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Impact of Chlorophyllin Dye on some Enzyme Systems of the Cotton Leafworm, *Spodoptera littoralis* (Boisd.)

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ABSTRACT

The second larval instar of the cotton leafworm, *Spodoptera littoralis* (Boisd.) as a series polyphagous pest was treated, in laboratory, by ingestion of two chlorophyll derivatives namly copper chlorophyllin (Chl-Cu) and magnesium chlorophyllin (Chl-Mg) to study the possible role of mixed function oxidases (MFO) in detoxifying the dye, and determine the efficancy of treatment on protein metabolizing enzymes or transaminases. Moreover, to clarify whether the photoactive dyes have non phototoxic effect. The larvae were treated for 24 hrs with LC_{50} of the dye followed by exposure to direct sunlight for 2 hr. Copper chlorophyllin was more potent than magnesium chlorophyllin and represented by LC_{50} = 0.130 M and LC_{50} = 0.299 M, respectively. The percentage mortility of larvae treated by LC_{50} of the dyes and kept in dark during the experimental time was up to 9%. The dark process appear to be less active than the light induced photo dynamic reactions. Biochemical analyses revealed that treatment led to induction of MFO system. It was 733, 926 and 687 µ mole substrate oxidized /min/g. b. wt for Chl-Mg, Chl-Cu and control, respectively. Effect of treatment on transaminases showed that GPT activity was significantly changed by both chlorophyll derivatives, while GOT activity was non significantly changed. Induced GPT level could be considered as a sing or secondary effect of chlorophyllin poisoning. This effect has been interprreted as suggestive of interinsic capacity of insect to repair damage. MFO might share in detoxifying chlorophyllins, and development of resistance to photodynamic pesticides. The observed dark effect suggests that the used photosynthetizers posses low interinsic chemical toxicity.

Keywords: *Spodoptera littoralis*, photoactive dye, Chlorophyllin, Detoxification, Mixed function oxidases (MFO) , transaminases, dark.

INTRODUCTION

Chlorophyllin refers to any one of a group of closely related water-soluble salts that are semi synthetic derivatives of chlorophyll. The structure of chlorophyll consists of nitrogen, hydrocarbons, carbon & oxygen around the magnesium atom. Sodium copper chlorophyllin was prepared through alkaline hydrolysis where the magnesium atom is replaced by sodium and copper atoms. Chlorophyll is capable of processing the sunlight into energy, therefore it is used as source of energy in various forms for different purposes including pest control (Castro *et al.*, 2009).

Photosenitizion employs the photochemical interaction betwean non-toxic photosenitiziers,such as xanthene derivatives and chlorophyllin, and light. All thes dyes require the prescence of molecular oxygen to express their phototoxic action, hence the overall photoinsecticidal process appears to be of a photodynamic type (Jori 1996). This interaction generates reactive oxygen species (ROS) that can cause structural disintigration and inhibition of functional activity of subcellular structures that leading to cell death (Al-Asmari *et al.*, 2017).

The mode of action of photodynamic type insecticidal agents including photodamaged midgut wall and photoinactivation of enzymes such as AchE (Ben Amor and Jori, 2000). Photosenitized insects show reduction in their body weight, water content and protein mass,suggesting the occurrence of lethal energy stress in the insects (Broome *et al.*, 1976). Main metabolites were reduced after treatment of 2nd larval instar of the cotton leafworm, *Spodoptera littoralis* by Chlorophyllin dyes (Abd El-Naby, 2017). However reports on the effect of Photosenitization on enzymes related to main energy storage molecules such as transaminases is few. *Ceratitis capitata* larvae treated with Phloxine B dye showed less active locomotion due to inhibition of glycogen phosphorylase and amylase activity, thus decreasing catabolism of glycogen (Berni *et al.*, 2009).

