Original Article

Digestive enzymes of Greater wax moth (Galleria mellonella) (Lepidoptera: Pyrilidae) through some strains of endophytic fungi infection

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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).

The fungal filtrate of A. nidulans, A. flavus and A. niger had satisfactory levels of mortality compared with those of fungal spore suspension that exhibited less levels of mortality. The results were recorded at different incubation intervals (1, 2, 3, 5, 7, 9 and 11 days) and the obtained data were recorded in table. The results revealed 8 % of fungal filtrate showed a great mortality (70%) than other concentrations. More than 8 % of fungal filtrate decreased larval mortality. It can be concluded that 4 % and 5 % of fungal filtrate of the three fungal strains produced significant increase in larval mortality, as compared with other concentrations.

A. flavus AUMC 13942 was superior for either trehalase and amylase activity of the experimental fungal strains. A. flavus AUMC 13942 was found to be the most effective fungal strains and produced significant differences ($P \leq 0.05$) in trehalase and amylase activity. The highest trehalase activity values obtained by A. flavus AUMC 13942 strain was 14.22 mg glucose\ g. b. wt\ min after 7 days and the highest invertase activity values obtained by A. nidulans AUMC 13941 strain after 9 days were and 13.42 mg glucose\ g. b. wt\ min by, respectively compared to control.

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Introduction

The fungus life cycle is associated with the synthesis and secretion of a number of toxic metabolites, including extracellular enzymes and the low-molecular weight compound (toxin). The potential for a successful pathogen relies on the ability to overcome the various host-defence systems. Interaction between the deuteromycetes, its secondary metabolite, and its host cellular defence was investigated using in vivo and in vitro studies. In vitro studies showed that toxins (efrapeptins) inhibit phagocytic activity of Galleria mellonella (Lep: Pyralidae) haemocytes. The effect of efrapeptins on phagocytosis was in a dose-dependent manner i.e: the amount of phagocytosis in a treated cell-culture with 0, 3, and 30 µg efrapeptins per well was about 12, 7.5, and 4.5 %, respectively. In vivo studies showed that injection of insects with 0, 0.25, and 0.025 µg toxin rendered percentages of phagocytosis of 13, 11.5, and 7.2, respectively. There was no significant reduction in the total haemocyte count (THC). THC was suppressed at 48 hrs post-treatment of larvae with spores. Considering that toxin suppresses phagocytosis, nodule formation, but not THC, it was suggested that efrapeptins may interfere with the ligand-receptor interactions that are likely to occur in the plasma membrane of specific haemocytes (Bandani, 2008).

Jahagirdar and Seligy (1991) speculated that the multiple trehalase bands in PAGE assay (8 % polyacrylamide) could be obtained due to post translational modification (glycosylation, phosphorylation and proteolytic degradation) of a single gene product or products of related genes expressed in different larval tissues. The physiological effects of the three fungal isolates have been also studied, in a trial for controlling the infection of wax moth.

According to Champion et al. (2016) in G. mellonella infection model, the survival rate needs to be monitored for up to 5 days post infection to allow for the calculation of a maximum half lethal dose.

Fungal acid trehalase may be involved in the hydrolysis of haemolymph trehalose into available glucose for the invading fungus to grow. Wang et al.

(2005) have investigated the differential gene expression in isolated haemolymph and insect cuticle and found that the genes concerned with the carbohydrate metabolism exhibited increased expression when grown invitro on extracted insect haemolymph.

Trehalase 1, 2 are shown to participate in the activation of chitin synthesis pathway in insects (Merzendorfer and Zimoch 2003; Xie et al., 2013). Growth, development, energy supply for flight, regulation of chitin biosynthesis and recovery from abiotic stress are important physiological roles of trehalase in insects. The enzyme has been functionally characterized using different inhibition approaches including RNA interference (RNAi) and potent inhibitors. Trehalose (α-D glucopyranosyl (1,1) - α-D glucopyranosidel) is non-reducing disaccharide which is rarely found in animals but is present at high concentration in most insects. It is predominant haemolymph sugar in many insects. Trehalose plays important roles in insect physiology (Elbein, 1974 and Thompson, 2003) and the major carbohydrate metabolic fuel used for flight (Becher et al., 1996).

