Postharvest phytosanitary disinfestation of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in citrus fruit: Comparative tolerance of larvae reared in synthetic diet and oranges to ionizing radiation

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Abstract

It had been previously established that ionizing irradiation either of eggs or 5th (final) instars of *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) with 50–70 Gy would suppress the flight ability and reproduction of moths that emerged. A mean dose of 40 Gy was used to γ-irradiate larvae in oranges and in artificial diet in rearing jars to determine if the larvae reared on a synthetic diet would differ in radiotolerance from those reared in oranges. The mortality of irradiated larvae was low and very similar in rearing jars and oranges. A significantly larger number of eggs were produced by moths from 5th instars irradiated in rearing jars compared to that from oranges. However, the reduction in fecundity caused by irradiation was not significantly different for moths originating from both sources. Thus the fertility of the moths from larvae irradiated in oranges and in rearing jars from 4th and 5th instars was reduced by 64.6, 37.7 and 15.1%, respectively, compared to moths from non-irradiated control larvae. These differences were statistically significant and indicated that wild *T. leucotreta* were less radiotolerant than their artificially reared counterparts. Significantly fewer eggs were produced by moths from non-irradiated larvae in oranges than by moths from non-irradiated larvae in rearing jars. It is unknown whether the wild moths underperformed, or conversely, if performance of the insectary moths was enhanced by artificial rearing. Nonetheless, this aspect complements the experimental differences observed between the 2 larval sources in the experiment. The use of artificially reared *T. leucotreta* in research aimed at the eventual validation of ionizing radiation for the phytosanitary suppression of this pest in export citrus fruit is thus justified.

Key Words: false codling moth; relative radiotolerance; phytosanitary irradiation; artificial medium, feral host

Resumen

Se había establecido anteriomente que la irradiación ionizante de huevos o del 5º estadio (final) de *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) con 50-70 Gy suprimiría la capacidad de vuelo y la reproducción de las polillas que emergen. Se utilizó una dosis promedio de 40 Gy para gamma-irradiar larvas en naranjas y en una dieta artificial en frascos usados para cria para determinar si las larvas criadas en una dieta sintética diferiría en radiotolerancia de las larvas criadas en naranjas. La mortalidad de larvas irradiadas fue baja y muy similar en la crianza de los frascos que en las naranjas. Un número significativamente mayor de huevos fueron producidos por las polillas de 5º estadios irradiados en frascos de cría en comparación con los de las naranjas. Sin embargo, la reducción de la fecundidad debido a la irradiación no fue significativamente diferente para polillas de ambas fuentes. Sin embargo, la fertilidad de las polillas de larvas irradiadas en las naranjas y de las 4º y 5º estadios en frascos de cría se redujo en 64.6, 37.7 y 15.1%, respectivamente, en comparación con las polillas de larvas de control no irradiadas. Estas diferencias fueron estadísticamente significativas e indican que las polillas salvajes de *T. leucotreta* son menos radiotolerantes que sus homólogos criadas artificialmente. Significativamente menos huevos fueron producidos por las polillas de las larvas no irradiadas en las naranjas que por las polillas de las larvas no irradiadas criadas en frascos. No se sabe si las polillas salvajes desempeñaron inferiormente, o por el contrario, si el desempeño de las polillas del insectario fue mejorado por la cría artificial. No obstante, este aspecto complementa las diferencias experimentales observadas entre las 2 fuentes de larvas en el experimento. El uso de *T. leucotreta* criados artificialmente en la investigación dirigida a la eventual validación de las radiaciones ionizantes para la supresión fitosanitaria de esta plaga en cítricos de exportación de frutas es, pues, justificada.

Palabras Clave: falso gusano de la manzana; radiotolerancia relativa; irradiación fitosanitaria; medio artificial, hospedero salvaje

The motivation for a research project on the irradiation of *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) that would result in an internationally approved phytosanitary measure was discussed by Hofmeyr et al. (2016). The project was divided in 3 successive phases: (i) identification of the life stage of *T. leucotreta* most radiotolerant and where applicable, the most radiotolerant age within that particular life stage, (ii) the validity of using artificially reared *T. leucotreta* in radiation research as an alternative to feral insects occurring in citrus fruit and (iii) validation of a treatment for phytosanitary application. The radiation biology of eggs and larvae of *T. leucotreta* was reported in the first phase of this project (Hofmeyr et al. 2016). Final (5th) instars were shown to be most radiotolerant and were, as such, essential for continued research.