Insect adaptation or defense against chemical dyes is not clear. Xenobiotics, including insecticides, undergo extensive metabolic transformation in living organisms. The microsomal mixed function system is primary system for phase one recations because they can attack the lipophlic compound directly. Essentially all insecticides interact with it as substrate, inhibitor and inducer (Kulkarmi and Hodgson, 1980).

The cotton leafworm, *S. littoralis* (Boisd.) as a series polyphagous pest was treated in laboratory, by ingestion of two chlorophyll derivatives namely copper chlorophyllin (Chl-Cu) and magnesium chlorophyllin (Chl-Mg) to study the following:

1. The possible role of mixed function oxidases (MFO) in detoxifying the dye
2. Determine the efficiency of treatment on inhibition of protein metabolizing enzymes or transaminases
3. Detect whether the photoactive dyes have non phototoxic effect.

The 2nd larval instar was chosen for treatment by LC_{50} of the dyes.

MATERIALS AND METHODS

1. Chemicals

Copper chlorophyllin and magnesium chlorophyllin from Sigma (Sigma-Aldrich chemical company).

p-nitroanisole was purchased from Stanbio laboratory (Texas, USA).Glucose-6- phosphate and glucose-6-phosphate dehydrogenase were from Sigma (Sigma chemical company). Transaminases substrates; L- aspartate and D,L alanine were from BDH chemicals Ltd.(Pool, England). Enzyme units (U) as standard was obtained from Ubichem Ltd. (Hampshire, England). The international unit defined as the ammount of enzyme which, under defined assay condition will catalyze the canversion of 1µ mole of substrates per minute. Other chemicals such as HCl and α-Ketoglutaric acid was purchased from native companies.

2 Apparatus:

Eldonet Dosimeter : The values of fluence rates of natural sun or solar simulator were measured on Three Channels Eldonet Dosimeter (Germany). This instrument has an ability to measure the irradiance of UV-B (280-315 nm), UV-A (315-400 nm) and Visible light (400-700 nm). The analog data are converted (12 Bit A/D conversion) and processed by the digital output (RS232) for automatic computer measurements, compatible with any PC computer with serial port.

Larvae were homogenized in a chilled glass Teflon tissue grinder (ST-Z Mechanic - Preczjna, Poland). Centrifugation was done in a refrigerated Centrifuge at 4°C (Beckman GS-6R Centrifuge). Double beam UV/visible spectrophotometer (spectronic 1201, Milton Roy co., USA)

was used to measure the absorbance of enzyme reaction products.

3. Bioassay

Serial concentrations (ranged from 1 to10^{-4} M) of Chl-Cu and Chl-Mg in distilled water was prepared for treating the 2nd larval instar of the cotton leafworm, *S. littoralis* (Boisd.). Castor–bean leaves were dipped for 30 sec in each concentration and then left to dry in room temperature before being offered to fed by newly moulted larvae for 24 hrs. Control larvae fed on leaves previously dipped in water. After treatment, the larvae were transferred onto untreated leaves and exposed to direct sunlight for 2 hr.The average of light intensities during exposure time was 450 w/m^2.

In dark experiment, which done to show the non toxic effects of the dyes, the larvae were treated by LC_{50} of the dye and kept in dark during the experimental time. The experiment was replicated three times each one had 20 larvae.

Preparation of homogenate sample for analysis

Whole larval body was homogenized in phosphate buffer (0.1 M, pH7). Each 100 mg larvae were crushed in 1 ml buffer. Homogenates were centrifuged at 8000 r.p.m. for 15 min at 4°C. After centrifugation, the deposits were discarded, and the supernatant was stored at –20 °C in freezer for about one week till use for analysis.