The depletion of blood trehalose may be detrimental to the insects. Acid trehalase may associate with other proteins during purification e.g a highly glycosylated protein invertase (Mittenbuhler and Holzer, 1988; Nilima and Ghosh, 1998). This enzyme is a glycoprotein with a molecular weight of 170 KDa, while after deglycosylation, its molecular mass decreased to about 130 KDa. The acid optimum pH of 5.5 is very similar to those of comparable enzymes from Saccharomyces cerevisiae (Thevelein, 1988; Alizadeh and Klionsky, 1996) and A. nidulans (d,Enfert and Fontaine, 1997). The invertase enzyme is responsible for regulating the toxic substances in the alimentary canal of insects (Ishaaya and Swirsky, 1976). Hence, an observed increase in the activity of invertase in the initial concentration of the alkaloids may be due to the toxic stress.

The temperature optimum 30°C of the enzyme is the same as the optimum temperature for pathogenesis, the temperature stability at 45°C would help the fungus maintain integrity during the expression of behavioural fever in the host. The pure acid trehalase efficiently hydrolyzed haemolymph trehalose into glucose invitro (Wyatt, 1967).

Acid trehalases are required for growth on trehalose as sole carbon source and knockout mutants of several filamentous fungi are unable to grow on trehalose. These enzymes reported in fungi are generally present on the surface of spores and mycelium, or in vacuoles (d,Enfert and Fontaine, 1997; Sussman et al., 1971; Nwaka et al.,1996), and less frequently secreted into the mycelium (Jorge et al., 1997; Lucio et al., 2000 and Almeida et al., 1997). Trehalase activity rapidly decreased with temperature above 50°C and no activity was detectable at temperatures of 70 and 100 °C. Quantification of the intensity of trehalase bands further revealed the relative differences in concentration and specific activity.

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 utilized to gauge the activity of carbohydrate hydrolyzing enzymes.

Carbohydrate hydrolyzing enzymes

The method used to determine the digestion of trehalose, starch and sucrose by trehalase, amylase and invertase enzymes, respectively were similar to those described by (Ishaaya and Swiriski 1976). The free aldehydic group of glucose formed after trehalose, starch and sucrose digestion were determined using 3, 5 dinitrosalicylic acid reagent. The trehalase reaction mixture consisted of 0.2 ml of 3% trehalose (substrate), 0.2 ml phosphate buffer (pH 5.4) and 0.2 ml larval homogenate, the invertase reaction mixture consisted of 0.2 ml of 4% sucrose (substrate), 0.1 ml phosphate buffer (pH 5.4) and 0.2 ml of insect homogenate, the amylase reaction mixture consisted of 0.2 ml of 2% starch (substrate), 0.160 ml phosphate buffer (pH 5.4) and 0.2 ml of larval homogenate, the dinitrosalicylic acid reagent was prepared by dissolving one gram of 3,5-dinitrosalicylic acid in 20 ml of 2N NaOH and 50 ml of distilled water with the aid of a magnetic stirrer. Potassium sodium tartarate (30 g) was added, and magnetic stirring was continued until a clear solution was obtained. Distilled water was then added to bring the final volume to 100 ml. All test tubes were incubated at 37ºC for exactly 60 min, 0.8 ml of 3, 5 dinitrosalcylic acid reagent were the added. The reaction mixture was heated 5 min at 100ºC in a boiling water bath followed by immediate cooling in an ice bath. The optical density (O.D.) of the produced color is measured at 550 nm using spectrophotometer.

Results

The efficacy of fungal filtrates on larval mortality of G. mellonella:

The fungal filtrate of A. nidulans, A. flavus and A. niger had satisfactory levels of mortality compared with those of fungal spore suspension that exhibited less levels of mortality. The results were recorded at different incubation intervals (1, 2, 3, 5, 7, 9 and 11 days) and the obtained data were recorded in table. The results revealed 8 % of fungal filtrate showed a great mortality (70%) than other concentrations. More than 8 % of fungal filtrate decreased larval mortality. From these data, it can be concluded that 4 % and 5 % of fungal filtrate of the three fungal strains produced significant increase in larval mortality, as compared with other concentrations.

This experiment aimed to study the effect of different dilutions of fungal filtrates on wax moth larvae mortality by fungal isolates. (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9 %) of A. nidulans, A. flavus and A. niger were separately added as 1 ml of fungal filtrate to wax moth diet. One ml of saline solution added without fungal filtrate in aseparate pettri dish served as control.

