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Irradiation as a phytosanitary measure for grapes infested with the European grapevine moth pupae

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Abstract

Grapevine moth, *Lobesia botrana* (Denis & Schiffermuller), late pupae were exposed to gamma radiation of 50 to 200 Gy and effects on adult emergence were examined. In addition, moths resulting from male and female pupae exposed to the same doses were crossed to non-irradiated insects of the opposite sex. The effects on fecundity, fertility and survival of F_1 insects to the adult stage were investigated. Results showed that, while adult emergence was not significantly affected at the examined dosages, fecundity of females, whether irradiated or crossed to irradiated males was negatively affected. The effect, however, was stronger when females were the irradiated sex. Fertility of both males and females also decreased with increasing irradiation dose; percentage fertility decreased from about 88% for the control to 37.6 and 0 % for irradiated males and females exposed to 200 Gy, respectively. Furthermore, irradiation reduced survival of the progeny of irradiated insects and at 200 Gy, all F_1 insects died before reaching the adult stage. This study supports a dose of 200 Gy as a phytosanitary measure against *L. botrana* and a generic dose of no more than 400 Gy for pupae of Lepidoptera.

Keywords: quarantine, irradiation, pupae, *Lobesia botrana*, grapes

1. Introduction

The European grapevine moth, *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae), is a serious pest of vineyards in Southern Europe and the Mediterranean basin and North and West Africa [1, 2, 3]. More recently, the pest was introduced into certain areas in Japan, Chilly and the United States of America [4, 5]. In addition to vineyards, the pest may attack pears, roses and many ornamental and wild plants [6, 7]. In Syria, *Lobesia botrana* is a key pest on grapevine, *Vitis vinifera* L., and causes great losses to the grape industry every year. In neglected orchards, infestation rate exceeds 40%, and with control, bunch infestation could be as high as 10% [8]. The pest also requires quarantine treatment in many countries [4, 5, 9, 10]. *L. botrana* larvae feed mostly on grape berries and can easily be transmitted from one country to another on fresh fruits. Fully grown larvae of the last generation leave grape bunches and pupate on the woody parts of the plant (diapausing pupae). Non-daipausing pupae of the penultimate generation, however, are typically found in rolled leaves and grape bunches [4, 11] and can be transmitted to *L. botrana* free countries with fresh fruits, particularly in early table grape varieties. As a result, grape exports to *L. botrana* free countries are difficult without effective phytosanitary measures as immature stages of this species, including pupae, can be transmitted with fresh grapes to importing countries [4, 11, 12].

The most used chemical fumigant for disinfesting fresh agricultural products, methyl bromide, is phased out globally [13, 14] with no other chemical to replace at present. Ionizing radiation has been suggested as a possible alternative to methyl bromide for treating fresh agricultural products [15] with little or no harm to fresh commodities [16]. This requires, however, determining the irradiation dose which ensures death or, at least, inability of the irradiated insects to reproduce without any unacceptable changes in the treated products [17].

Effects of gamma irradiation on the grapevine moth egg and larval stages have been reported before [18, 19]. Because the sensitivity of insects to ionizing radiation decreases as they develop [20, 21], pupae are, most likely, more resistant to irradiation treatment than eggs and mature larvae. Consequently, *L. botrana* late pupae are the most resistant stage potentially present in the shipped fresh grapes and other fruit hosts.

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This makes it necessary to study the sensitivity of this stage to gamma irradiation.

Some information on the effects of gamma radiation on the grapevine moth pupal stage is available. Steinitz et al. (2015) [22] reported data on the effects of gamma irradiation on some fitness parameters, particularly adult emergence, flight ability, male response to sex pheromone and mating success of *L. botrana* adults exposed as pupae to 150 Gy dose. Because the study examined the effects of a single dose of gamma radiation (150 Gy) and concentrated on measuring parameters that are basically more useful for control (not phtosanitary) purposes, no specific dose for quarantine purposes was recommended. Consequently, a more detailed study on the effects of gamma radiation on *L. botrana* late pupae is needed.

The goal of this study was to examine the effects of gamma irradiation on *L. botrana* late pupae, as the most radiotolerant stage that may occur in the shipped commodity. In particular, it intended to study the effects of gamma irradiation on adult emergence, fecundity and fertility of emerged adults and survival of F_1 insects to the adult stage. In addition, it discusses, on the bases of the obtained data, the irradiation dose needed as a phytosanitary treatment against this pest.