Biochemical analyses

Larvae that were treated with LC_{50} of Chlorophyllin, were subjected to enzyme activity assays

Determination of Mixed function oxidase activity

Mixed function oxidase activity were estimated according to the method described by Hansen and Hodgson (1971). P-nitroanisole o-demthylation was assayed to determine the mixed function oxidase activity with slight modification. The standard incubation mixture contained 1 ml solution phosphate buffer (0.1M, ph7.6), 1.5 ml enzyme solution, 0.2ml NADPH (final concentration1mM), 0.2ml glucose-6- phosphate (G-6.P final concentration1mM) and 50 ug glucose-6- phosphate dehydrogenase (G-6.PD). Reaction was initiated by the addition of p-nitroanisole in 10 μl of aceton to give a final concentration of 0.8 mM and incubated for 30 min at 37°C. incubation period was terminated by addition of 1ml HCl (1N).). p-nitroanisole was extracted with $CHCL_3$ and 0.5 N NaOH and Absorbance of NaOH solution was measured at 405 nm. Anextinction coefficient of 14.28mM^{-1} was used calculate 4- nitrophenol concentration.

Determination of Transaminases activities

Glutamic pyruvic transaminase (GPT) and Glutamic oxaloacetic transaminase (GOT) were estimated according to Reitman and Frankle (1957). The reaction mixture consisted of 100 µl sample, and1 ml. of mixture of phosphate buffer (PH7.2), 0.2 mM α-Ketoglutaric acid and 200 mM D, L alanine or L- aspartate. The mixture was incubated for 30 min at 37°C, then 1 ml of 2,4 dinitro phenylhdrazine was added. After 30 min,10 ml of 0.4N NaOH were mixed with the solution to form the brown color which measured at 520 nm. The enzyme activity was compared with enzyme standard.

4.Statistics

Toxicological data including LC_{50} of each dye were detected by the probit procedure (Finney 1971) using Ldp line program (http://www.ehabsoft.com/ldpline/). Mortality counts were corrected by Abbott's formula (1925). All enzyme determinations were made in triplicates. The data presented as the mean ±SE, and analyzed by one way analysis of variance (ANOVA) completely randomized. Means were compared using Duncan's Multiple Range test. A probability value of ($P<0.01$) was considered to denote the statistics significant difference. All statistical analysis were done using the software package Costat program.

RESULTS AND DISCUSSION

The newly moulted 2nd larval instar of *S. Littoralis* was treated for 24hrs with serial concentrations of Chl-Cu or Chl-Mg, follwed by exposing of larvae to direct direct sunlight for 2h. The used concentrations ranged from 10^{-4} to 1M. Molecular weight of Chl-Mg is 684.91 while that of Chl-Cu is 724.15.Toxicological data in Table (1) and Figure (1) indicated that copper chlorophyllin was more potent than magnesium chlorophyllin. LC_{50} was 0.130 and 0.229 M, respectively. The used concentrations caused percentage mortality ranged from35-52.3% after treatment with Chl-Mg, and 28.3-57.2% in Chl-Cu treatment. In both treatments, slope was relatively low. It was 0.108 and 0.206 for Chl-Mg and Chl-Cu treatment, respectively.

Table 1. Toxicological values after treatment of the 2nd larval instar of *S. Littoralis* for 24 hrs with serial concentrations of two photoactive dyes.

Dyes	Concentration (M,w/v)	Mortality percentage	LC_{50} (M,w/v)	Slope	X^2
Chl-Mg	10^{-4}	35	0.299	0.108	0.0135
	10^{-3}	40			
	10^{-2}	43.3			
	1	52.3			
Chl-Cu	10^{-4}	28.3	0.130	0.206	0.938
	10^{-3}	28.3			
	10^{-2}	43.4			
	1	57.2			
Control	0.0	1.5	-	-	-

- **α = 0.05 - Data were corrected using Abbott's formula (1925)**
- **The result after exposure to direct sunlight for 2hr.**

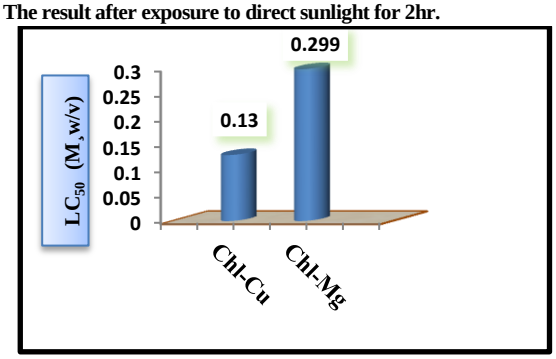

Figure 1. Comparison between the toxicity of magnesium chlorophyllin and copper chlorophyllin against the 2nd instar larvae of *S. Littoralis*.

