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ENTOMOPATHOGENIC FUNGI AS BIOPESTICIDES AGAINST THE EGYPTIAN COTTON LEAF WORM, *SPODOPTERA LITTORALIS*: BETWEEN BIOCONTROL-PROMISE AND IMMUNE-LIMITATION

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ABSTRACT

In this study, a preliminary virulence test on three concentrations (1×10^6, 1×10^7 and 1×10^8 conidial spore ml^{-1}) of the aqueous conidial suspension of the four entomopathogenic fungi isolates, *Beauveria bassiana* IMI 382302, *Beauveria bassiana* IMI 386701, *Trichoderma harzianum* T24 and *Aspergillus flavus* Link was carried out against both larval and pupal stages of *Spodoptera littoralis* within 5 days post-treatment. *T. harzianum* T24 showed 80% larval mortality only when applied at its highest conidial concentration (1×10^8/ conidial spore ml^{-1}), while *A. flavus* showed 100% pupal mortality only at all of its conidial concentrations. However, *B. bassiana* IMI 382302 showed relatively high dose-dependant larval and pupal mortalities. While, strain IMI 386701 of *B. bassiana* showed a very weak mortality against pupae at its higher concentrations but no virulence against larvae was recorded. Enzymatic and antibiosis bioassays of the four fungal isolates showed relatively high activities against the sensitive fungus, *Fusarium oxysporum*, for most of the tested isolates. The humoral antifungal response of insect host relatively high compared to the anti-bacterial one. Injection of larvae with the immune sensitive bacteria, *Micrococcus luteus*, (5×10^3 bacteria/larva) showed a detectable humoral response by 2h, peaked around 12h and become hardly detectable by 24h post-injection. Injection of larvae with conidial suspension (5×10^3 conidia/larva) from each of fungal isolates showed humoral antifungal activity against *Beauveria bassiana* IMI 386701and *Aspergillus flavus* only. This activity was detectable by 12h, peaked around 24h and become hardly detectable by 36h post injection. Although the humoral antifungal response was started slowly compared to the antibacterial one, it lasted for longer and enabled larvae to withstand the infection with these immune-sensitive fungal strains. However, no humoral activity was detected against *Beauveria bassiana* IMI 382302, although however, weak activity was detected against *T. harzianum* T24 only at the low conidial concentration but not at the higher one ($1 \times 10^8 ml^{-1}$). Thus, this study concludes that larvae of *S. littoralis* showed immune-dependant sensitivity to *T. harzianum* T24 and *B. bassiana* IMI 382302. Therefore, this study may recommend these two fungal isolates as mycoinsecticides in the battle against cotton leaf worm in Egypt. And hence, they have been selected for future comprehensive bioassays in the laboratory under conditions similar to that in the field. This, in fact, may help developing effective mycoinsecticides against this pest.

INTRODUCTION

Development of an effective control method against the cotton leaf worm, *Spodoptera littoralis* is urgently needed since it does serious damage to many important agricultural crops in Egypt. There is a serious interest in the use of microbial insecticides for biological control of insect pests, as alternatives to chemical control, since they neither leave toxic chemical residues in the environment nor do they induce resistance in their insect hosts (Evans, 1999). And hence, the public awareness and concern for environmental quality, has led to more focused attention on research aiming at developing biological agents (Hidalgo *et al*., 1998). A promising strategy with good potential to control insect pests and, at the same time, to minimize the adverse effects of chemical insecticides is the use of entomopathogenic fungi as well as other microbial agents. Entomopathogenic fungi are among the most promising group of biological control against insect pests (Lacey & Goettel 1995). Currently, 66 products representing at least 38 taxonomically diverse species or varieties of entomopathogenic fungi have been developed or are being developed (Liu & Li 2004).

The entomopathogenic Hyphomycete fungi, such as *Beauveria bassiana*, are naturally occurring in the soil (Klingen *et al*., 1998) and is being developed as biocontrol agents against soil dwelling pests like scarabs and weevils (Keller, 2000) with no effect on the non-targeted insects (Goettel and Hajek, 2001). On the other hand however, the insect host has developed effective immune systems composed of both cellular and humoral responses (Boman and Hultmark, 1987) for fighting back against different kinds of microbial agents. In the cellular response, insect hemocytes react to a foreign body by degranulation, phagocytosis, and/or encapsulation (Gotz and Boman, 1985). The insect cellular responses against fungi, and other microorganisms, are well established. In humoral response, insects respond to microbial infection by the production of humoral immune factors including an array of potent antibacterial and antifungal peptides (Hoffmann *et al*., 1999).

Although numerous antibacterial peptides have been identified (Hetru *et al*., 1998), only a small number of antifungal peptides have been reported in insects. For example; drosomycin (Fehlbaum *et al*., 1994), cecropin A and B (Ekengren and Hultmark, 1999), and andropin (Samakovlis *et al*., 1991) from *Drosophila melanogaster*, heliomicin from *Heliothis virescens* (Lamberty *et al*., 1999), cecropin A from *Hyalophora cecropia* (Ekengren and Hultmark, 1999), thanatin from *Podisus maculiventris* (Fehlbaum *et al*., 1996), holotricin 3 from *Holotrichia diomphalia* (Lee *et al*., 1995), termicin and spinigerin from *Pseudacanthotermes spiniger* (Lamberty *et al*., 2001) and scarabaecin from rhinoceros beetle, *Oryctes rhinoceros* (Tomie *et al*., 2003). In fact, these antifungal factors may affect the capability of a fungus to kill the insect host, and thus, affect its participation as a biocontrol agent. Thus, it is urgently needed to study the immune responses, particularly the humoral response that might be induced against these biocontrol agents which may help improving the utilization of entomopathogenic fungi in the biological control strategy.

