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Physiological responses of Greater wax moth (Galleria mellonella) to inoculation with some strains of endophytic fungi

Ahmed A. Ismaiel[1], Gamal El-Didamony[1], Ali El-Sheikh[2], Rehab El-Gendy[2], Doaa Fekry[2]

[1]Botany department, Faculty of science, Zagazig university, Zagazig, Egypt.
[2] Plant protection research institute, Agriculture Research Centre, Cairo, Egypt.
Corresponding author: Doaa El-sayed Fekry

***ABSTRACT*: This study was carried out to investigate the effect of endophytic fungi on physiological responses of Galleria mellonella (G. mellonella). The analyses of some parameters have been recorded as influenced by the filtrate of A. nidulans, A. flavus and A. niger. For, A. niger, the total larval toxicity index reached 99.4 % for the least used median lethal concentration of LC50 (11.96).**

After 3 days, A. flavus AUMC 13942, A. niger AUMC 13944 strain produced the highest GOT activity of up to 1.80 and 1.14 µg pyruvate\ g. b. wt\ min, respectively. The increase in incubation period caused a notable decrease in GOT activity of G. mellonella larvae with the treated fungal strains. A. flavus AUMC 13942 causes a significant decrease (P<0.05) in GOT enzyme activity then causes disturbance in its values to the end of experiment. A. nidulans, A. niger and A. flavus caused the highest increase in the enzyme activity after 3 days and their values were 42.27, 21.91 and 8.81 µg/ g.b.wt respectively, then decreased again until the end of experiment. It can be seen that, the highest increase was recorded after treatment of LC50 of A. nidulans after 3 days, while the lowest value of the enzyme recorded 0.11 after treatment with LC50 of A. niger after 9 days of treatment. Sub lethal concentrations of A. nidulans AUMC 13941 produced the highest increase in ACP enzyme activity at the 3rd day with value of (98.16 µg/ g). A. niger AUMC 13944 causes gradual increase of ALP activities and the values were 87.99, 64.19, 53.56 and 69.55 µg/ g. respectively after the same time interval.

KEYWORDS: G. mellonella, transaminase enzymes, phosphatase enzymes

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I. INTRODUCTION

The response to fungus or other microorganisms is frequently assessed using an artificial infection pathway. The goal of the oral force-feeding approach is to replicate how commensals naturally colonise hosts' intestines **(Lange *et al.*, 2018)**. Ingested xenobiotics may enter an insect's body through the midgut, a crucial organ. The detoxification, endocrinology, reproduction, and nutrient needs of insects are all greatly influenced by the fat body. Therefore, any disruption in these two organs may result in significant, non-lethal modifications to the insect's physiology and behaviour **(Adamski *et al.*, 2005).**

The retention of a lipophilic substance in the fat layer is caused by the high lipid content of *G. mellonella*. The poor distribution of increasing lipophilic ionic liquids causes the lipophilicity to grow as the length of the alkyl chain increases. Due of the stress of infection, this causes the injection of ionic liquids to concentrate in certain areas to encourage melanization, which results in the larvae transforming from cream to dark brown or black **(Kavangh and Fallon, 2010)**. In addition to gallerimycin, *G. mellonella* has a number of naturally occurring antifungal peptides (galiomycin, cecropins, and moricins), as well as peptides that block fungal virulence factors **(Wiesner and Vilcinskas 2010 and Bergin *et al.*, 2006).**

All of Lepidoptera's digestive enzymes aside from those needed for early digestion are immobilised at the surface of the midgut cells **(Terra and Ferreira, 2012)**. In addition, the Lepidoptera's digestive tract is distinct due to its very alkaline pH **(Berenbaum, 1980; van Wielendaele *et al.*, 2013).** The multifunctional detoxifying enzymes known as glutathione-s-transferase are found in both invertebrates and vertebrates **(Vontas *et al.*, 2001)**.

