INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-4, Issue-1, Jan-Mar-2014
ISSN 2231-4490
Coden : IJPAES www.ijpaes.com

Received: 21st Dec-2013 *Revised: 23rd Dec-2013* *Accepted: 28th Dec-2013*
Research article

INSECTICIDAL ACTIVITY OF THE ENTOMOPATHOGENIC FUNGUS AND THE STRATEGY OF HORIZONTAL TRANSMISSION FOR CONTROL OF COCKROACHES

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ABSTRACT: Among several methods of application of entomopathogenic fungal conidia of *Metarhizium anisopliae*, injection method was proved to be reliable for generating mycosed cadavers of cockroach. Biochemical changes instigated by the infection of *M. anisopliae* on *Periplaneta Americana* were understood by injecting conidia at LC50 dose in to the haemolymph of the insects. Evaluation of antioxidant enzymes Catalase, peroxidases, ascorbate peroxidase and superoxide dismutase from 2nd to 10th hour post treatment revealed that the oxidative stress induced by infection of *M. anisopliae* was counteracted by insect's antioxidant defense system in a time dependant manner. Transmission of *M. anisopliae* among American cockroaches was tested by ingestion of mummified insect through cannibalism and by the method of surface contact with sporulated cadaver. Effective transmission was recorded through ingestion (84% mortality) compared to surface contact demonstrating 23% mortality. Spread of the lethal 'green muscardine disease caused by the fungus by horizontal transmission through cannabalism among *P. americana* colonies forms the first report.
Key words: LC50, *Metarhizium anisopliae,* Oxidative stress *Periplaneta americana,* Horizontal transmission.

INTRODUCTION

Metarhizium anisopliae (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), is a ubiquitous insect parasitic fungus [1]. The entomopathogenic fungus, *M. anisopliae* has been reported to infect more than two hundred species of insects belonging to different orders [2]. *Periplaneta americana* (Linnaeus) (Blattodea: Blattidae) is the well-known pest species and ubiquitous throughout the world. Cockroaches are implicated as vectors of several human disease agents and prefer warm temperatures around 29°C . Cockroaches are medically important as they have been associated with many illnesses and health problems [3] and carry viral and bacterial pathogens on their bodies and in their faeces which can cause poisoning, diarrhea and dysentery [4]. An important reason for the need to eliminate this vermin is that sensitization to cockroaches is associated with asthma [5]. German cockroaches have developed resistance to a wide range of insecticides including organochlorine, organophosphate, and pyrethroid insecticides (Jeffrey et al. 1990). Therefore, an imminent need to explore alternate management devices of this important pest [6,7]. To minimize the potential threats of ROS, the cells are equipped with numerous antioxidant defense systems to regulate and maintain low steady state levels of ROS and other radicals in the cell. The antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and Peroxidase (POX) form a part of the defense system [8]. Insects appear to rely on Ascorbate POX (APOX, E.C. 1.11.1.11) activity, which catalyses the oxidation of ascorbic acid with the concurrent reduction of hydrogen peroxide (H_2O_2) [9]. SOD dismutates superoxide anions directly [10], but in this process, potentially toxic H_2O_2 is generated. Among insect pathogens, transmission is usually direct and follows a passage from host to host or host to environment to host pathway. Transmission can be horizontal or vertical. Horizontal transmission is the transfer of the pathogen from individual but not directly from parent to offspring [11]. Direct transmission is when the pathogen is transferred from an infected to a susceptible host without the intervention of any living agent [12]. It has been noted that insect pathogens have a variety of mechanisms and adaptations to perpetuate themselves. A fundamental aspect of epizootiology is the transmission pathways adapted by these pathogens to ensure their survival [13] and the continual occurrence of a pathogen in a host population. In horizontal transmission, the normal route into the host is through the integument in fungi and nematodes. Oral transmission is by ingestion of food contaminated with the infective stage of pathogen or by predation or cannibalism of infected insects. Transmission of *Beauveria bassiana* and *Metarhizium anisopliae* among the populations of *Tetranychus kanzawai* mites was reported [14].