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The required life stages for the initial research were available as artificially reared insects. For practical reasons this simplified the research, and studies were conducted that would otherwise have been very difficult or impractical to attempt using wild larvae. Ultimately, it had to be resolved how the performance of the artificially reared insects compared with their counterparts in naturally infested oranges when irradiated. Differences in the larval sources could, if they existed, hypothetically be due to inherited or acquired features. The dissimilar physical environments in which the insects developed, namely artificially controlled versus naturally variable ambient conditions, inclusive of a synthetic diet, could result in insects differing in radiotolerance. It was logical that the most robust insects with the best chance of retaining their biological attributes following irradiation had to be used in continued research. The 2 larval sources differed substantially in important practical aspects. Generally, elements such as sporadic seasonal availability, degree of fruit infestation, time restraints, and instar range would be difficult or impossible to manipulate. In contrast, large numbers of artificially reared larvae of a certain age could be easily obtained. An experiment was consequently conducted to determine whether the use of artificially reared larvae, rather than wild insects, would in any way jeopardize a definitive conclusion in respect of phytosanitary treatment acceptability for validation purposes.

Materials and Methods

LARVAL SOURCE AND TEST PROCEDURES

All larvae were irradiated in the medium in which they had developed, i.e., naturally infested oranges and artificial diet in rearing jars.

Oranges

A consignment of 300 navel oranges with symptoms of probable *T. leucotreta* infestation was collected consecutively with 2–3-wk intervals from orchards on each of 3 farms in the Citrusdal region, Western Cape, South Africa. The consignments were again examined in the laboratory to discard fruit subsequently regarded to be not infested. A sample of 20 oranges was collected at random from each consignment and cut open to determine the infestation level. Head capsules of the retrieved larvae were measured with an electronic caliper to establish the instar categories in each consignment (Daiber 1979). Fifty randomly collected oranges per consignment were retained as controls. The balance of the fruit, respectively 133, 156 and 98 oranges per consignment, was irradiated. Control and irradiated oranges were placed individually into 500 mL plastic containers and incubated at 26 °C. Pupae were collected daily, counted, and placed separately into glass vials, 50 × 15 mm, with foam rubber stoppers. Moths were collected as they eclosed, and as many female and male pairs as possible were put together. A mean of 8.3 and 22 mating pairs was accumulated, respectively, per consignment in the control and radiation treatments. Protracted emergence periods, or gender ratios that were female or male biased prevented utilization of all moths. Individual mating pairs were placed into 100 mL plastic oviposition cages and eggs were deposited directly onto the top, bottom, and sides of the containers. To assess mating status each female was removed after 5 d, dissected and examined for spermatophores in the bursa copulatrix. All eggs were incubated for 14 d before the moths' fecundity and fertility were assessed.

Rearing Jars

Thaumatotibia leucotreta were reared at 26 °C on an artificial diet consisting predominantly of corn flour (Moore 2002). Wide-necked glass jars (500 mL volume; Consul Glass BN8175701, Stellenbosch, South Africa), each filled with 280 g of the medium, were used as rearing containers. Each jar was closed with a screw-on metal lid provided with a 40 mm diam aperture. A paper membrane (112 g/m^2 High Yield Fluting; Sappi Kraft, Milnerton, South Africa) was fitted into the lid to close the aperture, regulate moisture loss, and prevent putrefaction of the diet. Five hundred to 800 eggs, 24 h old, on wax paper were inoculated into each jar and up to 700 larvae were obtained per jar 12–14 d later. The larvae pupated in rolled-up strips of corrugated cardboard, 30 mm wide, which replaced the metal lids. The cardboard strips were pulled apart to collect the pupae or were put into emergence containers for moth eclosion and oviposition.

