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Evaluation of *Aspergillus niger* as a Biocontrol Agent in the Insect Pest Management of Red Cotton Bug, *Dysdercus koenigii* (Heteroptera: Pyrrhocoridae)

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Abstract

The present work evaluates the efficacy of *Aspergillus niger* as a biocontrol agent against *Dysdercus koenigii***.** Three concentrations (C1:1 × 10^4, C2:1 × 10^5 and C3:1 × 10^6 spores/mL respectively) were applied topically to the arthrodial membrane of adult insects and virulence of the fungi in terms of mortality was obtained, which was highest (66.66%) after 96 h post infection with 1 × 10^6 spores/mL. Its effect on cellular immune response was investigated. The decline in hemocyte count was highest in C3 group treated, i.e. 6992.66±216.34, followed by other groups. Differential hemocyte counts showed that granulocytes and plasmatocytes were the most affected hemocytes with count 26.33±0.66, 24±1.15 respectively in "C3" treated group in comparison to control, 33.66±2.07, 39.66±0.88 respectively. The humoral response was also studied by assessing the activity of four important antioxidant enzymes (superoxide dismutase, catalase, cytochrome p450 and glutathione-S-transferase) and lipid peroxidation that showed significant changes. Based on mortality test we can say that even the sub-lethal concentration can pose significant threat on insect pest. Our results further revealed that *Aspergillus niger* compromises the immunological parameters of insects and help us understand the underlying mechanism of insect response towards pathogens.

Keywords: *Aspergillus niger*; *Dysdercus koenigii*; Superoxide dismutase; Catalase; Cytochrome p450; Glutathione-S-transferase.

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1. Introduction

Dysdercus koenigii (red cotton bug) is a notorious cotton pest. It is distributed in cotton growing region of India [1,2]. It is polyphagous in nature; feeds on different host plants like lady's finger, sambhal, hollyhock etc. Nymphs and adults feed on developing or mature cotton seeds [3]. In recent years, synthetic insecticides are used frequently to control this pest. However, rapid dispersal of nymphs and adults makes insecticide partially ineffective. Failure of insecticide and focus on lesser use of chemical insecticide

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in agriculture practices, propel the researchers to develop alternative ways to control the insect pests.

Implementation of microbes in pest management is a sustainable method. Organisms used in microbial control belong to several taxa namely bacteria, viruses, fungi, nematodes and protozoa [4]. Amongst all, more than 700 species of fungi are used against arthropods and insects [5]. Despite the broad range of entomopathogenic fungi, there are fewer mycoinsecticides available against *Dysdercus koenigii* [6]. Till date, fewer works have been reported regarding the importance of *Aspergillus* sp.; especially *A. niger* against *D. koenigii*. Consequently very little is known about the potential of entomopathogenic fungi to control *D. koenigii*.

Aspergillus niger is a deuteromycetes, generally asexual but perfect forms (sexually reproducing) of fungi falls within this family. Genus *Aspergilli* are cosmopolitan and can be easily found across a wide range of the habitats. They can grow on the stored grains, compost piles, dead and decaying substrates like leaves and vegetation as saprophytes [7]. Entomopathogenic fungi penetrate and enter through the insects' cuticle. There is immense relationship between the entomopathogens and the immune response of the insects, which needs to be explored in order to study the pathogenesis. To elucidate this relationship, one has to explore the mechanism by which hemolymph imparts the defense response against pathogens in insects. The active participation of the hemocytes in performing the vital activities against the pathogenesis reveals its role as milestones in understanding the immune defense against the pathogens. They are the crucial mediator of the cellular and humoral immune response.

Under the influence of fungal infestation, reactive oxygen species (ROS) levels may elevate drastically causing significant damage to cell structures. This phenomena is commonly called as oxidative stress [8,9]. Reactive oxygen species comprises of free radicals, e.g. superoxide anion radical (O_2^-), hydroxyl radical (OH) and hydroperoxyl radical (HO_2). They are highly reactive as well as unstable, which finally produce non-reactive species, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [10-14]. In order to defend ROS damage, the host cells activate different antioxidants, which for example, decrease protein damage and the level of lipid peroxidation [15,16]. The principal components of the antioxidant defense system of insects include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), cytochrome P450 (Cyt P450) and glutathione-S-transferase (GST) [15].

