Being a tropical country, India is blessed with a variety of timber yielding tree species and as many as 1500 species are commercially utilised for diverse purposes. Some of the important plantation tree species grown in India are *Tectona grandis*, *Eucalyptus* spp., *Acacia* spp., *Dalbergia sissoo*, *Swietenia* sp. *Santalum album*, *Melia dubia*, *Ailanthus excelsa*, *Leucaena leucocephala* etc. Productivity of forests in general, and particularly that of commercial forest plantations, is very much affected by frequent outbreak of pests and diseases, besides human interventions and various natural calamities. Among the pests, largely insects belonging to the orders Coleoptera, Lepidoptera, Isoptera and Homoptera are the major ones of significant economic importance that attack trees, right from the seeds to the final product. Wood deterioration by xylophagous insects leads to unhealthy tree growth and malformation of wood in timber yielding forest trees. Some such major pests belong to Lepidoptera, Coleoptera and Isoptera.

In India, *Hypsipyla robusta* Moore is a serious pest of meliaceous forest trees such as the exotics, *Swietenia macrophylla* King, *S. mahagoni* Jacq. and native *Toona ciliata* M. Roem. Although *Swietenia* spp. is grown in plantations in many states, the establishment is difficult because of shoot borer attack during the sapling stage. The most important host response associated with borer attack is the sprouting of multiple shoots on infested plants which affects the growth and economic value of the timber. Similarly, termites are another group of highly serious and economically important pests destroying wood and wooden products in houses, building materials and growing trees in forests.
Termites are abundant in tropical and subtropical environments and are able to deteriorate wood of trees in plantations and natural forests.

Entomopathogens have been used in the control of forest pests as alternatives to chemical insecticides. They are considered to be safer than chemical insecticides having little effect on man or other vertebrates and non-target invertebrates. Among the entomopathogens, fungi are of great importance because, unlike bacteria and viruses that need to be ingested for causing diseases, fungus enters the host by penetrating the insect cuticle and spread the infection which is an important aspect of successful use of entomopathogens as insecticides.

*Metarhizium*, commonly known as “green muscardine” fungus has a few species which are infectious to hexapods. Species such as *M. anisopliae* and *M. flavoviridae* have wide geographical distribution which has enabled them to be exposed to a broad diversity of environmental factors and insects host species. *Metarhizium* sp. was capable of infecting insects living in diverse habitats including fresh water, soil and air and infective to more than 200 insect species of various orders. However, its potentiality against only a few insect species belonging to Orthoptera, Isoptera, Homoptera and Coleoptera has been experimentally proved and exploited in different parts of the world.

In the present study, the notorious pest *Hypsipyla robusta* Moore on mahogany and the arboreal termites, *Odontotermes* spp. on several timber yielding trees were targeted to test the efficacy of *Metarhizium anisopliae* isolates as biocontrol agents, with the following objectives:
Screening the pathogenicity of *Metarhizium anisopliae* isolates against *Hypsipyla robusta* and *Odontotermes* spp.

Study the field efficacy of highly virulent *Metarhizium anisopliae* strains

Characterization of virulent isolates of *Metarhizium anisopliae*

6.2 Salient findings of the work

6.2.1 Incidence of Mahogany shoot borer and *Odontotermes* sp.

The incidence of the mahogany borer *Hypsipyla robusta* and the termite *Odontotermes* sp. was recorded in forest nurseries and plantations of ten districts of Karnataka, viz., Bangalore rural, Ramanagaram, Chikmagalur, Mandya, Mysore, Coorg, Hassan, Mangalore, Shimoga and Uttar Kannada. Visual observations were taken in the nurseries of mahogany throughout the year during the periods of infestation. On an average, infestation levels of the borer attack in the stands of mahogany was highest in the forest divisions of Hassan and Mangalore, 78 and 77.4 % respectively in the two divisions. Most plants were attacked in the height class between 2 to 4 ft and incidence of borer attack was noticed throughout the year with peak infestation during May to October. The mean percentage infestation of *Odontotermes* sp. in the plantation of teak, sandal and eucalyptus was highest in Bangalore rural and Yellapur forest divisions.