The study presented herein was conducted to hypothesise three objectives; the first, was to preliminarily evaluate the susceptibility of *Spodoptera littoralis* to the four entomopathogenic fungi, *Beauveria bassiana* IMI 382302 (Kenya/ Kisii), *Beauveria bassiana* IMI 386701 (Kenya/KIB West 1S), *Trichoderma harzianum* T24 and *Aspergillus flavus* Link. The second objective was to investigate the enzymatic activity of these four isolates. The third objective was to investigate the humoral antifungal activity induced by this pest against these fungal isolates (with reference to the humoral antibacterial activity) which may act as an immune-limiting factor that might affect the competency of fungi as biocontrol agents against this dangerous Egyptian pest.

MATERIALS AND METHODS

Rearing of insect

Eggs of *Spodoptera littoralis* (Lipidoptera, Noctuidae) were kindly gifted from The National Research Centre, Dokkey, Cairo, Egypt. Eggs hatching and larval rearing took place at 27 ± 1°C, 65 ± 5% R.H. and a photoperiod of LD 14:10h according to (Hegazi *et al*., 1977). Emerged adult moths were maintained together on 80% bee-honey solution until egg laying. Early fifth larval instars and newly formed pupae were used for performing the experiments.

Maintenance of microorganisms

i)- bacteria

The bacterium *Micrococcus luteus* (NCTC 2665) (Sigma, UK) was incubated in nutrient broth (13 g l^{-1}) at 37°C for 48h and checked for a density of 1×10^6 ml^{-1} as detailed in Nimmo *et al*. (1997). After incubation, 1.5 ml of bacterial broth was centrifuged at 4000 rpm for 10 minutes, washed twice in distilled water then re-suspended in a similar amount of sterile distilled water, and finally used for the experimental purposes.

ii)-fungi

Before they were used in the experiments, the four fungal isolates were grown and held on suitable growing agar media in 9 cm Petri dishes for one week at 30°C in Botany Department, Faculty of Sciences, El-Minia University for use as fresh renewed isolates. The two *Beauveria bassiana* isolates (Hyphomycetes**)** were kindly provided by Marilena Aquino de Muro (CABI Bioscience, Bakeham Lane, Egham, TW20 9TY, UK) who isolated them from the weevil, *Sitophilus zeamais*, in Kenya (de Muro *et al*., 2003). *Aspergillus flavus* Link (Ascomycetes) has been isolated from *Spodoptera* egg batches and identified by the Mycological Unit, Botany Department, Faculty of Science, El-Minia University, Egypt. *Trichoderma harzianum* T24 (Deuteromycetes) (identified by DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was obtained from Botany Department, El-Minia University. *Fusarium oxysporum*, the soil-borne plant pathogen, (identified by Mycological Unit, Assiut University, Egypt), was obtained from the Botany Department, Faculty of Science, El-Minia, University, Egypt, for use as a target in the test of antifungal effect of the four fungi strains of this study as detailed below. All fungal isolates were cultured on potato dextrose agar medium (PDA) media (200g grated potato, 20g glucose, 20g agar) (g l^{-1}). Cultures of fungi were incubated at 30°C for **7** days. Stock cultures (from each isolate) were stored on agar slants at 5°C until further use.

Preparation of fungal inocula

For fungal bioassays, conidia were harvested by scraping the sporulating colonies and suspended in sterile distilled water containing 1.0% Tween80 (v/v aqueous solution, as wetting agent) (Hicks, *et al*., 2001). While for immunity assays, conidia were suspended in sterile ddH_2O. The resulting conidial suspensions were first cleared for hyphal debris by filtration, using a suitable sterilized piece of clothes, and centrifugation for 5 min at 3000 rpm then washed twice with 0.05% Tween80 or ddH_2O with intervening centrifugation. Resulting conidia were re-suspended in 1.0% Tween80 or ddH_2O and the concentrations were determined using a haemacytometer before they were diluted with sterile water containing 1.0% Tween80 or ddH_2O to reach the appropriate concentrations (1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}). Conidial viability was examined prior to the experiments by placing three droplets of a 1×10^6 conidial spores suspension ml^{-1} onto suitable agar plates followed by incubation for 24h at 30°C, after which, their germination was examined under a light microscope by observing > 90% growth for all isolates.

Preliminary test of fungal virulence

Virulence of the experimental fungi against early 5th larval instars and newly formed pupae was performed by testing three concentrations of spore suspension from each of the four fungal isolates (1×10^6, 1×10^7 and 1×10^8 ml^{-1}). Larvae or pupae were randomly divided into four groups (one group for each fungus), each group was then subdivided into three subgroups (50 larvae or pupae each) in three different plastic dishes (30 cm in diameter each). Ten ml from each conidial suspensions (from each fungus) were sprayed individually onto one of the three larval or pupal subgroups (one concentration against one subgroup) using sprayer fitted with a hollow cone nozzle with a volume diameter droplet of 1.4mm. A mist of distilled water was sprayed into each pot at the beginning of the experiment, and when needed during the experiment, in order to ensure a continuous and adequate moisture for spore germination (Hicks, *et al.*, 2001). A group of control larvae or pupae were treated with a 1.0% Tween80 solution and maintained under the same conditions as the experimental groups. During incubation at 25ºC, treated and control groups were allowed to feed on the leaves of the Castor Bean plant, *Ricinus communis*. Dead larvae or pupae were counted daily and mortality was calculated. Dead insects were then transferred into a Petri dish lined with moistened filter paper and mortality due to fungi was confirmed by light-microscopic examination of hyphae and conidia on the surface of the dead insect (Fig. 1). All test insects were maintained in the incubator at 35°C and a photoperiod of L: D; 12h: 12h.

Electron microscopy study

A. flavus-infected pupae were clearly showing ideal fungal growth (10 days post-infection), and thus, samples were handled over to the Electron Microscope Unit at El-Minia University. Dead infected pupae were directly subjected to critical point drying for 15 min to remove water contents. Paupae were then mounted on SEM holders for sputter-coating with gold and viewed using a JEOL JSM-T200 scanning electron microscope (Fig. 2).

