Chapter III:

MATERIALS AND METHODS
This investigation aimed at studies on diversity and ecology of microfungi found growing on insect and mite hosts and associated substrates collected from a few urban and rural localities of Kerala, Karnataka, Maharashtra and Goa, besides forests of Western Ghats in Goa State (Fig. 3.1) and their activity in relation to control of mosquito developmental stages, was carried out for a period of over two years, from March 1999 to June 2002.

The materials used and methods followed are elaborated in this Chapter.

3.1. Sourcing and documentation of entomogenous fungi

3.1.1. Sampling sites and sample types

A variety of substrates collected from a wide range of habitats were scanned to recover maximum number and type of entomogenous fungi. The substrates included (i) live and dead larvae and dead adults of mosquitoes from breeding and resting sites from 8 localities in Goa (Bicholim, Cuncolim, Curchorem, Panaji, Pernem, Porvorim, Taleigao and Vasco), (ii) dead and live non-mosquito insects such as hemipteran, coleopteran, orthopteran, phasmida and dipteran and arachnids such as mites and spiders from foliage of forests and scrub-jungles of 24 localities in Goa (Alorna, Ambe, Anmod, Bondla, Chandreshwar, Chorlem, Codal, Colem, Cotigao, Cudnem, Curchorem, Dhudhsagar, Edda, Endrem, Goa University (GU) campus, Karmali, Kesarval, Melka, Molem, Pernem, Sonauli, Taleigao, Tambdi Surla and Valpoi), 1 locality in Maharashtra (Amboli), 3 localities in coastal Karnataka (Kodachadri, Kollur and Subrahmanya) and 1 site in Kerala (Kasaragod), (iii) healthy and later infected larval baits introduced, under laboratory set-up, into water samples collected from puddles, paddy fields and ponds from 7 sites in Goa.
Fig. 3.1. Map of Western Ghats and Goa showing collection sites.

1. Alorna
2. Ambe
3. Amboli
4. Annod
5. Bicholim
6. Bondla
7. Chandreshwar
8. Chorlem
9. Codal
10. Colem
11. Cotigao
12. Cunnilm
13. Cuncolim
14. Curchorem
15. Dhudhsagar
16. Edda
17. Endrem
18. GU campus
19. Karmali
20. Kasaragod
21. Kesarval
22. Kodachadri
23. Kollur
24. Melka
25. Molem
26. Panaji
27. Penem
28. Porvorim
29. Sonauli
30. Subrahmanya
31. Taleigao
32. Tambdi Surla
33. Valpoi
34. Vasco
(Bondla, Cotigao, Cudnem, Curchorem, GU campus, Karmali and Taleigao), and (iv) healthy and later infected mosquito larval baits used in simulation float chambers maintained in ponds at GU campus and Panjim and slow running streams at Pernem. Baits used in the latter two exercises were 2nd instar larvae of *Culex quinquefasciatus*, reared in the laboratory. The mosquito breeding sites examined were well, pond, ditches, puddle, paddy field, slow flowing stream, curing water at construction site, overhead water-tank, sump, open septic tank, drain, abandoned container, barrel, abandoned tyres, etc. and transient rain water pools on building terraces (Plate: I-IV). The localities and habitats visited and substrates and samples scanned are given in Table. 3.1.

Typical of coastal belts in southern India, ambient temperature at the collection sites ranged between 22-35°C, the temperature seldom falling below 19°C. Mean annual rainfall was 200-300 cm and humidity ranged between 60-95% (Sourced from Mat. Dept., Panaji, Goa).

3.1.2. Sampling methods

Live mosquito larvae and pupae were collected from breeding sites following the methods described by Service (1976). A plastic container with 9 cm diam at the base, 11.5 cm wide in the middle and of a height of 7cm (Plate: VI-a,b,c & d) was used as a ‘dipper’ to collect mosquito larvae and pupae that appeared in small or large pool of water and other aquatic habitats. Wherever necessary, a 2 m long plastic stick was attached to the dipper. The dipper was gently scooped through surface water or lowered to subsurface level and lifted out. In very shallow waters, the dipper was pressed firmly to the bottom and lifted. From small pools three dipper-samples whereas in large-sized ponds, up to 20 dipper samples were taken.
Dead mosquitoes of *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* were collected with help of an aspirator from adult mosquito cages in the insectory and from their resting sites. Dead and live non-mosquito insects such as hemipterans, coleopterans, orthopterans, dipterans and arachnids such as mites and spiders from litter and foliage were collected from forests at altitudes ranging from sea level up to approx. 650 m (Plate: IV). Humid places on either side of streams and rivers were scanned for infected insects or spiders. Water samples were also gathered from puddles, paddy fields and ponds without mosquitoes.

