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STUDIES ON THE SYNERGISM BETWEEN ENTOMOPATHOGENIC FUNGI, ASPERGILLUS NIGER AND INSECTICIDE, SPIRODICLOFEN IN *Dysdercus koenigii* (Heteroptera: Pyrrhocoridae)

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Abstract: Controlling insect pests, which continue to be agricultural, medical, and economic threats worldwide, requires constant human innovation. One promising pest control method is to simultaneously attack insects with both an insecticide and an insect pathogen. This “dual-attack” approach can result in much higher insect mortality than using either of these methods independently. *Dysdercus koenigii* is a serious pest of cotton and other plants of economic importance. We challenged *Dysdercus koenigii* with simultaneous doses of both the insecticide spirodiclofen and the fungal pathogen, *Aspergillus niger*. Our results showed synergistic and antagonistic effects on host mortality and enzyme activities. To elucidate the biochemical mechanisms that underlie detoxification and pathogen-immune responses in insects, we monitored the activities of 3 enzymes. After administration of insecticide and fungus, activities of glutathione-S-transferase (GST) decreased in the insect during the initial time period and activities of superoxide dismutase (SOD) and catalase (CAT) decreased at a later time period post treatment. Our study illuminates the biochemical mechanisms involved in insect immunity to xenobiotics and pathogens as well as the mechanisms by which these factors disrupt host homeostasis and induce death. We expect this knowledge to lead to more effective pest control.

Keywords: Synergistic, antagonistic effects, glutathione-S-transferase, superoxide dismutase and catalase

Introduction: *Dysdercus koenigii* is a well-known pest of cotton and other economically important plants. Most of the nymphal stages as well as adults feed on the seeds within the developing cotton bolls producing a stain, which reduces economic value of the cotton ^[1]. Although cultural procedure, sanitation and chemical control are used to decrease population outbreaks of the pest but it annually causes severe damages worldwide.

Integrated Pest Management (IPM) is a powerful tool for the pest management ^[2]. It bestows protection of beneficial insects, as well as suppression of secondary pest outbreaks, pest resurgence and pathogenesis. It incorporate various control methods in order to improve economic, epidemiology, and environmental outcomes. Furthermore, simultaneous attack of insecticide and entomopathogenic fungi is one of the encouraging pest control method. This integrated approach for pest control requires a

proper knowledge of physiological changes underlying the synergism between insecticides and entomopathogenic fungi.

Aspergillus niger is a “deuteromycetes,” It is a cosmopolitan fungus that propagate rapidly. Strains can be screened from many different sites; soil, decaying fruit, as well as surrounding environments. *Aspergillus* genus consist of 180 species, out of them 33 species are pathogenic ^[3-4].

Spirodiclofen is derivative of tetrionic acids and falls in the categories of ketoenols. It inhibit lipid biosynthesis via interfering acetyl-CoA-carboxylase activity ^[5]. It is not a neurotoxic compound, but its mode of action is similar to insect growth regulators (IGRs) that inhibits cell cycle of the insects. It is widely used against phytophagous mites and whiteflies ^[6-7].

Entomopathogenic fungi take prolong time to infest insect pest. Combination of insecticide and fungus can surpass this obstacle,

since the entomopathogenic fungi and insecticide often act synergistically to increase mortality and shorten the lifespan of insects [8, 9, 10].

Insects protect themselves against both insecticide and pathogen infestation via detoxifying and stress enzyme system [11, 12, 13, 14]. GST plays a crucial role in detoxification of toxin and defenses against ROS. GST conjugate RGS (reduced glutathione) to the electrophilic centers of insecticides [15-16]. Stress enzymes such as superoxide dismutase (SOD), catalase (CAT) [17] provide defences against pathogenic fungi and insecticides. These enzymes can be up-regulated in response to toxin material and that improved activities of these enzymes are linked with insecticide resistance and immune response in insects [18, 19, 20]. In this study, we have tried to unravel the effects of simultaneous *A. niger* infection and Spirodiclofen poisoning on different insect enzyme systems.