The present study was aimed at assessing the role of antioxidant enzymes in *P. americana* produced in response to infection by the high virulent isolate of the entomopathogenic fungus *M. anisopliae* in terms of profiles of Ascorbate peroxidase (APOX), POX, CAT and SOD which were estimated till 10th hour with 2 hour time interval after infecting with conidia at LC_{50} dose. We report for the first time the phenomenon of horizontal transmission of the green muscardine disease in *P. americana*, through cannibalism in particular.

Fungal culture and maintenance

From among a collection of 30 isolates, based on laboratory bioassays [15], *M. anisopliae sensu lato* [16] (ARSEF–1823), M20 isolated from *Nilaparvatha lugens* obtained from ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungi), Ithaca type culture collections was selected as high virulent.

The cultures were grown on Sabouraud's dextrose agar yeast extract medium ((SDAY) – 4% dextrose, 1% peptone, 1% yeast extract, 2% agar, pH 7.0), incubated at 25 ± 1° C. The 14 day old sporulated cultures were stored at 4° C. Virulence of the isolate was maintained by strain passage by infecting the cockroaches through spraying with conidial suspension of 1 x 10^8 conidia/ml. After 48 hours, the insects were washed with 0.1% Tween80 solution for removing the ungerminated conidia adhering to the insect cuticle. The treated insects after death were transferred to humidity chambers for inducing mycosis. The conidia harvested from the sporulated insects after the reisolation were used in the experiments. Germination assays of the conidia were conducted prior to experimentation using SDAY plates. All ingredients for preparation of the culture media were purchased from Merck (India) ltd.

Insects

Rearing of cockroaches was followed with slight modifications to the method reported [17]. *Periplaneta americana* (American Cockroach) adults were initially collected from their natural habitats and were transferred to wooden framed boxes of 30 x 30 cm with iron mesh on one side for aeration. Saw dust was placed at the bottom of the container for absorbing excess moisture. The cages were kept dry and the insects were fed hardened bread. An absorbent cotton squab soaked in water was kept in a bowl to serve as a source of water and moisture. The food was changed every two days and the containers were cleaned every 15 days. Once in a month the oothecae were removed from the saw dust and placed in separate containers and maintained as batches of 5. The oothecae were maintained in a wooden framed box with 15 x 15 cms dimensions with iron mesh on two sides for aeration and glass on the remaining. The bottom was lined with filter paper. The container was covered with wet cloth for retaining moisture and the temperature was maintained at 29±1 ^{0}C and RH at 60-65%. The nymphs that emerged from the oothecae were maintained as batches of 25 until maturity. The healthy adult roaches measuring 3.25 cm (±0.1) in length and 1.5 g (±0.1) in weight were selected one week before starting the bioassays. The bioassay set up comprising of the selected roaches in the wooden boxes were stationed in a growth chamber at 29 ±1°C with a 12 h L:12 h D photo-period.

Mode of treatment

The injection method [6] was adopted with slight modifications for treating the cockroaches among other methods of administering fungal conidia to *P. americana* [15]. Twenty micro liters (µl) of spore suspension at LC50 was injected in to the haemocoel of cockroach using a 1 ml disposable syringe with a 0.30 mm x 8.0 mm needle. The suspension was injected in to the ventral side of the roach body piercing through inter segmental region of the 5th and 6th segments [18]. The suspension was released gently so as to ensure effective spread of spore suspension into haemocoel of the insect. The control insects were maintained in two batches, each in triplicates. The dead hardened and mummified insects were kept in Petri dishes (90 mm X 15 mm diameter) lined with wet filter paper so as to provide moisture to promote mycosis and the Petri dishes were sealed with parafilm before keeping in the incubator at 25°C.