Enzymatic bioassay

Types and activities of extracellular hydrolytic enzymes of the four tested fungal isolates were investigated on solid media. Mycelial disks of each of the four isolates were placed on agar medium containing the relevant enzyme substrate, 1.5% colloidal chitin-agar or 3.0% milk-agar for chitinase or ptorease test respectively. Zones (halos) of degraded substrate that formed around the growing colony were then measured 3 days-post inoculation. Chitinase and protease activities were evaluated according to Hsu and Lockwood, (1975) and Sarath, *et al.*, (1989) respectively.

Effect of fungal isolates on the growth of *Fusarium oxysporum*.

i) Effect of volatile inhibitors

The *in vitro* antagonistic effects of the four fungal isolates on the growth of *Fusarium* by producing volatile substances were evaluated using the method of inverted plates (Dennis and Webster 1971a). Mycelial discs (4 mm in diameter each) from each of the four tested fresh isolates were separately inoculated in the centres of PDA plates (one disk/plate). A separated group of plates were inoculated with *Fusarium*. Lids-free plates, inoculated with the *Fusarium*, were individually inverted over that inoculated with the tested isolates. Petri dishes of *Fusarium* inverted on fungi-free agar media dishes were considered as control. Each pairs of Petri dish were sealed together with paraffin tape and incubated at 25°C. Four replicates were carried out for all tests and controls (n = 4). Colonies diameters of the *Fusarium* were measured at 1, 2, 3, and 4 days post-incubation, and percentages of inhibition in daily growth rate were calculated.

ii) Effect of non-volatile inhibitors

The effect of non-volatile metabolites produced by the four tested fungal isolates on the growth of *Fusarium*. was determined according to Dennis and Webster (1971b). Each of the four fungal isolates was grown on a sterile cellophane disk lying on PDA in 9 cm Petri dishes for 2 days. Then, the

Cellophane with the fungal mycelium was removed and immediately replaced with a mycelial disk of *Fusarium*. The growth diameter of the *Fusarium* colony was determined after 72 h and was compared daily with that grown on PDA without metabolites (control). The studies of non-volatile and volatile metabolites were conducted in four replications (n = 4). The percentages of inhibition in daily growth rates of the *Fusarium* in all above experiments were recorded as the differences in the daily growth rates in the presence or absence of the antagonists as detailed in El-Katatny, (2001).

Injection of larvae for immunity assay

i)- Injection with bacteria

This experiment was conducted in order to visualize the humoral anti-bacterial activity in this insect as a reference to the anti-fungal activity. Larvae were injected with 5µl from stock *Micrococcus luteus* (NCTC 2665) suspension (≈1×10^6 ml^{-1}) (Sigma, UK) as detailed by Nimmo *et al*., (1997). Larvae were first chilled on ice for 5 minutes prior to thoracic injection. A group of 20 larvae was injected with bacterial suspension (5µl each) using a Hamilton microsyringe (Sigma) fitted with a 25 guage needle. Control larvae were injected with the same amount of sterile distilled water. Haemolymph was collected at 2, 4, 8, 12 and 24h post-injection by puncturing one of the pro-legs with a sterile needle and bleed haemolymph was received in a clean 1.0 ml appendorf tube. Two micro-litres of fresh haemolymph were subjected to inhibition zone assay (2µl/well).

ii)- injection with fungal conidia

Four groups (10 larvae each) were injected with 5µl from stock conidial suspension (1×10^6 ml^{-1}) (suspended in sterile ddH_2O), from each of the four fungi separately as described above. Control larvae were injected with ddH_2O only. Haemolymph was collected 12, 24 and 36h post-injection and subjected to inhibition zone assay as described above.

Humoral antimicrobial activity test

Haemolymph was collected from chilled *M. luteus*-injected or control larvae at 2, 4, 8, 12 and 24h post-injection for visualizing the humoral antibacterial activity. Collected haemolymph was immediately subjected to inhibition zone assay against the same bacteria according to Nimmo *et al*., (1997) and as detailed in Ahmed *et al.*, (2002). Ten replicates (in two dishes, 5 wells each, were assigned at each time point of the experiment) were carried out in test and control groups.

Humoral antifungal activity was visualised according to Ekengren and Hultmark (1999). One ml of conidial suspension (1×10^6 ml^{-1}) from each of the four fungi was individually mixed with 7 ml of PDA media in a sterile Petri dish (9 cm; Sterilin) for carrying out the inhibition zone assay. Haemolymph from conidial-injected larvae (for each of the fungal isolates) was collected at 2, 4, 8, 12, 24 and 36h post-injection. Collected haemolymph was immediately subjected to the inhibition zone assay using PDA seeded with the same conidia used for injection. A separate assay loaded with the antifungal cecropin B (sigma-Aldrich, UK) (Ekengren and Hultmark, 1999) was carried out at the same time and under the same condition as a reference in order to calculate the equivalent amount of antifungal peptide in the same way as detailed in Ahmed *et al.*, (2002).

Statistical analysis

Means and standard errors of 10 replicates of humoral antibacterial and antifungal activity, and fungal bioassay were undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002).

RESULTS

This study investigates the competency of the entomopathogenic fungi, *B. bassiana* IMI 382302, *B. bassiana* IMI 386701, *A. flavus* Link and *T. harzianum* T24 to be used in the biocontrol regime against the Egyptian cotton leaf worm, *S. littoralis*. The virulence of each of these fungi was preliminarily tested, and the humoral immune response of larvae against them was investigated.