Materials and Methods

Insect Rearing: *Dysdercus koenigii* were collected from the fields having okra (Lady's finger) plants in the Banaras Hindu University campus and agricultural fields abounding Varanasi city. In the laboratory, insects were maintained in a BOD incubator (Narang Scientific, NSW 152) on wet cotton seeds (pre-soaked in water for 24 hrs) under long day (16L: 8D); photoperiod at $25 \pm 2^\circ\text{C}$ and relative humidity of 70% - 80% [21]. One day old adults were used for fungal inoculation.

Fungus and Synthetic Insecticide: The entomopathogenic fungus *Aspergillus niger* was cultured on potato dextrose agar yeast extract (PDAY) at $27 \pm 2^\circ\text{C}$ and $75 \pm 10\%$ RH for 14 days under constant light. Conidia were harvested from culture plates by scraping the surface of the PDAY with a sterile mounted needle and were then placed into plastic centrifuge tubes containing 0.1% Tween in sterile water. Aggregates were broken down by vortexing the solution. The spore concentration was determined using an improved Neubauer haemocytometer. Different dilutions of insecticide, spirodiclofen (Sigma- Aldrich Co., Austria) were prepared in distilled water. For experiment, the spore concentration of 2.5×10^7 spores/ml and 1.36 mg/L of Spirodiclofen was given to the adult insects independently and in combination of the two also.

Sample Preparation: Haemolymph from adult male and female (*D. koenigii*) was collected in a

chilled, calibrated microcapillary through amputated leg/ antenna. Haemolymph was centrifuged at 10,000 rpm for 10 minutes at 4°C . The supernatants was used for further analysis [22].

Protein Estimation: Protein estimation was done by the method of Lowry [23]. Bovine serum albumin was used as a standard. Absorbance was taken at 660 nm using UV-Vis spectrophotometer, Systronics.

Estimation of Superoxide Dismutase: The SOD activity was measured according to the method of Beauchamp and Fridovich, (1971), with slight modifications. 1.5 ml of the assay mixture consisted of 50 mM sodium phosphate buffer (pH 6.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and with 20 μl enzyme extract. Riboflavin was added in the last in the 15 W fluorescent lamps. After 15 min, reaction was stopped by switching-off the light and the sample were placed in the dark. The absorbance was taken at 560 in a UV-VIS spectrophotometer. A reaction mixture placed in the dark was taken as the control. The blank reaction mixture lacking enzyme developed the dark blue color while reaction mixture with enzyme having less or no color. One unit of SOD activity is taken as the amount of enzyme required to inhibit the photo reduction of NBT by 50%.

Estimation of Catalase: Catalase activity was measured in a reaction mixture (1.5ml) containing 50 mM phosphate buffer pH 6.8 (900 μl), 180 mM H_2O_2 (50 μl) and 10 μl of enzyme extract [24]. The decrease in absorbance was recorded at 240 nm in UV-VIS spectrophotometer. Catalase activity was calculated by using the extinction coefficient of 40 M⁻¹cm⁻¹ for H_2O_2 at 240 nm.

Estimation of Glutathione-S- Transferase: 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 (= 9.6 M^{-1}) (Habig et al 1974).

Statistical Analysis: Data were presented as mean \pm SEM (n=3). Statistical significance was analysed by two-way ANOVA followed by post hoc, Duncan multiple range test [25] using SPSS 16.0.

