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Research article

CHANGES IN THIOL CONTENT, GLUTATHIONE AND HYDROGEN PEROXIDE LEVELS AS A MEASURE OF OXIDATIVE STRESS IN *PERIPLANETA AMERICANA* TREATED WITH *METARHIZIUM ANISOPLIAE* SPP.

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ABSTRACT: *Periplaneta americana* (American cockroach) treated with conidia of entomopathogenic fugal isolates of *Metarhizium anisopliae* revealed a decrease in the thiol content and an increment in the levels of oxidized glutathione as well as hydrogen peroxide (H_2O_2). T-SH levels decreased to 48% and 50% at 24 hours and 48 hours post treatment respectively at LC90 of M20 isolate. Low virulent isolate M48, on the other hand, recorded a decrease of 76% and 78% in the T-SH levels at 24 and 48 hours post treatment respectively with LC30 treatment. Remarkable increase of 200% in the levels of H_2O_2 recorded at LC90 of M20 and 102% increase at LC30 of M19 was remarkable and focuses on the extent of oxidative stress induced by the fungal infection. Dynamics in the levels of GSH, GSSG, H_2O_2 and thiols in the insects treated with different fungal isolates in a time and dose dependant manner reveals oxidative stress induced by the fungal infection and the information would facilitate to explore the antioxidant defense system in augmented resistance or susceptibility of cockroach against entomopathogenic fungal conidia.
Key words: *Periplaneta americana, Metarhizium anisopliae,* Glutathione, Hydrogen peroxide, Total thiols.

INTRODUCTION

Metarhizium anisopliae, formerly known as *Entomophthora anisopliae,* now categorized under Ascomycetae, Clavicepitales, is an ubiquitous insect parasitic fungus and the causal organism of green muscardine disease. The entomopathogenic fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin has been reported to infect more than two hundred species of insects belonging to different orders (Zimmermann, 1993). Kershaw, *et al.,* (1999) have reported that, in the pathogenesis of *M. anisopliae* var *anisopliae*, there is a relationship between the titer of DTX production of isolates *in vitro* and the killing power. The action of dtx has been reported in different target organs in the larval body. This toxin was found to possess uncompetitive inhibitory effect on the hydrolytic activity of vacuolar-type ATPase in the brush border membrane vesicles of the midgut of *G. mellonella* larvae (Bandani, *et al.,* 2001). Catalase appears to be important in the prevention of excessive accumulation of cytotoxic H_2O_2 (Ahmad and Pardini, 1990). CAT and POX, more appropriately the specific APOX, act to remove ROS and cellular haemostasis is also regulated by non enzymatic antioxidants such as thiols, ascorbate and glutathione (Joanisse and Storey, 1996). Insecticidal activity of destruxin was examined in insects belonging to all insect orders (Pedras, *et al.,* 2002). The morphology and cytoskeleton alterations of plasmocytes observed in *M. anisopliae* infected larvae were predominantly caused by destruxins released by fungus during mycosis (Andreas Vilcinskas, 1997).

The detection, characterization and analysis of the role of reactive oxygen species (ROS) is well established in both normal and pathological processes of cellular metabolism. The antioxidant enzymes, such as superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6) and peroxidase (POX, E.C. 1.11.1.7) form a part of the defence system (Joanisse and Storey, 1996). 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 (Mathews, *et al.,* 1997). Reduced glutathione (GSH) is involved in both enzymatic and non enzymatic biological processes. Glutathione reacts non-enzymatically to maintain ascorbate in its reduced and functional forms. However, GSH also reacts rapidly and non-enzymatically with hydroxyl radical, the cytotoxic Fenton reaction product, as well as with N_2O_3 and peroxynitrite, cytotoxic compounds produced from the reaction of nitric oxide with O_2 and superoxide (Griffith, 1999).