Test of fungal virulence

The pathogenic effects of the four fungal isolates were preliminarily tested on both early fifth larval and newly formed pupal stages of *S. littoralis*. Only the higher conidial suspension of *T. harzianum* T24 (1×10^8 ml^{-1}) showed 80% and 0% larval and pupal mortality respectively (Table 1). This may indicate that *T. harzianum* T24 has only larvicidal effect at a minimum concentration of 1×10^8 conidia ml^{-1}. Moreover, only the higher conidial concentrations of *B. bassiana* IMI 386701 showed a very weak mortality against pupae but not against larvae (Table 1). However, all conidial concentrations of *B. bassiana* IMI 382302 showed dose-dependant larval and pupal mortalities (Table 1 and Fig. 1 A1 & A2), which may indicate stronger larvicidal and pupicidal toxicity compared to the other strain. All conidial concentrations of *A. flavus* (isolated from *Spodoptera* egg patches) showed 0% and 100% of larval and pupal mortality respectively (Table 1 and Fig. 1B), which may indicate a lethal effect on the silent stages only (eggs and pupae). Control insects showed no mortality, as all larvae managed to pupate and all pupae managed to emerge to adults.

Table (1): Mortality of larval and pupal stages as a result of infection with the three concentrations of the four fungal isolates. No mortality was recorded within the control group as 100% of larvae succeeded to pupate. **n**: number of tested individuals in each case.

Fungal strains	Treated stages	Mortality (%) against conidial concentrations (ml^{-1})			n
		1×10^6	1×10^7	1×10^8	
T. harzianum T24	Larvae	0	10	80	50
	Pupae	0	0	0	50
A. flavus Link	Larvae	0	0	0	50
	Pupae	100	100	100	50
B. bassiana IMI 382302	Larvae	50	60	90	50
	Pupae	40	60	80	50
B. bassiana IMI 386701	Larvae	0	0	0	50
	Pupae	0	10	10	50

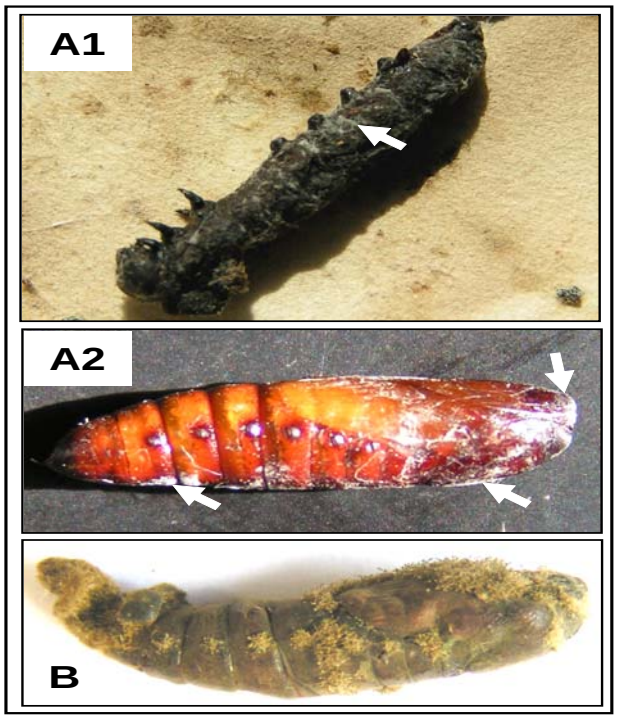

Fig. 1. Photographs showing dead larval and pupal cadavers at 5 days post-fungal infection. **A1 and A2**: dead larva and pupa infected with *B. bassiana* IMI 382302 (Kenya/ Kisii) showing few white fungal hyphae appear spread on the outer surfaces of the cadavers (white arrows). These hyphae completely covered the cadavers 5 days later. Larval cadaver appears black as a result of inducing the prophenoloxidase system activity that causes melanization of the internal body cavity. **B**: Dead pupa infected with *A. flavus* Link showing better fungal growth on its outer surface at 5 days post-infection.

Electron microscopy study

Investigation of *A. flavus*-infected pupae (since it was showing the best fungal infection features in the study) with scanning electron microscope 7 days post-infection showed heavy infestation with fungal hyphae (Fig. 2A). Fungal hyphae were covering most of the cuticular surface and were having fully developed conidia within 7 days post-pupal infection (Fig. 2B & C). Higher magnification (at × 2800) showed numerous numbers of mature infective conidia trapped within the hyphae 10 days post-infection (Fig. 2D). Similar infection features were observed in *B. bassiana*-infected pupae. However, killed larvae were black in colour with poor external fungal growth at 5 days post-infection (Fig. 1 A1), and become fully covered with hyphae by 10 days-post-infection.

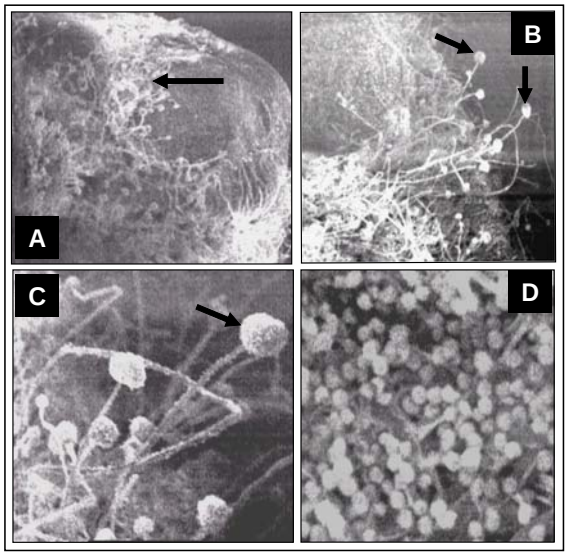

Fig. 2. Scanning electron microscopy sowing a part of *A. flavus*-infected pupa 7 days post-conidial infection. **A**: shows the posterior part of the larval cadaver covered with fungal hyphae (× 35). **B** & **C**: show fully grown conidial vesicle loaded with infective spores (× 58 & ×150 respectively). **D**: shows thousands of infective conidia (× 2800) trapped within the hyphae on the cuticle of the cadaver after falling down from their carrying vesicles 10 days post-infection.