Healthy 2\textsuperscript{nd} instar larva of *Culex quinquefasciatus* was used as bait in simulation float chambers to source entomogenous fungi in their natural habitats. A 500 ml plastic dish (9 cm diam at base and 11.5 cm diam in the middle and 7 cm height) with 2 windowsills of 6 x 3 cm covered with mesh and fitted to a wooden plank served as a float chamber (Plate: V-a). The float was tied to a stationary object in the field by a nylon rope and allowed to drift in water for 2-3 days. About 20 larvae were placed in each float chamber (Plate: V-b,c & d).

### 3.1.3. Collection of samples in the field

Live mosquito larvae collected using the dippers were concentrated by decanting excess water and subsequently transferred into small screw cap plastic jars (Plate: VI-a,b,c & d). Adult mosquitoes were transferred to 15 ml test tubes and plugged with non-absorbent cotton. Insects and arachnids were placed in clean polythene bags of 18 x 14 cm size or plastic boxes depending on size of the sample. Moribund insects found clasping to leaves and fungal fructifications were collected along with host foliage, twigs or litter in polythene bags. Larval baits used in simulation float chambers were transported to laboratory in plastic jars.
3.1.4. Processing in the laboratory

Mosquito larvae of different developmental stages were maintained in 250 ml water in small plastic containers. These were administered with larval feed (Baker's yeast and powdered dog-biscuit in 1:3 ratio) at regular intervals. The mouth of the container was covered by a mosquito net. A 2 cm diam hole was made in the centre of mosquito net so as to enable insertion of a dropper or aspirator to pick up larvae or adults. The hole was covered by a cotton plug (Plate: VI-e). The set up was observed every second day for sluggishness, mortality or associated symptoms until all the larvae/pupae either emerged as adults or dead. The sluggish or dead larvae/pupae were observed under a stereoscope (Zeiss Stemi 1000) and a light microscope (Olympus CH30) for signs of fungal infection. Infected material was used as source of entomogenous fungi.

Dead/live non-mosquito insects and arachnids were examined under the microscope for the signs of fungal infection. Infested hosts were used as source of entomogenous fungi.

Twenty healthy 2nd instar larvae of *C. quinquefasciatus*, reared in insectory, were introduced as baits into a small plastic container containing 300 ml of water sample. Larval feed was provided at intervals. For each water sample 5 replicates were maintained. The mouth of the container was covered with mosquito net so that emerged mosquito remained in the container itself. A 2 cm diam hole was made in the centre of the net to enable insertion of a dropper or aspirator and to pick up the larvae or adults as the case may be. The hole was plugged by non-absorbent cotton. The set up was observed every second day under the microscope for sluggishness, mortality or associated symptoms until all the larvae/ pupae emerged as adults. The material found infested was used as a possible source of entomogenous fungi.
The larvae from simulation float chambers were transferred to fresh water containers and fed with larval feed. The infested larvae were used as possible source of entomogenous fungi.

3.1.5. **Culture media for isolation and maintenance fungi**

Malt extract (5%) agar (MEA), Sabouraud dextrose (6.5%) agar (SDA) and Corn meal (1.7%) agar (CMA) media were used for isolation of fungi. A mixture of antibiotics consisting of bacitracin 0.02 g, neomycin 0.02 g, penicillin G 0.02 g, polymixin 0.02 g, streptomycin 0.02 g and terramycin 0.04 g dissolved in 10 ml of sterile distilled water added to 1 L of medium was used in isolation plates to prevent bacterial growth. Malt extract (2.5%) agar and Sabouraud dextrose (3%) agar were used for maintenance of cultures in slants.

### 3.1.5.1. Malt extract (5%) agar (MEA) medium

Five g of dehydrated malt extract (Himedia Laboratories Pvt. Ltd.) and 20 g of agar (E. Merck India Ltd.) were boiled in 1000 ml of distilled water to dissolve, pH adjusted to 6.0 and sterilized in an autoclave at 121°C for 15 minutes.

### 3.1.5.2. Corn meal (1.7%) agar (CMA) medium

Corn meal agar, 17 g (Himedia Laboratories Pvt. Ltd.) in 1000 ml distilled water boiled to dissolve completely and sterilized in an autoclave. It contained ingredients, viz., corn meal infusion from 50 g L\(^{-1}\); Agar, 15 g L\(^{-1}\) and final pH of 6.0 ± 0.2.