The insecticidal property of *A. niger* has been reported earlier against other insects like *Spodoptera littura, Aedes sp., Culex sp.* etc [17,18]. But for the first time *A. niger* was used against *D. koenigii* as biocontrol agent. The present work was aimed to evaluate *Aspergillus niger* as a biocontrol agent against *D. koenigii*. Lethal dose determination in terms of mortality as a result of infestation as well as the cellular response in terms of total and differential hemocytes count of circulating hemocytes was evaluated. Also, the activities of four principal antioxidant enzymes, SOD, CAT, Cyt P450 and GST, as an enzymatic antioxidant response system were assessed.

2. Materials and Methods

2.1. *Maintenance of generation of insect and fungus*

2.1.1. *Dysdercus koenigii*

Dysdercus koenigii was collected from Okra field in the Banaras Hindu University campus and agricultural fields abounding Varanasi city. Colony was maintained in BOD incubator and fed on the water-soaked cotton seeds under photoperiod of 16L: 8D at 25±2 ºC and relative humidity 70% - 80% [19].

2.1.2. *Aspergillus niger*

Aspergillus niger (MTCC no. 9652) was obtained from the culture collection of Microbial Type Culture Collection and Gene Bank (M.T.C.C.), Chandigarh, India. Stock cultures of the isolates were stored at -80 °C. *A. niger* culture was maintained according to Kulshrestha and Pathak [20] on sabouraud dextrose agar medium. Fungal cultures were incubated at 30 °C for **7** days. Stock cultures were stored on agar slants at 4°C until further use.

2.2. *Preparation of fungal inocula*

For fungal bioassays, conidia were collected by scraping the spore colonies and suspended in distilled water containing 0.5% Tween 80 [21]. The resulting spore suspensions were vortexed and centrifuged for 5 min at 2500 rpm. Spore count was determined by using a hemocytometer (C1:1 × 10^4, C2:1 × $10^5$and C3:1 × 10^6 spores/mL). Spore viability was examined before the experiments by placing droplets of a 1 × 10^6 spores/mL suspension on sabouraud dextrose agar medium. After 24 h of incubation at 30°C, spore germination was examined under light microscope.

2.3. *Fungal susceptibility tests*

Topical applications of 2 µL of groups of fungal spore suspension were given once on the arthrodial membrane of one day old adult *Dysdercus koenigii*. The control group of insect was treated with 0.5% tween 80. Mortality in treated and control groups were noted periodically. The dead insects were further kept in petri dishes having water soaked blotting paper and incubated for four-five days for observation**.** The insects covered with spores and mycelia were considered as the death due to fungal infection. Abbott's formula [22] was used to calculate the mortality percent.

$$Abbott's\ corrected\ mortality = \frac{\%\ mortality\ in\ treatment\ - \%\ mortality\ in\ control \times 100}{100 - \%\ mortality\ in\ control}$$

2.4. *Collection of hemolymph and smear preparation*

Hemolymph for smear preparation was taken out at 24-h of post treatment**.** It was collected by amputing the hind leg and antenna of the red cotton bug (control group and treated group). The membrane was first wiped with 70% ethanol, allowed to air dry. The hemolymph was dripped on glass slide, and then smeared. The slides were left for air drying and fixed in methanol for five minutes and stained with Giemsa for 30 min. The excess stain was removed and slides were rinsed with tap water for fifteen minutes. The hemocytes nucleus was dyed red or blue, while the cytoplasm was more transparent. Slides were observed and photographed under the Lieca DM2000 microscope.