Enzymatic and antibiosis activities

The hydrolytic activities of chitinase and protease of the four tested fungal isolates were demonstrated in agar plates. The four fungal isolates showed chitinolytic and proteolytic activities when grown in agar plates amended with the chitin and protein sources substances respectively (Table 2). *T. harzianum* T24 showed the highest chitinolytic but the low proteolitic activities compared to the other three isolates. *A. flavus* Link showed the highest proteolytic and moderate chitinolytic activities. *B. bassiana* IMI 382302 showed relatively high chitinolytic and low proteolytic activities, while *B. bassiana* IMI 386701 showed the lowest chitinolytic and moderate proteolytic activities (Table 2).

When *F. oxysporum* was cultivated in the same Petri dish previously seeded with any of the four tested fungal isolates, an inhibition in its daily

growth rate was observed (Table 3). Metabolites of the fungal isolates that produced in the agar culture did inhibit the growth of *F. oxysporum* with varying degrees. As shown in Table (3), growth inhibition of *F. oxysporum* by the volatile and non-volatile compounds of *A. flavus* Link reached to 20 and 100% respectively. As a comparison, this activity was weaker in case of the two strains of *B. bassiana*, but relatively high for *T. harzianum* (Table 3). On the other hand, the two *B. bassiana* strains showed the lowest inhibition activity. *T. harzianum* showed a moderate or weak inhibition activity by non-volatile or volatile compounds respectively (Table 3).

Antagonist	Clear zone (mm)[a] (mean ± SE)		n
	Chitinase[b]	Protease[c]	
***T. harzianum* (T24)**	6 ± 0.033	1 ± 0.011	4
***A. flavus* Link**	3 ± 0.013	3 ± 0.021	4
***B. bassiana* IMI 382302**	4.5 ± 0.02	1 ± 0.01	4
***B. bassiana* IMI 386701**	2.5 ± 0.012	2 ± 0.02	4

Table (2): Hydrolytic activities of extracellular chitinase and protease enzymes in agar-plates shown by the tested four fungal isolates.
a: Fungal isolates were incubated for three days, before measuring the degradation halos. **b**: 1.5% of colloidal chitin-agar plates. **c**: 3% milk-agar plates

Antagonist	% of inhibition in daily growth rate of *Fusarium*[a]		n
	Volatile compounds test	Non-volatile compounds test	
***T. harzianum* (T24)**	5.0 ± 0.02	50.0 ± 0.08	4
***A. flavus* Link**	20.0 ± 0.04	100.0 ± 0.01	4
***B. bassiana* IMI 382302**	0.0	10.0 ± 0.03	4
***B. bassiana* IMI 386701**	16.5 ± 0.03	25.0 ± 0.05	4

Table (3): 1. Effect of volatile and non-volatile metabolites produced by the tested four fungal isolates against *Fusarium oxysporum* mycelial growth (mm).
a: Colony diameter of the pathogen was measured at 1, 2, 3, and 4 days after incubation

Humoral anti-microbial activity

We focused on the humoral activity as one of the most effective immune weapons against microorganisms. This insect showed humoral anti-bacterial and anti-fungal responses when injected with bacteria and fungal spores respectively (Fig. 3). Humoral anti-*M. luteus* activity, as a result of injecting larvae with the same bacteria, was visualised by inhibition zone assay. This activity was strong and easily detectable as early as 2h post-injection which was equivalent to 316.64 ± 6.66 ng μl^{-1} haemolymph (n = 10) at 2h post-injection (Fig. 4). This activity peaked around 12h post-injection as it was equivalent to 503.3 ± 22.6 ng μl^{-1} haemolymph (n = 10) (Fig. 3A1 and 4). Then, become to decline sharply until it reached 296.66 ± 6.66 ng μl^{-1} haemolymph (n = 10) at 24h post-injection (Fig. 4) after which it become hardly detectable. Thus, this pest seems to respond quickly and strongly to bacterial infection. Control insect did not show any detectable humoral anti-bacterial responses.

Humoral anti-fungal activity was used as an indicator for whether or not cotton leaf worm strongly responds to fungal infection (injection with 5µl from stoke conidial suspension 1×10^6 ml^{-1}). Injection of larvae with the three concentrations (1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}) of conidial suspension of *B. bassiana* IMI 382302 did not show any detectable humoral anti-fungal activity. However, injection of larvae with the lowest conidial concentration (1×10^6) of *B. bassiana* IMI 386701 showed easily and clearly detectable humoral anti-fungal activity that was equivalent to 37.32 ± 7.78 ng μl^{-1} haemolymph (n = 10) at 12h post-injection (Fig. 3B1, B2 & B3 and Fig. 5A). This activity peaked at 36h post-injection which was equivalent to 303.3 ± 17.0 ng μl^{-1} haemolymph (n = 10) (Fig. 5A). Then, started to decline sharply until it become equivalent to 23.28 ± 6.68 ng μl^{-1} haemolymph (n = 10) (Fig. 5A) at 48h post-injection, after which, it become hardly detectable. The rest of conidial concentrations showed similar non-dose dependent activities (data not shown).

In addition, injection with the lowest conidial concentration (1×10^6) of *A. flavus* showed humoral anti-fungal activity (Fig. 3C) that was detectable at 12h post-injection which was equivalent to 46.7 ± 10.8 ng μl^{-1} haemolymph (n = 10) (Fig. 5B). This activity peaked at 36h post-injection which was equivalent to 323.3 ± 12.5 ng μl^{-1} haemolymph), and declined sharply at 48h post-injection (equivalent to 36.64 ± 8.18 ng μl^{-1} haemolymph) (Fig. 5B), after which, it became hardly detectable. The rest of conidial concentrations of this fungus showed similar non-dose dependent activity (data not shown). Moreover, injection with *T. harzianum* T24 showed a very week humoral activity only at 24h post-injection with 5 µl from stock conidial concentration of 1×10^6 or 1×10^7 conidia ng μl^{-1}, which was equivalent to 20.22 ± 5.60 or 28.81 ± 6.06 ng μl^{-1} haemolymph respectively. However, injection with the higher concentration (1×10^8 conidia ml^{-1}) did not show any humoral activity (Fig. 3D). This may indicate that the anti-fungal activity was very week against *T. harzianum* T24 and that the higher concentration could have suppressed this weak response of insect host. Control insect did not show any detectable humoral anti-fungal responses.

