CHAPTER V
DISCUSSION

Mosquitoes are very common vector of several tropical diseases. Many chemical pesticides (xenobiotics) are still in use as the substance of choice in the mosquito-control programmes, as contact pesticides which targeting the adult mosquitoes. However, frequent and indiscriminant use of chemical insecticides led to the development of pesticide resistance, pest resurgence and secondary pest outbreaks along with other environmental pollution. Moreover, extensive use of these xenobiotics may lead to cause of opportunistic disease like cancer. The growing concern about the ill effects of chemical insecticides has necessitated for development and adoption of eco-friendly measures for vector mosquito control. Moreover, the strategies which target the immature stages of mosquitoes in the breeding habitat require more environmentally safe alternative substances for effective control of mosquito species. Therefore, biological control can be utilized as an effective and environmental friendly alternative approach to minimize the mosquito population. Research on alternative pesticides results the development and use of biopesticides including plant based and other pathogenic microorganisms with biocontrol potentiality. The microbial biopesticides include fungal, viral and some bacterial pathogens among which fungal entomopathogens constitute as one of the important component with added advantage over other microbial pathogens. Fungal biopathogens and its derivatives are highly toxic to mosquitoes with low toxicity to nontarget organisms. Though these naturally occurring microbial entomopathogens are virulent, genetically stable and host
specific however, these are constrained with efficacy due to low inoculums and other environmental factors. Considering the seriousness of the vector mosquito species and the increasing consciousness of toxic residues caused by chemical insecticides and development of vector resistance, the present investigation was undertaken to evaluate few indigenous entomopathogenic fungal pathogens for the control of the primary DF mosquito vector *Ae. aegypti*. As the North-eastern part of India is conductive for supporting the adaption and growth of different mosquito vectors so, effective control of mosquito vector is of utmost important for vector borne disease management in the region.

The findings of the present investigation presented in the Results Chapter have been discussed here, which is based on the supports of previous findings and few reports in this field and other relevant areas.

### 5.1. Isolation, screening and identification of fungal strains from soil:

Soil samples were collected randomly from five different locations of Guwahati city based on nearby areas of open drainages, high population density, dumping of garbage’s, water logging areas etc. Entomopathogenic fungi are reported to be the largest group of microorganisms colonizing the soil environment next to the bacteria. They form diseases and play an important role as one of the natural factors, limiting populations of soil insect pests that overwinter or pupate in the soil environment (Ignoffo *et al.*, 1978; Ferron 1981; Miętlickiewski *et al.*, 1994; Bajan *et al.*, 1995). Entomopathogenic fungi spread mycosis followed by death of the host insect. Thus they exist in the soil environment and the remaining part of the life cycles of these fungi exist as dormant conidia in the soil or in the vicinity of the dead host insects. Conidia
produced on the surface of dead host insects are relatively present long in the soil. It has been reported that soil existing resting conidia of entomopathogenic fungi are responsible for the natural biocontrol of insects including mosquitoes (Ignoffo et al., 1978; Bajan et al., 1995).

Several authors also reported the sampling of soil for entomopathogenic fungi from different habitats (Quesada-Moraga et al., 2007; Imoulan et al., 2011; Vega et al., 2009). Tkaczuk et al., (2012) conducted sampling of soil samples from mid-field woodlots and adjacent small farmlands to compare the species composition and the intensity of entomopathogenic fungi. Similarly, Mora et al., (2016) also reported the sampling of soil samples for composition and distribution of native entomopathogenic fungal species in Brazil. Entomopathogenic fungi were isolates by serial soil dilution method as described by Meyling, 2007. Altogether, twenty two isolates were obtained after the subculture of the primary culture.

After screening the twenty two fungal isolates, seven were selected based on the mortality percentage of each of the isolate. Several workers have been reported the similar type of study (Lee et al., 2015; Ihara et al., 2001; Vu et al., 2007).

5.2. Efficacy study of different fungal pathogens against *Aedes aegypti* larvae:

In the Bioassay treatment, all the seven fungal isolates showed various degree of virulence against 3rd instar larvae of *Ae. aegypti* at different spore concentrations. The percentage of larval infection resulting moulting or even mortality of *Ae. aegypti* by the fungal isolate *Aspergillus niger* on 2nd instar larvae
of *Ae. aegypti* had been recorded. The highest percentage of larval mortality was recorded (65.7%) after 7 days and also recorded larval mortality (55.7%) after 4 days of treatment at the spore load of $10^9$ spores per ml. Similar results also reported at the spore load of $10^8$ spores per ml with larval mortality 62.8% after 7 days and 47.1% after 4 days of treatment. Similar results also reported by Soni and Prakash (2011), with the application of the *Aspergillus niger* metabolites on *Ae. aegypti* mosquito larvae and recorded significant mortality on the 4:6 ethanol : metabolite ratio.