Although glutathione's antioxidative function is its most significant characteristic, it also participates in the rebuilding of damaged cellular components, including the lipids and proteins of cell membranes. In addition, glutathione aids in the control of intracellular metabolism and takes role in cell development, differentiation, and death **(Hall, 1999 and Pastore *et al.*, 2003)**. A rise in GST activity has been seen in insects resistant to pesticides; Insects resistant to pesticides have been discovered to have increased GST activity **(Papa- dopoulos *et al.*, 2000)**. GST has a reputation for being a detoxifying and antioxidant enzyme that eliminates cellular lipid peroxidation byproducts or hydroperoxides **(Dubovskiy *et al.*, 2008)**.

Due to enhanced resistance to oxidative stress and an increase in both female and male lifespan, oxidative stress caused an increase in GST activity in Drosophila melanogaster **(Aslan *et al.*, 2019)**. The greatest GST activity indicated that the GST enzyme helped insects detoxify the antifungal drug (terbinafine) **(Kastamonuluoglu *et al.*, 2020)**. Esterases' outstanding function in the breakdown of toxins from various sources is attested to by the large range of substrates that they can metabolize. Specifically, the lipids and proteins of cell membranes, glutathione-s-transferase participates in the remodelling of damaged cell components. There is a physiological mechanism of ecdysone activation and deactivation via hydrolysis of ecdysone conjugates and esterification of free ecdysteroids as mentioned by **Rees (1995).**

Tyrosine, the building block of dopamine and octapamine, and its vital synthesising enzyme alkaline phosphatase are known to have a role in controlling the levels of juvenile hormone, 20-hydroxyecdysone, and insect developmental hormones **(Wright, 1987 and Rauschenbach *et al.*, 2007 a, b)**. The enzyme also participates in the transphosphorylation process **(Sakharov *et al.*, 1989)**. Any modification to the way an insect's stomach works will have an impact on its physiology. This enzyme is found in the muscles, nerve fibres, midguts, and malpighian tubules of lepidopteran insects **(Horie, 1958).** The activity peaked before the fifth instar's full appetite gluttonous stage and peaked to its lowest point during the mature larval stage **(Miao, 2002; Senthil- Nathan *et al.*, 2005)**. An important factor in insects developing pesticide resistance is alkaline phosphatase **(Srinivas *et al.*, 2004)**.

When ecdysteroid levels were at their greatest, acid phosphatase activity was discovered to be at its peak on the midgut of wax moths **(Lambremont, 1960)**, which is particularly true when transformation takes place **(Lockshin and Williams, 1965)**. Conjugation of ecdysone with phosphate and fatty acids (esterification on C-3 and C-22) is most frequent among other metabolic reactions **(Connat and Diehl, 1986, Lafont and Connat, 1989; Grau and Lafont, 1994).**

II. MATERIALS AND METHODS

1-Rearing technique for the insect:

On medium made by **Ibrahim *et al.* (1984)**, *Galleria mellonella* larvae were raised. This medium contains 22% polenta (corn groats), 22% full-com (wheat flour), or 22% brushed-grain (wheat) wheat. 11.5% yeast powder (brewer's yeast, beer yeast), 11.5% honey, 11.5% glycerol, and 11.1% skim milk powder. 7.5% bee wax. The larvae were originally obtained from bee hives and transferred to transparent plastic rearing jars (17× 17× 27 cm), containing 250 g from the previous prepared media, closed with a lid of muslin for aeration and incubated at 28± 2°C with a photoperiod (L:D) 8:16 and relative humidity 65± 5% in the insect rearing chamber. When larvae grown to the pupal stages and then to the adult moths, a piece (15 × 15cm) of paper tissue was folded and placed in the container to promote egg laying.

Researchers examined the endophytic fungus's poisonousness to *G. mellonella* larvae. 30 grams of fictitious food were placed in each sterile petri dish, which was then coated with 1 ml of the experimental treatment and left to dry. Each treatment was triplicated 3 times. The larvae of *G. mellonella* were transferred to the surface of the treated diet in petri dishes using sterilized fine brush. The petri dishes were incubated at 28±2 °C and 65% R.H. Another group of petri dishes was prepared containing the same diet but treated with water only used as control and left to dry and an equal number of the maintained larvae were placed on their surface.