After incubation at 30ºC for 11 days, each sample was tested for its mortality concentrations and the data presented in table indicated that mortality was strong by using 7 % of A. flavus and A. niger filtrates (70 %). However, the mortality was weak with 1% of A. nidulans (10% at 11 days). In the same time, 2% of A. nidulans showed weak effect on the percents of mortality of wax moth larvae (20% at 11 days). However, the percents of mortality were greatly affected at 8% of A. nidulans recording (60 %) as shown in table and fig.. The results of this experiment indicated that fungal filtrate was effective in wax moth larvae mortality by the two isolates A. flavus and A. niger and the effect was dose- dependant.

Conc (% v/v)	**Mortality percentages (%) during different incubation periods (days)**														
	A. flavus					***A. niger***					***A. nidulans***				
	1	**3**	**5**	**7**	**9**	**1**	**3**	**5**	**7**	**9**	**1**	**3**	**5**	**7**	**9**
1%	0	20	20	40	40	0	20	40	40	40	0	0	0	10	10
2%	0	30	30	50	50	0	40	50	50	50	0	0	10	20	20
3%	0	40	40	60	60	0	40	50	60	60	0	30	30	30	30
4%	10	40	40	50	50	0	40	50	60	60	0	10	40	40	40
5%	20	50	50	60	60	10	40	50	60	60	0	20	50	50	50
6%	20	50	50	60	60	10	50	60	60	60	20	20	50	50	50
7%	30	60	60	60	70	20	50	60	60	70	20	50	60	60	60
8%	30	60	60	70	70	40	60	60	60	70	20	50	60	70	70

9%	10	20	20	20	20	10	0	20	20	20	10	20	30	30	30
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Physiological investigations

The present experiment was designated to study the changes in the activities of transaminase enzymes, carbohydrate hydrolyzing enzymes and the changes in the effects of total soluble protein and total lipid against last instar larvae of G. mellonella using treated diet of larvae (diet dipped in tested fungal filtrates (LC50). The attributes were measured at different time intervals (3 & 5& 7& 9 days after treatment).

Carbohydrates hydrolyzing enzymes

The results given in Table and Figure showed the effect of Aspergillus fungal species on the activity of carbohydrate hydrolyzing enzymes of G. mellonella larvae (last instar larvae) and indicated that A. flavus AUMC 13942 was superior for either trehalase and amylase activity of the experimental fungal strains. A. flavus AUMC 13942 was found to be the most effective fungal strains and produced significant differences ($P \leq 0.05$) in trehalase and amylase activity. The highest trehalase activity values obtained by A. flavus AUMC 13942 strain was 14.22 mg glucose\ g. b. wt\ min after 7 days and the highest invertase activity values obtained by A. nidulans AUMC 13941 strain after 9 days were and 13.42 mg glucose\ g. b. wt\ min by, respectively compared to control.

Amylase enzyme

Table: Specific activity of amylase enzyme in last instar larvae of G. mellonella treated with different fungi after 3, 5, 7 and 9 days under laboratory conditions

Days	**Specific activity of amylase enzyme with different fungi during different periods (mg glucose\ g. b. wt\ min)**			
	A. flavus	***A. niger***	***A. nidulans***	**Control**
3	1.90±0.52[b]	2.05±0.07[b]	2.13±0.58[ab]	3.43± 0.10[a]
5	3.55±0.15[a]	1.95±0.16[b]	3.78±0.25[a]	3.75±0.01[a]
7	4.63±1.07[a]	2.15±1.42[b]	3.48±0.28[ab]	4.18±0.27[a]
9	7.68±0.79[a]	2.49±0.47[b]	4.06±0.22[b]	3.58±0.99[b]

Days	**Specific activity of trehalase enzyme with different fungi during different periods (mg glucose\ g. b. wt\ min)**			
	A. flavus	***A. niger***	***A. nidulans***	**Control**
3	6.50±1.77[a]	4.92±2.01[a]	5.76±0.14[b]	8.54± 0.27[a]
5	14.30±0.55[a]	11.51±0.07[b]	7.84±0.58[c]	10.02±0.30[b]
7	12.12±1.06	12.40±0.26	10.69±0.26	12.27±0.78
9	6.93±1.64	9.89±1.41	9.41±0.59	10.08±2.91

Days	**Specific activity of invertase enzyme with different fungi during different periods (mg glucose\ g. b. wt\ min)**			
	A. flavus	***A. niger***	***A. nidulans***	**Control**
3	6.34±0.86	7.17±1.01	8.83±0.27	9.45± 0.74
5	11.02±0.75	9.84±0.28	9.33±0.61	12.74±0.41
7	11.85±0.37	10.02±0.49	12.58±0.39	14.71±0.35
9	9.73±1.19	10.19±0.45	11.63±1.03	10.68±3.07

Discussion

In the present study, it can be concluded that 4 % and 5 % of fungal filtrate of the three fungal strains produced significant increase in larval mortality, as compared with other concentrations. The fungal filtrate exhibits an immunomodulation effect on G. mellonella larvae.