The dead and mummified cockroaches that were obtained by injecting fungal conidia were used as a source of inoculum to investigate the horizontal transmission of *Metarhizium* conidia among the cockroaches in a colony. The mummified cockroaches and healthy cockroaches were kept in a ratio of 1:5. In the second mode of transmission sporulated cadaver was mixed with healthy cockroaches (1:5). Triplicates were used for each trail and the experiments (trails) were repeated for 5 times. The mean of total deaths in all the trails was taken as average mortality obtained for that mode.

Scanning Electron Microscopy (SEM)

The mummified and sporulated cockroach cadavers obtained after injection with M20 were sampled for SEM study. The samples were fixed in 2.5% gluteraldehyde in 0.1 M Phosphate buffer (pH 7.2) for 24 hours at 4^{0}C and post fixed in 2% aqueous osmium tetroxide for 4 hours in the same buffer. After the fixation, samples were dehydrated in series of graded alcohols and dried to critical point drying with Electron Microscopy Science CPD unit. The dried samples were mounted over the stubs with double-sided carbon tape and applied a thin layer of gold coat over the samples by using an automated sputter coater (JOEL JFC-1600) for 3 minutes. The samples were scanned under scanning Electron Microscope (Model: JOEL-JSM 5600) at various magnifications [19].

Sample preparation

For qualitative assay of antioxidant enzymes, cockroaches at 2nd, 4th, 6th, 8th and 10th hour of post injection with conidia (LC50) from high virulent isolate M20 were sampled and homogenized in a small Chinese clay crucible using a glass, hand held homogenizer. The homogenization buffers for each assay were used as specified in the protocols. The enzyme activity was expressed in katals. One katal is the amount of enzyme that converts one mole of substrate per second. The total soluble protein levels in the enzyme extracts were determined according to [20] with bovine serum albumin as standard.

Antioxidant enzyme assays

Preparation of enzyme extracts

The treated and the control insects were homogenized (1 : 5 w/v) in 50 mM potassium phosphate buffer (pH 7.8) along with 1 mM EDTA for SOD activity determination and 50 mM potassium phosphate buffer (pH 7.0) with 1 mM EDTA and 10 mM dithiothreitol for CAT, POX and APOX assays. The homogenate was centrifuged at 12 000 g for 20 min at 4^0C and the supernatant was used to measure the qualitative and quantitative activity of the antioxidant enzymes.

Catalase

The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0), 19 mm H_2O_2 and 50 µg of protein in a final volume of 3 ml. The activity was determined by the oxidation of H_2O_2 at 240 nm (ε = 0.03941/mM). One unit is defined as the activity of the enzyme that catalysed the reduction of 1 mM of peroxide/min [21].

Peroxidase

The reaction mixture contained 50 mM phosphate buffer, 0.2 mM guaiacol, 10 mM H_2O_2 and distilled water in a total volume of 3 ml. The reaction was initiated by adding 50 µg of protein. The change in the absorbance of one unit per minute at 470 nm (extinction coefficient of 26.6/mm/cm) gave the activity of POX [22].

Ascorbate peroxidase

The reaction mixture for measuring APOX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mm ascorbic acid, 250 mM H_2O_2 and 50 µg of protein. The activity was recorded as the decrease in absorbance at 290 nm for 1 min. The amount of ascorbate oxidized was calculated from the extinction coefficient of 2.6/mm/cm [23].

Superoxide dismutase

The required cocktail for SOD activity estimation was prepared by mixing 27 ml of potassium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg/ml), 1 ml of Nitroblue Tetrazolium (NBT) (14.4 mg/10 ml), 0.75 ml of triton X-100 and 1.5 ml of 2 mm EDTA. To 1 ml of this cocktail, 10 µl of riboflavin (4.4 mg/ 100 ml) and 50 µg of protein were added into a cuvette. After mixing, the contents taken in a cuvette were illuminated for 8 min using three comptalux bulbs (100 W, Philips India Ltd., Kolkata). The temperature was maintained at 25^0C using a water bath. Protein sample kept in the dark served as blank, while the tube kept in the light without the enzyme as control. The absorbance was measured at 560 nm. NBT reduction under illumination was measured without the enzyme and with enzyme. The activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction with protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light [24].

