CHAPTER – 4: ROUTE OF INFECTION AND HAEMATOLOGY

4.1. Introduction

Insects are infected when getting into contacted with fungal conidiospores (Stolz, 1999; Scholte *et al.,* 2004; Wang *et al*., 2005). After attaching to the insect's body (Bateman *et al.*, 1996), conidia penetrate the cuticle (Clarkson and Charnley, 1996; Charnley *et al.,* 1997; Wang and Leger, 2005) with the help of enzymatic degradation and pressure of the germ tube (Bateman, 1998; Ferron, 1981; Starnes *et al.*, 1993; Pirali-Kheirabadi *et al*., 2007). Similarly *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes), infect its host with spores that adhere and germinate to form a series of infection structures during penetration (Wang *et al*., 2002; Wang and Leger, 2005). Structures and processes for the invasion of insect tissues are similar to plant pathogens, including the formation of germ tubes, appresoria and penetration pegs (Samson *et al*., 1988). The infection may also take place via the respiratory system-spiracles (Burges and Hussey, 1971). The fungus germinates and penetrates into the respirical siphon, blocking the breathing mechanism (Lacey *et al.,* 1988) of the insects. Plugging of the spiracles usually leads to death before significant invasion of the haemocoel has occurred, so hyphal body formation is minimal. Invasion of the insect body and circulatory system (haemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton.

After infection, yeast-like hyphal bodies (blastospores) (Samson *et al.,* 1988) are produced and spread throughout the haemocoel (Ferron, 1981; Flexner *et al.*, 1986), take nutrients and lead to the death of the host by physiological starvation about 3–7 days after infection depending on species and their size (Boucias and Pendland, 1998; Stolz, 1999;

Pirali-Kheirabadi *et al*., 2007). However, comparatively little is known about the development of the fungus in the haemolymph, in particular assimilation of nutrients. On death of the insect host, the fungus emerges from the dead host and sporulation or conidiogenesis usually occurs on the outside of the cadaver (Whitten and Oakeshott, 1991; Starnes *et al.*, 1993; Shah and Pell, 2003), which can subsequently infect other susceptible hosts (Bateman *et al.*, 1996). Because the normal route of infection is through the cuticle, fungi are especially suitable microbial control agents for sucking insects of Hemiptera (Dolinski and Lacey, 2007).

Recently, Destruxins (dtx) A, B, D, E, and E-diol were identified from this fungi (Seger *et al.,* 2006) and was predicted that this might depress the cellular immune reaction. Saxena and Tikku (1990) performed studies to show deformity in haemocytes due to application of plumbagin, a plant-based phytochemical. Circulating haemocytes play important roles in defense mechanisms against microorganisms in the haemocoel. Cellular defenses refer to haemocyte-mediated responses such as phagocytosis, nodulation, and encapsulation (Schmidt *et al*., 2001). Histopathological studies of beetles tissues infected by *M. anisopliae* suggest that toxins like Destruxins A, B, D, E, and E-diol, Swainsinone, and Cytochalasin C kill the host by inciting degeneration of the host tissues due to loss of the structural integrity of membranes and then dehydration of cells by fluid loss (Ferron, 1981; Strasser *et al.,* 2000; Scholte *et al.,* 2004; Seger *et al.,* 2006). With these facts in mind, an experiment was conducted to examine the route of penetration and distribution of *M. anisopliae* at various parts of *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae). Moreover haemocyte count and haemolymph quantity were also performed.

4.2. Materials and Methods

4.2.1. Re-isolation of *Metarhizium anisopliae*

The insect cadavers that had fungal sporulation on the surface after 72 hours of treatment with 10^7 conidia/ml of *M. anisopliae* CPRC18 were considered for analysis to confirm that the death of the insects was due to the pathogenicity by *M. anisopliae*. The water washed dead insects were immersed individually thrice in 0.1% (v/v) sodium hypochloride solution, 0.1% (v/v) Tween 80 solution, 0.85% (w/v) sodium chloride solution separately and then rinsed finally in sterile distilled water with gentle agitation to remove non-attached fungi. The antennae, rostrum, head (after removal of antennae and rostrum), tergal plate, sternal plate, leg, haemolymph, muscle, fat body, foregut, midgut, hindgut and testis were individually dissected and separated aseptically. Each body part was ground separately using pestle and mortar along with 1ml distilled water. From each crushed suspension, 0.1ml was transferred using 100 µl sterile micropipettes, onto separate potato dextrose agar (PDA) plates and spread using a sterile, L-shaped glass rod. The body parts of the untreated insects were also plated individually as control. The plates were then incubated at 26°C ± 2°C in an incubator and observed for total fungal population after 7 days. After eminent growth was observed, the fungal colonies suspected to be *Metarhizium* spp. were sub-cultured to obtain pure cultures of the suspected entomopathogen.