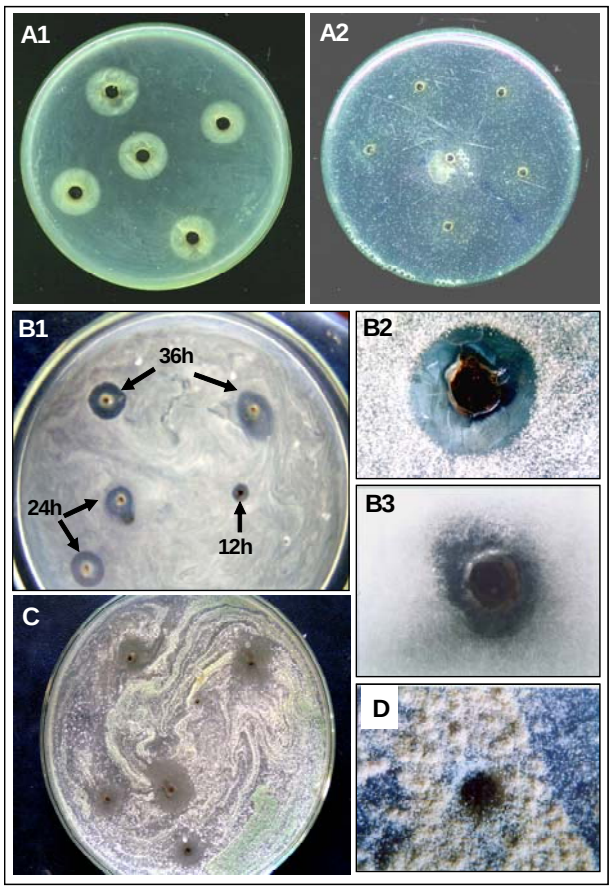

Fig. 3. Inhibition zone assay (IZA) showing humoral activities in the haemolymph of *S. littoralis* larvae against bacteria and fungi at different times post-injection.

A1: IZA showing humoral anti-bacterial activity against *M. luteus* at 12h post-injection with the same bacteria and after 12h incubation period at 25°C.

A2 control IZA against the same bacteria using haemolymph from control larvae (injected with ddH_2O) showing no humoral activity.

B1: IZA showing humoral anti-fungal activity against *B. bassiana* [IMI 386701 (Kenya/KIB West 1S)] at different hours post-injection with its conidial spores into larval haemocoels, and after 2 days incubation at 25°C. **B2** and **B3** show the same IZA after 5 and 10 days incubation period respectively showing the progress in hyphal growth everywhere except in the inhibition zone where anti-fungal peptides exist.

C: IZA showing humoral anti-fungal activity against *A. flavus* at 36h post-injection with its conidial spores into larval haemocoels, and 2 days incubation at 25°C.

D: IZA showing no humoral anti-fungal activity against *T. harzianum* T24 12h post-injection with its highest concentration (1×10^8 conidia/ml^{-1}) and after 5 days incubation at 25°C.

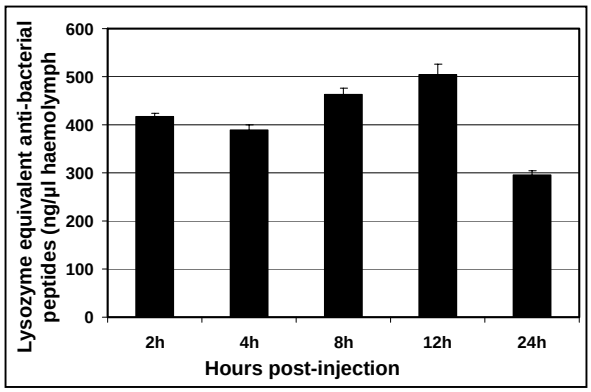

Fig. 4. A profile for the humoral anti-bacterial activity induced against *M. luteus* by *S. littoralis* larvae. Last larval instars were injected with 5µl *M. luteus* (5×10^3 bacteria ml^{-1}), then haemolymph was collected at different times post-injection and subjected to an inhibition zone assay. Error bares represent the means of 10 replicates (10 different samples from 10 different larvae) at each time point.

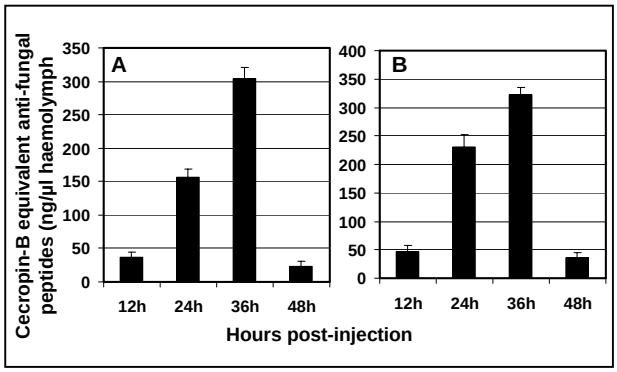

Fig. 5. Humoral anti-fungal activity induced against *B. bassiana* [IMI 386701 (Kenya/KIB West 1S)] (**A**) and *A. flavus* Link (**B**) by *S. littoralis*. Last larval instars were injected with 5 µl from the lower conidial concentration (1×10^6 conidia ml^{-1}), then haemolymph was collected at different times post-injection and subjected to an inhibition zone assay. Error bares represent means of 10 replicates (10 different samples from 10 different larvae) at each time point.