The bioassay results and proteomic profiling indicate that the fungal culture filtrate has the greatest impact on the insect immune system. Differences in proteomic profiles for different treatments may reflect or even partial explain the differences observed in bioassays. For example, higher levels of detoxifying enzymes in B. bassiana treated larvae than in those treated with M. anisopliae and B. caledonica may be one alteration reflective of their differences in immunomodulation (Namara et al., 2017).

The results obtained indicated good potential of using some fungal filtrates of various fungi for wax moth management as greater wax moth individuals, pupae and egg stages don't damage to effervesced honey combs but their larvae's do different levels of damage to honey combs in the appropriate environmental conditions (Haewoon et al., 1995). Overall, this study demonstrated a comparative finding for the effect of fungal filtrate with its derivatives on some biological activities of wax moth larvae. Moreover, the inoculation with fungi may control wax moth in its habitat or laboratory as an alternative for the synthetic insecticides. Also, the presence of an enzyme system which will convert glucose into trehalose and the presence of trehalase which splits trehalose into glucose suggest that trehalose formation is adevice used by insects to store dietary carbohydrates in areadily mobilizable reverse. In the present study, it was obvious that on using 3 fungal strains, the lowest activity of carbohydrate hydrolyzing enzymes of G. mellonella larvae (last instar larvae) were observed after 3 days by A. flavus AUMC 13942 and A. niger AUMC 13944 strain and the lowest amylase and invertase activity (1.01mg glucose\ g. b. wt\ min by A. flavus and 4.89 mg glucose\ g. b. wt\ min and the lowest trehalase activity by A. niger AUMC 13944 strain recording 1.53 mg glucose\ g. b. wt\ min after 3 days. Invertase could have abiological role in hydrolysis of sucrose rich diets or of other glycosides as maltooligosaccharides (Carneiro et al., 2004). Glycerine is made chiefly of glycerol(80%) (Swiatkiewicz and Koreleski, 2009), which is normally

part of metabolism of animal cells.

The various diet may regulate amylase levels differentially. The substitution of a conserved arginine into glutamine in unrelated amylase, this arginine is involved in the fixation of an activating chloride ion which changes the protein conformation and without which a deterimental salt bridge interaction would form. The glutamine is found in all lepidopteran amylase (Pytelkova et al., 2009). Lepidopteran amylase generally has alkaline preferences. In some species, several amylases are produced with different pH optima, due to different tissue specificities (Yezdani et al., 2010 and Abraham et al., 1992) or storage specificities (Shabarari et al., 2014).

Continuous feeding would stimulate the continuous discharge of enzymes into the gut lumen, where they would accumulate (Teo et al., 1990). Lepidopteran larvae completely void the gut when they cease feeding and become prepupae; therefore, the gut tissue of prepupae contained no amylase.The larvae of lepidoptera often have strongly alkaline midgut contents (Wiggles- worth, 1972), the amylase of spodoptera litura is corresponded very well with the pH of midgut contents (Ishaaya et al., 1971), also the natural diets would contain large quantities of starch is sufficient to saturate the enzyme, this may compensate for the lowest activity of amylase at the normal midgut pH of 7.6. This indicates that amylase of lepidoptera is well adapted to an environment of fluctuating temperature.

It was found that the fourth and fifth instar larvae had the highest amylase activity, but the obivious differences were found in the sites of enzyme activity known as soluble and membrane bound fractions. By using line regression, the slope showed larval dependency of enzymes along the developmental and feeding processes. Optimal pH of soluble and membrane bound α- amylase was found at 9 (Zibaee, 2012).

Insects are known to cope with threats of pathogens through the action of different enzyme systems. Howden and killby (1960) have shown that trehalose concentration in the hemolymph of the fifth instar nymph increases throughout 10 days of the stadium but falls sharply during the moulting period. Before the time of moulting, chitin is required for the formation of the new cuticle, and it seems possible that N- acetyl glucosamine units in it may be ultimately derived from the trehalose carbohydrate store.

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