### 3.1.5.3. Sabouraud dextrose (6.5%) agar (SDA) medium

Sabouraud dextrose agar, 65 g (Himedia Laboratories Pvt. Ltd.) was boiled in 1000 ml of distilled water to dissolve and sterilized in an autoclave. The ingredients included mycological peptone, 10 g L\(^{-1}\); Dextrose, 40 g L\(^{-1}\) and Agar, 15 g L\(^{-1}\) adjusted to pH of 5.6 ± 0.2.
3.1.6. **Isolation of fungi**

Source materials were washed thoroughly in sterile distilled water and plated at equidistant in plates containing MEA, CMA and SDA with antibiotics. The plates were incubated at a temperature of 23-25°C and observed every second day for growth of fungi. Small portions of fungal colonies emerging out in different zones were aseptically transferred into fresh MEA plates cut into 9 equal sectors and incubated for 7 days (Plate: VII). Growing colonies were compared and dissimilar ones were transferred to MEA slants. Part samples were placed in sterile moist chamber and examined for sporulation.

3.1.7. **Documentation of fungi**

3.1.7.1. **Microscopic observations**

Description of each species was based on material collected and/or cultures established.

Semi-permanent slides of fungi from colonies with sporulating structures such as sporangiophores and sporangia (Zygomycetes), ascocarp, asci and ascospores (Ascomycetes), conidiophores and conidia (Hyphomycetes), pycnidia, conidiogenous cells and conidia (Coelomycetes) were prepared using water, lactophenol or lactophenol and cotton blue as mountant. The edges of cover slip were sealed with DPX mountant. The slides were numbered and maintained in the Herbarium of Botany Department, Goa University. Illustrations were made using camera-lucida drawing tube attached to a binocular microscope. Photomicrographs were taken using an automatic camera (Nikon Make) fitted to the microscope. Fungi growing on hosts were photographed using an automatic SLR camera fitted to the stereoscope (Plate: VIII-XIII).
3.1.7.2. **Identification**

Each fungal colony was considered as a ‘morphotype’. The isolates were grouped into ‘sporulating’ and ‘nonsporulating’ forms. Sporulating structures were considered as features for identification of fungi. Using standard taxonomic keys and monographs (Carmichael et al., 1980; Domsch et al., 1980; Tzean et al., 1997), isolates were identified and assigned to respective genera and species. Along with morphological features, ecological and cultural characters of each taxonomic entity were compiled. Descriptions of fungi were written in a standard diagnostic form.

3.1.7.3. **Ex situ maintenance and preservation of fungi**

A representative pure culture of each identified species or morphotype was maintained in the collection of ‘Goa University Fungus Culture Collection’ (GUFCC).

3.1.7.4. **Herbarium preparation**

Dried herbarium material of fungi in culture or on host/substrate was prepared by air-drying the specimen in a drier. The specimen was assigned with an accession number, well-packed in paper bags, sealed, labeled and maintained in the herbarium of Department of Botany, Goa University. Holotypes of new taxa were deposited at the International Mycological Institute, Cabiscience, U.K. Where the new taxon is based on a live fungus, dried and dead culture mats in herbarium sheets were maintained in the Herbarium of the IMI, U.K. and/or the GUBH, Goa University, so as to satisfy the nomenclatural rules.

3.2. **Screening fungi for biocontrol potential against mosquito larvae**

3.2.1. **Composition of media used for mass production of fungal conidia**

3.2.1.1. **Malt Czapek agar (MCzA):** Czapek solution A, 50 ml; Czapek solution C, 50 ml; sucrose, 30.0g; Malt Extract, 40g; Agar, 20.0g; in 900 ml distilled water. Malt Extract was dissolved by boiling in distilled water. Sucrose was added followed by Czapek solution A
& C. At the end agar was added and dissolved. pH was adjusted to 5.5. Medium was sterilized by autoclaving at 121°C for 15 minutes. [Cz solution A: Sodium nitrate NaNO₃, 4.0g; Potassium chloride, 1.0g; Magnesium sulphate, MgSO₄·7H₂O, 1.0g; Ferrous sulphate, FeSO₄·20.0 mg; all dissolved in 100.0 ml of distilled water; Cz solution C: Dipotassium hydrogen phosphate, K₂HPO₄, 2.0g dissolved in 100.0 ml of distilled water].

3.2.1.2. **Corn Meal agar**: As in 3.1.5.2.

