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Potential Methods for Identification the Gamma Irradiated from Unirradiated Larvae of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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ABSTRACT

To verify the irradiated from unirradated *Helicoverpa armigera* larvae through the two methods, the 1st , 2nd , 3rd and 4th instar larvae were irradiated with gamma radiation at 0, 75 and 150 Gy and observed in the late 5th instar. Irradiated at 75 Gy, the total haemocyte counts (THCs) of the irradiated 1st, 2nd , 3rd and 4th were found to be 6207±178, 6990±215, 9014±244 and 9991±279 h/mm^3 respectively while those irradiated at 150 Gy averaged 3939±156, 4988±157, 5704±203 and 5904±218 h/mm^3 respectively. It was also revealed that phenoloxidase (PO) activities of the irradiated 1st, 2nd, 3rd and 4th larval stages were 2.6±0.7, 9.7±0.7, 16.6±2.6 and 23.7±2.6 units/mg protein respectively whereas those irradiated at 150 Gy averaged 0.8±0.1, 4.6±0.5 , 10.4±1.3 and 15.0±1.4 units/ mg protein respectively. The results of the effects of radiation on THC and PO activity supported each other. The changes in such experiments on each instar at each dose were found to be significantly less than THC (12921±605 h/ mm ^{3}) and PO activity (31.6 units/mg protein) of the controls. In both doses, percent reduction of THC and PO activity also increased from the 4th to 1st instars.

Key words: *Helicoverpa armigera*, gamma radiation, haemocyte count, phenoloxidase activity

INTRODUCTION

Many fresh fruits and agriculture products are quarantine commodities, offering an armor against insect pests which are necessary to ensure that potentially damaged pest species can not enter the countries where the pests do not exist. Insect disinfestation by irradiation, while it satisfies quarantine requirements, needs not necessarily result in immediate mortality of target pests. Therefore, verification of irradiation as an insect disinfestation can not in most cases be based on dead pests. There are possible avenues to the regulatory problem of intercepting live pests in irradiated consignments. The method could conceivably be developed to verify that live pests have been appropriately irradiated. However, development of such methods for a broad range of target pests has proved to be difficult to date

An elegant and promising technique for identifying irradiated fruit fly (Tephritidae) larvae was elaborated but had limited use in a commercial setting. Some methods to detect previous exposure of insect pests to ionizing radiation are needed.

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Nation *et al*. (1995a) suggested that an indicator that could be used easily for identifying irradiated insects might lie in the irradiation causing inhibition of the darkening or melanization that usually followed death of injury in a living insect

Nation *et al.* (1995b) also dissected the larvae of the Caribbean fruit flies irradiated with gamma radiation for measurements of the ganglion (brain) which were used as indicators. Elbadry (1964) demonstrated the effects of gamma irradiation on total haemocyte counts (THCs) and differential haemocyte counts (DHCs) of the potato tuberworm. The haemocyte number was found to change by the levels of irradiation. The function of enzyme in cotton bollworm might also be inactivated when larvae exposed to radiation similarly to the Caribbean fruit fly when irradiated with gamma radiation from a Cs-137 source (Nation *et al.*, 1995a) Assays of phenoloxidases in irradiated larvae showed greatly decreased enzyme activity and substantial reduction at lower doses as well.

The cotton bollworm, *Helicoverpa armigera* Hübner, has been one of the most destructive pests of many field crops in Thailand since the 1960s causing great economic losses as a result of crop destruction and costs for its control (Mabbett, 1983). The larval stages of the insect cause a great damage to many plants. Consequently, chemical control still remains the backbone of management tactics for *H. armigera* . However, there has been an attempt to control this insect by sterile insect technique (SIT) employing gamma radiation. The verification of the irradated insects would be quite beneficial for such control method. The aim of this study was to identify the irradiated from unirradiated insects through the effect of different doses of gamma radiation on *H. armigera* larvae in terms of the changes of total haemocyte count and phenoloxidase activity.

MATERIALS AND METHODS

Laboratory rearing on artificial diet

The larvae of the cotton bollworm, *Helicoverpa armigera* and artificial diet were obtained from the mass rearing laboratory at the Entomology and Zoology Division, Department of Agriculture, Bangkhen, Bangkok. The insects were reared under laboratory condition with the average temperature of 26 ± 2°C, 70 ± 10 % relative humidity and 10:14 (light:dark) photoperiod. The full grown larvae were then allowed to pupate, to emerge into adults, to mate and to lay eggs as normal.