2. Material and methods

2.1. Laboratory rearing of *L. botrana*

Pupae used in this research were obtained from a colony of *L. botrana* that had been reared in our lab on an artificial medium similar to that reported by Mohamad *et al.* (1997) [23]. In this rearing system, mature larvae pupate on the dry upper surface of the medium. The colony originated from insects collected in 2009 from the grape production area in the southern part of the country. To avoid interbreeding and adoption to laboratory conditions, wild *L. botrana* males from the natural populations in the same area were introduced into the colony every summer since. Larvae were reared in plastic trays (19×14×5 cm), containing each about 700 g of the rearing medium. Rearing conditions were maintained at 26±1 °C, 60 ± 5% RH and 16:8 L:D cycle. Under these conditions, eggs hatch after 4 days, larvae pupate in about 20 days and pupae start to emerge as adults in 7 days.

2. 2. Obtaining *L. botrana* pupae.

When larvae finished feeding, strips of sterile light yellow cardboard paper (25×1 cm), folded at one centimeter distance to take a zigzag form “larval holding strips”, were distributed at the top of the rearing medium. Mature larvae searching for a place to pupate, spun their cocoons in the folds of the paper strips. The "larval holding strips" were collected at 24 h intervals, incubated under the same environmental conditions (26±1 °C, 60 ± 5% RH and 16:8 L:D cycle) for pupation and 6 days later, the pupae (late pupae) were prepared for irradiation.

2. 3. Preparing pupae for irradiation

For studying the effects of gamma irradiation on adult emergence the "larval holding strips" with pupae inside were carefully examined, the pupae were counted and the strips were placed in 9 cm Petri dishes. In experiments to study the effects of gamma irradiation on fecundity, fertility and survival of F_1 insects to the adult stage, 6-day-old pupa were carefully removed from the larval holding strips using a forceps, examined, sexed and each sex (males or females) was placed separately in 9 cm Petri dishes. In both experiments, care was taken to include only undamaged, healthy looking insects of a uniform stage of development and any unsuitable pupae were excluded. To reduce temperature fluctuations during transportation to and from the gamma cell, the dishes were placed inside an insulating box.

2. 4. Irradiation

The insect pupae were exposed to gamma radiation dosages in a gamma cell with a Co-60 source around the cylindrical (15×25 cm) irradiation chamber (Issledovatel Gamma Irradiator, Techsnabexport, www.tenex.ru). The average dose rate at the time of irradiation was about 8.7 Gy/minute with a factor of homogeny (the ratio of the max. to the min. received dose) of about 1.14 and the absorbed dose was calibrated using a Fricke solution dosimeter. For studies on effects of gamma irradiation on adult emergence, Petri dishes containing 50 *L. botrana* pupae each, were irradiated. In studies on the effects of gamma irradiation on fecundity, fertility and survival of F_1 insects to the adult stage, an excess number of pupae (males or females) were irradiated separately in 9 cm Petri dishes.

In both experiments, 4 Petri dishes were placed at the center of the irradiation chamber and simultaneously irradiated each with one of four different doses (i.e., 50, 100, 150, 200 Gy).

2. 5. Effects of gamma radiation on adult emergence

Following irradiation, the Petri dishes containing the pupae were returned immediately to the laboratory and incubated under the same previously mentioned conditions (26±1°C, 60 ± 5% RH and 16:8 L:D cycle) for adult emergence. The dishes were checked daily, emerging adults were collected and their number was recorded.

2. 6. Effects of gamma irradiation on fecundity and egg hatching

In this experiment, irradiated *L. botrana* pupae were returned to the laboratory and incubated under the same conditions for adult emergence. Emerged moths were crossed to untreated insects of the opposite sex by confining them inside oviposition cages similar to that reported by Al-Motny (2003) [24]. Twenty five irradiated insects of one sex were placed in an oviposition cage with the same number of non-irradiated insects of a similar age and the opposite sex. Cages were provided with cotton wicks soaked in 5% sugar solution as a source of food and water. The cages were placed randomly on shelves in the rearing room under the same conditions for the colony. Eggs were deposited on a polyethylene sheets "egg sheets" covering the opening of the cage and the "egg sheets" were collected daily (for 5 days) and replaced with new ones. The "egg sheets" were incubated under the same conditions for the colony and placed at the appropriate age (73-96 h old), on the surface of the larval rearing diet with eggs on the side facing the diet in 19×14×5 cm plastic dishes. The dishes were incubated under the same rearing conditions for the colony. Five days later, the "egg sheets" were removed and examined under a binocular microscope for egg hatching. Fertilized eggs were separated from unfertilized ones by counting all deposited eggs and all eggs with signs of embryo development. The number of deposited, fertilized and hatched eggs in each replicate and treatment was recorded and percentage egg hatching was calculated by dividing the number of hatched eggs in each replicate on the total number of eggs with signs of embryonic development (fertilized).