Photosynthetizers depend on light to exert their phototoxic effect. However, when the larvae were treated by LC_{50} of Chl-Mg and Chl-Cu and kept in dark during the experimental time, they casud chemical toxicity to the larvae (Fig. 2). Percentage mortality was 6 , 9 and 1.5% for Chl-Mg and Chl-Cu treatments, and control, respectively. The observed toxicity is relatively low and Chl-Cu was more toxic than Chl-Mg.

Biochemical analyes (table, 2) revealed that treatment of the second larval instar of *S. littoralis* by LC_{50} of Chl-Mg and Chl-Cu for 24h and activated by direct sunlight for 2h, led to induction of MFO system activity. It was 733, 926 and

687 µ mole substrated oxidized /min/g b.wt for Chl-Mg and Chl-Cu treatments, and control, respectively.Statistic at P <0.01 showed that Chl-Cu photoactive dye caused significant increse in MFO activity. The activity was increased more than control by 34.7 and 10.6 for Chl-Cu and Chl-Mg treatments, respectively.

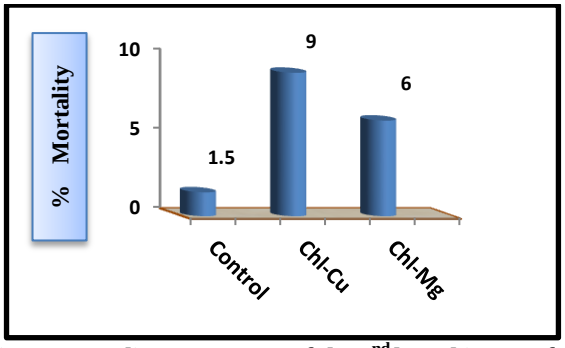

Fig. 2. Mortality percentage of the 2nd larval instar of *S. littoralis* treated by LC_{50} of Chl-Cu or Chl-Mg for 24 h and kept in dark conditions.

Table 2. Enzyme activities of the 2nd larval instar of *S. Littoralis* treated by LC_{50} of two photoactive dyes for 24 hrs and exposed to direct sunlight for 2hr.

Dye	GPT (Ux 10^3 / g.b.wt.)	GOT (U / g.b.wt.)	MFO µ mole substrated oxidized /min/g b.wt
Chl-Mg	2324±68 a	127±5.4 bc	733±22.5 b
Chl-Cu	2534±57 a	150±2.4 ab	926±22 a
Control	1854±57 b	141.7±4.7 b	687±36 bc

- U= Enzyme Unit - Date are presented as the mean ± SE
- means were analyzed by completly randamized ANOVA, and compared by Duncan's multiple range test at P <0.01. Means, whithin column, followed by the same letter are not significantly different.

Effect of treatment on transaminases showed that GPT activity was significantly elevated by the dyes treatments, while GOT was non significantly changed (table, 2). GOT activity was 127, 150 and 141.7 (U /g b.Wt) for Chl-Mg and Chl-Cu treatments, and control, respectively. On The other hand GPT activity was 2324, 2534 and 1854 Ux 10^3 /g b.Wt. for Chl-Mg and Chl-Cu treatments, and control, respectively.

Figure (3) illustrates percentage decrase or increase in different enzyme systems as compared to control. The figure shows that GOT activity was slightly changed as compared to control, while both dyes caused relatively high changes with respect to GPT activity as compared to control after treatment by Chl-Mg and Chl-Cu, respectively. MFO was highly changed as compared to control (34.7% increases) after treatment by Chl-Cu.

