INVESTIGATING PHYSIOLOGICAL METHODS TO DETERMINE PREVIOUS EXPOSURE OF IMMATURE INSECTS TO IONIZING RADIATION

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Abstract. Effect of gamma radiation on phenoloxidase activity in codling moth, *Cydia pomonella* L., larvae was investigated. Phenoloxidase activity was determined spectrophotometrically by measuring the increase in optical density at 490 nm, or by observing the degree of melanization in larvae killed by freezing. Results showed that, in unirradiated larvae, phenoloxidase activity could be detected in 7 day old larvae and activity continued to increase throughout the larval stage. This increase was not observed when larvae were irradiated with a minimum dose of 50 Gy during the 1st week of their development. However, irradiating larvae in which enzyme activity was already high (24 week old) did not eliminate the activity but reduced further increase. Larval melanization studies were in general agreement with the results of the phenoloxidase assay.

1. INTRODUCTION

The codling moth, *Cydia pomonella* L., is a pest of pome and stone fruits throughout most deciduous fruit growing areas of the world. Its larvae infest apples, pears, walnuts, and many other deciduous fruit crops and causes hundreds of millions of dollars in losses to the fruits industry every year. This species is also of quarantine importance in Japan, Korea, Taiwan, Brazil, and parts of China [1] and strict quarantine measures are applied to prevent its entry and/or establishment in these countries. The insect overwinters as diapausing larvae in cocoons which, sometimes, are present within the shell of inshell walnuts. Therefore, inshell walnuts could not be exported to countries where codling moth is quarantined. The insect could also be transmitted as eggs or larvae on or in fruits. As a result, quarantine regulations are imposed on fruits exported to codling moth free countries.

Ethylene dibromide (EDB) was accepted to disinfest nuts and fruits exported to codling moth free countries [2]. Concern over its carcinogenic, mutagenic and environmental effects led the US government to phase out this chemical and several other countries have also adapted the same policy [3].

Ionizing radiation has been proposed as a possible alternative to chemical fumigation against commodities subject to quarantine regulations because of infestation by insects pests and a generic dose of 300 Gy was recommended for any pest other than fruit flies [4]. Studies on the codling moth has indicated that gamma radiation is an effective treatment against larvae in apples [5, 6]. However, quarantine doses do not necessarily cause immediate death and larvae may continue to develop to the pupal stage. Consequently, if a living larva is detected in a shipment, it is very important to determine if it has been irradiated [7] and whether the minimum required dose has been received.

Several studies have been done to distinguish irradiated larvae from those not irradiated. The size of the supraoesophageal ganglion in relation to the proventriculus was reduced when eggs and young larvae of several Tephritid fruit fly species were exposed to ionizing radiation [8, 9, 10]. Tsiropoulos [11] found that melezitose metabolism in adults of the olive fruit fly,

Dacus oleae (Gmelin), was affected by irradiation. Irradiated flies were unable to hydrolyse this sugar into its basic components (2 glucose and 1 fructose). He suggested that a hydrolytic enzyme produced in gut cells had become inactive because of the irradiation treatment. The enzyme phenoloxidase plays an important role in the melanization of insect cuticle through the conversion of aromatic quinones and its products into melanin [12]. The enzyme is also responsible for the darkening (melanization) of insect tissue at injury sites or after death [13]. Nation et al. [14] studied the effects of gamma radiation on phenoloxidase and melanization in the larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Loew). They found that 20 Gy applied to the late egg stage or young larvae drastically decreased the activity of this enzyme in 3rd instars and prevented melanization in larvae killed by freezing. Similar results on the Mediterranean fruit fly, *Ceratitis capitata*, were also reported by Mansour and Franz [15, 16].

The objectives of this investigation were to study the effects of gamma radiation on melanization and phenoloxidase activity in *C. pomonella* larvae killed by freezing and to investigate the possibility of using the level of phenoloxidase in irradiated larvae as an indication of irradiation dose.

2. MATERIALS AND METHODS

2.1. Insects. Larvae used in this research were obtained from a colony of *C. pomonella* that has been reared on an artificial media similar to that reported by Brinton et al. [17]. The larvae were reared in plastic boxes (18 × 15 × 6 cm) at 28±2 °C, 40 60 % RH and a photoperiod of 16:8 (L:D). Under these conditions, the larval stage requires approximately one month.