1. Physiological effects of three fungal species on G. *mellonella* larvae under laboratory conditions:

1.1. Preparation of samples for biochemical assays:

Larval samples of *G. mellonella* used for conducted biochemical assays were collected at 3-, 5-, 7- and 9-days post treatment with 3 fungal concentrations, the treated larvae were weighted and homogenized in distilled water using a Teflon homogenizer. Centrifuging the homogenates for 30 minutes at 10°C and 5000 rpm. After the precipitate was removed, After the precipitate was eliminated, the supernatants were gathered and kept in a deep freezer until they were utilised to gauge the activity of two digestive enzymes (AST and ALT), alkaline phosphatase enzyme (ALP), acid phosphatase enzyme (ACP), α- and β- esterase enzymes.

2 .Transaminase enzymes (GOT& GPT):

Transaminases aspartate aminotransferase (AST)(GOT) and alanine aminotransferase (ALT)(GPT) enzyme activities were determined calorimetrically according to the method of **(Reitmen and Frankel 1957)**. GOT transfer the amino group from L-aspartate to α-Keto glutaric acid) producing a new amino acid L-glutamate) and a new keto acid (oxaloacetic acid), GPT transfer the amino group from D, L alanine to

α-keto acid (α-keto glutaric acid), resulting in a new amino acid (L-glutamate) and a new keto acid (pyruvic acid). Oxaloacetate or pyruvate reacts with 2, 4-dinitropheyl hydrazine forming oxaloacetate or pyruvate hydrazine which in alkaline medium form a brown color that can measured spectrophotometrically. The reaction mixture was consisted of 1 ml of a mixture of phosphate buffer (pH 7.4) 0.2 mM α-keto glutaric and 200 mM D-L- alanine or L-aspartate, 0.2 ml of larval homogenate was then added to the reaction mixture. The mixture was incubated for 30 min. then after; 10 ml of 0.4 N NaOH was added. The optical density of the produced brown colors is measured after 5 min using spectrophotometer at 520 nm. The enzyme activity is expressed as mM Pyruvate/ gm body weight/min.

3.α &β esterase enzymes:

α-estrases and β-estrases, as non-specific estrases, were determined colorimetrically according to the method described by **(Van Asperen, 1962)** using α-naphthyl acetate and β-naphthyl acetate as substrate, respectively. Naphthol produced as a result of hydrolysis of substrate can be identified by the addition of diazoblue sodium lauryl sulphate solution to insect homogenate as enzyme resource which producing a strong blue color in the case of α-naphthol a strong red color in the case of β-naphthol at which colors are measured spectrophorometrically at an absorbency of 600 and 555 nm for α-naphthol and of β-naphthol.

4.Acid and alkaline phosphatases:

The activities of acid and alkaline phosphatase were determined using the method of **Powell and Smith (1954)**. In this procedure, the phenol released by enzymatic hydrolysis from disodium phenyl phosphate (substrate), under defined condition of time, temperature and pH reacts with 4-amino antipyrine and potassium ferricyanide producing a reddish brown color which was estimated at 510 nm.

$$\text{Phenyl phosphate} = \xrightarrow[\text{Phosphatase}]{\text{Phenyl phosphate}} \text{phenol} + \text{phosphate}$$

The reaction mixture consists of 1 ml of citric buffer (pH 4.9) for acid phosphatase or 1 ml of sodium carbonate and bicarbonate buffer (pH 10:14) for alkaline phosphatase, 1 ml Disodium phenyl phosphate (substrate) and 0.2 ml of larval homogenate. The reaction was mixed gently and incubated for 30 min at 37°C. At the end of incubation period, 0.8 ml of 0.5N NaHCO3 followed by 1 ml of 4-amino antiphrine solution and 1 ml potassium ferricyanide were added to the reaction mixture. In the control experiment, 0.2 ml homogenate was added while in blank test 0.2 ml distilled water was used. The produced brown color was measured immediately by spectrophotometer at 510 nm against blank.

5. Statistical Analysis:

The significance of the main effects was determined by analysis of variance (ANOVA). The mortality percentages *G. mellonella* were corrected according to **(Abbott, 1925)** formula. The LC50s and the slope values were determined according to **(Finney, 1971)**. Toxicity index (T.I) at LC_{50} levels were determined using **(Sun, 1950)** equation.