Enzymatically, GSH acts as an electron donor to detoxify hydrogen peroxides and lipid peroxides in reactions catalyzed by multiple isoforms of GSH peroxidases. These reactions can lead to the production of GSH's oxidized form, glutathione disulfide (GSSG) (Griffith, 1999). Thiols defend cells from damage by hydroxyl radical ($OH^{\bullet-}$), nitroxyl radical ($NO^{\bullet-}$), and superoxide radical $O2^{\bullet-}$ (Udupi and Rice- Evans, 1992). The oxidation of SH-containing compounds results in a decrease in reduced SH-groups (RSH) and an increase in oxidated SHgroups (RSSR) (Wang, *et al.*, 2001). Hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot -}$), the ubiquitous products of single electron reductions of dioxygen, are amongst the most reactive compounds known to be produced during oxidative stress (Dietz, *et al*., 1999). To minimize the potential threats of ROS, the cells are equipped with numerous antioxidant defense systems. Their function is to maintain low steady state levels of ROS and other radicals in the cell, a process involving precise regulation of their location and amount. Cockroaches are insects of the order Blattaria with about 4,500 species, is widely distributed throughout the world. 30 species of cockroaches are associated with human habitations and about four species are well known as pests (Valles, *et al.,* 1999). Cockroaches are implicated as vectors of several human disease agents. *Periplaneta americana* (American Cockroach) is the well known pest species and ubiquitous throughout the world. The present study aims at studying the consequences of the infection of conidia from entomopathogenic fungal isolates of *M. anisopliae*, when subjected to injection in to the haemolymph of American cockroach, in terms of changes in the levels of antioxidants and free radicals as a measure of oxidative stress in the treated cockroaches.

MATERIALS AND METHODS

Fungal Culture and Maintenance

Metarhizium anisopliae isolates, M-20 (*M. anisopliae sensu alto*, ARSEF – 1823, isolated from *Nilaparvatha lugens*), M-48 (*M. anisopliae sensu lato,* ARSEF – 1882, isolated from *Tibraca limbativentres*), and M-19 (*M. anisopliae sensu stricto* (Bischoff, *et al*., 2009)*,* ARSEF – 1080, isolated from *Helicoverpa zea*), obtained from ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungi), Ithaca_culture collections. The microscopic cultures were grown on SDAY medium (Sabouraud dextrose Agar with yeast extract medium) – 4% dextrose, 1% peptone, 1% Yeast extract, 2% Agar, pH 7.0, incubation of slants at 25 $\pm 1^{o}$ C. The sporulated cultures seen with green colored powdery coating on the white mycelial mat were stored at 4° C. Virulence of the isolates was maintained through strain passage by infecting Cockroaches by spraying with conidial suspension. After 48 hours, the insects were washed with distilled water for removing the ungerminated conidia adhered to the insect body. The treated insects after death and mummification were transferred to humid chambers for promoting mycosis. The mycosed and sporulated insects were used to reisolate the fungus. The isolates at the first cycle after the reisolation were used for evaluation. Germination of the conidia tested on SDAY prior to experimentation revealed a range of 90% - 95% values for all the isolates used in the study. All the solvents used as medium components of the culture media were from Merk (India) ltd.

Experimental Insects

Rearing of cockroaches was followed with slight modifications to what was followed by Gilbert (1964). *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 assays were conducted on the house hold pest *Periplaneta americana* using three isolates (M20, M48 and M19) of *M. anisopliae*. Injection method as adopted by Gunnarsson and Lackie (1985) was followed with slight modifications for treating the cockroaches with fungal conidia.

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Twenty micro liters of conidial suspension at $5x10^7$ ($1x10^6$ conidia in 20 µl), $5x10^6$ ($1x10^5$ conidia in 20µl), $5x10^5$ ($1x10^4$ conidia in 20 µl), $5x10^4$ ($1x10^3$ conidia in 20 µl), $5x10^3$ ($1x10^2$ conidia in 20µl) conidia/ml was injected in to the haemocoel of the cockroach using a 1 ml disposable syringe holding a 0.30 x 8.0 mm needle on the ventral side of the roach body piercing through inter segmental region of 5th and 6th segments as was done by Andreas Vilcinskas (1997). The suspension was released gently so as to ensure effective spread of conidial suspension in to the haemocoel of the insect.
Three lethal concentrations LC30, LC50 and LC90 were selected to treat the cockroaches. The lethal concentrations were evaluated statistically based on bioassay data (Naren and Padmaja, 2013). The changes in the levels of thiols and glutathione were recorded at 1hour, 24hour and 48hour time interval post treatment. LC50 and LC90 for M19 isolate and LC90 for M48 isolate were too high to count with haemocytometer and hence were not included in the study. The control cockroaches were treated with 0.02% tween solution.

Measurement of Total, Protein-Bound and Nonprotein Thiol content

The treated and the control insects were homogenized in 0.02M EDTA under cold conditions. The total, protein-bound and non-protein thiol (NP-SH) content of the homogenates was measured using Ellman's reagent 5,50dithio bis (2-nitrobenzoic acid) (DTNB) following the method of Sedlak and Lindsay (1968).