RESULTS

In the antioxidant assay, APOX activity increased with increase in post treatment time from 2nd to 10th hour compared to control. There was an increase of 22% in APOX performance as early as 2nd hr post treatment and by 10th hr an increment up to 50%. Activity of POX revealed a decreasing trend till 6th hr and recorded an increase by 8th hr (23%) and 25% by 10th hr compared to control. CAT revealed a dynamic pattern in the activity till 8th hr where, there was 10% decrease by 2nd hr followed by an increase up to 145% compared to control. The activity of SOD initially increased by 10% at 2nd hr and decreased by 50% at 8th hr and subsequent increase of 5% by 10th hr post treatment (Table 1, Figure 1).

Table 1: The activities (% increase/decrease over control) of APOX, POX, CAT and SOD in the haemolymph of the household pest, Cockroach at 2 hour interval from 2nd to 10th hour post injection with LC50 of M20 isolate of *M. anisopliae* in comparison with the control.

	2nd hr	4th hr	6th hr	8th hr	10th hr
POX	-20 (±0.23)*	-17 (±0.058)*	-20 (±0.23)*	+23 (±0.12)*	+25 (±0.25)*
APOX	+22 (±0.18)*	+39 (±0.14)*	+43 (±0.18)*	+44 (±0.06)*	+51 (±0.12)*
CAT	-10 (±0.26)*	+145 (±0.31)*	-20 (±0.23)*	+100 (±0.08)*	+5 (±0.24)*
SOD	+10 (±0.20)*	-20 (±0.29)*	-47 (±0.13)*	-54 (±0.25)*	+5 (±0.27)*

+: %increase over control, -: %decrease over control, APOX: Ascorbate peroxidases, POX: Peroxidase, CAT: Catalase, SOD: Superoxide dismutase, *Mean of Standard error.

***Foot notes:** The ± values indicate the standard error means (SEM) from the triplicates of the activity values obtained for each enzyme at different time intervals.

With respect to horizontal transmission, single mummified (Figure 2*a*) but not yet mycosed and sporulated cockroach cadaver was kept along with healthy cockroaches in a container in the first experiment. Before 24 hours, the cadaver was consumed by the healthy ones which were subsequently succumbed to the fungal disease. The deaths started by 2nd day and continued till 8th day. 84% mortality was demonstrated in the cockroaches that ingested the cadaver of cockroach in view of the prevailing cannibalism in cockroach populations. LT50 values ranged between 4.2 to 5 days (Table 2). On the other hand, in the second experiment, when the mycosed/sporulated insect cadaver (Figure 2*b*), was used as a source of inoculum, the healthy cockroaches did not ingest the bait. However, mortality of cockroaches was recorded though in few number amounting to an average of 24% with LT50 values ranging between 25 to 33 days (Table 2). The deaths started by 5th day and continued till 8th day. In both the experiments no mortality was evident even after 10th day in controls and treated.