The eggs were daily collected, soaked in 10 % formalin solution for 10 minutes to prevent disease infection then were allowed to dry. As soon as the larvae hatched, they were individually placed on a cube of artificial diet in plastic cups. Pupae were collected, soaked in 10 % formalin solution for 10 minutes and allowed to air-dry. The pupae of different sexes were placed in separate dry plastic box until adult emergence. The emerged adults were transferred into egg-laying plastic container with cotton cloth as an oviposition site covering the top. Ten males and ten females were paired. The moths were daily provided with 10 % honey solution. After mating and eggs laying, the cotton with attached eggs was collected daily.

The irradiation process

First, second, third and fourth instars were irradiated at doses of 0, 75 and 150 gray (Gy), with Gamma Mark I Research Irradiator having cesium-137 as a gamma source at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University. After that, they were normally reared with artificial diet to the fifth instar.

Total haemocyte count (THC)

Irradiated larvae were heat-fixed (60°C for 1 min) and bled from a first pair of proleg on the abdominal segment, cut with fine scissors.

Haemolymph was allowed to flow onto a clean glass slide and drawn into a Thoma white blood cell pipetted, diluted 1:100 by physiological versene (ethylene diamine tetra-acetic acid (EDTA), 1 g : 100 ml distilled water). After vigorously shaken and discarded the first three drops, the haemocytes were counted in a haemocytometer (counting chamber). Haemocytes from 1-mm^2 (the four corner square) were counted (EI-Mandarawy *et al*., 2000). This experiment was replicated 3 times. Data obtained for THCs were statistically analyzed and Duncan's new multiple range test at 0.05 level of probability was used for mean separation.

Measurement of phenoloxidase activity (PO)

Control (0 Gy) and irradiated larvae with 75 and 150 Gy were killed by placing them in a freezer (-73°C) for a few days. Larvae were then removed from the freezer until used in the experiments. PO activity was to be determined by homogenizing instar individually in 150 µl 0.1 M phosphate buffer solution (pH 6.5) in glass apparatus. This tissue homogenate was added with polyvinylpyrrolidone (oxidation protection) and centrifuged 12,000 x g for 10 min. The supernatant was pipetted from homogenate solution and the resulting volume used as the enzyme source. Enzyme was kept on ice until tested to avoid possible auto oxidation. The substrate was 3 mg/ml of L-dihydroxyphenylalanine (2-methyl dopa, Sigma) in 0.1 M phosphate buffer solution (pH 6.5). One hundred microlitre of the supernatant were added to 150 µl of the substrate solution, mixed for a few seconds and incubated at 25°C for 30 min. The intensity of the red colour produced was measured by light absorption at 490 nm in a 6400 Spectrophotometer (Jenway). A unit of PO activity was defined as the amount of enzyme at pH 6.5 that caused a change in light absorbance at 490 nm. Specific activity was measured as units per milligram of protein (units/mg protein). Data from spectrophotometric analyses were statistically analyzed by Duncan's new multiple range test at 0.05 level of probability.

RESULTS AND DISCUSSION

Types of haemocyte

In the experiment, heat fix was employed by submersing the insects into hot water. The method was considered satisfactorily when (1) haemolymph was easily obtained, (2) haemolymph did not agglutinate or precipitate, (3) cells did not agglutinate and (4) cells did not changed in size, shape or form. However, they changed their appearances and shapes from time to time even in the same insect and could be distorted in shape by fixation, staining including the other procedures used in collecting and processing haemolymph (Jones, 1962).

Six types of haemocytes found in the haemolymph of *H. armigera* were identified by appearance and size according to the classification of Gupta (1979) namely, prohaemocyte (PR), plasmocyte (PL), granulocyte (GR), spherulocyte (SP), oenocyte (OE) and podocyte (PO).

Since the types of haemocyte of *H. armigera* were not the main concern of the study, percent accounts and function of each type were not studied. However, the effect of gamma radiation on the haemocyte features of GR, OE, and SP such as cytoplasm appearing to be granulated and vacuolized were still noticed. The cellular types observed in the haemolymph of *H. armigera* were similar to the ones described by several authors for other species in spite of the existent controversies among them about classification. This was in agreement with Elbadry (1964) who tried low doses of gamma irradiating the potato tuberworm and found morphological changes in haemocytes. Younes *et al*. (1999) also showed the malformation of haemocytes of the treated larvae with some insecticides as the blood cells appeared larger and irregular in shape compared to the control. In addition, the irradiated larvae in the study were found to have turbid haemolymph and lesser

volume than the controls. The unclear haemolymph indicated the burst of cell while cytoplasm partially gush-flowed into plasma. In addition, the different colours between irradiated and disease- infected haemolymph, if could be separated, may make the identification practical in the field.