Total thiols (T-SH): Aliquots of 0.5 ml of the homogenate were mixed with 1.5 ml of 0.2M Tris buffer (pH 8.2) and 0.1 ml of 0.01MDTNB. The mixture was brought to 10 ml with absolute methanol. The color was allowed to develop for 15 min. Absorbance of the clear supernatant was read at 412 nm (ε=13,100).

Non-protein thiols (NP-SH) and protein bound thiols (PB-SH):

Aliquots of 5ml of the homogenate were mixed with 4ml of distilled water and 1ml of 50% TCA. After 15 min the tubes were centrifuged at 10,000g for 15 min. About 2ml of the supernatant was mixed with 4ml of 0.4MTris buffer (pH 8.9), 0.1 ml of DTNB and absorbance was read within 5 min at 412nm against a reagent blank. The level of PB-SH was calculated by subtracting the level of NP-SH from T-SH.

Cellular glutathione content

The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were estimated fluorimetrically (Hissin and Hilif, 1976). The treated and control samples were ground in 1ml of 25% H_3PO_3 and 3ml of 0.1M sodium phosphate–EDTA buffer (pH 8.0). The homogenate was centrifuged at 10,000g for 20 min. The supernatant was used for the estimation of GSH and GSSG in a Hitachi spectroflourimeter, F-3010. The supernatant was further diluted five times with sodium phosphate– EDTA buffer (pH 8.0).

***Estimation of reduced glutathione (GSH)**:* The final assay mixture (2.0ml) contained 100 ml of the diluted supernatant, 1.8ml of phosphate–EDTA buffer and 100 ml of O-phthalaldehyde (1mg/ml). After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence at 420 nm was measured after excitation at 350 nm.

***Estimation of oxidized glutathione (GSSG)**:* An aliquot of 0.5 ml of the supernatant was incubated at room temperature with 200 ml of 0.04M N-ethylmaleimide for 30 min. To this mixture, 4.3 ml of 0.1N NaOH was added. A 100 ml portion of this mixture was taken for the measurement of GSSG, using the procedure outlined for GSH assay, except that 0.1N NaOH was used as the diluent rather than phosphate–EDTA buffer.

Measurement of Hydrogen peroxide (H_2O_2) levels

Hydrogen peroxide was extracted and estimated according to the method of Mukherjee and Choudhari (1983). The treated and the control insects were ground in ice-cold 100% acetone. The peroxide– titanium complex was precipitated by the addition of 5% (w/v) titanyl sulfate and concentrated NH_4OH solution to the extract. The precipitate thus obtained after centrifugation at 10,000g for 10 min was dissolved in 15 ml of 2M H_2SO_4 in a final volume of 20 ml made up with cold distilled water. The absorbance of the resultant was read at 415nm. The H_2O_2 content was calculated from a standard curve prepared in a similar way.

RESULTS

The cockroaches treated with conidia of *M. anisopliae* spp., when exposed for 1 hour, displayed a dose dependent increase in the T-SH levels and a decrease in PB-SH levels with increase in the dose from LC30 to LC50 and recorded an increase with LC90. Infection with high virulent isolate M20, at 24 hours and 48 hours post treatment, revealed decrease in the T-SH levels with increase in the lethal concentrations.

With LC50, there was 47% decrement in the levels of T-SH after 24 hours post treatment and at LC90, decrement of 48% and 50% in the T-SH at 24 hours and 48 hours post treatment respectively. The PB-SH levels showed a decrement from LC30 to LC50 and an increase at LC90. As the post treatment time progressed, the PB-SH levels showed a decrement from 1 hour to 24 hour and an increase by 48th hour post treatment with LC50 as well as LC90 treatments (Table 1). With LC30 the PB-SH levels showed a gradual decrease with increase in the post treatment time from 1 hour to 48th hour. The NP-SH levels in treatment with M20 displayed a gradual decrease from LC30 to LC50 in time and dose dependant manner and a similar trend was observed in the samples treated with low virulent isolate M48 (Table 1). The insects treated with M19, also high virulent, recorded a decrement in thiols (T-SH, PB-SH and NP-SH) with progression in the post treatment time. The PB-SH levels displayed a decrease of about 45% and 56% at 24th and 48th hour post treatment respectively in M19 infected insects, compared to that of the untreated ones (Table 1).