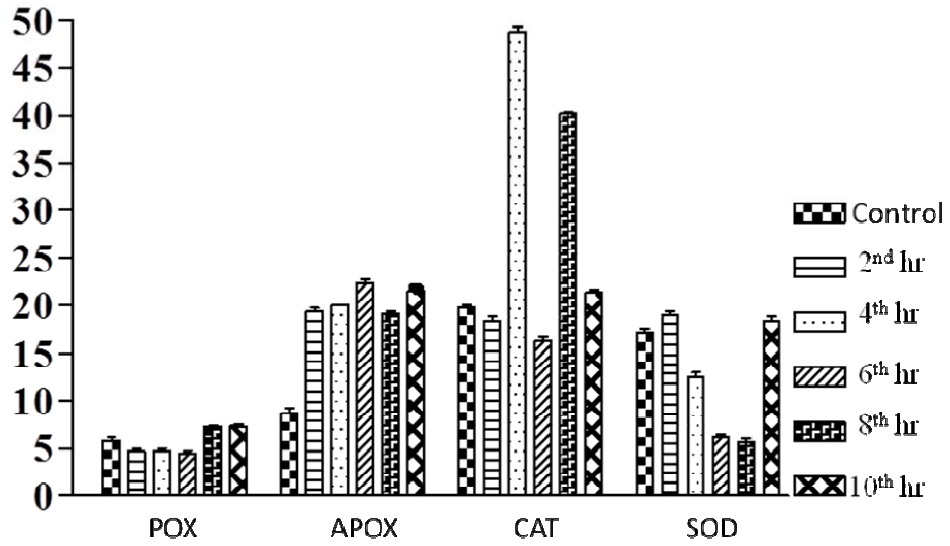
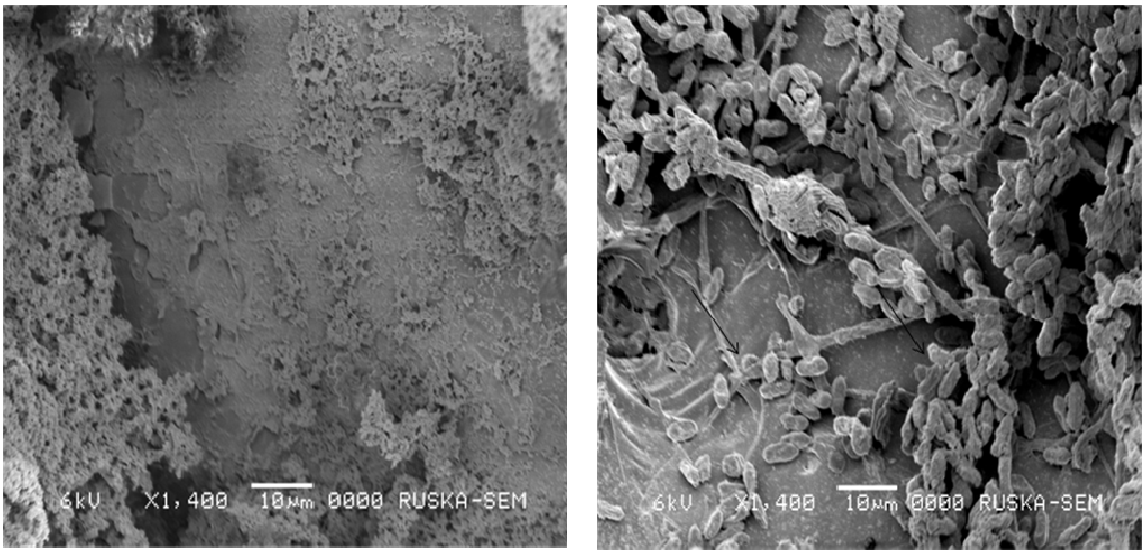


Fig 1: Activity (µg/min) of APOX (Ascorbate Peroxidase), POX (Peroxidase), CAT (Catalase) and SOD (Superoxide dismutase) from 2nd hr to 10th hr post treatment in the cockroaches injected with conidia from *M. anisopliae* isolate (M20).



a **b**

Fig. 2: a. Scanning Electron Micrograph of the abdomen of the mummified insect cadaver without mycelium and conidia of *M. anisopliae*. b. Scanning Electron Micrograph of the abdomen of the sporulated insect cadaver covered with mycelium and conidia of *M. anisopliae* (arrows indicate conidia and mycelium).

Table 2: Percent mortality, Percent mycosis and LT50 values obtained in experiments tested for transmission of *M. anisopliae* among *P. americana* populations.