2.5. *Determination of total hemocyte count (THC) and differential hemocyte count (DHC)*

2.5.1. *THC (Total hemocyte count)*

Hemolymph was diluted with Tauber-Yeager´s fluid [23] and then counted in four corners of WBC chamber (1mm²) of the improved Neubauer haemocytometer. The number of circulating hemocytes cubic millimeter (mm³) was calculated using the following formula of Jones [24].

$$= \frac{Hemocyte\ in\ four\ squares\ (1mm^2)\ \times Dilution \times depth\ factor\ of\ chamber}{No.of\ squares\ counted}$$

2.5.2. *DHC (Differential hemocyte count)*

It was made by counting at least 200 cells of different categories selected from random areas of stained smears of insect hemocytes and the percentage of different cell types was calculated.

2.6. *Sample preparation*

Hemolymph from adult *D. koenigii* was collected in a chilled, calibrated microcapillary through amputated leg and antenna. Hemolymph was centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatants were stored at -20 ºC till further analysis [19]**.**

2.7. *Enzyme activity assays*

2.7.1. *Lipid peroxidation*

Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage have been implicated in the pathogenesis of various diseases. Lipid peroxidation is assessed indirectly by the measurement of the secondary products, such as malondialdehyde (MDA). Lipid peroxidation would be measured by the method of Ohkhawa *et al*. [25].

2.7.2. *Superoxide dismutase (SOD)*

The SOD activity was measured according to Beauchamp & Fridovich [26] with slight modifications in a Systronic UV-VIS spectrophotometer 119. One unit of SOD activity is taken as the amount of enzyme required to inhibit the photo reduction of NBT by 50%.

2.7.3. *Catalase (CAT)*

Catalase activity was measured in a reaction mixture (1.5 mL) containing 50 mM phosphate buffer pH 6.8 (900 µL), 180 mM H_2O_2 (50 µL) and 10 µL of enzyme extract [27]. The decrease in absorbance was recorded at 240 nm in Systronic UV-VIS spectrophotometer 119. Catalase activity was calculated by using the extinction coefficient of 40 $M^{-1}cm^{-1}$ for H_2O_2 at 240 nm.

2.7.4. *Glutathione-S- transferase (GST)*

Reaction mixtures for assays contain 1.350 ml of 0.1 M phosphate buffer (pH 6.8), 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB), 1 mM GSH 75 µL, and 20 µL of sample. Absorbance were noted down for 5 min at 340 nm due to the formation of S-(2, 4-dinitrophenyl)-GSH ($\varepsilon = 9.6\ M^{-1}$) [28].

2.7.5. *P450 assay*

Cytochrome P450 activity was quantified by an indirect measurement of cytochrome P450 by using heme peroxidation [29] with slight modification. Absorbance was noted down at 630 nm. Standard curve for heme peroxidase activity was prepared using different concentrations of cytochrome C from horse heart. Cytochrome P450 (general oxidase) activity was expressed as equivalent units (EU) of cytochrome P450 per milligram of protein.

2.8. *Determination of protein concentration*

Protein concentration of hemolymph was estimated [30]. Bovine serum albumin was used to construct the calibration curve.

2.9. *Statistical analysis*

Probit analysis using POLO-PLUS software [31,32] was used for the determination of lethal concentrations. Survival analysis was done by Kaplan Meier survival curve by Graph pad Prism 7 software. Data were presented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by post hoc, Duncan multiple range test and significant levels were set at $p < 0.05$ using IBM SPSS Statistics for Windows (Version 16.0. Armonk, NY: IBM Corp.).

3. Results and Discussion

Consequences of the synthetic chemical insecticides had led us to rethink over this serious issue to say *bid adieu* to these insecticides by developing the application of the biological control agents in insect pest management. Fungi and their xenobiotics can be highly toxic to insects [33]. Therefore biological control with entomopathogenic fungi can thus be an effective and environmental friendly approach, which can be used to curb the insect population [34].