Results

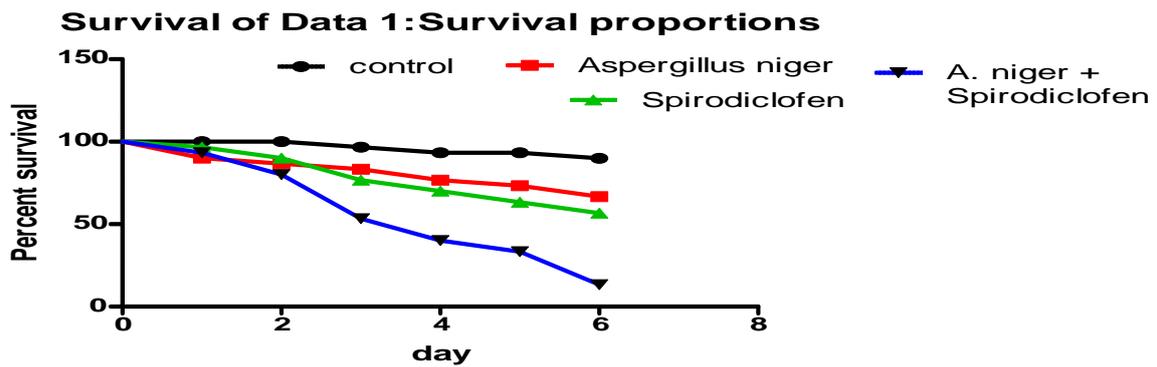


Fig1. Mortality in *Dysdercus koenigii* following 1-d exposure to *Aspergillus niger* followed by addition of Spirodiclofen by Kaplan–Meier survival curve by Graph pad Prism 7 software with log-rank analysis to examine the level of significance and $P < 0.05$