Changes of total haemocyte count (THC)

The total haemocyte count (THC) was made on individuals of the larvae irradiated at the 1st, 2nd, 3rd and 4th and observed in the late 5th instars of *H. armigera* from a haemocytometer grid using a haemocytometer. The biometric measurements of haemocyte counts and percent reduction of haemocytes in the unirradiated (the control) and irradiated 5th larval instar are shown in Table 1. It was demonstrated that irradiation had an effect on the amount of haemocytes present in the haemolymph of larvae. Irradiated at 75 Gy, THCs of the group of the 1st and 2nd instar larvae were found to be significantly different from the others but not between each one in the group while those of the 3rd and 4th instar larvae were significantly different from all instars. However, the reverse result was observed between the group of the 1st and 2nd and another of the 3rd and 4th instar larvae irradiated at 150 Gy. The values obtained at each dose for THC showed a decrease in the number of haemocytes during the larval development from the 4th to the 1st instars. In all doses, THC of each larval instar was significantly lower than that of the control. The results of both doses of gamma radiation supported each other, that was, the younger larval instars showed more sensitivity to radiation than the older ones while all instars showed more sensitivities to high than low doses. Percent reduction of the larval stages at each dose also decreased from the 1st to 4th instars as expressed in Table 1.

According to the theory of Bergonie and Tribondea, the most radiosensitive cellular populations are cells that 1) are primitive in their relative degree of maturity, 2) are rapid division during irradiation, and 3) have the ability to divide for long period of time. Because the blood cells were considered primitive cells as in 1), the results were well explained as the blood cells were damaged by irradiation

The decreased haemocyte counts of the irradiated compared to the unirradiated larvae could possibly be explained as follows. Insects, in general, are sensitive to radiation. As with other organisms, the effect of radiation on insects are closely related to the effects on constituent cells.

Table 1 Total haemocyte counts (THCs) and percent reduction of the 5th unirradiated larvae and the ones irradiated at the 1st, 2nd, 3rd, 4th instars of *Helicoverpa armigera* at different gamma radiation doses.

Dosage (Gy)	Mean±S.E. of THCs (h/mm^3) (% reduction)			
	1st instar	2nd instar	3rd instar	4th instar
0 (control)	12921±605a[1/]A[2/]			
75	6207±178d (51.96)	6990±215d (45.90)	9014±244c (30.24)	9991±279b (22.68)
150	3939±156D (69.51)	4988±157C (61.40)	5704±203BC (55.85)	5904±218B (54.31)

[1/]and [2/]Means followed by the same types of letter in the same rows are not significantly different from each other as determined by Duncan's new multiple range test at 0.05 level of probability

During the larval period of insects, very little cell differentiation occurs (Molin, 2001). According to Tubiania *et al.* (1990), the cell cycle of the treated insects were delayed after irradiation and the younger stages were greater affected than the older ones. The same assumption could be applied with the study resulting in the small amount of haemocytes in the irradiated larvae.

Changes in total haemocyte count (THC) during growth and development of healthy insects have been reported by a number of workers. The THC differences between the efficacy of unirradiated and irradiated insects could be compared to the experimental results of Tu *et al.* (2002) who studied the effects of heavy-ion radiosurgery on the haemopoietic function of a silkworm, *Bombyx mori*. The haemocyte densities of the irradiated larvae at different developmental stages compared to the later stages had a significant suppressive effect on haemocyte densities. The percentages of dead haemocyte were obviously higher for irradiated larvae than unirradiated controls during the late 5th instar.

This was also similar to the work of Elbadry (1964) who demonstrated the effects of gamma irradiation with 3,000 and 9,000 rad on the potato tuberworm on both morphological changes and changes in total haemocyte counts. The THCs were found to decrease in the treated larvae. The same result was reported by Hoffmann (1972) who studied the X-irradiation of 25,000 rad to larvae and adults, *L. migratoria* on the dorsal haemocytopoietic tissue. The THCs fell by approximately 50%.

In addition, Eppensteiner and Karp (1989) reported the effect of gamma irradiation on the american cockroach, *P. americana* exposed to 5,000 rad which depleted 1/3 haemocytes from normal. Grégioire (1974) also found that after irradiation by X rays of adult *L. migratoria* (10,000 rad), the alterations decreased in the number and size of haemocyte.

