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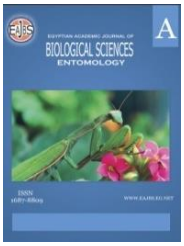

Changes in Antioxidant Enzymes During the Development of the Cotton Leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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ABSTRACT

Herbivorous insect pests are continuously suffering reactive oxygen species (ROS) from either endogenous or exogenous challenges and they possess an antioxidant system responsible for protecting insect tissues during its development. The present work aims to offer a comprehensive view of antioxidant activity of the destructive pest, the cotton leaf worm, *Spodoptera littoralis* (Boisd.). The level of four antioxidant enzymes namely, Catalase (CAT), Phenoloxidase (PO), Peroxidase (POX) and Glutathione-S-transferase (GST) have been assessed throughout the insect developmental stages. Generally, the late larval stages hold most of the antioxidant activity in all tested enzymes while the adult showed the least ones. Catalase was completely absent during egg stage while both PO and POX showed a similar trend of activity. The overall results showed a stage-specific antioxidant enzyme activities and we concluded that *S. littoralis* possess an efficient antioxidant enzyme system which able to withstand oxidative challenges and overcome oxidative stress threat. This study is an essential step in elucidating how antioxidant system develops with age in *S. littoralis* and eventually how can we make use of these information in future in insect control.

INTRODUCTION

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) is one of the most devastating polyphagous insect pests being widespread in most countries like Africa, several areas of Asia and Mediterranean Europe feeding upon a long list of economic crops (Smagghe and Degheele, 1997 and Azab *et al.*, 2001). It's a voracious pest responsible for destroying a broad range of ornamental, industrial and vegetable crops (Lanzoni *et al.*, 2012 and Azzouz *et al.*, 2014). Larvae damage the vital plant parts such as leaves and buds, and consequently causing retardation in crop growth and severe yield losses (Pineda et al., 2007 and Rawi *et al.*, 2011). Applying massive pesticides indiscriminately on the long run enhanced pest resistance leading to adverse hazards on environmental and non-target animals (Smagghe *et al.*, 1999 and Aydin and Gürkan 2006). Accordingly, the need to create a new promising novel alternative in pest control became a must.

On the other hand, insects in general and herbivorous species in particular are challenged permanently with reactive oxygen species (ROS) generated from either

endogenous or exogenous sources as aerobic metabolism, exposure to xenobiotic compounds, pathogenic infections or even adverse temperatures as well as being exposed to allelochemicals produced by host plant as a result of herbivory (Bi and Felton, 1995 and Krishnan and Kodrík, 2006). These ROS may cause an oxidative stress leading to oxidative damage to different important biomolecules such as proteins, nucleic acids and lipids especially those in cell membrane leading to cellular death unless they are trapped and eliminated (Orozco-Cardenas and Ryan 1999 and Cnubben *et al.*, 2001).

Insects are equipped with efficient antioxidant defense system to reduce harms caused by free radicals and have a regulatory balancing between ROS species and antioxidant defense mechanisms (Ahmad, 1992 and Molina-Cruz *et al.*, 2008) and this balance affect development and aging (Klichko *et al.*, 2004; Jena *el al.*, 2013 and Sahoo *et al.*, 2015). Moreover, ROS and antioxidants have been reported to influence larval development as well as many other life activities (Allen, 1991 and Orr and Sohal 1994). In order to reduce oxidative damage and keep normal homeostasis to cells, a well-synchronized antioxidant system of enzymes and non-enzymatic molecules have been evolved (Klichko *et al.*, 2004; Fahmy, 2012). These antioxidants are able to eliminate ROS before they oxidize vitally important biomolecules or at least minimize their deleterious impact (Steenvoorden and Henegouwen 1997; Howe and Schilmiller; 2002 and Blokhina *et al.*, 2003). Many enzymes are involved in this battle of defense. One of the front-line antioxidant enzymes is Catalase (CAT), which consumes hydrogen peroxide (H_2O_2) converting it into water and oxygen (Kono and Fridovich, 1982; Felton and Duffey, 1992 and Felton and Summers, 1995). Phenoloxidase (PO) which is also called tyrosinase is a copper-containing enzyme present nearly in all living organisms (Chase *et al.*, 2000). In insects, it is involved in melanization of foreign organisms, defensive encapsulation and wound healing (Ashida and Brey, 1995). It undergoes hydroxylation of monophenols producing diphenols then it oxidizes them into quinones (Nappi and Christensen, 2005). Enzymatic reactions of PO cascade and some of the non-enzymatic reactions would lead to formation of melanin drived from quinone ending with encapsulation (Nappi and Christensen, 2005 and Cerenius and Söderhäll, 2021).