Chlorophylls are important dyes of light harvesting complex. The 2nd larval instar of *S. littoralis* (Biosd) was chosen, in present study, to evaluate dyes biochemical stress. In general earlier instars apper to be more photosuscptible than later instar form (Heitz,1987). On the other hand, two Chlorophylls dervatives were selected for the study to compare their effects. Chemical structure detect dye activity, since it can modulate the nature of the subcellular photodamged sites (Ben Amor and Jori, 2000).

Cytochrome P450-dependent monoxygenases, commonly called mixed function oxidases (MFOs). Cytochrome P-450 catalyzes a variety of oxidative reactions, involving both exogenous and endogenous chemicals, which are described as function oxidases or mono-oxygenations.These terms refer to the fact that in the reaction one atom of molecular oxygen is reduced to water while the other is corporated into the substrate (Hodgson1983). They are by for the most important enzyme system because they detoxify a wide range of foreign chemicals e.g.,drugs and pesticids (Ahmed, 1986).

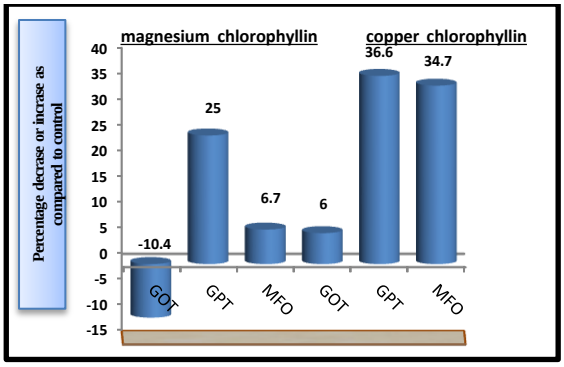

Fig. 3. Percentage of decrase or increase in different Enzyme systems as compared to control after treatment of *S. Littoralis* larvae by LC_{50} of the dyes.

The results showed that treatment of 2nd larval instar of the *S. littoralis* with LC_{50} of copper chlorophyllin dye led to induction of MFO activity. Insects enhanced detoxification could be via activation of some enzyme systems. Incrased cytochrome P450 levels have been reported for a number of resistant house fly strains possessing high oxidase level (Hodgson1983). MFO activity in field populations were between 1.8 and 4.6 times higher than those observed in the susceptible strain (Reyes *et. al.*, 2012). This might be due to field population are exposed to a variety of chemicals. MFO may act as a barrier enabling *S. littoralis* to reduce effect of chlorophyllin dye, consequently it may share in resistance to this type of chemicals. Resistance to toxicants is usually defined as an increase in the ability to survive the effects of toxicant. Oxidative metabolism is particalar relevance in this aspect. Like other chemicals pesticides there is a possibility to develop resistance to photosynsitizers. *Musca domestica* were able to establish resistance levels to erythrosin B (Mangan and Moreno,2001).

One group of enzyme related to main energy storage molecules is amino transferases which catalyzes protein metabolism. GPT activity in *S. littoralis* larvae was induced by both dye treatment, while GOT was not affected by the dye ingestion. There is evidence that photodynamic reaction mechanisms can involve biochemical components, especially, binding to macromolecules including nucleic acids and binding to vital enzyme components necessary to crucial metabolic pathways (Mangan and Moreno, 2001).

Induced GPT level could be considered as a sign or secondary effect of chlorophyllin poisoning. This effect has been interprreted as the suggestive of an interinsic capacity of the insect to repair damage. However, this elevated enzyme level, if continued, may case drastic metabolic disturbances with abnormal physiological consequences.

Induced activities of various metabolic enzymes after treatment by pesticides have been reported by several studies. Saleem and Shakoori (1987) found increased level of transaminases and phosphatases in the sixth instar larvae of *Tribolium castaneum* treated with cypermrthrin. Significatly raised enzyme activites presumblay indicate (i) higher

concentration of enzymes due to decresed body wight of treated larvae (ii) to defend against insecticidal stress conditions (iii) to increase the source of energy production through break down of phosphate bonds of energy rich nucleotides and amino acids.