Table 1: Levels of total, protein-bound and non-protein thiols in the control and cockroaches treated with conidia of *Metarhizium anisopliae*.

Time of Exposure			M20			M19	M48	
		Control	LC30[a]	LC50[b]	LC90[c]	LC30[a]	LC30[a]	LC50[b]
1 hour	T-SH#	5.79	5.39	5.02	4.69	4.64	2.92	2.85
	NP-SH*	9.95	0.00011	8.43	0.0001	0.00011	4.91	2.7
	PB-SH^	4.16	5.38	3.41	4.68	4.63	1.99	0.15
24 hour	T-SH#	5.79	5.02	4.01	2.96	4.28	1.35	3.33
	NP-SH*	9.95	8.39	6.93	6.09	6.55	5.19	4.45
	PB-SH^	4.16	3.37	2.92	3.13	2.27	3.84	1.12
48 hour	T-SH#	5.79	3.58	3.03	2.85	3.51	1.22	1.79
	NP-SH*	9.95	6.18	6.01	7.03	5.34	1.32	1.18
	PB-SH^	4.16	2.6	2.98	4.18	1.83	0.1	0.61

[a] Lethal concentration at which 30% deaths occurred, [b] Lethal concentration at which 50% deaths occurred, [c] Lethal concentration at which 90% deaths occurred. #Total thiols, *Non- protein bound thiols, ^Protein bound thiols,

Low virulent isolate M48, with LC30, revealed a decrement of 78% in T-SH levels at 48th hour post treatment. PB-SH levels displayed a decrement of about 97% at 48th hour post treatment with LC30 compared to control. There was a prominent decrement of about 86% and 88% respectively at 48th hour post treatment that was recorded compared to that of the NP-SH levels in the control insects (Table 1).

The dynamics in the levels of GSH and GSSG revealed the induction of oxidative stress in the insects treated with the conidia of *M. anisopliae* isolates. In the treatments with high virulent isolate M20, the GSH levels recorded a decrease with increase in the conidial concentrations from LC30 to LC90 at 48th hour post treatment. At 24th hour post treatment, the GSH recorded a decrease from LC30 to LC50 and displayed an increase by 48th hour post treatment. There was an increment in GSH levels in the insects of about 13% and 3% over control at 24th and 48th hour post treatment respectively in the treatment with LC30. The levels of GSSG displayed a gradual increase of 23%, 41% and 66% at 1 hour, 24th hour and 48th hour post treatment respectively in with LC90 of M20 isolate (Fig. 1).

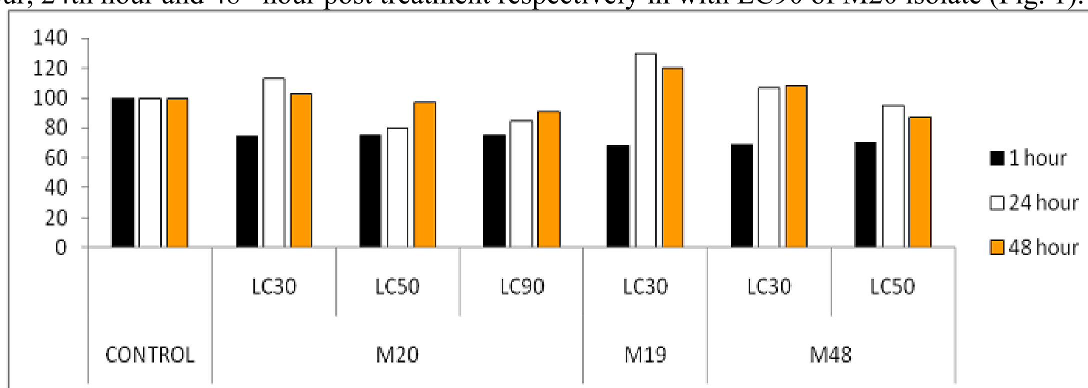

Fig 1: Graph showing the levels of Reduced Glutathione (GSH) (%over the control at 1hr, 24hr and 48hr post treatment in the cockroaches treated with conidia from *M. anisopliae* isolates.