4.2.2. Haemosomic index

Twenty *D. cingulatus* adults treated with CPRC18 isolate at concentrations of 2.8 x 10^5,3.1 x 10^6,1.9 x 10^7 and 2.3 x 10^8 conidia/ml were used in this study. Ten live adults were selected randomly from each concentration after 24, 48, 72 and 96 hours of

infection and their initial weights were recorded individually. Base of the antenna was amputated using fine sterile scissors and the oozing out haemolymph was collected in an eppendorf tube. The final weight of the individual insects was recorded after the haemolymph collection. Untreated control adults were also weighed before and after haemolymph collection. The haemosomic index was calculated using the following formula (Jones, 1962):

$$\text{Haemosomic index (\%)} = \frac{\text{Initial weight of the animal - Final weight of the animal}}{\text{Initial weight of the animal}} \times 100$$

4.2.3. Total haemocyte count

Haemolymph collected in the previous experiment was swabbed with 70% ethanol and allowed to air dry. In another experimentation, haemolymph was directly drawn into the WBC pipette up to 0.5 marking for the total haemocyte count (THC). Then it was diluted with an acidified physiological saline (NaCl - 4.65 g; KCl - 0.5 g; $CaCl_2$ - 0.11 g; Gentian violet - 0.005 g and acetic acid 0.125 ml and make up into 100 ml with double distilled water). The diluting fluid was drawn into the pipette up to mark 1.1 giving 20 times dilution. The inner surface of the WBC pipette was rinsed several times with diluting fluid before drawing the haemolymph. At least one minute of gentle stirring was required for complete dispersion of the cells to prevent agglutination and plasma clot formation. Neubauer haemocytometer was loaded immediately with the haemolymph taken in WBC pipette. The cells in all the four 1.0 mm squares were counted. The haemocyte population per mm^3 of haemolymph was calculated (Jones, 1962) as follows.

$$\text{Total Haemocyte count (cells/mm}^3\text{)} = \frac{\text{Haemocytes in four 1 mm}^3\text{ X depth factor X dilution factor}}{\text{Number of squares counted}}$$

Where, Depth factor = 10; Dilution factor = 20

THC was determined in 10 individuals from both control and *M. anisopliae* treated experimental *D. cingulatus* separately .

4.2.4. Enumeration of *M. anisopliae* in haemolymph

The haemolymph (0.1ml) from each concentration was transferred using 100 µl sterile micropipettes, onto separate potato dextrose agar plates and spread using a sterile, L-shaped glass rod for every 24 hours till 96 hours. The haemolymph of the untreated insects were also plated individually as control. The plates were then incubated at 26°C ± 2°C in an incubator and observed for total fungal population after 7 days. After eminent growth was observed, the fungal colonies that were suspected to be *M. anisopliae* were counted.

4.2.5. Cuticle mounting

1. *D. cingulatus* adults treated with CPRC16 were longitudinally opened on its lateral side.
2. The dissected abdomen was heated in 15% KOH over low flame until the cuticle appeared transparent.
3. Then it was fixed on clean glass slides.
4. It was spread evenly using a dissection needle to differentiate the upper and the lower cuticle using dissection microscope.

5. Cuticle was were mounted temporarily in a drop of 15% KOH and covered with a coverslip and examined using an inverted microscope (MOTIC M210, Motic China Group Co., Ltd. China) to observe the changes in the cuticle and also in the spiracles after *M. anisopliae* treatment.

4.2.6. Statistical analysis

Correlation analysis was performed for haemosomic indices at various time intervals and total haemocyte counts during different days after treatment of *D. cingulatus* adults was performed using STATISTICA/w 5.0. software.