Wild larvae in oranges occur in unsynchronized instars that differ from the mostly synchronized artificially reared larval populations. To partially simulate the natural fluctuations in oranges, 2 batches of 6 rearing jars containing 4th and 5th instars were prepared as counterparts for each consignment of oranges. Each batch was subdivided into 2 sub-batches consisting of 3 rearing jars each for control and irradiation purposes. All larvae were incubated at 26 °C post-treatment to pupate, and the pupae of each sub-batch were combined. From each of these, 160 pupae were collected at random and placed separately into glass vials, 50 × 15 mm, with foam rubber stoppers. The first 10 pairs of moths to eclose were collected for fecundity and fertility studies as described for the oranges. The remaining 140 pupae were retained until all viable moths had eclosed to assess pupal mortality. Thirty larvae were collected at random from an additional representative rearing jar per batch per larval age group to confirm instar distribution with head capsule measurements (Daiber 1979).

COMPARISONS BETWEEN WILD AND INSECTARY-REARED LARVAE

The numbers of wild larvae obtained from oranges were limited. All available moths from irradiated wild larvae were therefore used to assess reproductive issues, viz., mating incidence, fecundity, and fertility. Acute larval mortality was recorded post-treatment.

DOSIMETRY

A cobalt-60 point source, panoramic irradiator at the *T. leucotreta* rearing facility of Xsit (Pty) Ltd in Citrusdal, Western Cape, South Africa, was used. The targets were placed on 8 secondary turntables, each 300 mm in diam that were located on a primary turntable, 1,370 mm in diam. The turntables rotated at 15 and 2 rpm, respectively.

PVC containers, 300 mm high × 200 mm OD × 190 mm ID with 5 mm wall thickness, each holding 16 infested oranges, were used for dosimetry and treatment purposes to provide sufficient dose build-up and ensure charged particle equilibrium. Artificially reared larvae were treated in the diet contained in the rearing jars as the glass sides provided sufficient dose build-up. Stacks of 3 jars with diet and larvae were used for dosimetry and treatment applications.

The initial output factor for a dose absorbed from the irradiator was conducted using Fricke dosimeters, applying the standard G-value for cobalt-60 of Fe^{3+} = 15.5/100 eV. Optical density readings were performed using a Hewlett Packard diode array spectrophotometer. For this instrument a molar extinction coefficient of 2,081 L/Mole/cm at 304 nm was determined using spectrosol grade Fe^{3+} solutions. This instrument specific constant relates optical density readings in a quartz glass flow cell with an optical path of 10 mm to [Fe^{3+}] concentrations, and is used to calculate dose absorbed values in Gy (J/kg). Different Fricke solutions read within 1% when irradiated in a cobalt-60 reference field. The dosimeter response was checked in a cobalt-60 reference radiation field of 300 × 300 mm with 2 mL Fricke solutions placed in a Perspex container producing 6 mm build-up and 50 mm backscat-

ter. The output factor for the reference field was determined using a Farmer tissue equivalent ionization chamber calibrated in a standard field of the South African National Metrology Laboratory.

Mapping was conducted at the top, middle, and bottom of the PVC containers and in the top and bottom of the rearing jar stack. Fricke dosimeters were inserted into the centers of the oranges and rearing diet. No reference position was used; the dosimetry was conducted for various positions in the irradiation set-up and the mean was calculated. All doses assessed in the various experiments therefore had to be regarded as means. For oranges in PVC containers the dose uniformity ratio from top to bottom was 1.32, varying 1.6% above and below a specified dose. For larvae in rearing jars the dose uniformity ratio from top to bottom was 1.17, varying 8.5% above and below a specified dose.

Repeated readings taken on the same day at each position showed differences of less than 0.5% for both PVC containers and rearing jars. Each radiation set-up was calibrated using a range of doses that included the radiation levels to be used. Uncertainties in the dose rate from repeated dose readings were less than 4% for both PVC containers and rearing jars and the 95% confidence intervals of a reading at any position were 2% and 3%, respectively.