In case of the treatment with the high virulent isolate M19, the GSH levels displayed an increase from 1hour post treatment to 24th hour and then decrease by 48th hour. An increment of 30% and 20% in the levels of GSH was recorded at 24th hour post treatment. For the same isolate, the GSSG levels displayed a decreasing trend with progress in the post treatment time. Low virulent isolate M48, at LC30 instigated an increment in the GSH levels of about 8% at 24th hour compared to control. At LC50, for the same isolate, the GSH levels displayed a decrease in their levels for all the time intervals tested (Fig 1). The GSSG levels, at 1 hour post treatment, recorded an increment of 12% and 19% that was recorded at LC30 and LC90 respectively. There was an increase in the levels of GSSH in all the treatments with entomopathogenic fungal isolates in the study except for LC50 of M48 where there is decrement of 0.5% over the control that was recorded (Fig. 2).

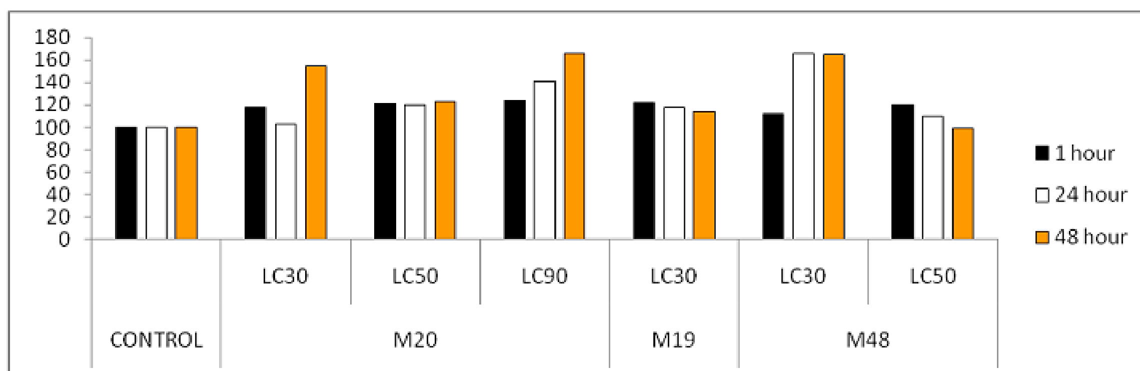

Fig 2: Graph showing the levels of Oxidized Glutathione (GSSG) (%over the control at 1hr, 24hr and 48hr post treatment in the cockroaches treated with conidia from *M. anisopliae* isolates.

The H_2O_2 levels recorded an increment of 44% over the control after 1 hour post treatment and a decrement in the levels with increase in the lethal concentration. At LC30, the insects displayed a decrease in the H_2O_2 levels with the advancement of time of exposure with the two high virulent isolates M20 and M19. But at LC50 and LC90 of M20, the levels increased from 1hour to 24th hour post treatment and then displayed a decrease by 48th hour (Table 2). On the contrary, the insects treated with LC30 of low virulent isolate M48 displayed a decrease in H_2O_2 levels from 1 hour to 24th hour post treatment and then recorded an increase by 48th hour post treatment. Prominent increase of 200% and 102% in the levels of H_2O_2 was recorded at 24th hour post treatment with M20 at LC90 and M19 at LC30 respectively (Table 2).

Table 2: Table showing the levels of Hydrogen peroxide (H_2O_2) in the treated cockroaches (% over control).

	M20			M19	M48	
TIME OF EXPOSURE	**LC30[a]**	**LC50[b]**	**LC90[c]**	**LC30[a]**	**LC30[a]**	**LC50[b]**
1 hour	+44	+4	-10	+106	+11	+19
24 hour	+42	+17	+200	+102	-38	+2
48 hour	+13	-25	+54	+54	+2	-61

a-Concentration that caused 30% deaths, b- Concentration that caused 50% deaths, c- Concentration that caused 90% deaths.

DISCUSSION

The present study investigated the biochemical effects in the cockroaches treated with fungal conidia. The evaluation of redox status shifts in terms of H_2O_2, levels of thiols and glutathione were evaluated. The extent of reduction in the thiol content in the treated cockroaches appear to be by and large corresponding to increase in the dose suggesting the increase in the induced oxidative stress with increase in the dosage of the fungal inoculums. Increased levels of lipid peroxidation and the thiols ratio (RSSR/RSH) are considered to be markers of oxidative stress (Wang, *et al*., 2001). A thiol group can stabilize protein structures by forming covalent disulfide bonds and can mediate cysteine-regulated redox reactions. At the same time, however, the high reactivity of thiol groups makes them also particularly vulnerable to nonspecific reactions during conditions of oxidative stress (Leichert, 2004).