Data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA. A Shapiro–Wilk test was conducted to check for normality as described by **Razali and Wah (2011)**. General linear model of statistical analysis system (proc GLM ; SAS Institute Inc., 2012) was used to examine the effect of fixed factors included fungi straines, time , and their effect on enzymes activites in last instar larvae of *G. mellonella* treated with different fungi after 3, 5, 7 and 9 days under laboratory conditions. Results were expressed as means ± SE. The differences between means were tested by Tukey HSD test with the level of significance set at $\alpha = 0.05$. Figures were fitted by the Graph-Pad Prism software 9.0 (Graph Pad, USA). Statistical significance was set at p-value less than 0.05.

III. RESULTS AND DISCUSSION

1- Total larval mortality

The total larval mortality recorded by the lowest median lethal concentration LC50of A. flavus and the toxicity index reached 100 % however; *A. nidulans* recorded 26.46 % in compared to control that recorded 0 % as in Table (1). For, *A.niger*, the total larval toxicity index reached 99.4 % for the least used median lethal concentration of LC_{50} (11.96).

Table 1: The effect of *A. nidulans, A. niger* and *A. flavus* on total larval mortality

Tested fungi	Values of LC50	Lower	Upper	Toxicity index	Slope
A. nidulans	44.93	25.04	85.09	26.46	1.57
A. niger	11.96	0.055	2.59	99.41	0.259
A. flavus	11.89	2.11	67.02	100	0.815
Control	0	0	0	0	0

2-Biochemical effects of *A. flavus, A. niger* and *A. nidulans* on the last instar larvae of *G. mellonella*:

The present experiment was designated to study the changes in the activities of transaminase enzymes, alpha and beta esterase enzymes and acid and alkaline phosphatase enzymes of *G. mellonella.*

1.1. Transaminase enzymes

To demonstrate the potential impact of 3 fungal species *A. flavus* AUMC 13942, *A. nidulans* AUMC 13941 strain and *A. niger* AUMC 13944 strain on the changes in transaminases (GOT and GPT) activities compared to control of larval supernatants of full-grown *G. mellonella* larvae as the absence of fungal strains represented control. Results of the GOT and GPT activities with the endophytic fungal strains were presented. After 3 days, *A. flavus* AUMC 13942, *A. niger* AUMC 13944 strain produced the highest GOT activity of up to 1.80 and 1.14µg pyruvate\ g. b. wt\ min, respectively.

The increase in incubation period caused a notable decrease in GOT activity of *G. mellonella* larvae with the treated fungal strains. There was significant effect of fungi treatment on the activities of GOT, and GPT enzymes ($p<0.001$), With regard to the activities of GOT and GPT, all fungi treated groups were significantly higher than the control (Table2). Treatment by time did not affect GOT activities ($p= 0.2703$), however there was a significant increase in *A. flavus*- treated group compared to the control ($p<0.05$) post treatment. For GPT activity, all fungi treated groups were significantly higher than the control post treatment.

2-Alpha and beta esterase enzymes:

Table (2) illustrate the variations in values of α-esterase activity in treated *G. mellonella* larvae exposed to sublethal concentrations of some fungi compared to control.

Regarding to fungi, both *A. flavus* and *A. nidulans* show significant increase ($P<0.05$) in the activity of α-esterase enzyme compared to control at the same time interval.

Fungi treatment showed highly significant effects on the activities of alpha and beta esterase ($p<0.0001$; Table 2) in last instar larvae of *G. mellonella*. Both of *A. niger* and *A. nidulans*- treated groups were significantly higher than the control and *A. flavus*- treated groups. Also, Treatment by time had significant effects on both two enzymes, with respected to beta esterase, both of *A. flavus* and *A. niger*- treated groups were significantly lower than the control post treatment ($p<0.05$; table 2). However, both of *A. niger* and *A. nidulans*-treated groups were significantly lower than the control group ($p<0.05$) during the same aforementioned days for the activity of alpha esterase.