4.3. Results

4.3.1. Re-isolation

Metarhizium anisopliae colonies in various parts of *Dysdercus cingulatus* adults after 72 hours is presented in figure 4.1. The fungal population in haemolymph was found to be maximum (13 colonies) followed by leg (8 colonies) and tergum (7 colonies). The antennae, rostrum and testis were free from *M. anisopliae* infection.

4.3.2. Haemosomic index

The haemosomic index (HSI) and percentage of haemolymph reduction of *D. cingulatus* adults is presented in table 4.1. The HSI decreased gradually as the period of incubation after treatment increased. Highest mean HSI was recorded in the control (20.28 ± 0.92), while in treated categories, maximum reduction in haemosomic index was observed after 96 hours of treatment at a concentration of 2.8×10^5 conidia/ml (4.53 ± 0.52). HSI was nil after 96 hours of *M. anisopliae* treatment (2.3×10^8 conidia/ml) which indicates the complete reduction of total haemolymph (Table 4.1). The percentage of total haemolymph reduction in *D. cingulatus* adults treated with *M. anisopliae* at different time

intervals reveals that least reduction (11.29%) of haemolymph was observed after 24 hours of treatment at 2.8 x 10^5 spores/ml. Haemolymph reduction was absolute (100.00 %) in the insects treated with 2.3x 10^8 conidia/ml after 96 hours of treatment. Highest correlation (0.99) was observed between HSI at 72 hours and 96 hours of treatment.

4.3.3. THC

The total haemocyte count (cells/ mm^3) in *D. cingulatus* adults at various time intervals after the treatment of *M. anisopliae* is shown in table 4.2. The haemocyte count decreased suddenly after 24 hours of treatment. However THC count increased to some extent after 48 hours followed by gradual decrease at 72 and 96 hours of treatment. Maximum number of haemocytes (11300 ± 93.0 cells/ mm^3) was observed after 96 hours of treatment in the control *D. cingulatus* adults. But in the case of treated adults, the count was highest after 48 hours (1387 ± 62.0 cells/ mm^3) at 2.8 x 10^5 conidia/ml. At 96 hours the count was least (223 ± 10.0 cells/ mm^3) at a concentration of 2.3 x 10^8 spores/ml. Highest percentage of total haemocyte count reduction was recorded in the *D. cingulatus* adults treated with *M. anisopliae* (99.12 %) at 2.3 x 10^8 spores/ml after 96 hours of treatment (Figure 4.2). Highest correlation (0.99) was observed among the THC at all the incubation periods except among 48 hours and 96 hours.

4.3.4. Enumeration of *M. anisopliae* in haemolymph

The total number of *M. anisopliae* colonies in haemolymph of *D. cingulatus* adults at different time intervals was differed (Figure 4.3). The *M. anisopliae* population in haemolymph was found to be maximum (63 colonies) at a concentration of 1.9 x 10^7 conidia/ml followed by 3.1 x 10^6 conidia/ml (48 colonies) after 96 hours of treatment.

4.3.5. Cuticle mounting

Whole mount of both the sternal (dorsal) and tergal (ventral) abdominal segments of *D. cingulatus* adults examined under an inverted microscope are shown in figure 4.4. The observations showed the following: conidia adherence to the host cuticle and germinate after 24 hrs post-infection (plate 4.1d), falling down of bristles was recorded in 24 hrs (plate 4.1d), the entry of spores through the spiracles (plate 4.2b), three sacs were recorded on the dorsal side of the abdomen (plate 4.3; 4.4; 4.5) and *M. anisopliae* were trapped at the base of the bristles (plate 4.1e) of sternum of the *D. cingulatus* (plate 4.1f). Within 24–48 h post-infection showed, trapping of the spores nearer to the spiracle and trachea (plate 4.6b, c and d). Moreover, colonization and emergence of the fungus to cuticular surface (plate 4.2d and e; 4.6c and d) within 72–96 hrs post-infection was also recorded.