Peroxidases catalyze oxidation-reduction reactions using H_2O_2 have many functions in insects as detoxification and defensive mechanism against pathogens (Shellby and Popham, 2006). It is a hematin-containing oxidase which catalyze oxidation of a broad variety reduced substrates such H_2O_2 and converting it into water and oxygen, thus protect cells from peroxidative damage (Das *et al.*, 2019 and Vengateswari *et al.*, 2020). On the other hand, Glutathione-S-transferase (GST) is a detoxifying enzyme which eliminate lipid peroxidation products in order to protect cells (Krishnan and Kodrík, 2006). It catalyzes the conjugation of reduced glutathione with a range of metabolites possessing electrophilic sites (Habig *et al.*, 1974) and has an important role in detoxification of many xenobiotic compounds eliminating H_2O_2 from cells (Ranson and Hemingway, 2005; Dubovskiy *et al.*, 2008 and Büyükgüzel *et al.*, 2010). Earlier studies reported that antioxidant enzymes are involved in combating the accumulation of ROS in insect tissues and they have a stage-specific oxidative activity during insect life span affecting insect development and any disturbance in this integrated antioxidant system may alter its function which ultimately deprive the pest its survival weapon and thus could be used as a key for insect control (Klichko *et al.*, 2004; Jena *et al.*, 2013 and Sahoo *et al.*, 2015).

Accordingly, the goal of present study is to follow changes of antioxidative defensive enzymes namely, CAT, PO, POX and GST in the developmental stages of *S. littoralis* as well as the changes of the previously mentioned enzymes within selected developmental stages (egg, 4th larval instar and pupal stage at different time intervals) as a trial to pave the road to make use of this study in insect control in the near future.

MATERIALS AND METHODS

Insects:

Different developmental stages of *S. littoralis* were obtained from the cotton leaf worm rearing laboratory, Plant protection research institute, Agricultural research center. They were maintained under crowded conditions at 28±2ºC and 16h light: 8h dark photoperiod.

Sample Preparation:

Samples for the present study included all developmental stages, namely, eggs, larval instars, prepupae, pupae and adult males of *S. littoralis* in addition to samples of egg stages at 12, 24 and 48 hrs. after being laid to follow up enzymes activity with egg stage. Similarly, 4th larval instar at 12, 24, 36 and 48 hrs after being molted. Pupae samples also were studied at 1, 4, 8 and 12 days post the onset of pupation.

The insect samples were homogenized in distilled water (50 mg/ml) in chilled glass Teflon tissue homogenizers (ST-2 Mechanic-preczyina, Poland) on ice jacket. The homogenates were centrifuged refrigerated 5415 (Hamburg, Germany) for 15 minutes at 5ºC in a refrigerated centrifuge at 8000 r.p.m. The supernatants were kept frozen at -20°C in a deep freezer till use.

Determination of Enzyme Activities:

Catalase activity was measured using Biodiagnostic Kit No. CA 25 17 which is based on the spectrophotometric method described by Aebi (1984). CAT enzyme activity was determined by measuring the rate of H_2O_2 consumption.

Phenoloxidase activity was determined according to Ishaaya (1971) with modifications made by Amin *et al.,* (2013). The reaction mixture consisted of 0.5 mL phosphate buffer (0.1 M), 200 µl of Catechol solutions as substrate concentration and reaction temperature was determined to detect the optimum conditions of the reactions.

Peoxidase activity was detected according to Fehrman, and Dimond (1967). Reaction mixture: 0.1 M phosphate buffer (pH 7.0), extract, distilled water, 0.2 M pyrogallol, and 3% H_2O. The whole was incubated at 30 °C for 25 min, and then a 25% trichloroacetic acid (TCA) solution was added.