3.1. *Virulence of different sub-lethal concentration of entomopathogenic fungi, Aspergillus niger on Dysdercus koenigii*

In the present study, the fungal lethal concentration was determined by Probit analysis using POLO-PLUS software. Lethal concentrations were found as LC_{50}: 9.3×10^6, LC_{60}: 3.2×10^7, LC_{80}: 6.1×10^8, and LC_{90}: 5.4×10^9 spores per mL respectively. The three different sub lethal concentration (< LC_{50}) of *A. niger* (C1:1×10^4, C2:1×10^5 and C3:1×10^6 spores/mL) were tested against adult *D. koenigii* to unravel mortality and immunological response of the insect with regard to cellular response via circulating hemocytes and oxidative stress. Mortality was checked for every 24 h for next four days (Fig. 1).

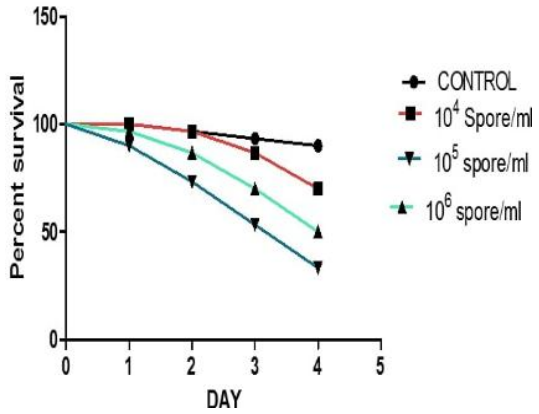

Fig. 1. Survival analysis of *Dysdercus koenigii* as response to exposure to *Aspergillus niger* by Kaplan–Meier survival curve by Graph pad Prism 7 software with log-rank analysis to examine the level of significance and $p < 0.05$.

Dead insects were collected and further placed in the petri dish containing water soaked blotting paper and suitable medium for providing moist conditions at 25±2 °C for next 2 days to check for symptoms of mycosis. The experiment was conducted in triplicate as per material and method.

The mortality rate increased with the increase in concentration of the fungal spores (Fig. 1). Highest mortality of the 66.66% was observed after 96 h of treatment with *A. niger* at the concentration of 1×10^6 spores/mL. In contrast to the fast acting neurotoxic

chemical insecticides, the fungal pathogens do not account for rapid mortality or any other immediate effects to the insects. Rather it may take some time to penetrate the cuticular barrier and proliferates inside the hemocoel [35]. Various species of *Aspergillus* viz. *A. flavus, A. leporis, A. nomius, A. ochraceus, A. sulphureus, A. tubingensis,* have been reported to possess the insecticidal activity against *Helicoverpa zea and Culex sp. and Anopheles sp.* [18, 36-39].

3.2. *Effect of fungal infection on total hemocyte and differential count of D. koenigii*

Treatment of sub-lethal concentration, C3:1 × 10^6 spores mL^{-1} showed the significant reduction in the total hemocyte count after 24 h of the treatment which is followed by the lower sub-lethal concentration. The total count was 11623.67±120.05 in the control group, whereas it was 10399.67±277.40, 8513±29.54, 6992.667±216.34 in the groups treated with different concentration, C1, C2 and C3 respectively (Table 1). This was further statistically analyzed by one way ANOVA (F= 120.102, df = 3, p = 0.05). Decline in hemocyte count might be due to the cytotoxic fungal activities which decrease defensive action of hemocytes so as to make the fungal infection successful. Similar results were reported by Kaur *et al*. [17] with decrease in hemocyte counts of *S. litura* after treatment with the fungus *Alternaria alternate* (Fr.) Keissl.

Our results clearly revealed the active participation of the primary hemocytes i.e., granulocytes and plasmatocytes, which showed significant decrease in their count. Both these cells are actively involved in the phagocytosis of the pathogen [40]. An increase in the mitotically divisible prohemocytes (precursor cells of hematopoiesis) count at the higher concentration of the treatment (Table 2).