4.4. Discussion

Conidial adhesion to host surface and integument penetration are the initial events during the pathogenic process (Askary *et al.,* 1999). McCauley *et al.* (1968) reported that infection sites varied within host species and most infections occur in the membranous inter-segmental regions. *Metarhizium anisopliae* conidia readily adhere to the surface of most insects (Arruda *et al*., 2005). *M. anisopliae* generally enters into the insects through spiracles and pores in the sense organs. Once inside the insect, the fungus produces a lateral extension of hyphae, which eventually proliferate and consume the internal contents of the insect. Hyphal growth continues until the insect is filled with mycelia. When the internal contents have been consumed, the fungus breaks through the cuticle and sporulates, which makes the insect appear "fuzzy." *M. anisopliae* can release spores

(conidia) under low humidity conditions (<50%). In addition, *M. anisopliae* can obtain nutrition from the lipids on the cuticle. The fungus can also produce secondary metabolites, such as destruxin, which have insecticidal properties on moth and fly larvae (Cloyd, 2005; Purwar and Sachan, 2006). The observations of the present study also revealed that *M. anisopliae* conidia adhered to all the body parts of *D. cingulatus* (except antennae, rostrum and testis). However, the degree of preference was maximum at the legs. Similar findings were also reported by Vestergaard *et al.* (1999). They reported that *M. anisopliae* conidia were capable to bind to cuticle of adult *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), but were frequently trapped by the setae on the wings and legs. Other studies with *M. anisopliae* (Goettel *et al.*, 1989; Sosa-Gomez *et al.,* 1997) showed that the cuticle regions of *Nezara viridula* (L.) (Hemiptera: Pentatomidae) contain a large number of setae where conidial adhesion was maximum. Polar *et al.* (2005) have indicated that oil formulations can be more effective than water-based formulations. In the present investigation also the use of castor oil might have lead to the attachment of *M. anisopliae* to *D.cingulatus.* A high propensity for attaching to the cuticle of insects may increase infectivity (Jeffs and Khachatourians, 1997; Altre *et al.,* 1999).

In the presence of nutrients and water, conidia of *M. anisopliae* form germ tubes. The germ tube continues undifferentiated hyphal growth on a soft surface or if nutrient quality and quantity is not conducive to differentiation. On a host cuticle, however, apical elongation terminates and the germ tube swells distally to form an appressorium, a major site of adhesion and for production of enzymes that help breach host cuticle and establish a nutritional relationship with the host (Wang and Leger, 2005). The entomopathogenic

fungus *M. anisopliae,* produces variety of hydrolytic enzymes such as proteases (Campos *et al*., 2005), chitinases and lipases on the host cuticle during the infection process (Clarkson *et al*., 1998; Krieger de Moraes *et al*., 2003; Pinto *et al*., 1997; St. Leger *et al*., 1987, 1996a, b; Tiago *et al*., 2002). *M. anisopliae* produce a complex mixture of chitinolytic enzymes during the growth on insect cuticle (St. Leger *et al*., 1996b). Since many such enzymes have been detected and isolated from different strains of *M. anisopliae*, the fungus is considered as a model organism for the study of genes and mechanisms involved in insect pathogenesis (St. Leger *et al*., 1996a, b; Leal *et al*., 1997; Bidochka *et al*., 2001). Previous work showed that *M. anisopliae* infects Elateridae larvae (McCauley *et al.*, 1968) and the conidia germinated within 24–48 hrs after post-infection. In the current investigation, conidial germination was observed on the surface of the host within a period of 24–48 hrs. However, other reports reveal that *M. anisopliae* conidia germinated within 12–18 hrs in *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) (Gunnarson, 1988), 24 hrs in *F. occidentalis* (Vestergaard *et al.*, 1999), 40 hrs in *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) (St. Leger *et al*., 1996a and b; Vestergaard *et al.,* 1999). Vestergaard *et al.* (1999) studied the infection of *F. occidentalis* by *M. anisopliae* and reported that colonisation was observed after 72 h post-infection. But in the present work, colonisation of *M. anisopliae* in trachea was observed after 24 h might indicate that rapid colonisation of host would lead to quicker mortality of the host.

The microscopic examination revealed that the route of infection of *D. cingulatus* by *M. anisopliae* was through the spiracles and root of the bristles since spores of the fungi were observed on them. Spores nearer to the spiracle and also at different areas of

trachea suggest that this fungus could enter into the host through the respiratory tract. This result corroborates with Burges and Hussey (1971) who suggested that insect infection due to fungi might also take place via the respiratory system. Several authors (Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Vilcinskas and Götz, 1999; Kurtz and Franz, 2003; Seger *et al.,* 2006) have reported that entomopathogenic fungi are characterized by their ability to replicate internally after entering into the insect, either by penetrating the cuticle using a combination of physical forces and the secretion of enzymes, or in some cases by entering the haemocoel through the insect gut after ingestion. Our study reveled that there is no possibility of entry of *M. anisopliae* into *D. cingulatus* through the food because cotton seeds soaked in sterile water were provided for *D. cingulatus*.

