the female has mated.

Whether the adult female has eggs cannot always be determined unless the scale cover is removed, which usually kills the female. Therefore, the reproductive status of the adult females at the time of treatment was mainly determined by their age. Adult females generally do not have eggs at less than 35 d, but they have eggs at more than 35 d. The effect of radiation on eggs was determined by irradiating them within the females when these females had attained an age of 36–40 d (A3 stage). On the other hand, the effect of radiation on crawler-producing females was determined by irradiating them when these females had attained an age of 45–50 d (A4 stage). Survivorship and reproductive status of adult females were determined at the end of the study by turning scales over and examining for evidence of eggs. Live scales typically have a pinkish color underneath their cover, whereas dead scales are brown.

Approximately 400 scales in each stage (1st, 2nd and 3rd) were used. When a large number of scales was present, the surface of the 'Kinnow' fruit was divided into equal sections. Next, counts were made on 2 or 3 randomly selected sections, and then the average was multiplied by the number to sections to estimate the total. Tests were usually terminated after 2 mo, at which time most irradiated scales had died and the fruit had hardened or rotted. Final counts were made of all live adult females at each stage of development and of the live forms under their scale covers.

IRRADIATION TREATMENTS

All radiation treatments were conducted at the Nuclear Institute for Food and Agriculture (NIFA) Peshawar, Pakistan, which has a self-shielded cobalt-60 research irradiator (Issledovatel GIK-7-2 from Russia).The doses were administered by placing samples inside a 15 × 23 cm treatment chamber that was lowered into the radiation field via an electric hoist for a predetermined length of time based on the dose rate (0.367 Gy·min^{-1}during experiments). A dose mapping of the area within the chamber where samples were placed was conducted to verify the accuracy and range of the doses applied. The absorbed dose was measured using Fricke dosimeters at 3 irradiation positions. To optimize the dose uniformity ratio (the ratio of the maximum to the minimum dose), the nymph-infested 'Kinnow' fruits were placed in the center of the chamber.

Dose response tests were conducted with 1st and 2nd stage immatures, 3rd stage adult females with eggs but not with crawlers, i.e., A3 stage, and females actively producing crawlers (A4 stage). A series of irradiation doses between 100–300 Gy was used to determine the most tolerant stage. An individual 'Kinnow' citrus fruit with scales served as 1 replicate, and treatments included 3–4 replicates depending on the number of scales on the fruits. After treatment, fruits were placed in individual cages and scales on fruit were examined weekly under a stereomicroscope to determine their developmental stages. Untreated controls from each stage were held under identical conditions and examined similarly.

LARGE SCALE VALIDATION TESTS

Large scale confirmatory tests were conducted to validate the estimated dose to prevent reproduction of the most tolerant stage, i.e., adult female. A total of 36,531 and 32,101 adult female scales were irradiated with 200 and 220 Gy, respectively, using the same methodology as above.

DATA ANALYSIS

Data on the effect of gamma irradiation on mortality and survival were subjected to analysis of variance (ANOVA) after testing for equal variances and normality. Means separations were done using a Tukey's test. All statistical analyses were done using Statistix 8.1 (Analytical Software, Tallahassee, Florida). In addition, percentage mortality and adult inhibition data were subjected to probit analysis using PoloPlus LeOra software version 2002 to estimate the log dosage response of exposed eggs and nymphs.

Results

The absorbed dose measurements, minimum dose, maximum dose, average dose and the dose uniformity are given in Table 1. The dose uniformity ratio (DUR) did not exceed 1.06 during all treatments. Thus, the variation in the range of doses absorbed during this experiment was small.

The inhibition of development of each stage of the life cycle of *A. aurantii* at d 60 after irradiation treatment was determined (Table 2). Thus, when the pre-imaginal stages were irradiated with 150 Gy, 99% of treated eggs did not hatch into 1st instars (crawlers), 97% of treated 1st instars (crawlers) did not molt into 2nd instars, and 96% of treated 2nd instars did not molt into 3rd stage scales, i.e., adult females or male pupae. Further when adult females with eggs were irradiated with 150 Gy, 71% of these treated females failed to produce 1st instars (crawlers) and they had died by d 60.

When the pre-imaginal stages were irradiated with 200 Gy none of them developed to the subsequent stage. Further, when adult females with eggs were irradiated with 200 Gy, 99% of these treated females failed to produce 1st instars (crawlers) and they died by d 60. Also the 1% that had produced 1st instars (crawlers) died by d 60.

In the control (0 Gy), the naturally occurring failure of a stage to develop to the subsequent stage varied in the range of 16–27% depending on the developmental stage (data not shown).

The dose-response data on prevention of adult emergence from various nymphal stages analyzed by ANOVA showed mortality at all stages and a significant correlation with the irradiation dose ($F = 49.10$, $P < 0.0001$). Therefore, all of the radiation doses affected the survival of the various developmental stages of *A. aurantii*. The percentage mortality to the adult stage—manifested by the inability of a pre-imaginal stage to develop into the subsequent stage—increased with increasing radiation doses (Table 2). Immature stages were found to be more sensitive to irradiation than 3rd stage scales, i.e., adult females. The pattern of tolerance to irradiation in *A. aurantii* was: 1st instar (least tolerant) < 2nd instar < 3rd instar-adult female. Linear regression was used to estimate the radiation dose needed to cause 99.99% inhibition of development of each immature stage to adulthood, or to prevent adults with eggs from producing 1st instars (crawlers). The 3rd stage, i.e., the adult female, was the most radio-tolerant. The range of the $ED_{99.99}$ values was 146–199 Gy (Table 2).