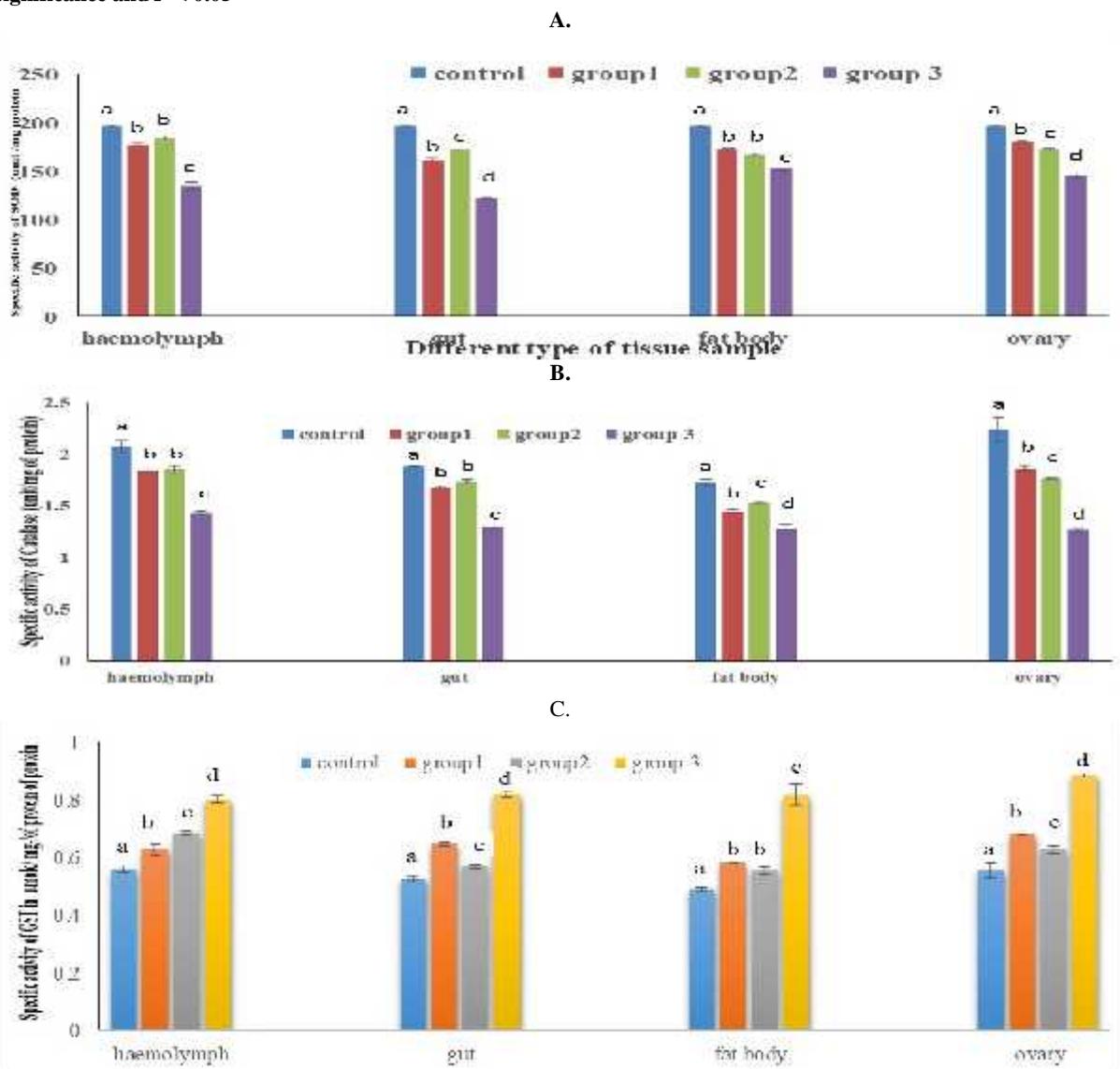


Fig.2 Effect of *A. niger* on the activity of antioxidant enzymes in haemolymph of *D. koenigii*. (A)SOD; B) Catalase; C) GST. The values are Mean \pm SEM (n=3). Data were analyzed by one way ANOVA ($P < .05$) followed by Duncken test ($p < .05$). The bars superscripted with different letters are significantly different from each other, and bars superscripted with same letters are not significantly different from each other. Group1: *A. niger*, Group 2 Spirodiclofen treated and Group 3 *A. niger* + 2 Spirodiclofen

The efficacy of spirodiclofen mixed with *A. niger* against *D. koenigii* can be seen in Fig 1 which shows the survival analysis of the adult insects in between the control and the treated groups. As it is clearly seen that the combination of the insecticide and entomopathogenic fungi results in the higher mortality rate in comparison to the control treated with Tween 80 and the groups 1 (*A. niger* treated) and 2 (Spirodiclofen treated).

The enzymatic study shows that the detoxifying enzymes, SOD and CAT shows decrease in its specific activity in post treated adults as compared to the control group. The combination of the insecticide and entomopathogenic fungi gives the significant increase in the enzyme activity and analyzed by two way ANOVA (df =9; f = 22.731 at p< 0.05) for SOD and for CAT (df =9; f = 6.346 at p< 0.05)

The activities of GST of different group were analysed as shown in Fig 2 C. When treated with Spirodiclofen and *A. niger* independently, the adult *D. koenigii* exhibited high GST activity in the different samples like haemolymph, gut, fat body and ovary in comparison to the control group. The combination of the insecticide and entomopathogenic fungi gives the significant increase in the enzyme activity and analysed by two way ANOVA (df = 9; f = 5.303 at p< 0.05)

Discussion

Successful incorporation of chemical and biological control is the need for the implementation of IPM programs and this approach is effective with only certain insecticides. Improved activities of detoxifying enzyme against fungal infection in insect's leads to detoxification of fungal metabolites or the tissue degradation products [26]. Hall has found that the entomo-pathogen debilitated the insect pest and so on lower down the insecticide tolerance. Furthermore insecticide in turn, weaken the insect resulting in the increased susceptibility to pathogen infection. Cooperation between fungi and insecticides are develop during a particular phase of the infection, such as conidial attachment germination and cuticular penetration [27].

Insect's antioxidant defense system contain stress enzymes; superoxide dismutase (SOD), catalase (CAT) [28]. Both enzymes eliminate harmful reactive oxygen species (ROS) [19]. ROS are continuously synthesized in the insect guts followed by pathogenic infections. ROS production and its removal is a constant and

necessary phenomenon in insects [29, 28]. *Oxya chinensis* display elevated SOD and CAT activities after phoxim, malathion and chlorpyrifos treatment [30-31]. Correspondingly in *Nilaparvata lugens* planthoppers, SOD and CAT activities were increased after chlorpyrifos selection [32].

Moreover, SOD activity improved in the midgut of *Galleria mellonella* followed by *Bacillus thuringiensis* infection, whereas CAT activity was lower down [14]. SOD and CAT are involved in stepwise ROS reduction. Enhanced SOD activity results in the elevate H₂O₂ level and H₂O₂ is further detoxified by CAT. Our results establish a theoretical basis for the interaction between *A. niger* and insecticides and future studies may provide the mechanism by which *A. niger* overcomes host immunity. We hope to use the information from this study to design better control methods for harmful insects.

Conclusion: Our findings based on the analysis of different enzyme systems and survival analysis of insects prove that effective combination of chemical and biological control might be a significant approach in implementation of integrated pest management (IPM) programs.

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