DISCUSSION

There is increasing interest in the use of entomopathogenic fungi for the biocontrol of insect pests (Hajek & St. Leger, 1994 and Evans, 1999). Hyphomycete fungal species, for example *B. bassiana*, were reported to infect insect pests from several orders (Leatherdale, 1970 and Zimmerman, 1993) and thereby play an important role in the regulation of pest populations (Smith *et al*., 1999). Four well known entomopathogenic fungi were tested against *S. littoralis* in the current study herein. The first is *B. bassiana* [two strains; IMI 382302 and IMI 386701] which has already showed a great potential for use as a biological control agent against wide

range of insect pests (Hicks *et al*., 2001). This fungus is a naturally occurring mitosporic and characterised by a wide arthropod host range, its conidia retain high viability and virulence for effective biological control (McClatchie *et al*., 1994) and have greater conidial mass-production potential (Wraight *et al*., 1998). However, in the current study, only strain IMI 382302 showed larval and pupal pathogenicity, while strain IMI 386701 did not.

The other two fungal isolates used in the current study were *A. flavus* and *T. harzianum* T24, both have also been reported as an important entomopathogenic against several insect pests (Glare *et al*., 1996 and Shakeri & Foster 2007). The preliminary virulence test of the current study showed larvicidal effect for *T. harzianum* T24 when applied at the higher conidial concentration (1×10^8 ml^{-1}). Although *A. flavus* has been isolated from died egg batches, it showed pathogenic effect on pupal stages only. This could be as a result of the highest antibiosis activity that induced against *F. oxysporum* comparing to the other isolates, which might constitute a key role for killing these silent stages. These findings may indicate that both *B. bassiana* IMI 382302, *T. harzianum* T24 and *A. flavus* have effective killing mechanism that enabled them to successfully grow on *Spodoptera* larvae and/or pupa, however, ***B. bassiana*** IMI 386701 lack this ability.

Catalyzing activity using specific enzymes is considered one of the main mechanisms of fungal infection to insect host. Evidence for this have been provided by Shakeri and Foster (2007) who reported production of protease (31 kDa) and chitinase (44 kDa) during the growth phase of *Trichoderma*. These proteases are believed to facilitate the penetration of fungal hyphae into the host tissue by degrading the protein linkages in the insect cuticle and/or the utilization of the host proteins for fungal nutrition (Pozo *et al*., 2004). This seems to be the same strategy undertaken by the virulent isolates of the current study as they produced chitinase and protease. Moreover, *Trichoderma* are known to produce a number of antibiotics, such as trichodermin, trichodermol, harzianum A, harzianolide and peptaibols (Claydon, *et al.,* 1991, Dickinson, *et al.,* 1995 and Hoell *et al*., 2005). These components are proved to be insecticidal when fed to larvae of *Tenebrio molitor* or when applied to the cuticle together with the serine protease (Shakeri and Foster 2007). Thus, they suggested these components as virulence factors involved in insect pathogenicity. We would therefore suggest that enzymatic and antibiotic activities detected from *Trichoderma* could have occurred by one or all of these compounds. Furthermore, extracellular subtilisin-like chymoelastase designated protease and other components are considered amongst the main pathogenicity determinants for entomopathogenic fungi (St. Ledger *et al*., 1987 and Castillo *et al*., 2000). Based on these findings, the fungal strains *T. harzianum* T24 and *B. bassiana* IMI 382302 used in the current study showed similar activity and may have adopted these strategies to kill the experimental larvae and pupae, while *B. bassiana* IMI 386701 could not. Thus the current study reported an overall entomopathogenic activity for the virulent isolates that may probably due to the combination of enzymatic, volatile and non-volatile antibiotic activities, which thought to be closely related in virulence factors for some entomopathogenic fungi and play a role in their mycoinsectisides against the target insects (Fan, *et al.,* 2007).

On the other hand, the non-pathogenicity of *A. flavus* to larvae and *B. bassiana* IMI 386701 (Kenya/KIB West 1S) to larvae and pupae may refer to two reasons. The first, their disability or poor production of all or some of the infection catalyzing enzymes i.e. lack one of the main infection mechanisms. The second is that they may have been recognised, and hence, attacked by the host immune system. Because all isolates of this study have the ability to produce hydrolytic enzymes, we strongly support the second suggestion since insects are known to induce different anti-fungal immune responses to avoid infection (Lemaitre *et al*., 1997) (Fig. 6). Thus the current study focused on the humoral anti-fungal activity and showed that this activity peaked around 36h post-*B. bassiana* IMI 386701 and *A. flavus* injection, and was very week against low concentrations of *T. harzianum* T24 conidial suspension. Although this activity reported in this study was slower compared to that against *M. luteus*, it was sufficient enough to inhibit the growth of these two particular immune-sensitive fungi. These results are in consistence with the findings of Bidochka *et al*., (1997) who reported that challenging of fifth instar larvae of tobacco hornworm, *Manduca sexta,* by injection with the yeast, *Saccharomyces cerevisiae*, resulted in the induction of anti-fungal 14- and 33-kDa peptides at 24h post-injection.

In addition, several studies suggested that the immune defense reactions in some insects may be under hormonal control and that the parasite can alter the hormonal system, and subsequently, modulate the host immune response (Nappi & Stoffalono, 1972; Nappi, 1975). Evidences for this have been provided by some studies which showed that the juvenile hormone (JH) is an immuno-modulator as they reported more pronounced immune responses against parasitoids and pathogens when JH declines to low or undetectable levels (Blumberg 1977 and Khafagi & Hegazi 2001). If this is the case with *Spodoptera* larvae, we would suggest that virulent isolates could have modulated the hormonal system to suppress immune responses. Basically, there are two available suggestions for invading the insect host immune system by fungi; the first suggestion is that the entomopathogenic fungi may produce certain

peptides rather than enzymes that are able to modify the host immune reactions. Evidences for this have provided by Von Wartburg & Traber, (1988) and Reeves, *et al*., (2004) who reported cyclosporine-A from the fungus *Tolypocladium inflatum* and gliotoxin from *Aspergillus fumigatus* respectively as immunosuppressors. Furthermore, destruxins from the fungus *Metarhizium anisopliae* exhibit insecticidal properties to *Choristoneura fumiferana* (Brousseau *et al*., 1996) and is being more broadly cytotoxic, ultrastructural alterative for haemocytes and modulates the multicellular defense mechanism of the insect host (Vey *et al*., 2002), and thus, it plays a key role in the evolution of the fungal infection (Kershaw *et al*., 1999).