3.2.2. **Preparation of spore suspension**

Test fungus was grown in plates containing 1.7% CMA and MCzA at 22-25°C for 15-20 days. Spores were harvested by flooding the colony with 10ml of sterile 0.05% tween 80 saline solution and agitating with a glass rod or a sterile brush. The spore suspension was dispensed to a 30 ml sterile screw cap tube and vortexed for two minutes. Spore concentration was determined using a haemocytometer. In case of non-sporulating forms, mycelial mass was used without any estimation of dose.

3.2.3. **Preliminary bioassay**

Spore suspension was transferred to a 500 ml plastic container and made up to 50 ml with tap water. A final concentration of 10,000-100,000 conidia/ml was maintained. Twenty healthy late 2nd instar larvae of *Culex quinquefasciatus*, raised in the insectory (Plate: XIV-a & b), were introduced into the container. Observations were made for features such as sluggishness, mortality and other associated pathogenic symptoms of mosquito for 5 days at intervals of 24 h. As in other toxicological investigations, effect produced in mosquito larvae was measured as 'all or none' principle, considering severely affected larvae also as dead. Since mosquito larvae being sensitive to starvation, larval feed was provided during the bioassay. Control was maintained with 50ml of tap water (Plate:
XIV-c). Experiment was done with three replicates and repeated twice. Fungi leading to more than 50% mortality were considered as possible biocontrol agents of mosquitoes and referred to as promising isolates.

3.2.4. Corrected mortality

When control mortality was more than 20%, the bioassay was discarded and repeated in order to correlate and confirm the results obtained. The control mortality when ranged between 5-20%, the ‘corrected mortality’ was calculated using Abbott’s formula (Abbott, 1925)

\[
\text{Corrected Mortality} = \frac{\text{Observed mortality} \% - \text{Control mortality} \%}{100 - \text{Control mortality} \%} \times 100
\]

Corrected mortality was not calculated whenever the control mortality remained below 5%, (Plestina, 1984).

3.3. Assessment of mosquito-larvicidal activity of candidate species

Dose-response relationships of conidia of candidate isolates against mosquito larvae were determined in two successive steps. In the first, standardized bioassay were done in 250 ml water in 500 ml containers. Four representative promising isolates, viz. *Acremonium* sp. (E29), *Gliocladium* sp. (E16), *Penicilliunz* sp. (E9) and *Trichoderma* sp. (C54), were tested for dose-response relationship of mosquito-larvicidal activity against early 3rd instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. In the second step, standardized bioassay were done in 1500 ml water in 4000 ml containers. *Pezzicillium* sp. (E9) and *Gliocladium* sp. (E16) were evaluated for their potential against early 3rd instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. 
3.3.1. Mass production of conidia

*In vitro* production of conidia in large quantity was done by modifying the still-shallow-liquid-culture technique of Jenkins and Goettel (1997). Sporulation occurred at the liquid-gas interface in this setup. Fifty ml of the medium was poured into culture bottles with a surface area of about 115 cm² when kept horizontally (Plate: XIV-d).

3.3.1.1. Media for production of conidia

Malt Czapek (MCz) and corn meal (CM) liquid media with and without sugar were used for mass production of conidia. For MCz broth, standard ingredients were used. In case of CM or CM sugar liquid media, locally available corn and sugar were used.

**Malt Czapek (MCz) liquid medium:** Malt extract powder-40g; sucrose-30g; Czapek Solution A-50ml; Czapek Solution C-50ml; distilled water-900ml. Malt extract was dissolved in 900ml of distilled water by boiling. Sucrose and Cz. solution A & C were added and pH adjusted to 5.5. Fifty ml of each medium was dispensed into culture bottles and sterilized in an autoclave.

**Corn Meal Sugar medium:** Whole corn was finely powdered in a blender and corn meal infusion was prepared by boiling the powder in water. Sugar was added to the infusion before autoclaving. One percent corn meal liquid medium: corn meal infusion from 10g of corn powder made up to 1000 ml with distilled water; 4% corn meal liquid medium: corn meal infusion from 40g of corn powder made up to 1000 ml with distilled water; 4% Corn meal Sugar liquid medium: corn meal infusion from 40g of corn powder made up to 1000 ml with distilled water with 30 g sugar. pH was adjusted to 5.5 in each case. Fifty ml of each medium was dispensed to culture bottles and sterilized in an autoclave.
3.3.1.2. 'Starter inoculum' and preparation of spore suspension

Using a sterile needle, candidate fungus was point-inoculated into the culture bottle and incubated for 7 days at room temperature. Pre-sterilized 0.05% tween 80 was flooded into the culture bottle and thoroughly shaken to harvest the conidia. The spore suspension was filtered through 250, 100 and 50 μm sieve plates and diluted to a rough concentration of 10,000 conidia/ml. This was used as 'starter inoculum' for mass production of spores.