Table 1. Effect of the sub-lethal concentration of *A. niger* on the total hemocytes count of adult *D. koenigii*. Data were presented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by post hoc, Duncan multiple range test ($p < 0.05$).

	CONTROL	INOCULATED GROUP (spores per mL)		
		C1: 1 ×10^4	C2: 1 × 10^5	C3: 1 × 10^6
Total hemocyte count	11623.67±120.05**a**	10399.67±277.40**b**	8513± 29.54**c**	6992.667±216.34**d**

The differential hemocyte count remained insignificant in the treated adults but they were showing significant difference with the control group (Table 2) in case of the granulocytes (F = 6.586, df = 3, p< 0.05). The plasmatocytes decreased significantly when compared to control at the higher concentration of the fungal spores after 24 h of treatment (F= 60.564, df = 3, p < 0.05).

There was increase in the count of prohemocytes at the higher concentration of C3: 1× 10^6 spores/mL followed by the decrease in the count within the treated groups. They were showing significant difference in comparison to the control (F = 21.225, df = 3, p < 0.05). Spherulocytes and oenocytoids were not showing any significant difference between the groups with F = 5.333 and F = 10.933 at df = 3 and p < 0.05 respectively.

Table 2. Effect of the sub-lethal concentration of *A. niger* on the differential hemocyte count of adult *D. koenigii*. Data were presented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by post hoc, Duncan multiple range test ($p < 0.05$).

	TYPES OF HEMOCYTES				
	GRs (%)	PLs (%)	SPs (%)	Oes (%)	Pro (%)
Control	33.66 ±2.07aB	39.66 ± 0.88aA	3 ± 0.57aD	4 ± 0.57aD	23 ± 0.57bC
C1: 1×10^4	29.66 ± 0.33bB	31.33 ± 0.66cA	1± 0cD	1 ± 0cD	19.33 ± 1.20cC
C2: 1×10^5	28 ± 1.15bB	35 ± 0.57bA	2.33 ± 0bD	2.66 ± 0.33bD	15.66 ± 1.20dC
C3: 1×10^6	26.33 ± 0.66cA	24 ± 1.15dC	1.66 ± 0.33cE	2.33 ± 0.33bD	25.33 ± 0.33aB

GRs: granulocytes; PLs: plasmatocytes; SPs: spherulocytes; Oes: oenocytoids; Pro: prohhemocytes.
Lowercase alphabets shows the difference between the groups and uppercase alphabets shows the difference within the group.

3.3. *Effect of fungal infection on lipid peroxidation*

With increasing infection, the humoral response of the insect immune defense gets provoked to minimize the level of generated oxidative stress. These symptoms of oxidative stress were the consequences of the change in the enzymatic activities, which are involved in the detoxification of the pathogens as well as the change in the lipid peroxidation of the hemolymph [41]. Changes in the MDA is a major oxidation product of the peroxidized polyunsaturated fatty acids in the process of lipid peroxidation. It can be used as the biological indicator of the oxidative stress in plants and animals [42,43]. Increase in the level of lipid peroxidation in hemolymph could be attributed to decrease in catalase activity that the cells secrete to fight the oxidative stress induced by the fungal infection. This is in contrast to the findings of Babu [44] where there was decreased level of lipid peroxidation in cockroaches on treatment with the conidia of *M. anisopliae* after various post incubation time.

The treatment with the three different sub-lethal concentration of *A. niger* caused significant difference in the level of the lipid peroxidation in the treated group as compared to the control with $F = 28.264$, $df = 15$, $p < 0.05$ (Fig. 2). The different incubation time after the treatment did not pose any difference in the level of lipid peroxidation.