The dose rate was updated before every irradiation procedure using the decay half-life of cobalt-60 at 5.2714 yr. A relatively low dose of 40 Gy was used to ensure that irradiated larvae would develop into partially sterile moths able to produce viable eggs enabling comparison between treatments. Due to the dose uniformity ratios in the oranges and rearing jars, this dose had to be regarded as a mean. For larvae in oranges the applied dose varied from 39.4 Gy to 40.6 Gy. For larvae in rearing jars the applied dose varied from 36.6 Gy to 43.4 Gy. All treatments for calibration and experiments were applied at 15 °C.

DATA ANALYSIS

The experimental design was a completely random split plot with larval source (oranges, 4th, and 5th instars) as main plot factors and radiation treatment as the split plot factor. Each larval source was randomly replicated, viz., a consignment of *T. leucotreta* infested oranges from each of 3 orchards and 3 batches each of 4th and 5th instars from rearing jars. Each replicate (batch) was divided into 2 sub-batches for control and irradiation purposes. An experimental unit consisted of all the mating pairs obtained from a replicate of a larval source that received a particular treatment, viz., 10 pairs of moths in the case of rearing jars and as many as possible in the case of oranges.

The mean fecundity per mating pair was calculated for each experimental unit. The percentage fecundity resulting from treatment (40 Gy) compared to the untreated control of each larval source was also calculated.

The percentage hatched eggs from the total number of eggs produced, as well as the percentage reduction in fertility due to irradiation compared to the untreated control of each larval source were calculated.

The effects of larval source and ionizing radiation treatment were analyzed using analysis of variance (ANOVA). If a significant F-test was detected, the Fisher's protected LSD test was used to further elucidate treatment differences (SAS Version 9.2, SAS Institute, Inc., Cary, North Carolina). Count data were square-root transformed prior to analysis to improve normality (Snedecor 1980); non-transformed means are presented with transformed values in brackets. A probability level of 5% was considered significant for all significance tests.

Results

Larvae from rearing jars and larvae from oranges are henceforth referred to as 'RJ larvae' and 'OR larvae', respectively.

INFESTATION RATE

On average 53% of the samples of 20 fruit collected in each of the 3 consignments of oranges were infested (consignment 1 = 60%; consignments 2 and 3 = 50%). Two attempts were made to remove non-infested fruit and the low degree of infestation is indicative of the difficulties inherent in a large scale study using oranges. The actual numbers of pupae in the rearing jars were not recorded, but from previous experience on average 500–700 pupae could easily be obtained per rearing jar.

INSTAR CATEGORY

Instar categories were variable in and between the 3 consignments of oranges, including a mean of 24.4% unwanted younger instars (Table 1). This disparate distribution was due to 2 factors. Firstly, oranges are unevenly infested by wild *T. leucotreta* populations that often occur in poorly defined and partially overlapping generations throughout the citrus season. Secondly, similarly aged individuals have a natural tendency to develop at inherently different rates.

Eighty to 100% of the larvae collected from the different batches of rearing jars could be allocated to the required instar category (Table 1).

ACUTE LARVAL MORTALITY

A mean of 8.1% fewer pupae were collected from irradiated than non-irradiated oranges in the 3 consignments, representing a minor treatment effect. In an earlier experiment treatment of 5th instar RJ larvae with 40 Gy reduced the number of pupae relative to the control by 9.1% (Hofmeyr et al. 2016). The combined average pupal reductions of 8.1% in oranges and 9.1% in rearing jars do not represent a significant difference in pupation at 40 Gy.

Table 1. Ratio of instars of *Thaumatotibia leucotreta* in rearing jars and oranges collected from the field. Head capsules of the retrieved larvae were measured with an electronic caliper to establish the instar categories.

Instar categories in oranges from field (%)				
Instar	Consignment 1	Consignment 2	Consignment 3	Mean
2nd	8.3	0.0	10.0	6.1
3rd	25.0	0.0	30.0	18.3
4th	8.3	50.0	40.0	32.8
5th	58.4	50.0	20.0	42.8
Instar categories in rearing jars selected for 4th instar (%)				
Instar	Batch 1	Batch 2	Batch 3	Mean
2nd	0.0	0.0	0.0	0.0
3rd	20.0	6.7	0.0	8.9
4th	80.0	93.3	93.3	88.9
5th	0.0	0.0	6.7	2.2
Instar categories in rearing jars selected for 5th instar (%)				
Instar	Batch 1	Batch 2	Batch 3	Mean
2nd	0.0	0.0	0.0	0.0
3rd	3.3	0.0	0.0	1.1
4th	6.7	0.0	0.0	2.2
5th	90.0	100.0	100.0	96.7

Note: Thirty larvae from an additional representative rearing jar per batch per larval age group were assessed at random.