Effect of gamma radiation on phenoloxidase (PO) activity in *H. armigera*

Results of the preliminary experiments on the effects of radiation on PO in larvae of *H. armigera* indicated variability of the response of enzyme activity to gamma irradiation. Several sets of experiments were done to determine PO activity colourimetrically. For this assay frozen larvae were used to increase the activity of the PO system according to Mansour and Franz (1996). The results of PO activity measurement are shown in Table 2.

The values obtained for PO activity of both doses showed a decrease during the larval development from the 4th to the 1st instars. At each dose, Table 2 shows PO activity of each larval instar to be significantly lower than that of the control. Irradiated at 75 Gy, PO activities of the two pairs of 1stand 2nd as well as 3rd and 4th instar larvae were found to be significantly different from each other while between instars in each pair PO activity was not significantly different. There were significant differences noticed among PO activities of all instars irradiated at 150 Gy. The results of both doses of gamma radiation supported each other. Percent reduction of the larval stages at each dose also increased from the 4th to 1st as expressed in Table 2.

This PO activity observation was confirmed by Nation *et al.* (1995a) who suggested that ionizing radiation inhibited the production of one or more enzymes involving in melanization. Phenoloxidase was responsible for melanin formation by catalizing the oxidation of phenol to quinone that subsequently polymerized into melanin. There was > 85% reduction in PO activity of larvae irradiated at > 20 Gy and >50% reduced activity in larvae irradiated at 10 Gy. Preliminary analysis of PO in 1st and 2nd instar larvae showed that PO activities were very low and difficult to detect.

Lupa and Ignatowicz (1999) showed that the highest doses inhibited enzyme activity in larvae of Mediterranean flour moth (*Ephestia kuehniella*) and the confused flour beetle

Table 2 Phenoloxidase (PO) activities of larvae irradiated at the 1st , 2nd , 3rd and 4th *Helicoverpa. armigera* instars at different radiation doses compared to the 5th unirradiated control.

Dosage (Gy)	Mean±S.E. of PO activity {x10^3} (units/mg protein) (% reduction)			
	1st instar	2nd instar	3rd instar	4th instar
0 (control)	31.60a[1/]A[2/]			
75	2.60±0.67c	9.70±0.68c	16.60±2.58b	23.70±2.63b
	(91.77)	(69.30)	(47.47)	(25.0)
150	0.80±0.14E	4.60±0.50D	10.40±1.27C	15.00±1.44B
	(97.47)	(85.44)	(67.09)	(52.33)

[1/] and [2/] Means followed by the same types of letter in the same rows are not differently significant from one another as determined by Duncan's new multiple range test at 0.05 level of probability.

(*Tribolium confusum*) after irradiation. There was decreasing of PO activity and being significant when larvae were treated with 0.3 kGy or higher dose of gamma radiation.

PO in the haemolymph of *H. armigera* was localized in the haemocytes as reported for most insects. The lower enzyme level in the treated larvae was caused by the ability of plasma to suppress PO activities in haemocytes implicating possible inhibitors in the irradiation. That inhibition was lost by lower dose rate in the older instar larvae. PO activity decreased concomitant with a decrease in THC. This PO decrease could be a result of haemocyte dispersion induced by gamma radiation (Marmaras *et al*., 1996).

Marmaras *et al*. (1996) also demonstrated that PO occured as inert proenzyme which activated in response to some factors as well as hormone. There was related hormone secreted from ganglion according to Nation *et al.* (1995b) who studied the influence of gamma radiation on development of Caribbean fruit fly larvae with 50 Gy. They revealed reduction in brain growth as well as small and misshapen compound eye and leg imaginal disk. In accordance with the results from Rahman *et al*. (1990), they reported supraesophageal ganglion decrease in the eggs and larvae of Mediterannean fruit flies subjected to radiation treatment. Therefore, ganglion size reduction was due to the decreased quantitative and effectual hormone resulted from radiation. Since the hormone was decreasing, it did not respond to PO activation.

Nation *et al*. (1995b) and Rahman *et al*. (1990) identified the irradiated fruit fly larvae by the dissected method for measurement of the internal organs and was used as the indicators of radiation exposure. Morphological method was generally not very promising both as external and internal inspectors. Nevertheless, the processes of internal organ dissection which could be employed in identification, the requirement skill in dissection might be formidable barriers to practical application.

According to the results obtained, both THC and PO activity could be used to verify the irradited insects in the laboratory. However, in order to make the haemocyte count more complete, differential count of each haemocyte type should be studied since each one may be differently affected by radiation as reported by many authors. And since there have been no reports concerning THC and PO activity of *H.armigera*, the value of the investigation may provide a useful source for further studies.