2. 7. Effects of gamma radiation on F_1 survival to adults

Following recording egg hatching, the dishes containing diet with larvae resulted from the different crosses were incubated under the same temperature and relative humidity for insect development. Three weeks later, “larval holding strips”, were distributed at the top of the rearing medium to collect mature larvae leaving the medium and searching for a place to pupate. The dishes were covered with fine muslin mesh to prevent emerging moths from escaping from the dishes. The dishes were examined every day and emerging adults in each dish were collected, counted and their number was noted. Percentage larval survival to adults was calculated by dividing the number of recorded adults by the number of hatched eggs.

3. Data analysis

Data from these experiments were subjected to analysis of variance. Means (mean adult emergence, fecundity, fertility and survival of F_1 insects to the adult stage) were separated, at the 5% level of probability, by Fisher's protected least significant difference (PLSD test). Furthermore, percentage egg hatching and survival of F_1 insects to the adult stage (F_1 adult emergence) data were subjected to regression analysis. Linear regression was used because probit and logit models showed poor fit.

4. Results

Table 1 presents data on the effects of gamma irradiation on adult emergence from *L. botrana* late pupae and fecundity of irradiated insects crossed to non-irradiated individuals of the opposite sex. The data show that gamma irradiation did not significantly affect adult emergence from treated pupae at the 95% confidence level ($F = 2.42$; df = 4, 15; $P = 0.09$). Results on the effects of gamma irradiation on female fecundity clearly indicate that irradiation significantly reduced the mean number of eggs produced by females whether were irradiated ($F = 103.65$; df = 4, 15; $P<0.0001$) (IF x NM) or crossed to irradiated males ($F = 10.0$; df = 4, 15; $P<0.0001$) (IM × NF). The effect, however, was more severe when females were irradiated (IF × NM), particularly at 150 and 200 Gy.

Table 2 presents data on the effects of gamma irradiation on fertility of irradiated *L. botrana* females (female pupae) crossed to normal males (IF × NM) or irradiated males (male pupae) crossed to normal females (IM × NF) and survival of F_1 insects to the adult stage. The table clearly shows that irradiation significantly reduced egg hatching and this effect increased with increasing dose (F = 222.78; df = 4, 15; P<0.0001 and F = 71.69; df = 4, 15; P<0.0001 for irradiated females and males, respectively). Percentage egg hatching ranged from over 96% for the control to about 38% at 200 Gy dose when irradiated males were crossed with normal females. When females resulted from pupae exposed to the same dose (200 Gy) were crossed with normal males, however, no egg hatching was noted.

Table 2 also presents data on survival of the progeny (F1) of moths resulted from *L. botrana* irradiated pupae and crossed to non-irradiated insects of the opposite sex to the adult stage. The results clearly shows that, similar to the effects of gamma irradiation on % egg hatching, survival of F_1 insects to the adult stage was severely affected (F = 1421.2; df = 4, 15; P<0.0001 and F = 1317.7; df = 4, 15; P<0.0001 for irradiated females and males, respectively). The results also show that delaying the anticipated developmental response allows for lower doses to be used. For instance, when irradiated males were crossed with normal females, 150 Gy reduced survival of F_1 insects to the adult stage to less than 2% and, at 200 Gy dose, no adult emergence was noted. In the 2nd case where irradiated females were crossed with normal males, the effect was even more severe; at 150 Gy, survival of F_1 insects to the adult stage was close to zero and no survival was recorded at 200 Gy.

Linear regression analysis on the data presented in the table was used to predict the required irradiation doses to cause 100% failure in egg hatching and F_1 adult emergence. The data in the table show that, for egg hatching, the doses were estimated to be 335 and 288 Gy for crosses between (IM × NF) and (NM × IF), respectively. When adult emergence was used as a criterion for determining effectiveness, however, the estimated doses were 211 and 161 Gy for the same crosses and in the same order.