2.2. Irradiation. Larvae were irradiated in the larval rearing medium in plastic bags. Irradiation was done in a cobalt-60 source (Issledova Gamma Irradiotor, Techsnabexport Co. LTD, RUS). Radiation doses between 25 and 100 Gy with 25 Gy intervals were used and an untreated group was held as a control. The mean dose rate over the period of these tests was approximately 83.30 Gy/minute. After irradiation, larvae were returned to the rearing room and when they reached the desired age, they were killed by freezing.

2.3. Phenoloxidase assay. Larvae were homogenized individually in 150 µl of 0.1 M phosphate buffer solution (pH 6.5) in a 1.5 ml test tube. The homogenate was centrifuged for 5 min at 15,000 Xg, after which the supernatant, containing the enzyme, was kept on ice until tested to avoid possible autooxidation. The substrate was 5 mg/ml of L-dihydroxyphenylalanine in 0.1 M phosphate buffer solution. Fifty microliteres of the supernatant were added to 0.95 ml of the substrate solution, mixed with a vortex mixer for few seconds, and incubated at 25°C for 5 min. As a result of oxidizing L-Dopa to Dopachrome [18], a red color was produced that was quantified by measuring light absorption at 490 nm with a spectrophotometer [19]. Phenoloxidase activity was expressed as the increase in absorbance at 490 nm under the conditions described above.

2.4. Melanization test. Larvae exposed to 50 Gy dose were removed from the freezer and placed on a white background for observation. The color change (darkening) caused by the melanization reaction was compared with unirradiated controls.

2.5. Data analysis. Data from these experiments were subjected to analysis of variance. Means were separated by Duncan's [20] multiple range test.

3. RESULTS

3.1. Phenoloxidase Activity in *C. pomonella* larvae

Phenoloxidase activity in codling moth larvae was determined spectrophotometrically. Frozen larvae were used to increase the activity of the phenoloxidase system [18]. Results with unirradiated larvae (Table 1) showed that the activity of phenoloxidase was very low during the first week of larval development. Enzyme activity, however, continued to increase throughout the 4th week (end of larval stage). These results agree with those reported by Hackman and Goldberg [21] and Mnasour and Franz [16] for several species of insects.

TABLE 1. PHENOLOXIDASE ACTIVITY (OD UNITS $\times 10^3$) IN *C. POMONELLA* LARVAE*

Age of larvae (days) when assayed	Activity ± SD (OD units × 10^3)
7	023.4 ± 05.9[a]
14	210.3 ± 61.8[b]
21	321.6 ± 73.1[c]
28	533.8 ± 91.8[d]

*Data represent the average of 10 measurements on individual larvae. Means followed by the same letter in each column are not significantly different (P>0.05).

3. 2. Effect of gamma radiation on phenoloxidase activity

When one week old larvae were irradiated and tested as 2, 3, and 4 week old larvae, phenoloxidase activity was significantly affected. At a dose of 50 Gy and higher, enzyme activity was very low (Table 2). Statistical analysis indicates that irradiation significantly reduced the level of phenoloxidase in examined larvae (P <0.001). Comparisons between means showed that phenoloxidase activity was significantly reduced at a dose as low as 25 Gy (P <0.01) and this reduction reached its maximum at 50 Gy dose.

TABLE 2. EFFECTS OF GAMMA RADIATION ON PHENOLOXIDASE ACTIVITY (OD UNITS × 10^3±SD) IN *C. POMONELLA* LARVAE*

Dose (Gy)	Assay days 14	21	28
0	210.8 ± 62.3[a]	321.5 ± 73.3[a]	534.6 ± 90.8[a]
25	102.9 ± 23.6[b]	135.0 ± 33.4[b]	227.8 ± 45.9[b]
50	023.0 ± 09.0[c]	031.8 ± 08.3[c]	037.6 ± 07.6[c]
75	024.1 ± 05.0[c]	037.3 ± 05.9[c]	033.0 ± 09.6[c]
100	030.5 ± 10.7[c]	031.1 ± 07.0[c]	035.1 ± 10.0[c]

*Data represent the average of 10 measurements on individual larvae. Means followed by the same letter in each column are not significantly different (P >0.05).