CONCLUSION

Total haemocyte count and phenoloxidase activity were found to being able to separate the irradiated from unirradiated larvare of *H.armigera* under laoratory conditions. The results of both THC and PO activity supported each other, that was, at each dose they were significantly different to those of the control and both reduced from the 4^{th} to 1^{st} instars .

LITERATURE CITED

El-Mandarawy, M.B.R., N. Zohdy, A.A.El-Moursy and A.M. Abdel-Rahman. 2000. Effect of delfin and baythroids on the total haemocyte counts (THCs) and haemocyte percentage in healthy and parasitized larvae of the cotton leafworm. **Egyt.J.Agri.Res.** 7(4) : 1569-1585.

Elbadry, E. 1964. The effect of gamma irradiation on the haemocyte counts of larvae of the potato tuberworm, *Gnorimoschema operculella (Zeller)*. **J. Insect Pathol**. 6: 327–330.

Eppensteiner, J. M. and R. D. Karp. 1989. The effect of gamma irradiation on the response to primary xenograft in the american cockroach, *Periplanata amercana*. **J. Insect Physiol**. 35 (2): 81-86.

Grégioire, C. 1974. Haemolymph coagulation, pp. 309-360. *In* M. Rockstein (ed.). **The Physiology of Insecta**, Vol. 5, 2^{nd} ed. Academic Press, New York.

Gupta, A. P. 1979. **Insect Haemocytes: Development, Forms, Functions and Techniques**. Cambridge University Press. 614 p.

Hoffmann, J. A. 1972. Modification of the haemograme of larval and adult *Locusta migratoria* after selective X – irraiations of the haemocytopoietic tissue. **J. Insect Physiol**. 18: 1639-1652.

Jones, J. C. 1962. Current concepts concerning insect hemocytes. **Amer. Zool**. 2: 209-246.

Lupa, D and S. Ignatowicz. 1999. Changes of phenoloxidase activity in store product pests treated with ionization radiation. **Progress in Plant Protection** 39(2): 458-462.

Mabbett, T. H. 1983. The economic insect pest of cotton in Thailand. IV. The american bollworm *Heliothis armigera* (Hubner). **Thai J. Agri. Sci**. 16 (3): 233-252.

Mansour, M. and G. Franz. 1996. Effect of gamma radiation on phenoloxidase activity in Mediterranean fruit fly (Diptera: Tephritidae) larvae. **J. Econ Entomol**. 89(3): 695-699.

Marmaras, V. J., N. D. CHaralambidis and C. G. Zervas. 1996. Immune response in insects: the role of phenoloxidase in defense reaction in relation to melanization and sclerotization. **Arch. Insect Biochem. Physiol**. 31: 119-133.

Molins, R. A. 2001. **Food Irraidiation: Principles and Applications**. John Wiley and Sons, Inc., New York. 469 p.

Nation, J. L., B. J. Smittle and K. Milne. 1995a. Radiation-induced changes in melanization and phenoloxidase in Caribbean fruit fly larvae (Diptera: Tephritidae) as the basis for a simple test irradiation. **Ann. Entomol. Soc. Am**. 88 (2): 201-205.

Nation, J. L., B. J. Smittle, K. Milne and T. M. Dykstra. 1995b. Influence of irradiation on development of Carribbean fruit fly (Diptera :Tephritidae) larvae. **Ann. Entomol. Soc. Am**. 88(3): 343-352.

Rahman, R., C. Rigney and E. Busch-Petersen. 1990. Irradiation as a quarantine treatment against *Ceratitis capitata* (Diptera: Tephridae): anatomical and cytogenetic changes in mature larvae after gamma irradiation. **J. Econ. Entomol**. 83(4): 1449-1454.

Tu, Z.L., Y. Kobayashi, K. Kigushi and K. Yamamoto. 2002. Effects of heavy-ion

radiosurgery on the hemopoietic function of the silk worm *Bombix mori.* **J.Radiat. Res (Tokyo)** 43(3):269-75.

Tubiana, M., J. Dutreix and A. Wambersie. 1990. **Introduction to Radiationbiology**. Taylor and Francis, London. New York. 371 p.

Younes, M.W.F., M.G. Abou ElEla and M.E.El-Mhasen. 1999. Effect of certain insecticides on the haemocytes of the cotton leafworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). **J. Arab. Biol**. 11(A): 435-457.