3.4. *Effect of fungal infection on detoxifying enzyme activity*

The insect's response to detoxification of xenobiotics by metabolites or the host-tissue-degrading products of pathogens is demarcated by the changes in the detoxifying enzyme activities against the infections [45]. Several antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and cytochrome p450 constitute the major component of an insect's antioxidant defense system [15]. Reactive oxygen species are being scavenged by these antioxidant enzymes [46,47]. There was a gradual decrease in the activity of the detoxifying enzymes such as SOD, catalase and cytochrome P450 in the treated groups as compared to the control (Figs. 3-5). On the other hand, GST activity increased with the increasing days after treatment with respect to control (Fig. 6). SOD activity decreased significantly in the hemolymph of adult *D. koenigii* exposed to

different sub-lethal concentration of the *A. niger* as compared to control ($F = 90.223$, $df = 3$, $p < 0.05$) and the different groups also showed significant differences with the incubation time after the treatment also ($F = 32.835$, $df = 3$, $p < 0.05$). Similar studies have been reported in insect-fungal pathogenesis, where detoxifying enzymes play a crucial role in quashing ROS. Decline in activity of these enzymes compromises the defense mechanism in insects [46].

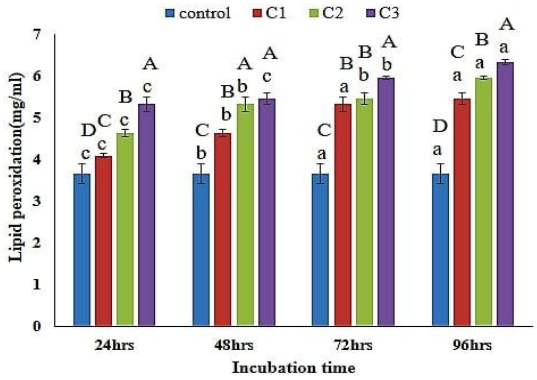

Fig. 2. Effect of sub-lethal concentration of *A. niger* on the lipid peroxidation of the hemolymph of adult *D. koenigii*. The values are Mean ± SEM (n=3). Data were analyzed by one way ANOVA followed by post hoc, Duncan multiple range test ($p < 0.05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other.

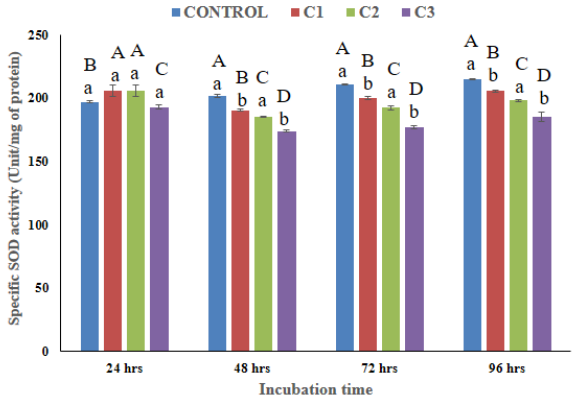

Fig. 3. Effect of sub-lethal concentration of *A. niger* on the superoxide dismutase activity of the hemolymph of adult *D. koenigii*. The values are Mean ± SEM (n=3). Data were analyzed by one way ANOVA followed by post hoc, Duncan multiple range test ($p < 0.05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other.

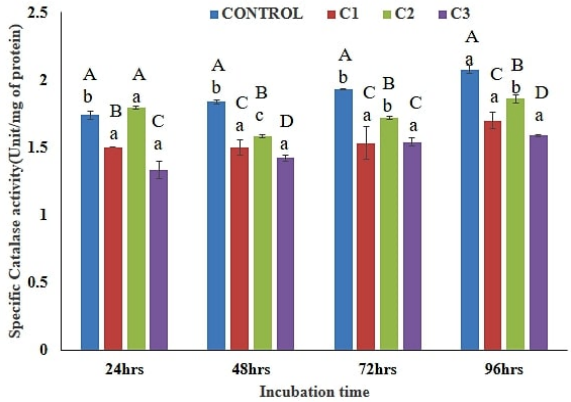

Fig. 4. Effect of sub-lethal concentration of *A. niger* on the catalase activity of the hemolymph of adult *D. koenigii*. The values are Mean ± SEM (n=3). Data were analyzed by one way ANOVA followed by post hoc, Duncan multiple range test ($p < 0.05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other.