3.3. Effect of age on phenoloxidase sensitivity to gamma radiation

Data on the effect of gamma radiation on phenoloxidase activity measured in 4 week old larvae, after irradiation as 1, 2, and 3 week old larvae showed that irradiation drastically reduced phenoloxidase activity, especially when larvae were treated during the 1st week (Table 3). However, when larvae were treated as 23 week old, the effect of radiation was less severe.

TABLE 3. EFFECTS OF GAMMA RADIATION ON PHENOLOXIDASE ACTIVITY (OD UNITS × 10^3±SD) IN MATURE *C. POMONELLA* LARVAE IRRADIATED AS 1, 2, AND 3 WEEK OLD AND EXAMINED AS 4 WEEK OLD*

Dose (Gy)	Irradiation days		
	7	14	21
0	500.8 ± 95.0[a]	500.8 ± 95.0[a]	500.8 ± 95.0[a]
25	121.2 ± 14.4[b]	201.6 ± 33.7[b]	270.0 ± 35.9[b]
50	025.3 ± 04.7[c]	121.5 ± 42.3[c]	204.3 ± 29.9[c]
75	019.6 ± 04.5[c]	154.2 ± 34.7[c]	217.4 ± 33.5[c]
100	022.0 ± 05.4[c]	132.5 ± 43.6[c]	211.9 ± 28.2[c]

*Data represent the average of 10 measurements on individual larvae. Means followed by the same letter in each column are not significantly different (P >0.05).

3.4. Effect of gamma radiation on melanization

Results of the larval melanization study (Table 4) agreed with results of the phenoloxidase assay. Untreated 1 week old larvae showed a clear lack of melanization, whereas 2, 3 and 4 week old larvae turned, partially or completely black after 2 hours of incubation at room temperature. In comparison with the unirradiated control, 24 week old larvae that had been irradiated as 1 week old larvae, particularly those that had been exposed to a dose of 50 Gy or higher, showed no melanization. However, when later stages were treated, irradiation did not prevent melanization in most of the examined specimens.

TABLE 4. RESULTS OF THE LARVAL MELANIZATION STUDIES*

Age of larvae (days) when irradiated	Age of larvae (days) when examined		
	14	21	28
7	+/	+/	+/
14		+/±	+/±
21			+/+

*Fifty larvae from each age group exposed to a dose of 50 Gy were examined.
+, melanized; _, not melanized; ±, inconclusive.

4. DISCUSSION

The irradiation dose of 300 Gy, as currently recommended for quarantine treatment against all insects other than fruit flies of the family Tephritidae [4], does not prevent *C. pomonella* eggs or larvae from developing to the pupal stage [5, 6]. Consequently, a method is needed to establish with certainty that larvae detected in imported fruits were indeed irradiated and exposed to the minimum required dose that prevents their development into sexually reproductive adults. In a search for a test system, Nation et al. [14] and Mansour and Franz, [15, 16] suggested that the effect of radiation on enzymes, particularly phenoloxidase, present in insect larvae, be examined. In this report, we present results of research conducted on the effects of gamma radiation on phenoloxidase activity and melanization in codling moth larvae killed by freezing.

Examination of phenoloxidase activity in codling moth larvae showed that the activity of this enzyme can be detected in unirradiated larvae starting with the 1st week and increases until it reaches its maximum immediately before pupation. These results agree with those reported by Hackman and Goldberg [21] and Mnasour and Franz [16] for other insect species. Irradiation during the first week, with a minimum dose of 50 Gy, reduced this enzyme activity to levels close to the detection level and prevented melanization. However, when 23 week old larvae were irradiated, the reduction in both phenoloxidase activity and melanization was not sufficiently pronounced to allow visual discrimination between irradiated and unirradiated larvae. These results indicate that measuring the activity of this enzyme in *Cydia pomonella* larvae can be used as an indication of irradiation treatment provided that the limits mentioned above are observed. Failure of codling moth larvae to melanize if exposed to a minimum dose of 50 Gy during the 1st week can also be used as an indicator of irradiation treatment. However, since a dose of 50 Gy affects the formation of phenoloxidase in developing larvae and the required dose for quarantine treatment is 300 Gy (4) this method will not be suitable to verify that larvae received the minimum required dose.

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