Glutathione-S-transferase activity was determined according to the method of Habig et al., (1974). 0.4 ml potassium phosphate buffer (50 mmol/l; pH 6.5), 0.1 ml of supernatant, 1.2 ml water and 0.1 ml CDNB (1-chloro-2, 4 dinitrobenzene, 30 mmol/l) were added and incubated in a water bath at 37◦C for10 min. After incubation, 0.1 ml of reduced glutathione (30 mmol/l) was added.

Absorbance was measured at 430 nm (TECAN Infinite 200) against a blank test in which 0.1 M phosphate buffer (pH 7.0) was added instead of the extract. The enzyme activity was: µmol × min^{-1} × mg protein^{-1} using a double beam ulteraviolet/visible spectrophotometer (spectronic 1201, Milton Royco., USA) was used to measure absorbance of metallic compounds or coloured substances.

The needed substrates, catechol and bovine serum albumin were purchased from Sigma chemical company (ST.Louis). The other needed chemicals for buffers were purchased from local companies

Statistical Analysis:

Obtained results were pooled from Triplicate. Using coStat statistical software (Cohort software, Brekeley), Data were expressed as means ± standard error of the mean (S.E.M.) and analysed using completely randomized one-way ANOVA test. Means were separated using Duncans multiple range test ($P<0.01$).

RESULTS AND DISCUSSION

Activity of Antioxidant Enzymes Throughout Developmental Stages:

The present work followed the activity of four antioxidant enzymes namely, CAT, PO, POX and GST during developmental stages (egg, larva, prepupa, pupa and adult male) of the cotton leafworm, *S. littoralis*. Adult female was excluded in the present work since viteollogensis and fecundity as well as other related reproductive aspects are intensively incorporated with antioxidant system (Dejong *et al.,* 2007) and needed to be independently studied.

Catalase was completely absent in the egg stage and its activity started to be detected in the 1st larval instar (42.3 U/mg protein) followed by an abrupt highly significant increase (9 folds) in the 2nd instar as shown in Figure (1). It started to decline gradually by 38, 68% in the 3rd and 4th instars, respectively. The greatest activity was recorded during the prepupal stage being 461 U/mg protein followed by a decrease by 26% in pupa while in adult, the activity was relatively low (87.3 U/mg protein). Generally, CAT didn't show a specific or regular trend all over the present study.

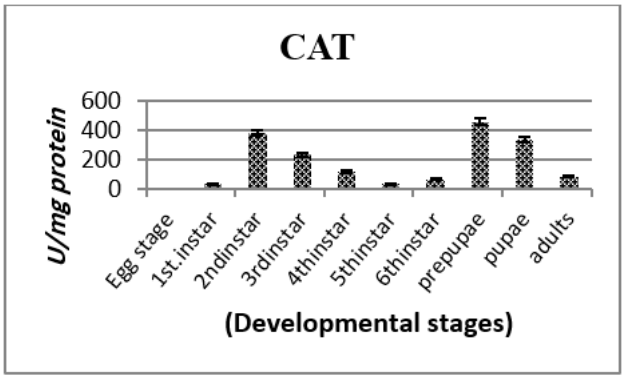

Fig. 1. Changes in Catalase (CAT) activity during the development of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

Phenoloxidase was traced during the developmental stages of *S. littoralis* showing a relatively low activity within egg stage (6.9 O.D. units/min/mg protein) as shown in Figure (2). It started to increase significantly by (69.5%) in the 1st larval instar. The activity of PO was nearly steady in the subsequent instars till the 3rd one. The highest activity of PO was recorded in the 6th instar being (46.1 O.D. units/min/mg protein) while the lowest one was detected in adult stage being (7.8 O.D. units/min/mg protein).

Peroxidase activity showed a similar trend to PO as shown in Figure (3) and they seem that both work parallel to each other. The activity of POX dramatically increased by 62.8% in the 1st larval instar compared to that in the egg stage. Peroxidase activity continued to increase significantly in the subsequent larval instars and recorded the highest activity in the 5th one being (135 ΔO.D. units/min/mg protein). Both egg and adult stage had the least activity of this enzyme being 16.9 and 16.5 ΔO.D. units/min/mg protein, respectively.