Table 1. Effects of gamma irradiation on adult emergence and fecundity in *L. botrana* late pupae.

Dose (Gy)	% Adult emergence ± SD	Female fecundity (Mean ± SD) in crosses between	
		Irradiated males and normal females (IM × NF)	Normal males and irradiated females (IF × NM)
0	93.0 ± 3.8^a	103.5 ± 9.9^a	103.5 ± 9.9^a
50	91.0 ± 3.8^a	75.8 ± 8.2^b	90.3 ± 4.3^b
100	89.0 ± 2.0^a	70.8 ± 7.1^b	86.1 ± 9.2^b
150	88.0 ± 3.3^a	69.5 ± 11.6^b	31.4 ± 8.0^c
200	87.0 ± 2.0^a	67.5 ± 9.6^b	15.2 ± 5.7^d

Means followed by the same letter within each column are not significantly different (P<0.05, Fisher's LSD test).

Table 2. Effects of gamma radiation on % egg hatching and adults emergence in crosses between irradiated males and normal females (IMxNF) or normal males and irradiated females (NM x IF).

Dose (Gy) & statistical analysis	Egg hatching and survival of F1 insects			
	% Egg hatching ± SD		% survival of F1 insects to the adult stage ± SD *	
	IM × NF	IF × NM	IM × NF	IF × NM
0	96.6 ± 2.2^a	96.6 ± 2.2^a	85.83 ± 4.5^a	85.83 ± 4.5^a
50	63.8 ± 4.9^b	72.9 ± 7.3^b	3.93 ± 0.5 ^{bc}	0.87 ± 0.2^b
100	56.1 ± 7.4^{bc}	68.4 ± 6.5^b	4.51 ± 0.5 ^{b}	0.58 ± 0.1^b
150	51.3 ± 4.9^c	45.5 ± 4.4^c	1.59 ± 0.3 ^{bc}	0.05 ± 0.1^b
200	37.6 ± 5.1^d	0.0 ±0.0^d	0.00 ± 0.0 ^{c}	0.00 ± 0.0^b
Slope ± SE	-0.2609 ± 8.66	-0.441± 11.095	- 0.0309 ± 0.003	- 0.0082 ± 22.4
R^2	0.83	0.90	0.89	0.87
Predicted dose (Gy) for 100% mortality	335	288	211	162

* Linear equation based on regression from dose 50 to 200.
Means followed by the same letter within each column are not significantly different (P<0.05, Fisher's LSD test).

5. Discussion

Phytosanitary measures are used to disinfest agricultural products carrying quarantine pests making it possible to ship these products out of quarantine areas. In the last few decades, ionizing radiation is being used increasingly as a phytosanitary measure to overcome biological barriers in trade [25]. This technique possesses several advantages over other treatments [13]. Some of these advantages are that this process is applicable to packed products, preserves the nutritive values of the treated product and commodities are often more tolerant to irradiation treatment than alternative treatments. In this report, *L. botrana* late pupae were irradiated and the effects of gamma radiation on adult emergence, fecundity and fertility of emerged adults were examined. Survival of F_1 larvae resulting from crosses between irradiated insects and normal ones to the adult stage was also investigated. In addition, we discuss the required dose for phytosanitary treatment against this pest in grape shipments coming from infested areas.

Practically, when shipping grapes from areas infested with *L. botrana*, the shipment may contain eggs, larvae and even pupae [4, 11, 12]. Because the radiosensitivity of insects decreases as they develop [20, 21], late pupae are, most likely, the most radiotolerant stage. Consequently, any irradiation treatment proven to be affective against this stage (late pupae), should also be able to kill, or at least prevent reproduction in all other stages potentially present in the shipped commodity.

The results of this study show that irradiation, up to 200 Gy, had no significant effect on adult emergence in irradiated *L. botrana* late pupae. This indicates that acute somatic damage to *L. botrana* pupae, at the examined dosages, was not considerable. It also suggests that preventing *L. botrana* adult emergence is not a good criterion for measuring effectiveness [25]. These results are in general agreement with data reported previously for *L. botrana* late pupae [22].

Contrary to the effects of gamma irradiation on adult emergence, the results show that fecundity and fertility of insects resulting from irradiated pupae and crossed to non-irradiated individuals of the opposite sex decreased with increasing radiation dose. The effect, however, was larger when females were the irradiated sex.