The reduced tripeptide glutathione (GSH) is a principal non-protein sulfhydryl compound in living tissues. In addition to its role as a substrate for glutathione peroxidase and glutathione S transferase (GST), it can reactivate some of the enzymes inhibited under oxidized conditions and is a potential scavenger of hydroxyl radicals (Halliwell and Gutteridge, 1989). GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress. Lack of Se-dependent glutathione peroxidases (GPOXs) in insects increases the potential importance of the putative Se-independent peroxidase function of GSTs in antioxidant defence, with the principle function of reducing organic hydroperoxides within membranes and lipoproteins. It is also involved in the process of vitamin E regeneration (Reddy, *et al*., 1982).

In the present study, an increase in the levels of oxidized antioxidants, i.e., GSSG and a reduction in the thiol content of the treated cockroaches is an indication of the extent of oxidative stress induced by fungal infection in the cockroaches. As revealed in the present study, the increase in the GSSG in the treated cockroaches when compared to that of the control at the expense of their reduced forms and dynamics in the GSSG levels among the treated insects is an indication of the extent of oxidative stress induced variably by high and low virulent isolates. It has been reported that elevated GSH and GSTs in the resistant insects, with a predominant peroxidase function, attenuated the pyrethroid-induced lipid peroxidation and reduced mortality (Vontas, *et al*., 2001). GSH is a key component of the cellular defence against injury and lipid peroxidation damage and a co-factor for the activity of GSTs. These have a crucial role in the termination of free-radical cascades and the lipid-peroxidation chain reaction (Klimek, *et al*., 1998). Parkes, *et al*. (1993) showed that GSTs and GSH play a vital role in prevention (by conjugating reactive species and activated compounds) and / or repair (by detoxifying lipid peroxidation products and oxidized DNA bases) of oxidative damage in *Drosophila melanogaster*, and suggested their possible involvement in insecticide resistance. GSH is one of the most important antioxidants in cells. It is used to detoxify reactive oxygen species and reduce peroxides in the gut. So, the depletion of GSH concentration in the treated insects in the present study is by increased ROS and is a sign of induced oxidative stress due to the fungal infection. When tissues are exposed to increased oxidative stress, the GSSG: GSH ratio will increase as a consequence of GSSG accumulation. Thus the GSSG: GSH ratio is a good indicator of the redox state of glutathione because it reflects the levels of oxidative stress in the tissue (Cristina, 2011). Sowjanya Sree and Padmaja (2008), reported an increase in the levels of oxidized oxidants i.e., GSSG and a reduction in the levels of thiols and an increase in the levels of H_2O_2 in the 9 day old lepidopteran larvae *Spodoptera litura* is an indication of extent of the oxidative stress induced by the mycotoxin, destruxin.

The response of the antioxidant enzymes, viz. catalase, total peroxidase, ascorbate peroxidase and superoxide dismutase in cockroaches treated with LC30, LC50 and LC90 dose revealed an increase in the enzyme activity after 1 h of treatment and a reduction thereafter till LD90 after 48 hour of exposure (Naren and Padmaja, 2013). To defend the insect body from the deleterious effects of the hiked free radicals, the activity of antioxidant enzymes and the level of antioxidants increase. Beyond a certain limit of time and dosage of the infectious propagules of the entomopathogenic fungus in the present study, the unbalanced generation of free radicals may suppress the innate defense mechanism of the insect. In the present investigation, the cockroaches treated with fungal conidia displayed a hike in the generation of the H_2O_2 levels which is an indication of increased oxidative stress due to the fungal infection. The greater intensity of increase of H_2O_2 levels in the treatment with M20 and M19 isolates compared to those treated with isolate M48 reveals difference in the virulence of the isolates.

CONCLUSION

The dynamics in the levels of GSH, GSSG, H_2O_2 and thiols in the insects treated with the various fungal isolates in a time and dose dependant manner reveals the involvement of immune defense mechanism, against oxidative stress induced by the fungal infection, prevailing in the form of antioxidant defense system working variably at various doses of inoculum which was revealed by the increased levels of GSH compared to GSSG as well the dynamics in thiols in treated insects. The results of the present study, being reported for the first time, will be helpful in exploring the role of innate antioxidant defense system in augmented resistance or susceptibility against entomopathogenic fungal conidia and the mode of action of the same in the insect body.

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