The present findings also established that higher pathogenicity of *Aspergillus* species was reported to be with higher mortality of *Aedes* larvae. This is also in conformity with the findings of other workers as reported similar results for higher pathogenicity of *Aspergillus* species like *Aspergillus clavatus*, shows higher mortality (100%) after 24 hours against both *Ae. aegypti* and *Cx. Quinquefasciatus* and *Anopheles gambiae* (S.L.) Giles larvae (Seye *et al.*, 2009).

Moreover, *Aspergillus niger* was also reported to be more susceptible to *Cx. quenquefasciates* and *An. stephensi* larvae in a moderately less concentration than *Ae. aegypti* larvae (Soni and Prakash, 2011). In the present findings a significant positive correlation was also recorded between spore density and mortality of *Ae. aegypti* larvae. Moreover, higher density of *A. niger* resulted in reduction of larval population of the *Ae. aegypti* with in short duration of time which is in conformity with the findings of other workers on different mosquito species (Al-Hussaini and Hergian, 2014). They had reported increasing mortality percent of *Cx. quinquefasciatus* larvae from 42.7% to 50.9 % after 24h and 48h
respectively while the mosquito mortality per cent increase to 78.75% after 72 hours of treatment of *A. niger* (AL-Hussaini and Hergian, 2014).

The bioassay study of *Beauveria bassiana* shows that the higher percentage (61.4%) of *Ae. aegypti* larval mortality was recorded at the concentration level of $10^9$ spore/ml after 7 days of treatment. Moreover, the efficacy of *Beauveria bassiana* with higher mortality of *Ae. aegypti* larvae was also recorded at the spore load of $10^8$ spore/ml and recorded 52.8% mortality after 7 days of treatment. In the earlier study the pathogenicity of *Beauveria bassiana* was also reported against the larvae of *Aedes aegypti* by Pinnock *et al*., (1973). However, Alves *et al*., (2002) reported minimum effect of *B. bassiana* against *Ae. aegypti*, compared to larvae of *Cx. pipiens*, *Cx. tritaeniorhynchus* and *An. albimanus*.

In the present findings, dose dependent mortality of the *Ae. aegypti* larvae with the fungal bioagents was also recorded which might be due to the secondary proliferation and transformation of *B. bassiana* among the larvae. This is in accordance with the findings of García-Munguía *et al*., (2011). Higher mortality response was also reported with the treatment of *B. bassiana* on other agricultural insect pests. Impact of the entoopathogenic fungus *B. bassiana* on the Honey Bees, *Apis mellifera* (Hymenoptera: Apidae) was described by Almazrawai, (2007). Similarly pathogenicity of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* on larvae of the legume pod borer *Maruca vitrata* (Lepidoptera: Crambidae) was evaluated by Mehinto *et al*., (2014).

Higher efficacy of *Metarhizium anisopliae*, was recorded against the larvae of *Ae. aegypti*. Larvae mortality rate was found to be increased with
increasing spore concentration of *M. anisopliae*. The present findings showed that the mortality of *Ae. aegypti* larvae treated with the different fungal concentrations varied from 4.28 to 91.2%. The higher efficacy of *M. anisopliae* was recorded with higher mortality of *Ae. aegypti* 2\(^{nd}\) instar larvae and recorded 91.2 % after 7 days and 62.8% after 4 days of treatment at higher dose (10\(^9\) spores/ml) of treatment. Higher mortality response for *Aedes* larvae in control was also recorded at the higher spores load (10\(^8\) spore/ml) and recorded 77.1% mortality after 7 days and 60% mortality after 4 days of treatment. Dose dependent mortality response of *Aedes* mosquitoes against *Metarhizium anisopliae* was recorded in the present findings which is in conformity with the reported results of other workers (Benserradj and Mihoubi, 2014, Priyadarshini and Lekeshmanaswamy, 2014). Significant virulence and efficacy of *M. anisopliae* against *Ae. aegypti* was proved in the present findings which is in accordance with the reports of other workers on some other mosquito species including *Aedes* sp. (Daoust and Roberts, 1983).