One ml of starter inoculum was added to each culture bottle and incubated for 15-18 days. The bottle was flooded with 0.05% tween 80. Spores were harvested, thoroughly washed in sterile distilled water and concentrated by centrifugation (Plate: XIV-e). Spore concentration was estimated using a haemocytometer. Production of conidia in different media was compared.

3.3.2. Standard Bioassay in 250 ml of water

A spore concentration gradient of $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, $5 \times 10^4$ and $1 \times 10^5$ conidia/ml was prepared leading to a final volume of 50 ml in 500 ml containers. Twenty five healthy early 3rd instar larvae of *Culex quinquefasciatus* were introduced into each container. Control was maintained. Larval feed was provided. Mortality and sluggishness were observed at 24, 48, 72, 96 and 120 h. Range of concentration which provides 0-100 % mortality was determined. Similar experiments were also done with *Anopheles stephensi* and *Aedes aegypti*.

A 'dosage range' for spores was decided based on the results of 'dose range testing' so as to cover 0-100% mortality. Accordingly experiments were set up with 5 replicates for each dose planned, in a final volume of 250 ml in plastic containers with a surface area of
~70 cm². Five controls were maintained each with 250 ml of water. Twenty five healthy early 3rd instar larvae of *Culex quinquefasciatus* were introduced to each container. Larval feed was provided. Mortality and sluggishness were observed and noted down for 24, 48, 72, 96 and 120 h (WHO, 1992). LD₅₀ values were determined using the Prism Demo Software from www.graphpad.com. The data were analyzed using sigmoid curve fitting with variable slope.

For *Penicillium* sp. (E9), standard bioassays were carried out against 3rd instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* at the Microbial Containment Complex, National Institute of Virology, Pune. Susceptibility of Goa and Pune strains to the spores of *Penicillium* sp. (E9) were compared by linear regression using Prism Demo Software from www.graphpad.com.

### 3.3.3. Standard Bioassay in 1500 ml of water

As a next step towards effective candidate species, bioassays were done in large volume of water in shallow plastic trays. Except for a reasonable increase in the dosage, experiments were performed as described in the section 3.3.2. Two positive isolates, viz., *Penicillium* sp. (E9) and *Gliocladium* sp. (E16), were further evaluated against early third instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. LD₅₀ values were determined using the Prism Demo Software from www.graphpad.com. The data were analyzed using sigmoid curve fitting with variable slope.

### 3.4. Larvicidal activity of culture filtrate of promising isolates

Cell-free extracts were tested for their insecticidal potential after 4, 7 and 20 day growth of fungal cultures.
3.4.1. **Medium used for culturing fungi**

Sabouraud dextrose liquid medium: Sabouraud dextrose broth powder (M033, Himedia Laboratories Pvt. Ltd.) 30 g was boiled in 1000 ml of distilled water. The medium contained ingredients, viz., special peptone, 10 g L\(^{-1}\); Dextrose, 20 g L\(^{-1}\); and final pH of 5.6 ± 0.2. Fifty ml of the medium was dispensed into culture bottles and sterilized in an autoclave.

3.4.2. **Preparation of cell-free extract**

Promising fungi were grown in plates containing 6.5% SDA. Culture blocks of approximately 1 cm\(^2\) were inoculated into the broth. Four sets of cultures were maintained for each fungus. One bottle each was picked up at the end of 4\(^{th}\) day, 7\(^{th}\) day and 20\(^{th}\) day for further processing. The culture was macerated using mortar and pestle, sonicated in a sonicator (Bandelin Make) and centrifuged at 10000 rpm to get a clear supernatant containing mixture of metabolites.

3.4.3. **Bioassay of cell-free extract**

Ten ml of cell free-extract was diluted to 50 ml by adding water. Ten healthy *Culex quinquefasciatus* larvae were introduced into each container. Simultaneously, two controls containing 10 ml of medium diluted to 50 ml were maintained. Observations were made at 24 and 48 h. Results were tabulated in percentage mortality for cell-free extract from 4, 7 and 20 day old cultures.