The activity of GST in general showed a gradual increase during early larval instars of *S. littoralis* as illustrated in Figure (4). The highest activity was recorded in the 5th and 6th instar being 83,7 and 87 m mol substrate conjugated/min/mg protein, respectively. The activity of GST then declined by 46.3% in prepupa and continued to decline till being nearly undetectable in the adult stage.

Generally, the antioxidant enzymes in the present study, had the lowest activity in both pupal and adult stage as shown in Figures (1, 2,3 and 4).

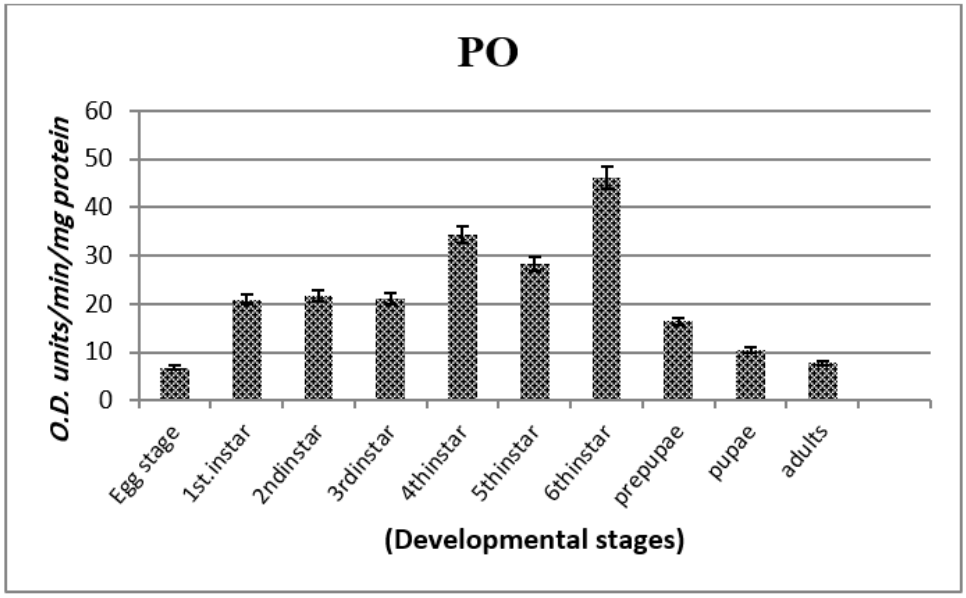

Fig. 2. Changes in Phenoloxiase (PO) activity during the development of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

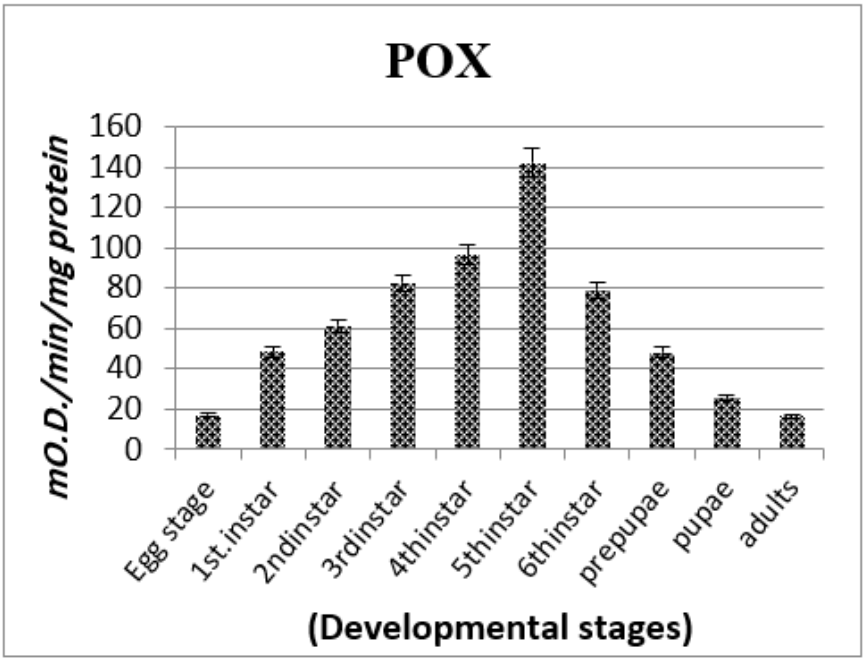

Fig. 3. Changes in Peroxidase (POX) activity during the development of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