	Trail No.	%MORTALITY# (SEM)	%MYCOSIS ψ(SEM)	LT50$ (FIDUCIAL LIMITS IN DAYS)	χ^2	Slope
Experiment 1^	1	79.7(±0.6)*	100	4.72 (4.12 – 5.42)	0.43	2.65 ± 0.32
	2	83.2(±0.3)*	100	4.81 (4.21 - 5.49)	1.94	2.76 ± 0.33
	3	86.5(±0.9)*	100	4.32 (3.91 – 5.00)	1.81	3.11 ± 0.34
	4	79.7(±0.9)*	100	5.09 (4.45 – 5.81)	0.39	2.69 ± 0.33
	5	89.8(±1.1)*	100	4.22 (3.74 - 4.76)	4.31	3.26 ± 0.35
Experiment 2$^{\alpha}$	1	23.1(±0.7)*	100	26.96 (11.70– 62.28)	2.27	1.58 ± 0.41
	2	26.4(±1.2)*	100	25.06 (11.81- 53.15)	4.17	1.77 ± 0.43
	3	23.2(±0.6)*	100	29.69 (12.12- 72.72)	4.45	1.63 ± 0.43
	4	19.8(±0.5)*	100	28.93 (11.96 - 69.98)	2.84	1.58 ± 0.42
	5	20(±0.6)*	100	33.60 (11.60- 97.34)	3.06	1.34 ± 0.40

^: Test for transmission of fungal infection using mummified cockroach cadaver as inoculum,
α: Test for transmission of fungal infection using sporulated cockroach cadaver as inoculum,
: Mean percent mortality observed by 10th day,
ψ: Percent no. of dead insects that sporulated.
$: Lethal time (in days) at which 50% deaths occurred 48 hrs post treatment,
*Mean of Standard error.