REPRODUCTIVE POTENTIAL

Mating Suppression

In the untreated controls a mean of 1.7% of the female moths from 4th and 5th instar RJ larvae remained unmated. The corresponding figure for females from non-irradiated wild (OR) larvae was 6.7%. The difference is small, but could indicate that the drastically different artificial environment that the OR moths were exposed to during evaluation, could have affected their mating behavior. The corresponding figures for the irradiated RJ and OR larvae were 15.5% and 23.4% respectively, inclusive of the possible environmental effect. Whether the last-mentioned factor was included or excluded, the discrepancy was considered too small as an exploitable difference between wild *T. leucotreta* and their insectary counterparts.

Fecundity

Analysis of variance on number of eggs indicated a significant interaction between larval source and radiation treatment ($F = 8.84$; df = 2.6; $P = 0.0163$). Significantly fewer eggs were produced by non-irradiated wild *T. leucotreta* than by non-irradiated insectary reared moths (Table 2). This could suggest that the wild moths were less fecund than the latter simply due to unfamiliarity with the egg laying substrate. Conversely, the wild moths may have been ovipositing at their normal rate, which would suggest that the insectary reared moths were more fecund. Specific empirical data are lacking for *T. leucotreta*, but it is conceivable that adaptation to less variable, more favorable ambient conditions and a possibly more nutritious diet could enhance the reproductive capability of insectary moths. It is an accepted practical problem that wild *T. leucotreta* collected in orchards have to be reared through several successive generations on artificial diet before their reproductive performance develops sufficiently to merit introduction into an existing insectary colony (Hofmeyr, unpublished; SJ Honiball, Vital Bugs, Tzaneen, South Africa, personal communication; SD Moore, Citrus Research International, Port Elizabeth, South Africa, personal communication).

Moths from irradiated OR and RJ larvae were significantly less fecund than their non-irradiated counterparts, a typical effect of irradiation (Table 2) (Hofmeyr et al. 2016). At the test dose of 40 Gy significantly fewer eggs were produced by moths from irradiated OR and 4th instar RJ larvae when compared to the moths from irradiated 5th instars. This was to be expected in part because 5th instar larvae are more tolerant of irradiation than younger instars (Hofmeyr et al. 2016), and agrees with Hallman et al. (2010) who reviewed the literature and found that radiotolerance increases with development. This means that the most radiotolerant stage for phytosanitary irradiation would be the most developed stage that could be found in the exported commodity.

However, when compared to their respective controls, the percentage reduction in fecundity of moths from larvae treated in oranges and rearing jars (both 4th and 5th instars), was not significant ($F = 0.54$; df = 2.6; $P = 0.6097$). This indicates that the radiotolerances of larvae in oranges and rearing jars were similar with respect to fecundity.

Fertility

Analysis of variance on the percentage hatched eggs indicates a significant interaction between larval source and radiation treatment ($F = 15.50$; df = 2.6; $P = 0.0043$). The fertilities of moths from non-irradiated wild and artificially reared larvae were normal for eggs deposited on wax paper and were similar to control treatments in general, viz., approximately 80% egg hatch (Hofmeyr et al. 2016) (Table 3).