In addition to their phototoxicity, Chl-Cu and Chl-Mg had low toxicity in dark conditions. Some photosenitizers also exert a toxic effect towards insects in the dark specially xanthene dyes, however, no similar effects were observed with furocoumarines, α terthiemyl and porphyrins (Ben Amor and Jori, 2000). The dark process appears to be less active than the light-induced reactions.

The dye activity in dark may affect metabolism in treated insects. Phloxine B ingested by *C. capitata* caused decrease in average of larval jumps. This was correlated with with a strong accumulation of glycogen, and inhabition of the glycogen phosphorylase activity. These results suggest that some of the non-phototoxic effects of Phloxine B might be caused by an alteration of the glycogen catabolism, which can eventually affect the viability of the flies (Berni *et. al.*, 2009). With respect to *S. Littoralis* larvae, the used dyes had non significant dark effects on the tested enzyme systems of the preseant study.

CONCLUSION

In conclusion, MFO might share in detoxifying chlorophyllin, and development of resistance to such pesticides. Transaminases could be considered as a sign or secondry effect of the photodynamic reaction. The observed dark effect of the dyes suggests that they posses low interinsic chemical toxicity.

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تأثير صبغة الكلوروفيلين على بعض النظم الإنزيمية لدوة ورق القطن

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معهد بحوث وقاية النباتات – مركز البحوث الزراعية – الدقى – الجيزة

تمت معالجة العمر اليرقي الثاني لدودة ورق القطن في المختبر ، عن طريق المعاملة باثنين من مشتقات الكلوروفيلين , كلوروفيلين النحاس وكلوروفيللين المغنيسيوم لدراسة الدور المحتمل لانزيم الأكسيديز المتعدد الوظائف (MFO) في إزالة السموم من الصبغة ، وتحديد فعالية العلاج على النمثيل الغذائى للبروتين أو الترانس امينيز بالاضافة إلى توضيح ما إذا كانت الصبغة الضوئية لها تأثيرات غير سامة. تم معاملة العمر اليرقي الثاني لدودة ورق القطن لمدة 24 ساعة ب LC_{50} من الصبغة تليها التعرض لأشعة الشمس المباشرة لمدة 2 ساعة. كان كلوروفيلين النحاس أكثر فعالية من كلوروفيلين المغنيسيوم ، LC_{50} = 0.299 M ، LC_{50} = 0.130 M على التوالي ، وكانت النسبة المئوية للموت من اليرقات التي تم معاملتها من قبل LC_{50}من الصبغة وتركت في الظلام خلال وقت التجربة يصل إلى 9 ٪. التجربة فى الظلام تكون أقل نشاطا مقابل التجربة فى الضوءمن حيث التفاعلات الديناميكية الناتجة عن الضوء ، وكشف التحليل الكيميائي الحيوي MFO أدى إلى 733 ، 926 و 687 (ميكرو مول مادة التفاعل المؤكسدة / دقيقة / جرام من وزن الحشرة) من الكلوروفيلين المغنيسيوم ، الكلوروفيلين والنحاس والكنترول ، على التوالي. وأظهر تأثير المعاملة لانزيم GPT زيادة محسوسة فى النشاط بمشتقات الكلوروفيلين. بينما لم يكن هناك نشاط محسوس لانزيم GOT مختلف اختلافا معنويا عن الكنترول ، لذلك يمكن اعتبار التأثير على مستوى نشاط GPT بمثابة تأثير ثانوي للتسمم بالكلوروفيلين. وقد تم تفسير هذا التأثير على أنه يوحي بقدرة الحشرات لإصلاح التلف الداخلى. قد تشارك MFO في إزالة السموم من الكلوروفيلين ، وتطوير المقاومة للمبيدات الضوئية. ام التأثير للمعاملة فى الظلام اشار إلى أن الديناميكية الضوئية المستخدمة تمتلك سمية كيميائية منخفضة.