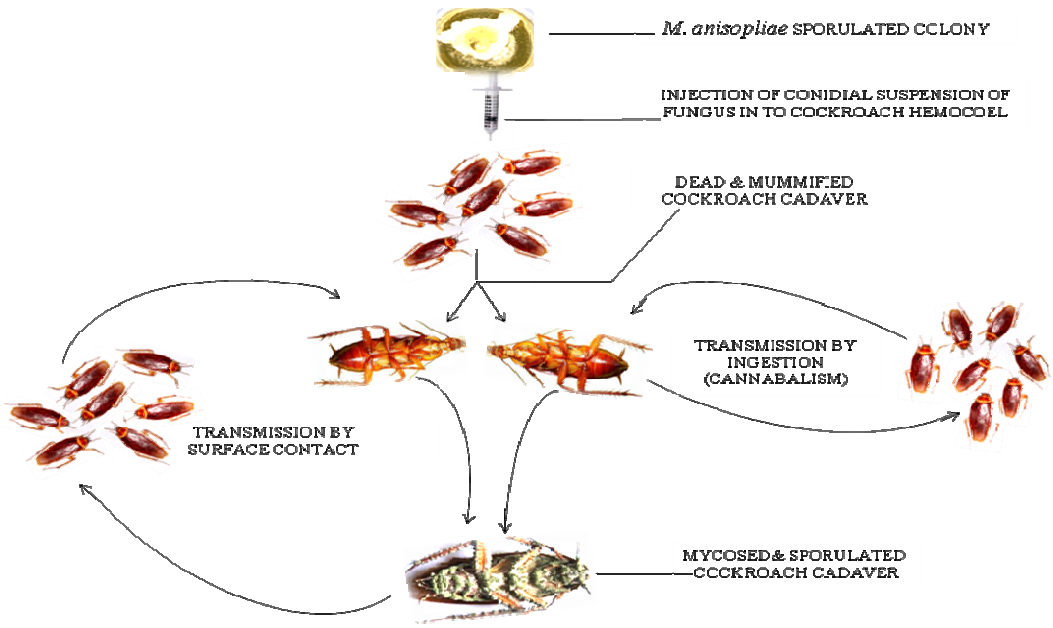


Fig. 3: Autodissemination of green muscardine disease in a cockroach colony

DISCUSSION

Increase in the activity of APOX in the insect body at 2 hour post treatment suggests the presence of an efficient ROS scavenging system at an early stage of the infection. However, with the advancement in time, the activity of the enzymes (CAT, POX, SOD) illustrated alterations till 10^{th} hr post treatment. Antioxidant defense was understood by the activity of these enzymes, more appropriately the specific APOX which accomplishes the removal of free radicals. Gradual increase in the activity with advancement of the post treatment time suggests steady participation of APOX in the detoxification mechanism of ROS and the key role of specific APOX in the removal of H_2O_2. Marginal differences in the activity of APOX and POX infer the absence or negligible activity of glutathione POX in the larval body [25]. POX appears to be contributing to the scavenging activity of ROS elicited during the infection and proliferation of the fungus which was visualized by elevated levels of POX at 8^{th} and 10^{th} hr.

Catalase, though displayed elevated levels, decrease in the activity of CAT with advancement in time implies ineffective role of this enzyme in managing the oxidative stress induced by the fungus. CAT and POX break down H_2O_2 to H_2O and O_2 and due to its sensitivity to $O_2^{\bullet}$ and can be inactivated at hiked levels [26]. Peak activity of CAT was recorded at 4^{th} hr, though there was steep decrease at 10^{th} hr indicating its retarded ability to encounter the increased oxidative stress prevailing at that hour in the insect. SOD displayed positive response as early as 2^{nd} hr post treatment but, could not sustain further suggesting increased oxidative stress. Increased levels of free radicals, as an indication of elevated oxidative stress was evident by reduced performance of SOD till 8^{th} hour. Decrease in the activity of SOD consequent to increased levels of H_2O_2 which inhibit SOD through the formation of excess hydroxyl radicals [27].

The phenomenon of auto dissemination through horizontal transmission of the infective propagules of *M. aniosopliae* s.l. (M20) causing green muscardine disease is an important attribute of the biocontrol agent. Similar instances of horizontal transmission and rapid spread of *M. anisopliae* infection was reported among German cockroach [28] and *Odontotermes obesus* [29]. In the present experiment, the possibility of horizontal transmission of the fungal infection among the American cockroaches was studied by providing the dead and mummified but unsporulated cockroach cadaver as food for the healthy cockroaches presuming cannibalism as is prevalent in the controls. The healthy cockroaches successfully ingested the mummified insect containing the fungal inoculums in the hemocoel of the bait as it was phenotypically not different from the inactive roaches and resulted in significant mortality among the healthy ones (Figure 3).

On the other hand, when a single mycosed/sporulated insect cadaver was used as a source of inoculum, though remained intact in the container even for 8 days, the healthy cockroaches did not ingest the bait. However, prevalence of auto dissemination of the conidia by topical contact was evident based on 24% insect mortality recorded.

Death of the host occurs shortly after inoculation and often without any sign of the fungus in the haemocoel, suggesting that toxins are secreted during very early stages of infection and that the pathogen colonizes its host as a nectrotroph [30, 31]. Activation of cockroach's antioxidant defense mechanism at early hours of infection with fungal conidia as observed in the present study is in accordance with the above interpretation.

The differential activity of antioxidant enzymes with advancement of post treatment time points to induction of oxidative stress at varied levels in the cockroaches. This study furnishes additional information on the role of antioxidant enzymes in arthropods in general and in American cockroaches in particular at an early stage of infection by *M. anisopliae*.

Administration of the biopesticide through ingestion mode was suggested for management of *P. Americana* [32] and injection method may be useful for effective spread of the infection among the cockroach colonies by horizontal transmission through cannibalism. The mummified cockroach cadaver in the initial stages could serve as an inoculum of fungal infection through ingestion mode by cannibalism and since cockroaches live in groups, the mummified cadaver will be available for more number of healthy insects as food. In the later stages, if not ingested in mummified form, the sporulated cadaver can serve as an inoculum infecting through surface contact.

ACKNOWLEDGEMENTS

The first author is grateful to the University Grants Commission, New Delhi, India for awarding RFSMS (Research Fellowship for Meritorious Students) fellowship. We are grateful to Dr. R. A. Humber, ARSEF culture collection, Ithaca for providing the fungal isolates.

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