3-Acid and alkaline phosphatase:

Table (2) show the changes of acid phosphatase (ACP) enzyme activities in *G. mellonella* larvae treated with LC_{50} of 3 fungal species: *A. flavus* AUMC 13942, *A. nidulans* AUMC 13941 strain and *A. niger* AUMC 13944 strain for 9 days compared to control. Results demonstrate that all tested fungal species reduce acid phosphatase levels at the third day until the nineth day of treatment compared to control. Sub lethal concentrations of *A. nidulans* AUMC 13941 produce the highest increase in ACP enzyme activity at the 3rd day with value (98.16 µg/ g). *A. niger* AUMC 13944 causes gradual increase of ALP activities and the values are 87.99, 64.19, 53.56 and 69.55 µg / g. respectively after the same time interval.

Changes in both of Alkaline and Acid phosphatase activities (µg phenole/g) in last instar larvae of *G. mellonella* treated with different fungi under laboratory conditions are shown in Table 2. Aforementioned two enzymes' activities were significantly higher in all considered fungi-treated groups than the control ($p<0.05$). Regardless of the treatment, treatment by time analysis showed that all fungi treated groups were significantly higher than the control ($p<0.05$) over all periods considered for alkaline phosphatase. Similarly, there were significant differences in Acid phosphatase ($p<0.05$) between the treated groups and the control post treatment in favor of fungi treated groups, meanwhile the non-significant differences were observed.

Table (2): GOT and GPT enzymes activities (µg pyruvate\ g. b. wt\ min), alpha (µg/g) and beta esterase (µg β-naphthol/ g), acid (µg / g) and alkaline phosphatase (µg Phenol/ g) in last instar larvae of *G. mellonella* treated with different fungi under laboratory conditions:

Items	Fungi (F)				p -Value		
	Control	*A. flavus*	*A. niger*	*A. nidulans*	F	Time (Ti)	F × Ti
GOT	0.354±0.10^{c}	1.072±0.15^{a}	0.789±0.09^{b}	0.633±0.11^{b}	<0.0001	<0.0001	0.2703
GPT	2.298±0.38^{c}	5.260±0.65^{b}	7.234±1.13^{a}	7.649±1.39^{a}	<0.0001	<0.0001	0.0055
Beta esterase	14.312±3.82^{a}	4.524±1.26^{a}	8.478±3.05^{b}	18.924±2.93^{b}	<0.0001	<0.0001	0.0007
Alpha esterase	1.4575±0.19^{a}	1.5042±0.15^{a}	0.805±0.11^{b}	0.7108±0.16^{b}	<0.0001	<0.0001	0.0150
Alkaline phosphatase	40.178±3.56^{b}	74.892±6.78^{a}	68.823±5.35^{a}	66.716±6.13^{a}	<0.0001	<0.0001	0.0413
Acid phosphatase	58.248±3.65^{b}	78.313±5.43^{a}	77.093±2.81^{a}	81.088±2.64^{a}	<0.0001	<0.0001	0.0104

a–c: rows with different superscripts are significantly different ($p<0.05$)

The main cause of increased activity of detoxifying enzymes during mycoses may be mechanical damage to the insect cuticle by the hyphae as they penetrate into the host organism and the action of fungal toxins released into the hemocoel. Mycoses and other infections represent the insect response to body intoxication with metabolites of the pathogen or with products of host tissue degradation **(Xie *et al.*, 2013; Serebrov *et al.*, 2001).**

As the assessment of change at the physiological level would be related to a rise in gluathione enzymes that reflects metabolic disruption in insects. In the present study, after 3 days, *A. flavus* AUMC 13942, *A. niger* AUMC 13944 strain produced the highest GOT activity of up to 1.80and 1.14 µg pyruvate\ g. b. wt\ min, respectively. The increase in incubation period caused a notable decrease in GOT activity of *G. mellonella* larvae with the treated fungal strains. After 9 days, *A. nidulans* AUMC 13941 strain reduced the GOT activity of *G. mellonella* larvae recorded 0.22 µg pyruvate\ g. b. wt\ min. On the contrary, after 3 days *A. nidulans* AUMC 13941 strain, *A. niger* AUMC 13944 strain produced the highest GPT activity of up to 19.36 and 12.97 µg pyruvate\ g. b. wt\ min, respectively.