Irradiation of both OR and RJ larvae caused a high degree of sterility in the moths resulting in reduced egg hatch. The differences in egg hatch were significant between both the larval sources (OR and RJ) and the instars of RJ larvae (4th and 5th instars). These differences were confirmed by comparison of the percentage reduction in egg hatch to the respective controls, viz., 64.6%, 37.7%, and 15.1%, respectively; all differences were statistically significant ($F = 14.40$; df = 2.6; $P = 0.0051$). The increased fertility of the 5th instars compared to the 4th instars was due to higher radiotolerance of the older larvae. Keeping the instar distribution in the 3 groups of larvae in mind, this age factor was largely irrelevant when comparing the significantly lower reduction in fertility of both instars to that of the OR larvae. The small differences in performance of moths from the controls of both larval sources were largely discounted by calculating the percentage reduction in fertility. Thus, the significantly reduced fertility of the moths from OR larvae could only be attributed to a lower tolerance of ionizing radiation, supporting the use of insectary reared insects in place of wild *T. leucotreta*.

Discussion

The objective of this study was to establish whether the use of wild *T. leucotreta* in a large scale assessment of an acceptable phytosanitary treatment was practically feasible. A factor counting heavily against wild *T. leucotreta* was the challenge of collecting and managing vast numbers of oranges, consisting of low and variable numbers of infest-

Table 2. Fecundity of *Thaumatotibia leucotreta* moths from larvae reared either in 'Navel' orange fruits or in an artificial diet in rearing jars and γ-irradiated with 40 Gy.

	Mean ± SE no. of eggs produced per ♀		
		Rearing jars: Moths developed from	
Dose (Gy)	Moths from larvae in naturally infested oranges	4th instars	5th instars
0	101.3 ± 47.2 (9.5)[x] c[y]	406.7 ± 37.9 (20.1) a	418.7 ± 14.3 (20.5) a
40	34.8 ± 20.8 (5.4) d	66.5 ± 7.2 (8.3) cd	176.4 ± 39.9 (13.1) b
LSD ($P = 0.05$)		(3.2)	
		Reduction in fecundity (%)[z]	
40	65.6 ± 20.5 a	83.7 ± 1.8 a	57.8 ± 9.5 a
LSD ($P = 0.05$)		44.1	

[x]Values in brackets indicate square root transformed counts.
[y]Values in rows and columns designated by the same letter do not differ significantly.
[z]Relative to the respective control treatments.

Table 3. Fertility of *Thaumatotibia leucotreta* moths from larvae reared either in 'Navel' orange fruits or in an artificial diet in rearing jars and γ-irradiated with 40 Gy.

	Mean ± SE % hatched eggs from		
	Moths from larvae in naturally infested oranges	Rearing jars: Moths developed from	
Dose (Gy)		4th instars	5th instars
0	82.0 ± 0.7 a[x]	78.5 ± 0.3 ab	80.1 ± 2.8 ab
40	29.0 ± 3.9 d	49.3 ± 6.3 c	68.0 ± 6.6 b
LSD (*P* = 0.05)		12.8	
	Reduction in fertility (%)[z]		
40	64.6 ± 4.7 a	37.3 ± 8.1 b	15.1 ± 8.2 c
LSD (*P* = 0.05)		16.0	

[x]Values in rows and columns designated by the same letter do not differ significantly.
[z]Relative to the respective control treatments.

ed fruit containing an assortment of required and unwanted instars. Assessments were made in a laboratory, and these artificial conditions resulted in the much reduced fecundity of moths from non-irradiated wild larvae compared to their non-irradiated insectary reared counterparts. This disadvantage could contribute to the validation of a dose too low to provide the highly effective treatment required for phytosanitary endorsement.

The study showed that the fecundities of moths from the 2 treated larval sources were similar. However, moths from naturally developed, irradiated wild larvae were less fertile than the moths from irradiated diet-reared larvae. The presence of unwanted young instars in oranges was an unpredictable variable that may have exaggerated the difference in fertility between moths of irradiated larvae in the 2 sources. However, 75.6% of the wild larvae were of the same age as their diet-reared counterparts, viz., 4th and 5th instars, and the fertilities of the moths should have been similar subsequent to irradiation of these larvae. This was not the case and the percentage of fertile eggs deposited by the wild moths were without exception much reduced compared to that of the diet-reared insects.

No experimental evidence was collected to support a conclusion that wild larvae were more radiotolerant than diet-reared larvae. Therefore, from the study it can be concluded that the use of artificially reared *T. leucotreta* in a validation study is experimentally justified and essential for technical reasons.

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