3.5. **Detection of enzymes of entomogenous fungi**

All viable isolates were tested for production of enzymes by determining the ability of cultures to grow on specific substrates in defined and semi-defined media. (Paterson and Bridge, 1994). These included qualitative analysis for protease, esterase and chitinase.
3.5.1. **Fatty acid esterase (presumptive test) activity** (Sierra, 1957)

**Principle:** Medium contained Tween 80 as Carbon source, bromocresol purple as a pH indicator and a calcium salt. Tween 80 is a mixture of fatty acids, predominantly elaidic, linoleic and palmitic, and the final pH of the medium is acidic. Growth on fatty acids results in rise of pH, turning the medium blue-purple, and further in the formation of insoluble calcium salts such as calcium stearate which appear as a white ring in the medium around the colony. It was noted that this is not a confirmative assay for lipase activity.

**Medium:** Mycological peptone 10.0 g; NaCl 5.0 g; CaCl₂.2H₂O 0.1 g; Bromocresol purple-25.0 mg; agar-15.0 g; distilled water to 1 L. pH was adjusted to 5.4 and medium dispensed into 90 ml aliquots.

Tween solution -A 10% (v/v) aqueous Tween 80 solution was prepared by slowly adding 10 ml Tween 80 to 90 ml warm (60-70°C) distilled water. Agar and Tween solution were sterilized in an autoclave. When the media had cooled to 65-70°C, 10 ml Tween solution was added to each 90 ml basal medium and mix. The completed medium was poured into petri dishes (10 ml/plate). The plates were point-inoculated and incubated for up to 14 days.

3.5.2. **Gelatin hydrolysis (presumptive protease) activity**

**Principle:** Gelatin medium was used as a presumptive test for protease. Culture medium was solidified with gelatin. Gelatin is known to be hydrolyzed by proteolytic enzymes; leading to liquefaction of the medium. Gelatin was chilled (4°C for 30 minutes) after the incubation period before the test was read. Liquefaction of gelatin was read as positive for protease activity.

**Medium:** Czapek solution A-50.0 ml; Czapek solution C-50.0 ml; Zinc solution-1.0
ml, Copper solution-1.0 ml; sucrose-10.0 g; gelatin-120.0 g; distilled water to 1 L. The medium was prepared in warm water (up to 50-60°C). Gelatin was stirred slowly in small amounts until all dissolved. The warm medium was dispensed into test tubes (7 ml/test tube) and sterilized in an autoclave. On cooling, the tubes were inoculated and incubated up to 21 days.

3.5.3. **Chitin utilization activity (presumptive chitinase)**

**Principle:** Chitin was added as a sole carbon source replacing sucrose in CDA medium. Ability of the fungus to grow in the medium by utilizing chitin was read as positive chitinase activity.

**Medium:** Chitin-10g; Sodium nitrate, NaNO₃-2g; Dipotassium hydrogen phosphate, K₂HPO₄-1g; Magnesium sulphate, MnSO₄- 0.5g; Potassium chloride, KCl-0.5g; Ferrous sulphate, FeSO₄- 0.01g; agar-15g; distilled water to 1 L. Chitin was slowly stirred in small quantities of 1 molar acetic acid to get a pasty texture until all the chitin was suspended to colloidal consistency. Other components of the medium were dissolved separately. Chitin was added, total volume of the medium was made up to 1000 ml and pH was adjusted to 5.5 before autoclaving. The medium was poured into petri plates, point-inoculated and incubated for up to 14 days. Results of enzyme activity were tabulated as positive or negative for each promising culture.

3.6. **Preliminary field trials of promising isolate**

Experiment was conducted in curing water inside a construction site near Bhavishya Nidhi Bhavan, Patto, Panjim. Four litres of curing water from the breeding habitat was transferred to each of 6 containers (60 x 40 cm). One hundred 3rd and 4th instar larvae of *Anopheles stephensi* were sampled and transferred to each container and a dose of ~80
million spores (=20,000 spores/ml) of *Penicillium* sp. (E9) was sprayed to each except control. Observations were made at 24 and 48 hours and percentage mortality was determined. Dead larvae and fungal mass were transferred to laboratory in screw cap containers for microscopic observations.

3.7. *In situ* observation of life cycle of species of *Aschersonia* and *Hirsutella*

Sampling was done from a pre-defined site at Sri Bhagawan Mahaveer Wildlife Sanctuary, Molem, Goa, for a period of one year from June 2001 to May 2002 at regular intervals (Table 3.2). The vegetation at collection site ranged from moist-deciduous and semi-evergreen.