The pathogenicity study of *Penicillium chrysogenum* was reported against the 2\(^{nd}\) instar larvae of *Ae. aegypti* and recorded higher per cent mortality (58.85%) at higher spore load of 10\(^9\) spores/ml after 7 days and 44.28% after 4 days of exposure period. The present study shows that the larval mortality percentage were increased with the increasing concentrations of fungal pathogens which is in conformity with the findings of other workers with different fungal isolates (Al-Hussaini and Hergian, 2014, Benserradj, O. and Mihoubi, I. 2014, Prasad, A. and Veerwal, B. 2010; Vyas, N. et al., 2015)

The fungal isolate *Penicillium chrysogenum* showed moderate efficacy against *Aedes* larvae. In contrast to the present findings Cruz, *et al*, (2016)
reported higher efficacy of other species of *Penicillium* on other mosquito species. Similarly, Maketon *et al.*, (2014) reported 100% mortality with other *Penicillium* sp. against *Cx. Quenquefasciates* larvae. Moreover, *Penicimmium citrinum* shows 100% mortality of third instar larvae of *Culex quinquefasciatus* within 2h using a conidial suspension of 1x10\(^6\) conidia/ml (Maketon *et al.*, 2014).

*Acremonium breve* spore suspension for pathogenicity studies on larvae of *Ae. aegypti* revealed the dose and duration dependent efficacy. There was no mortality of the *Aedes* larvae tested after 1\(^{st}\) day of treatment. However, after 3 days of application moderate response for larval mortality was recorded. Similar results also reported on other insect species (Diven and Mallapur, 2011). The pathogenicity response of *Acremonium breve* was found to be lower in comparison to other fungal isolates studied. The higher per cent mortality (40%) was recorded at higher spore load (10\(^9\) spores/ml) after 7 days and 20% mortality was recorded after 4 days of treatment. Dose dependent response for *Aedes* larval mortality was recorded with the lowest per cent larval mortality (1.4%) at 10\(^4\) spores/ml after day 4 of exposure which increased up to 12.8% after 7 days of exposure. Increasing concentration of fungal pathogen also enhance the larval mortality as reported earlier by different workers (Al Hussaini and Hergian, 2014). The rate of mortality varied with time and concentration of fungus tested. Diven and Mallapur, (2011) reported similar results on other insect pests. Evaluation of *Acremonium zeylanicum* (Petch) against major sucking insect pests was also reported by Divan and Mallapur, (2011). Moreover, no larval mortality was observed on day one in all the concentration levels except at higher dose like 10\(^8\) and 10\(^9\) spores/ml. The results are at par with the findings of Diven and
Mallapur, (2011) where other species of *Acremonium* (*A. zeylanicum*) were reported to be effective against few major sucking pests of important crops (Muegge, *et al.*, 1991).

From the present findings it was also revealed that amongst the fungal isolates studied the *Nomuraea releyi* shows significantly higher pathogenicity against the larvae of *Ae. aegypti* and recorded maximum (72.8%) mortality at the spores load of $10^9$ spores /ml concentration after 7 days of exposure. This result is in agreement with the findings of Onofre, *et al.*, (2002) where the entomopathogenic fungus *Nomuraea rileyi* was reported as highly active against 3rd instar larvae of *Anticarsia gemmatalis*. There was no significant difference of larval mortality was recorded in both day 1 and day 2 of treatments. Similar to the present findings of dose dependence mortality response of *Ae. aegypti* larvae, Namasivayam and Vidyasankar, (2014) reported the effectivity of *N. releyi* on *Spodoptera litura*. Another study conducted made by Namasivayam *et al.*, (2015) reported increase in mortality percentage with the increasing concentration of *N. rileyi* on *Spodoptera litura*.

It was established from the present study that the entomopathogenic fungal isolate *Aspergillus fumigates* was the most effective and virulent against larvae of *Ae. aegypti*. It was also observed from the present findings that all the concentrations of *Aspergillus fumigatus* isolate could influence at different degree on the rate of larval mortality of *Ae. aegypti*. It was also found to be significantly superior to other fungal isolates studied. *Aspergillus fumigates* recorded the 100% mortality of *Aedes* larvae from 4 to 7 days of exposure period at a concentration level of $10^7$ to $10^9$ spores/ml respectively.
Among the tested strains, *Aspergillus fumigatus* was found to be most potent and hence there is possibility of exploiting this native entomopathogenic fungal strain as a biological larvicide against *Ae. aegypti*. These results are in an agreement with the findings of Seye *et al.*, (2009) where other species of *Aspergillus* recorded as highly effective on *Ae. aegypti*. The larval mortality was recorded after the application of some other strains of *Aspergillus* sp. and 100% mortality reported after the application of 1.2 mg/ml dry fungal conidia against *Ae. aegypti* and *Cx. quinquefasciatus* larvae (Seye *et al.*, 2009). The similar type of result with the application of *Aspergillus clavatus* (Ascomycota: Trichocomaceae) on the Histopathology and on the larval mortality was observed on *Cx. quinquefasciatus* (Bawin, T. *et al.*, 2016).