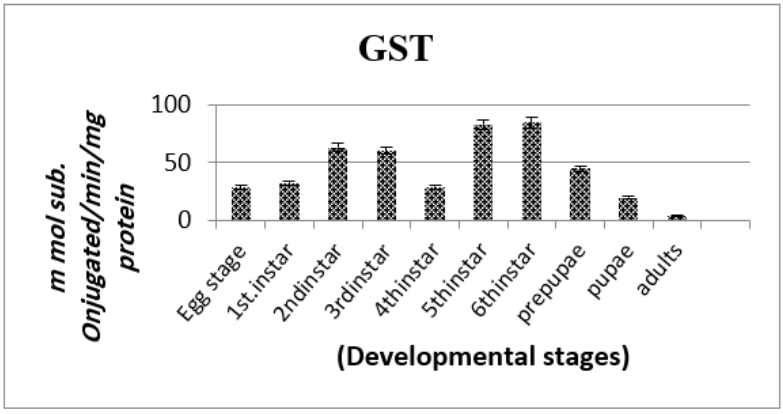

Fig. 4. Changes in glutathione-S-transferase (GST) activity during the development of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

Activity of Enzymes Within Selected Stages:

The present study determined the activity of the previously mentioned antioxidant enzymes within the chosen developmental stages namely, egg, 4th larval instar and pupal stage.

During the egg stage, variable trends recorded in the enzymes' activities. Catalase was completely undetectle in the egg stage while PO showed an ascending activity throughout the studied time intervals namely, 12, 24 and 48 hrs after eggs being laid. On the other hand, POX had a nearly stable activity during the entire egg stage. The activity of GST showed only a significant decrease after 48 hrs after eggs being laid as illustrated in Figure (5).