The results of large-scale validation testing (Table 3) showed that only 187 females out of 36,531 (0.5%) were able to produce crawlers and only 2 (0.005%) developed to F_1 3rd stage adults when exposed to 200 Gy, but none of the scales produced crawlers when exposed to 220 Gy.

Discussion

Tolerance to radiation in *A. aurantii* increased with age and developmental stage with respect to the efficacy of preventing the emergence of 3rd instars or adults. A similar increase in tolerance to irradiation was reported in the mango scale, *Aspidiotus destructor* Signoret (Homoptera: Diaspididae) (Khan & Salahuddin 2016), and in the pink hibiscus mealybug (*Maconellicoccus hirsutus* Green; Hemiptera: Pseudococcidae) (Jacobsen & Hara 2003), the green scale (*Coccus viridis* [Green]; Homoptera: Coccidae) (Hara et al. 2002) and *Dysmicoccus neobrevipe*s Beardsley (Hemiptera: Pseudococcidae) (Doan et al. 2012). Such trends of increase in insect radiotolerance with age and maturity were extensively reviewed by Hallman et al. (2010).

In order to confirm the validity of the estimated minimum dose required for quarantine security, it is necessary to treat a large number of individuals of the organism to either prevent their development or stop them from producing viable eggs. We exposed 36,531 adult female scales to 200 Gy, and 32,101 adult females to 220 Gy. Both doses prevented the successful development of F_1 adults (Table 3). In the 200 Gy treatment only 2 out of 36,531 (0.005%) adult females produced crawlers, some of which molted into 2nd stage nymphs but none continued their development to become fertile adults, and they died after their development was arrested.

In the present experiments with the target dose of 200 Gy, the highest dose measured was 222 Gy. Therefore, 222 Gy is proposed as the PI dose to stop the reproduction and turnover of generations of the red scale.

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Table 1. Dosimetry, i.e., measurement of absorbed doses of gamma radiation delivered by the Issledovatel GIK-7-2 cobalt-60 research irradiator at the Nuclear Institute for Food and Agriculture (NIFA) Peshawar, Pakistan for γ-irradiation of *Aonidiella aurantii* on 'Kinnow' mandarin orange hybrid fruits.

Target dose (Gy)	Minimum dose (Gy)	Maximum dose (Gy)	dose (Gy)	Dose uniformity ratio
100	94	100	97	1.06
150	146	150	148	1.03
200	197	211	203	1.07
250	247	253	249	1.02
300	297	308	303	1.04

Table 2. Probit analysis for percent inhibition of development or mortality of various developmental stages of *Aonidiella aurantii* at 60 d after various γ-irradiation doses were applied to either eggs, 1st instar nymphs, 2nd instar nymphs, or adult females with eggs. Generally individuals that had failed to develop to the subsequent stage by d 60 had died.

Stage irradiated	Stage to which development was inhibited	Percent inhibition at dose (Gy)[a]						Slope	$ED_{99.99}$ (GY)[b] (95% CI)[c]
		0	100	150	200	250	300		
Egg[d]	1st instar (crawler)	27.59c	72.21b	99.67a	100a	100a	100a	14.68 ± 0.60	146 (146–170)
1st instar (crawler)	2nd instar nymph	25.08c	71.63b	97.45a	100a	100a	100a	8.9 ± 0.50	169 (162–178)
2nd instar nymph	3rd stage ♀ (adult ♀) or male pupa	16.92c	80.74b	96.97a	100a	100a	100a	4.95 ± 0.35	174 (156–210)
Adult female with eggs[e]	Adult female with 1st instars (crawlers)	24.04 c	21.84c	71.43b	99.27a	100a	100a	16.02 ± 0.73	199 (192–210)

[a]Means within a column followed by the same letter are not significantly different from each other by Tukey's HSD test ($P < 0.05$).
[b]$ED_{99.99}$ is the dose (Gy) expected to cause either 99.99% inhibition of development of each pre-imaginal stage to the 3rd stage (adult)—hence, mortality, or 99.99% prevention of production of 1st instars (crawlers) by adult females with eggs—hence, prevention of reproduction.
[c]CI means confidence interval.
[d]The effect of radiation on eggs was determined by irradiating them within the females when these females had attained an age of 36–40 d (A3 stage).
[e]The effect of radiation on A3 stage females—which had eggs but did not yet produce crawlers—was determined by irradiating them when these females had attained an age of least 45-50d (A4 stage).

Table 3. Results of large-scale validation tests of irradiating *A. aurantii* adult females on 'Kinnow' mandarin orange hybrid fruits.

Target dose (Gy)	No. fruits	Measured dose	No. adult scales irradiated	No. F_1 generation 1st instars	No. F_1 generation adult females
Control	19	—	32,274	48,727	31,939
200	23	197–201	36,531	187	2
220	22	217–222	32,101	0	0

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