5.3. **Study of the effect of fungal pathogens on the developmental stages (egg hatching, larval period, pupal period, pupal mortality and adult emergence) of *Aedes aegypti***:

The bioassay study of the fungal isolate *Nomuraea releyi* against different developmental stages of *Ae. aegypti* revealed that the fungus *Nomuraea releyi* found to be highly effective. Concentration dependence variation on larval or even pupal mortality was also observed. The present findings also established that the developmental stages of the mosquito species increases with the higher fungal spore concentration as reported earlier by other workers (Sabbour and Abdel-Raheem, 2014). Larval period recorded in the spore concentration, $10^7$, $10^8$ and $10^9$ spores/ml recorded 8.4, 8.6 and 8.9 days respectively in comparison to the control 7.9 days, *i.e.* without fungal spore treatment. However, the egg hatching numbers declines with the increasing concentration of fungal (*Nomuraea releyi*) spore suspension. The egg hatching of *Ae. aegypti* was recorded in the spore
concentration, $10^7$, $10^8$ and $10^9$ spores/ml as 15.8, 14.2 and 9.0 numbers respectively in comparison to control 17.4 nos. The present findings are in conformity with the findings of other workers (Sabbour and Abdel-Raheem, 2014). As the application of entomopathogenic fungus *Nomuraea releyi* influences on the egg hatching of *Ae. aegypti* so it may be recommend for application in mosquito biocontrol.

The efficacy of entomopathogenic fungi *Metarhizium anisopliae* against *Ae. aegypti*’s developmental stages was recorded. The influence of fungal spores concentration ($10^7$, $10^8$ and $10^9$ spores/ml) for egg hatching and larval mortalities of *Ae. aegypti* was recorded at 24h interval and observed higher mortality with higher fungal spores load. It was observed in the present study that with the increasing spore concentration resulted in lowering the egg hatching rate of *Ae. aegypti*. This is in accordance with the findings of Luz et al, (2008) where survival of larvae inside egg due to fungal pathogens were reported earlier.

It was also observed in the present study that increased spore concentration results in lowering the egg hatching of *Ae. aegypti*. The egg hatching and larval development was completely prevented at the higher spore load ($10^7$ and $10^8$ spores/ml). It might be due to predation and infection of diapausing larvae or egg inside by the exposed fungal pathogen. Similar response of complete prevention of egg hatching due to fungal invasion through egg shell or production of toxic metabolites on the surface of mosquito eggs were also reported earlier (Russell et al., 2001, Luz et al., 2007).

The cumulative mortality of egg, larvae and pupal stages caused by *M. anisopliae* at various concentrations applied were recorded at different time of
exposure treatment. Extending the larval period with respect to fungal concentration was observed in the study. It was recorded as 8.06, 9.06 and 9.81 days in the spore concentration of $10^7$, $10^8$ and $10^9$ spores/ml respectively. Similarly, the pupal period also increased with the increasing fungal spore-load and recorded in $10^7$, $10^8$ and $10^9$ spores/ml as 2.9, 4.02 and 4.06 days respectively. From the study it was observed that there was an extension of the pupal period when an occasional interruption of the emergence happened. This is in accordance with the findings of other workers (Luz. et al., 2008). Larval death or proceed with deformities in pupation and even adult emergence were recorded with the treatment of *Metarhizium anisopliae*. Though some pupae specimens emerged to adult these were with deformed wings and a proliferation of the fungus was noted on the bodies of the emerged adult mosquito’s as reported earlier (Luz et al, 2008).