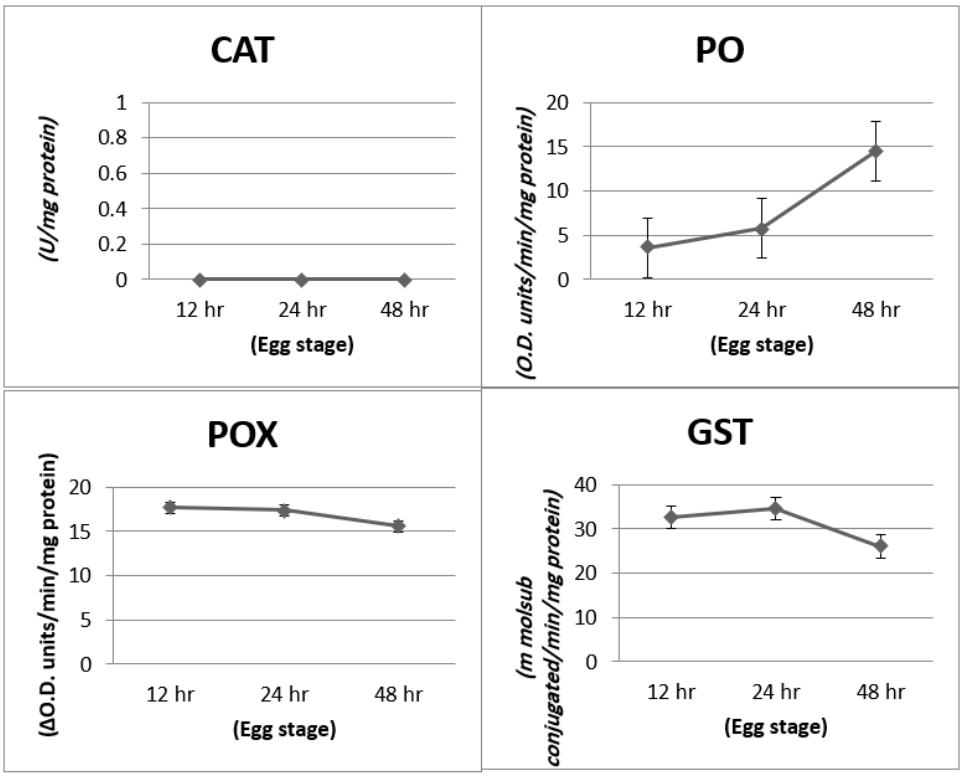

Fig.5. Changes in Catalase (CAT), Phenoloxidase (PO), Peroxidase (POX) and Glutathione-S-transeferase (GST) activity during egg stage of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

The activity of antioxidant enzymes in the 4th instar at 12, 24. 36 and 48 hrs post molting showed a general descending trend as shown in Figure (6) in both CAT and GST where a significant drop was recorded after 36 hrs reaching 100 U/mg protein and 23.2 m mol. sub. conjugated/min/mg protein), respectively. In contrast, an ascending activity was detected in PO and POX where the highest activity was recorded after 48 hrs post molting. Pupa of *S. littoralis* showed a tendency of a general decrease in activity of all the studied antioxidant enzymes as shown in Figure (7).

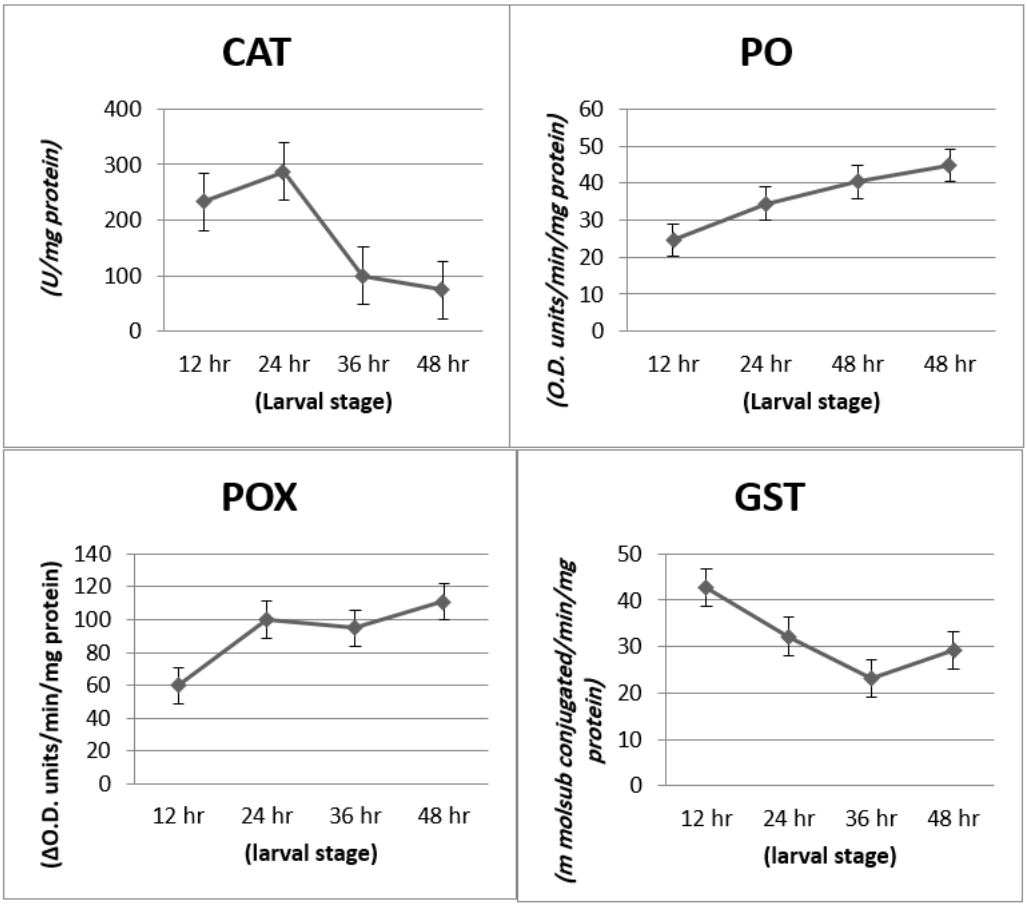

Fig.6. Changes in Catalase (CAT), Phenoloxidase (PO), Peroxidase (POX) and Glutathione-S-transeferase (GST) activity during 4th larval stage of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