The second suggestion is that, in addition to chitin and *β*-glucans, the major structural constituents (Cabib *et al*., 1988), fungal cell wall also contains the polysaccharides, α(1-3) glucans, and the glycoproteins, mannoproteins and galactomannoproteins, which may form antigenic surface layers that are recognized by insect immune system (San-Bias, 1982). Thus it has been reported that cell walls of virulent entomopathogenic fungi do not have these compounds when produced inside the insect host, and thus, escape from being recognized by the host immune system. This, in fact, has been proven by Hung and Boucias, (1992) who investigated that *in vivo* produced cells (hyphal bodies) of *B. bassiana* were freely circulating in *S. exigua* haemolymph and therefore appeared to evade recognition by the immune system. The same authors have provided an evidence for this when they reported the absence of the antigenically important galactomannan components as well as lack of chitin in *in vivo*-produced cells. Consequently, these cells escaped from recognition by the host immune system (Pendland *et al*., 1993). Thus, this might have happened with the virulent isolates of this study, however, *B. bassiana* IMI 386701 and *A. flavus* may lack these mechanisms since they have faced by strong antagonistic immune activity the by larval stage.

In conclusion, it is well established that, killing mechanism by fungus is complicated and involves a combination of enzymatic and antibiosis activities (Fig. 6). Basically, chitin [an abundant homopolymer of 1, 4-h-linked N-acetyl-d-glucosamine (GlcNAc)] is an important structural component of insect cuticle (Merzendorfer and Zimoch, 2003). Thus, chitinases are produced by the entomopathogenic fungi to degrade this protective structure (Herrera-Estrella and Chet, 1999). Therefore, entomopathogenic fungi kill their insect host by penetration of its cuticle *via* adopting a combination of enzymatic degradation, principally by proteases and chitinases and mechanical pressure by haustorium production (Jassim *et al*., 1990, Clarkson and Charnley, 1996 and Hoell *et al*., (2005). The current study may conclude that enzymatic activity and immune-suppression strategies may have been adopted by the virulent fungi in order to penetrate the cuticular barrier and avoid non-self recognition by the immune system of the experimental larvae. Furthermore, *B. bassiana* IMI 382302 and *T. harzianum* T24 have shown control-promise as mycoinsecticides against larval stage of *S. littoralis*. On the other hand, *B bassiana* IMI 386701 and *A. flavus* faced immune-restriction that blocked their capabilities to help in the battle against this pest. Finally, well-designed experiments for studying the fungal pathogenicity (on egg, larva and pupa), LC_{50} & LT_{50}, and suggesting an effective control program using *B. bassiana* IMI 382302 and *T. harzianum* T24 are still to be conducted in the future work.

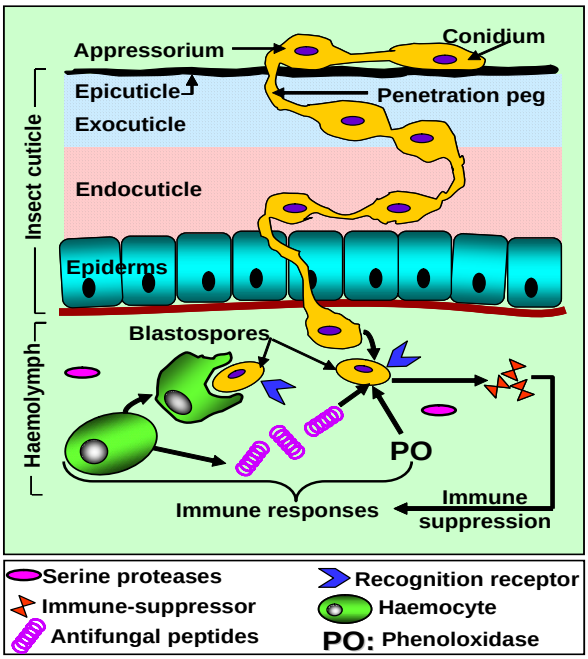

Fig. 6. A speculative illustration showing the mode of fungal penetration into insect host cuticle and the expected host immune responses against it. Conidium first lands upon the cuticle of the susceptible insect host and germinates to form appressorium that force a germ-tube (penetration peg) through the cuticle by both physical pressure and enzymic degradation of the cuticle. Eventually, it emerges into the haemocoel of the host, and consequently, grows inside the haemocoel as yeast-like blastospores, hyphal bodies or protoplasts. This may occur if the fungus has the ability to produce immune suppressors that block the host defensive immune responses, and consequently, kills the host by disrupting its physiological processes and/or consuming nutrients in the haemolymph (Samson, *et al*., 1988). Otherwise, the insect host will recognize the fungal blastospores and adopt an array of immune responses to block fungal growth. Of these, are the antifungal peptides, Drosomycin, metchnikowin & serine proteases which are the most effective weapons against fungi (Hetru *et al*.,1998), engulfing blastospores by haemocytes or induction of phenoloxidase cascade for melanizing fungal spores and as a result the internal body cavity of the host cadaver appears black (see Fig 1A) (Gillespie *et al*., 1997 and Marmaras *et al*., 1996 and Dean *et al*., 2004). Modified from Clarkson & Charnley (1996).

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