For example, in crosses where males were exposed to 150 and 200 Gy, egg hatching was reduced to 51.3 and 37.6 %, respectively, and this percentage decreased to 45.5 and 0 %, in the same order, when females were irradiated. This is in agreement with data reported for other Lepidopteron species in general [26, 27, 28] and for *L. botrana* in specific [22]. They are, however, little different from those reported by some other authors [12, 29]. Saour (2014) [29] found that 150 Gy dose reduced egg hatching to 0 % when *L. botrana* females were irradiated and crossed with normal males. To the contrary, Nadel et al. (2018) [12] irradiated 6-7 day old female pupae and found that 325 Gy dose was needed to produce the same effect (zero egg hatch). The differences with the current study may be related to several factors. Some of these may be attributed to the different irradiated stage (late pupae vs. adults) and some of the variations may be related to differences in experimental techniques [30]. It was also found that genetic variability among different strains and different laboratory colonies can be responsible for some of the differences in radiosensitivity [31, 32].

The results also show noticeable differences in radiosensitivity between sexes; irradiated female pupae were more susceptible to irradiation treatment than males. These results are in agreement with those reported for other lepidopteron species in general [26, 27, 28] including those reported for *L. botrana* [22, 29]. Erdman (1963) [33] explained this phenomenon by the fact that, in moths, males are homogametic while females are heterogametic. In other words, males possess a duplicate of the sex chromosome which makes them more radiotolerant. Hallman (1998) [34] offers two other reasons that may explain why female Lepidoptera are more sensitive to irradiation treatment than males. The 1st one is that eggs are more complex than sperms offering more possibilities for radiation-induced damage. The other reason is that males produce many more gametes than females. This means that 99.9% sterility of males leaves much more viable gametes than 99.9% sterility of females. One useful application of this phenomenon is that we may be able to reduce the irradiation dose (when desirable) to the level that ensures complete sterility (or F_1 sterility) of females, even if males retain some of their fertility.

Survival of F_1 generation to the adult stage was also severely affected. For example, 50 Gy reduced adult emergence in crosses between IM and NF to less than 4% and at 200 Gy dose no adult emergence was observed. However, when irradiated females exposed to 50 Gy were crossed with normal males (IF x NM), only about 1% of F_1 eggs were able to reach the adult stage. These results are in general agreement with those reported for *L. botrana* adults [29].

In general, the results of this study show that the predicted dose that reduces egg hatching in *L. botrana* adults resulting from irradiated pupae to 0 % (estimated to be 335 and 288 Gy for irradiated males and females, respectively) may be too high to be used commercially. This is because, in some commercial irradiators, the dose uniformity ratio could be as high as 3:1 [21, 29]. This dose could be harmful to grapes [16] and may exceed the 1000 Gy limit established by the US Food and Drug Administration [35]. However, when survival of F_1 insects to the adult stage was used, instead, as a criterion for measuring effectiveness [15, 17], the results are much more promising. A dose of 200 Gy applied to *L. botrana* late pupae (one day before the start of adult emergence) prevents F_1 insects from reaching the adult stage.

To summarize, *L. botrana* is a quarantine pest in many countries [4, 5, 9, 10] and can be transmitted from one country to another as eggs, larvae or pupae in exported host fruit. Results in previous studies [18] showed that 150 Gy prevented larvae resulting from *L. botrana* eggs, irradiated hours before egg hatching, from reaching the adult stage. Further studies [19] showed that 200 Gy prevented irradiated mature larvae from developing into adults. Results of the current study show that exposing late *L. botrana* pupae to 200 Gy prevented any F_1 generation individuals resulting from irradiated insects crossed with non-irradiated ones from reaching the adult stage. This may indicate that the use of gamma radiation as a phytosanitary treatment for grapes potentially infested with *L. botrana* late pupae (or any other immature stage potentially present in shipped commodity) is possible. In addition, this treatment requires a relatively low dose (200 Gy), provided that prevention of F_1 adult emergence is used as a criterion for effectiveness.

This dose (200 Gy) is less than the suggested generic phytosanitary irradiation dose of 400 Gy for Lepidopteron pupae [25], much less than the highest allowed dose for irradiation of fresh agricultural products in the US [35] and has no unacceptable effects on the treated grapes [16].

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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