Also, it is suggested that an increase in GOT and GPT reflects metabolic disruption in insects **(Verma and Rahman, 1984)**. Also, GOT and GPT enzymes activities associated with the amino acid metabolism. GPT catalyzes the two parts of alanine cycle. The amount of GPT and GOT is directly related to the extent of tissue damage. After severe damage, GOT levels rise 10 to 20 times and greater than normal, whereas GPT can reach higher levels up to 50 times greater than normal (6).

GST plays apivotal role in detoxification and cellular antioxidant defenses against oxidative stress by conjugating reduced glutathione to the electrophilic centers of natural and synthetic exogenous or endogenous activated compounds **(Ortelli *et al.*, 2003.; Enayati *et al.*, 2005.; Lumjuan *et al.*, 2005).** The extent of changes in GST activity can vary with change in the targeted insect species and the concentration of the used compounds. GST increased during the initial periods of treatment until 96 h post fungal application after which enzyme activities were restained leading to metabolic imbalance and *Spodoptera litura* mortality **(Wu *et al.*, 2016).**

In the present study, the highest value of α-esterase enzyme activity occurred by treatment with LC50 of *A. flavus* at the third day, while the lowest occured by treatment with LC_{50} of *A. nidulans* after 9 days of exposure. *A. flavus* AUMC 13942 causes a significant decrease ($P<0.05$) in β-esterase activity after 5 days then causes disturbance in its values to the end of experiment. Esterases play an important role in insect defense through catabolism of the esters of high fatty acids that influence flight and degradation of inert metabolic esters **(Terriere, 1984 and Roslavtseva *et al.*, 1993)**.

The esterases in the gut of the larvae convert various acids and alcohols in the honey comb into normal saturated and unsaturated fatty acids **(Neirmerko, 1959)**. The esters are hydrolyzed in the gut to an alcohol moiety. The alcohol moiety is oxidized to fatty acids which are further broken down **(Neirmerko and Weodower, 1950)**. The mechanism of esterase action is thought to be similar to that of proteolytic enzymes. The mechanism involving aserine and histidine residue on the enzyme and unknown acid.

Owing to different diets and developmental phases, the esterase activity in the gut of *G. mellonella* differs. In contrast to the acetone powder preparation, which lost its activity after 24 hours, the esterase completely lost its activity after ammonium sulphate precipitation **(Krieg, 1972).**

In the stomach and Malpighian tubules, acid phosphatase (ACP), also known as the alysosomal marker enzyme **(Csikos and Sass, 1997)**, is active **(Srivastava and Saxena, 1967)**. Additionally, cytolyzed tissues and organs that are dissolving contain large amounts of ACP **(Sahota, 1975)**. This enzyme may transphosphorylate processes to boost the phosphate pool for the synthesis of high energy molecules like ATP adenosine triphosphate while hydrolyzing a range of orthrophosphate esters.

The highest value of ACP activity occurs after 3 days by treatment with LC_{50} of *A. nidulans* while the lowest occurs after 9 days of treatment with LC_{50} of *A. flavus* AUMC 13942. Moreover, *A. niger* exert moderate effects on ACP activities but is less effective than A. flavus. The highest activity of ACP in cells localized in the cytoplasm, suggests that the enzyme participates in proteosynthesis **(Vorbrodt, 1958).** In the present study, *A. nidulans* AUMC 13941 produce the highest increase in ALP enzyme activity at the 3rd day with value (118.43 µg/ g). As in lepidoptera, alkaline phosphatase proteins have been characterized as functional binding receptors for bacterial toxins, ALP proteins first bind to bacterial toxins with alow affinity and subsequently carry the toxins to the microvilli membrane of the larval midgut. ALP interact with the oligomeric toxin structure which causes membrane insertion and pore formation, leading to osmotic lysis of midgut cells **(Lopez *et al*., 2006; Park *et al*., 2008; Escobar *et al*., 2013).** ALP provides phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes **(Etebari *et al*., 2007).** ALP is responsible for cytolysis of tissues during the insect development **(Dadd, 1970).**

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