In the current work, the entry of *M. anisopliae* into host was also observed through dorsal sac1, sac2, and sac3 of *D. cingulatus* which might be another possible route of infection. Once it enters into the body, the growth of *M. anisopliae* may be confined largely to the haemolymph prior to host death (Clarkson and Charnley 1996; Charnley *et al.,* 1997). Comparatively little is known about the development of the fungus in the haemolymph. Samson *et al.* (1988) reported that the invasion of the insect body and circulatory system (haemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton. The observation of *M. anisopliae* colonies in the haemolymph in this study suggests that the haemolymph of *D. cingulatus* could enhance the multiplication of *M. anisopliae*. Peveling and Demba (1997) have also recorded yeast-like cells found in the haemolymph of hosts *in vivo* when treated with *M. anisopliae.*

After entering into the haemocoel, *M. anisopliae* normally kills the host within few days, due to tissue penetration and nutrient depletion (Boucias and Pendland, 1998) leads to physiological starvation (Stolz, 1999). The present study also suggests that the death of the host might be due to nutrient depletion in the host after treatment with *M. anisopliae* by the reduction in the quantity of haemolymph of *D.cingulatus.* Hassan *et al.* (1989) showed that germination of *M. anisopliae* is initiated by water but progress to the first overt stage of germination (swelling) is depended on an exogenous nutrient. Within the haemocoel the main cellular response of the insect is a multi-haemocytic encapsulation of the fungal element following initial recognition of the fungus by the haemocytes (Huxham *et al.,* 1989).

Best and common bio-assay method of invertebrate immunity is blood-cell counts (Carton and Nappi, 2001; Kurtz and Franz, 2003). The current investigation highlights that immune response of *D. cingulatus* could be disturbed due to treatment of *M. anisopliae* since there were significant reductions in the total haemocyte counts in *D. cingulatus*. The results also reveal that the immunity of *D. cingulatus* gradually diminished from 24 to 96 hrs and finally lead to the death of the pest. It was proposed that the spores in the insect haemolymph reduce the effectiveness of the cellular defenses and not being as antigenic as the mycelium (Charnley, 1989). Recently, destruxin (dtx) A, B, D, E, and E-diol were identified from this fungi (Seger *et al.,* 2006) and was predicted that this might depress the cellular immune reaction of hosts. Saxena and Tikku (1990) studies to showed the deformity in haemocytes due to the application of plumbagin, a phytochemical.

In addition, the immune system of the host and the fungal response is likely to be another important factor governing the pathogenicity of *M. anisopliae* (Moorhouse, 1993). Many researchers hypothesized that the rapid killing ability of *M. anisopliae* on its host could be due to some enzymatic mechanisms of glucosidase, protease and acid trehalase which extra cellular hydrolysis of trehalose occurs in host haemolymph during fungal infection (Xia *et al.,* 2002).

4.5. Conclusion

The infection process in *Dysdercus cingulatus* by *Metarhizium anisopliae* observed in the present work involved the following events: (i) conidia adherence to the host cuticle and germination after 24 hrs post-infection accompanied by falling of bristles (ii) penetration into host through spiracles, root of bristles, haemolymph and three dorsal sacs within 24–48 hrs post-infection; (iii) colonisation of *M. anisopliae* occurred in both trachea and dorsal sacs within 24–48 hrs post-infection; (iv) cuticle colonisation and emergence of the fungus to cuticular surface within72–96 hrs after infection and (v) complete reduction in haemocyte count within 96 hrs. Morphological alterations of *M. anisopliae* during penetration of host, the identification of enzymes that solubilise the cuticle of *D. cingulatus* during the key step of host penetration, identification of sequence tags expressed by *M. anisopliae* during the infection process and other immune responses exhibited by the host to the fungi has to be further studied in the future. Studies on the ability of spore production by all the above isolates are also essential to mass-produce the organisms in large scale.