During the insect development, different cellular events occurred to improve the level of antioxidant system (Sahoo *et al.,* 2015). Among antioxidant enzymes, catalase is the primary scavenger of H_2O_2 in the cells (Kono and Fridovich, 1982). In the present study, CAT activity couldn't be detected in the egg stage while other enzymes, PO, POX and GST recorded relatively low activity compared to later developmental stages. Hu *et al.,* (2013) reported that the insect embryo before hatching, receives a limited amount of oxygen via egg membrane without direct exposure to ambient oxygen leading to a relatively low production of ROS while after hatching, larvae started to consume oxygen directly from the surrounding atmosphere and start to feed which eventually causes an increase in ROS generation rising the need of high activity of CAT. Similarly, Salama (2020) observed that the 1st nymphal instar of desert locust, *Schistocerca gregaria* consumes higher amount of oxygen than the pharate larvae in the egg shell. In general, activity of CAT started to be detected in larval instars in some insects and the highest

metabolic rate was recorded compared to later stages because metabolic efficiency declines as the insect ages (Rath *et al.,* 2006). As previously mentioed, the activity of CAT didn't display a clear trend as other tested enzymes in the present study. Our results go parallel with Sukhotin *et al.,* (2002) who concluded that CAT didn't show a clear age-related change during the development of *Mytillus edulis.*

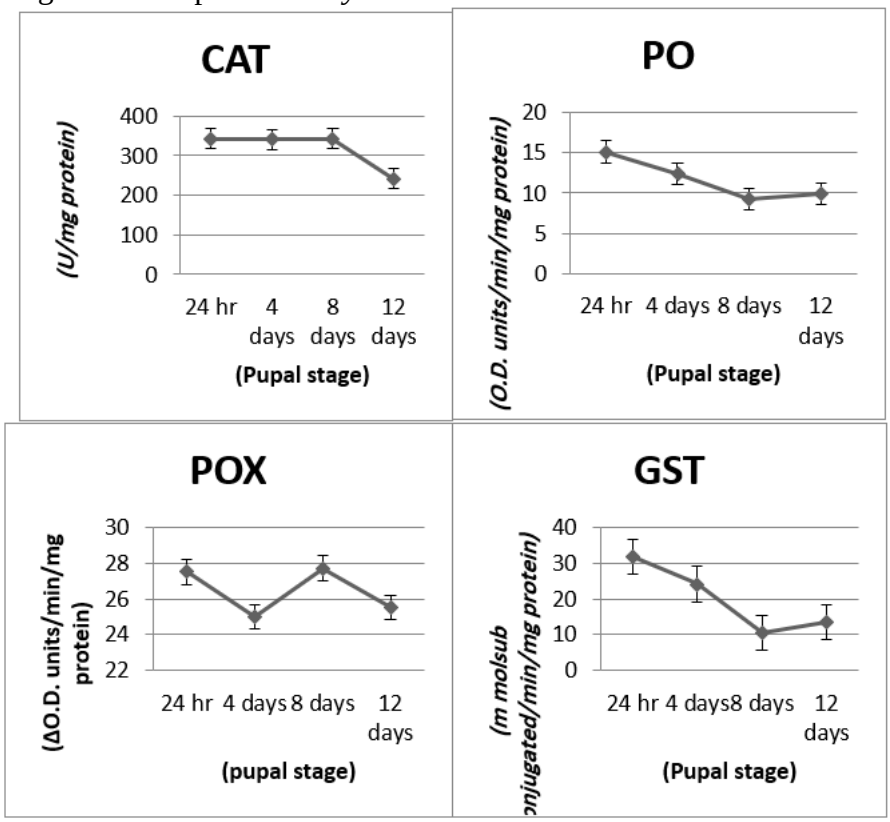

Fig.7. Changes in Catalase (CAT), Phenoloxidase (PO), Peroxidase (POX) and Glutathione-S-transeferase (GST) activity during pupal stage of the cotton leafworm, *S. littoralis*.
Data are presented as the mean±SE (n=3) and ($P < 0.01$) using one-way ANOVA.