The pathogenicity study of another screened fungal isolate *Aspergillus fumigates* was found to be most effective against various developmental stages of *Ae. aegypti*. In comparison to other entomopathogenic isolates *Aspergillus fumigates* shows a highly significant response for the microbial control of *Aedes* larvae at laboratory condition. Declining in hatching of *Aedes* eggs in response to increasing concentration of fungal spore suspension was recorded for *Aspergillus fumigates*. Compared to control (16.8 number) the dose dependent response for $10^7$, $10^8$, and $10^9$ spores/ml results decreasing the number of egg hatching and recorded 7.8, 5.0 and 1.4 numbers respectively. This is in accordance with the findings of other worker on other species (Seye et al., 2009, Luz et al., 2008).
Larval period was extended with the increasing fungal spore concentration and recorded as 8.9 and 9.5 days at the fungal spore load of $10^7$ and $10^8$ spores/ml respectively, whereas it was recorded as 8.05 days for control group. Influence of spore concentration on pupal period was also observed and recorded as 3.9 and 4.7 days respectively for the spore load of $10^7$ and $10^8$ spores/ml whereas for control group it was 3.05 days. The increasing the duration of the pupal periods with increasing fungal spore concentration was recorded. Likewise, mortality of the pupae was also observed at high concentration of fungal spore treatment. Complete prevention of egg hatching may be due to predation of fungal bioagent Aspergillus fumigates was observed at higher fungal dose ranging from $10^6$ spores/ml onwards. Similar results also reported earlier by other workers on different species (Luz et al., 2007).

5.4. **Scanning Electron Microscopic study on the growth and development of three potent fungal isolates (Aspergillus fumigates, Metarhizium anisopliae and Nomuraea releyi) on the Aedes aegypti larvae:**

Scanning electron micrographs (SEM) allowed the observation of entomopathogenic fungal spores and their adhesion and penetration structure on Ae. aegypti larvae. SEM study of the Aedes larvae treated with the $10^8$ spores/ml of the fungus, Aspergillus fumigates, revealed adhesion and penetration structures in the infected larvae. Growth of the fungus on the infected larvae and signs of hyphal penetration of insect cuticle as well as proliferation over the cuticle were also apparent. On the other hand, the fungus, Metarhizium anisopliae as declared by SEM showed a dense network together with numerous spores on Aedes larval cuticle. SEM also established the efficacy of Nomuraea releyi and allowed
observation and penetration of the spores and hyphae of the fungus in the cuticle and body cavity of infected *Aedes* larvae.

These observations agree with the SEM observation of *Asensioa recored* by Lopez-Llorca and Lo’pez-Jime’nez (2005) for *Beauveria bassiana* growing on the red scale insect of plams *Phoenicococcus marlatti* and they are in consistent with the following findings:

Infection of insects by fungus, *Metarhizium anisopliae* also requires adhesion, penetration into the host (St Leger, 1993) and establishment of the pathogen in the host cuticle (Hassan et al., 1989). Penetration through the host cuticle is the mode of entry for most of the entomopathogenic fungi (Hassan et al., 1989). During fungal infection, the first step prior to penetration is the adhesion of fungi to the host cuticle (Boucias and Pendland, 1991) which is at par with our findings.

Fragues (1984) also suggested that there must be adhesion to occur at three successive stages like fungi propagules adsorption to the host cuticler surface; adhesion of the interface between propagules and epicuticle; and fungal spore germination and development at the insect cuticler surface, until appresoria are developed to start the penetration stage.

The results obtained in these experiments establish the pathogenicity of entomopathogenic fungi *Aspergillus fumigates*, *Metarhizium anisopliae* and *Nomuraea releyi* on the larvae of *Ae. aegypti* as biological control agent. Biological control with pathogenic fungi is promising alternative to chemical control against the mosquito species.
5.5. Summary and conclusion:

1. The entomopathogenic fungi *Aspergillus fumigates*, *Metarhizium anisopliae*, and *Nomuraea releyi* were found to be more pathogenic to the larvae of *Ae. aegypti* and may be used as effective biocontrol agents.

2. *Aspergillus fumigates* was found to be most virulent isolate which is followed by *Metarhizium anisopliae*, *Nomuraea releyi*, *Aspergillus niger*, *Beauveria bassiana*, *Penicillium chrysogenum* and *Acremonium brevem*.

3. 100% mortality of *Ae. aegypti* larvae was recorded with *Aspergillus fumigates* within 4 days at a concentration of $10^7$ spore/ml.

4. The egg hatching rates, larval period, pupal period, pupal mortality and adult emergence were also found to be effected by the fungal pathogenicity.

5. The pathogenic fungal spores were found to be attached and effectively grow on the entire larval body surface causing ultimate death.

6. Histological study and surface topography by using SEM established higher pathogenicity of *Aspergillus fumigates*, *Metarhizium anisopliae*, *Nomuraea releyi* fungal pathogens in the host through adhesion, penetration into the host cuticle and establishment of the pathogen in the host.

By considering the present interesting findings regarding the potency of selected fungal isolates against *Ae Aegypti* and with the help of the modern tools and techniques of research, investigation may go further for the development of environment friendly and cost-effective biocontrol measures/product for vector mosquito management.