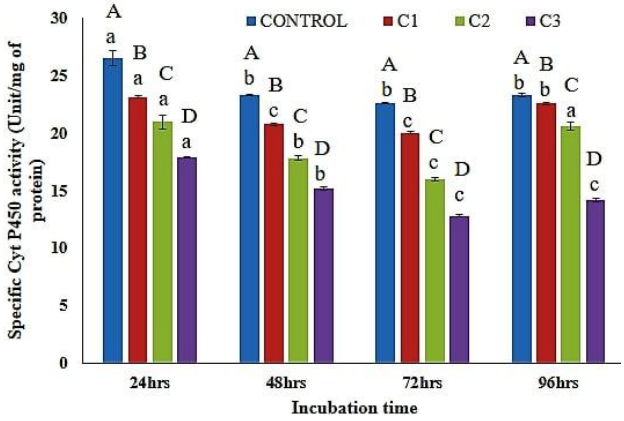

Fig. 5. Effect of sub-lethal concentration of *A. niger* on the activity of cytochrome p450 of the hemolymph of adult *D. koenigii*. The values are Mean ± SEM (n=3). Data were analyzed by one way ANOVA followed by post hoc, Duncan multiple range test ($p < 0.05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other.

Catalase and cytochrome P450 activity also decreased significantly in the treated groups as compared to control with $F = 76.235$, $df = 3$ and $F = 907.269$, $df = 3$ at $p < 0.05$ respectively while the different groups also showed significant differences with the incubation time after the treatment with $F = 22.526$, $df = 3$ and $F = 196.556$, $df = 3$ at $p < 0.05$ respectively. Decrease in the activity of CAT in the treated groups exposed to the different sub-lethal concentration of fungus may be due to the unbalanced generation of free radicals, which suppress the innate defense mechanism of the insect and also due to

the ineffective role of this enzyme in managing the oxidative stress induced by the fungus [48,49].

GST activity increased significantly in the treated groups as compared to the control ($F = 10.157$, $df = 3$, $p < 0.05$) and there is a significant difference between the incubation time ($F = 99.654$, $df = 3$, $p < 0.05$). Our results also suggest that the decline of detoxifying enzymes activity could hamper the defense mechanism, thereby reducing the capacity to scavenge the excess ROS. This, in turn deteriorate all cellular processes ultimately leading to insect death [15].

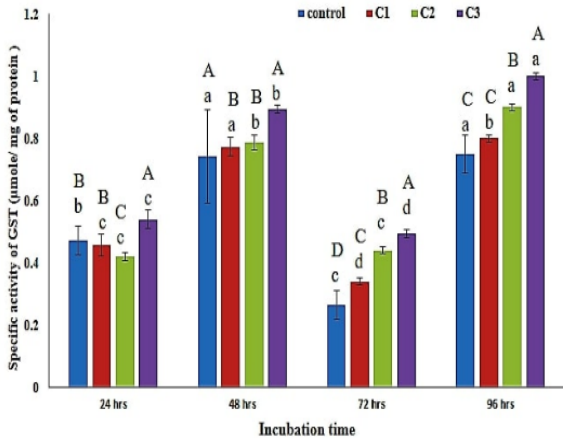

Fig. 6. Effect of *A. niger* on the activity of activity of glutathione-S- transferase in hemolymph of *D. koenigii*. The values are Mean ± SEM (n=3). Data were analyzed by one way ANOVA followed by post hoc, Duncan multiple range test ($p < 0.05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other.

4. Conclusion

A. niger affects *D. koenigii* by compromising its defense system. This also led us to know the underlying mechanism of the pathogenesis and the counter strategy by the host defense system. The bioassay results further suggest that *A. niger* can kill the host and thus proves its potential to be used as a biological control agent for management of *D. koenigii*. Further studies are also required to evaluate effect of fungal metabolites on host which may provide subsidiary information on potential of *A. niger* as biocontrol agent against *D. koenigii* and other insects.

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