The collection site was a 2 km stretch of riparian habitat along a slow flowing stream. Vegetation composition in the proximity included (i) Tree species: *Abrus precatorius* Linn. (F: Fabaceae); *Alstonia scholaris* (Linn.) R.Br.(F: Apocynaceae); *Careya arborea* Roxb. (Barringtoniaceae), *Cinnamomum zeylanicum* Bl. (F: Lauraceae); *Dillenia indica* Linn. (F: Dilleniaceae); *Flacourtia montana* Grah. (F: Flacourtiaceae); *Hopea ponga* (Dennst.) Mabb. (F: Dipterocarpaceae); *Lagerstroemia parviflora* Roxb. (F: Lythraceae); *Mangifera indica* Linn. (F: Anacardiaceae); *Morinda citrifolia* Linn. (F: Rubiaceae); *Myristica malabarica* Lamk. (F: Myristicaceae); *Olea dioica* Roxb. (F: Oleaceae); *Pongamia pinnata* (Linn.) Pierre (F: Fabaceae); *Pterospermum acerifolium* Willd. (F: Sterculiaceae); *Saraca asoca* Roxb. De Wilde (F: Caesalpiniaceae); *Syzygium cumini* (Linn.) Skeels (F: Myrtaceae); *Terminalia bellirica* (Gaertn.) Roxb. (F: Combretaceae); *T. crenulata* Roth. (F: Combretaceae); *T. paniculata* Roth. (F: Combretaceae) and *Xylocarpus xylocarpa* Taub. (F: Mimosaceae) (ii) Climbers and lianas: *Entada purga* D.C. (F: Fabaceae) and *Gnetum ula* Brongn.(F: Gnetaceae) (iii) Shrubs: *Calotropis gigantea* (Linn.) R. Br. (F: Asclepiadaceae), *Calycopteris floribunda* (Roxb.) Lamk. (F: Combretaceae),
Cassia tora Linn. (F: Caesalpiniaceae), Holarrhena antidysenterica (Roth.) A. DC. (F: Apocynaceae); Holigarna arnottiana (Wt. & Arn.) Hook.f. (F: Anacardiaceae); Ixora brachiata Roxb. (F: Rubiaceae); Ixora coccinia Linn. (F: Rubiaceae); Leea macrophylla Roxb. Ex Hornem (F: Hornaceae); Microcos paniculata Linn. (F: Tiliaceae); Psychotria dalzelli Hook.f. (F: Rubiaceae); Rauvolfia serpentina (Linn.) Benth. ex Kurz (F: Apocynaceae) and Strobilanthus ixiocephalus Benth. (F: Acanthaceae), (iv) Cane and bamboos: Calamus thwaitesii Becc. (F: Arecaceae); Dendrocalamus strictus (Roxb.) Nees (F: Poaceae) and Bambusa arundinacea (Retz.) Willd (F: Poaceae). Shrubs and herbs understory is abundant in this area.

Plant hosts such as Calycopteris floribunda, Dillenia indica, Holarrhena antidysenterica, Hopea ponga, Leea macrophylla, Morinda citrifolia and Strobilanthus ixiocephalus were found to harbour the fungi regularly and in sufficient numbers. Hence only these plants were observed for the fungi. A. aleyrodis and A. badia only occasionally seen in other plants. Equal number of leaves were collected randomly from a particular host plant in each collection. Ten individual plants were observed from each plant host species. Number of and diam. of colonies were recorded for each collection. Data were pooled together from all host plants to calculate the mean number of colonies per host plant, and mean colony diam (mm) of each fungus. Line graphs were plotted with mean number of colonies and mean colony diam (mm) against dates of collection for a year duration to observe variation along the season.

Sections of exosclerotium were done in a cryostat and mounted in lactophenol. Temporary mounts were prepared by sealing the coverglass with DPX. Representative herbarium specimens were prepared for each sampling. Illustrations of different stages of development of fruiting body were made.
Table 3.1. Localities and habitats visited and substrates and samples scanned.