The other studied enzymes in the present work namely, PO, POX and GST showed a significant increase in late larval instars specially 5th and 6th ones compared to earlier ones. Similarly, an enhanced activity of antioxidant enzymes was observed in the late larval stages of *Osmia bicornis* (Dmochowska-Slezak *et al.,* 2015) and *Depressaria pastinacella* (Lee and Berenbaum 1990).

Glutathione-S-transferase showed an increased activity in a number of herbivorous insects as they develop (Krishnan and Kodrik 2006). Xu *et al.,* (2015) reported also that Glutathione content was induced during larval development and simultaneously enhance GST activity, particularly in late instars. In general, the enhanced activity of GST as well as all CAT, PO and POX as larval age proceeds recorded in the present study may be due the intensive consumption of plant diet during late instars and ultimately accumulation of ROS (Zhao and Shi, 2009). The larvae of *Antheraea mylitta* which ingested more than 90% of the total food intake during the last two instars particularly in the 5th one and this eventually stimulated antioxidant system to scavenge extensively resulted ROS providing a potential protection against oxidative stress (Rath *et al.,* 2006). Generally, the relative increase in antioxidant enzyme activity causes reduction of hydroperoxides (Lukasik and Golawska 2007 and Gerardo *et al.,* 2010).

Moreover, polyphagous lepidopteran larvae consume a wide range of plants with variable allelochemicals and secondary metabolites including coumarins, alkaloids, flavonoids and phenols while insect feeds which disrupt the metabolic pathways of herbivorous insects (Alon *et al.,* 2012). Krishnan and Kodrik (2006) reported that the elevation of antioxidant enzymes of *S. littoralis* caterpillars was due to chlorogenic acid and tannin present in the host plant leaves which may substantially contribute in production of oxidative radicals. Similarly, antioxidant enzymes observed in southern armyworm *S. eridania,* were profoundly elevated from 3rd to 5th larval instar and this elevation strongly suggests that larvae use them as a defense against toxicity of plant prooxidant allelochemicals (pritsos *et al.,* 1988) and by the end of the feeding stage, accumulation of oxidative burden will be linked with up-regulation by antioxidant enzymes (Krishnan and Kodrik, 2006).

On the other hand, the present study showed *S. littoralis* recorded a relative increase in the activity of CAT, PO, POX and GST in Prepupa and pupa compared to adult male which had the least activity in all tested enzymes. The relative increase in antioxidant enzymes in pupae may be attributed to the protection of the cells against H_2O_2 produced by adult organogenesis and higher growth rate occurred during pupation which subsequently accompanied by higher oxygen consumption and ultimately increased ROS production (Rath *et al.,* 2006). Similarly, elevation of CAT was recorded in pupae of the European corn borer, *Ostrinia nubilalis* and in *A. mylitta* which coincide with our findings (Jovanovic-Galovic *et al.,* 2004 and Jena *et al.,* 2013).

In fact, accumulation food reservoir during immature stages for adult life is necessary accompanied by higher metabolic rate and accordingly higher need for antioxidant protection in contrast to adult stage where feeding may cease or food became scarce or even switched to simple sugars only and food intake activities and related metabolism were completed during the immature stages (Ahmad and Pardini, 1990).

The overall findings in the present study strongly suggest that the cotton leafworm, *S. littoralis* had an efficient antioxidant enzyme activity during insect development according to the need in each stage. larval stage is more susceptible to oxidative threat as it is the stage of initiation of feeding facing different challenges such as ambient oxygen and plant allelochemicals as well as successive molting which may be the reasons for higher levels of oxidative burden (Zhao and Shi, 2009) and consequently responsible for the enhanced activity of antioxidant enzymes.

Accordingly, our study provides an opportunity to understand the role played by antioxidant defense responses in *S. littoralis* during its development which may ultimately could be incorporated in its control via disturbing such protective system rather than depending upon the traditional methods of control. Further studies are needed for the entire antioxidant system in detail for each developmental stage independently.

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