<table>
<thead>
<tr>
<th>Places</th>
<th>No of visits</th>
<th>Substrates/Host</th>
<th>Habitats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alorna</td>
<td>4</td>
<td>✅</td>
<td>ii) Scrub jungle and forest</td>
</tr>
<tr>
<td>Ambe</td>
<td>1</td>
<td>✅</td>
<td>i) Forest</td>
</tr>
<tr>
<td>Amboli</td>
<td>1</td>
<td>✅</td>
<td>i) Forest</td>
</tr>
<tr>
<td>Annemod</td>
<td>4</td>
<td>✅</td>
<td>i) Forest</td>
</tr>
<tr>
<td>Bicholim</td>
<td>5</td>
<td>✅</td>
<td>i) Sump tank, cement tank, Overhead tanks, Curing water, wells, ponds, transient rainwater pools</td>
</tr>
<tr>
<td>Bondla</td>
<td>6</td>
<td>✅</td>
<td>i) Forest, National Park and water insects</td>
</tr>
<tr>
<td>Chandreshwar</td>
<td>1</td>
<td>✅</td>
<td>i) Scrub jungle</td>
</tr>
<tr>
<td>Chorlern</td>
<td>2</td>
<td>✅</td>
<td>i) Forest, foam trapped water insects from the stream</td>
</tr>
<tr>
<td>Codal</td>
<td>1</td>
<td>✅</td>
<td>i) Cashew plantation</td>
</tr>
<tr>
<td>Coleem</td>
<td>1</td>
<td>✅</td>
<td>i) Forest, riparian habitat</td>
</tr>
<tr>
<td>Cotigao</td>
<td>7</td>
<td>✅</td>
<td>i) Wildlife Sanctuary, dense forest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✅</td>
<td>i) Puddle in a scrub jungle, paddy field, stagnant water pool</td>
</tr>
<tr>
<td>Cudnem</td>
<td>1</td>
<td>✅</td>
<td>i) Degraded forest, scrub jungle in the mining area</td>
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<tr>
<td></td>
<td></td>
<td>✅</td>
<td>i) Water from a spring</td>
</tr>
<tr>
<td>Cuncolim</td>
<td>5</td>
<td>✅</td>
<td>i) Curing water, masonry tank, barrels, containers.</td>
</tr>
<tr>
<td>Curechorem</td>
<td>5</td>
<td>✅</td>
<td>i) Curing water, paddy field, drain, cement tank, drain, abandoned tyres, bottles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✅</td>
<td>i) Scrub jungle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✅</td>
<td>i) Water from paddy field</td>
</tr>
<tr>
<td>Dhedhsagar</td>
<td>1</td>
<td>✅</td>
<td>ii) Dense forest, water falls, riparian habitat</td>
</tr>
<tr>
<td>Edda</td>
<td>1</td>
<td>✅</td>
<td>i) Scrub jungle, dense forest</td>
</tr>
<tr>
<td>Endrem</td>
<td>1</td>
<td>✅</td>
<td>i) Scrub jungle, dense forest</td>
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<tr>
<td>GU campus</td>
<td>5</td>
<td>✅</td>
<td>i) University glass house, scrub jungle around a big water reservoir</td>
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<tr>
<td></td>
<td></td>
<td>✅</td>
<td>ii) Water from ponds, water reservoir</td>
</tr>
<tr>
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<td></td>
<td>✅</td>
<td>iv) Ponds</td>
</tr>
<tr>
<td>Karmali</td>
<td>1</td>
<td>✅</td>
<td>i) Vegetable field and scrub jungle</td>
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<td></td>
<td>✅</td>
<td>iii) Water from lake and ponds</td>
</tr>
<tr>
<td>Kasaragod</td>
<td>2</td>
<td>✅</td>
<td>ii) Mixed plantation</td>
</tr>
<tr>
<td>Kesarval</td>
<td>2</td>
<td>✅</td>
<td>ii) Mixed plantation, scrub jungle</td>
</tr>
<tr>
<td>Kodachadri</td>
<td>1</td>
<td>✅</td>
<td>i) Dense shola forest</td>
</tr>
<tr>
<td>Kollur</td>
<td>1</td>
<td>✅</td>
<td>ii) Forest, riparian habitats</td>
</tr>
<tr>
<td>Melka</td>
<td>3</td>
<td>✅</td>
<td>i) Dense forest</td>
</tr>
<tr>
<td>Molem</td>
<td>12</td>
<td>✅</td>
<td>i) Dense forest, riparian habitats</td>
</tr>
<tr>
<td>Panaji</td>
<td>3</td>
<td>✅</td>
<td>i) Curing water, asbestos tank, accumulated water over terrace</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✅</td>
<td>iv) Water logged area in batlem</td>
</tr>
</tbody>
</table>
Table: 3.2 Collection dates for *In situ* observation of life cycle of species of *Aschersonia* and *Hirsutella*

<table>
<thead>
<tr>
<th>Location</th>
<th>Collection Dates</th>
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<td>Pernem</td>
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<td>21.07.2001</td>
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<td>05.08.2001</td>
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<td></td>
<td>24.03.2002</td>
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<td>16.06.2002</td>
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