6.2.2 Collection and isolation of *Metarhizium anisopliae*

The occurrence of fungal infection in different species of insects were surveyed during the period of pest attack in different forest locations of South India. Eighteen *M. anisopliae* strains were isolated form forty nine cadavers of insects belonging to the orders, Coleoptera, Hemiptera, Isoptera, Orthoptera and Lepidoptera. Microscopic studies
showed that morphological features of the collected *M. anisopliae* isolates were matching with the valid described characteristics of the genus *Metarhizium*. Apart from these eighteen isolates, four isolates of *M. anisopliae* were collected from the type culture collection centre of the Agriculture Research Services of Entomopathogenic Fungi (ARSEF), Ithaca, New York and used as standards.

6.2.3 Pathogenicity tests

Infectivity of the twenty two *M. anisopliae* isolates were tested against the shoot borer *H. robusta* under laboratory conditions. In the single concentration assay, all the isolates caused mortality and mycosis in the first, second and third instar larvae. However, the final two stages of these larvae were less susceptible even to the highly effective isolates. Seven comparatively more virulent isolates were evaluated by a detailed bioassay against second instar larvae. Two isolates, IWST-Ma7 and IWST-Ma13 required lowest concentration of $1.19 \times 10^5$ conidia ml$^{-1}$ to kill 50% of the larval population. The potency indices for IWST-Ma7 and IWST-Ma13 were 0.41 and 0.47 respectively, which were indicative of their increased virulence. The average survival time for the larvae decreased as the conidial concentration increased and the isolate IWST-Ma7 required the shortest duration (6.2 to 7.3 d) to kill 50% of larvae at the four conidial concentrations.

Similarly, the pathogenicity of twenty two isolates of *M. anisopliae* was screened against the workers of two species of *Odontotermes* (*O. obesus* and *O. redemannii*). Almost all the isolates were pathogenic to the workers of the two species. The level of pathogenicity of each specific isolate was nearly similar for both species of the termites.
Among the twenty two isolates screened, five isolates that showed higher mortality and mycosis were selected to study their efficacy in multiple concentration assay against *O. obesus*. Two isolates, IWST-Ma2 and IWST-Ma13 were found to be more virulent than the standards, with significantly lower LC$_{50}$, AST and potency indices.

### 6.2.4 Horizontal transmission of fungal infection in *O. obesus* colonies

As the termites are highly social insects, they engage in a variety of activities that necessitate frequent, direct physical contact with other colony members. Trophallaxis (the exchange of regurgitated food), proctodeal trophallaxis (the consumption of anal excretions) and grooming are regular and necessary colony functions. Because of thorough grooming behavior, the infective propagules of *M. anisopliae* can be transferred from one individual to another. In our study on the transmission of fungal infection in a population of *O. obesus*, it was found that the mortality and mycosis caused due to active transmission was high in the case of isolates IWST-Ma13 (98%) and ARSEF 7413 (90%). Transmissions of fungal propagules were found to be lesser among soldiers compared to workers even for the highly virulent isolates.

### 6.2.5 Repellency of *O. obesus* to *M. anisopliae* conidia

The termites acted aggressively towards individuals carrying high loads of infective propagules and began to move rapidly back and forth in a characteristic alarm behavior. The foraging activity of termites in the presence of dry conidia of the most virulent isolates IWST-Ma13 and ARSEF 7413 was found to be repelled considerably and after 90 min of treatments, only 23 and 22% individuals showed activity. This could reduce the transmission of infection in the remaining population of termites. To
overcome this repellency behavior, conidia of the two isolates were mixed with attractants (mixture of sawdust, sugarcane bagasse and cardboard powder) which optimally decreased the repellency and improved the foraging activity to 59 and 60%. Thus the reorganization behavior of termites which prevailed when dry conidia were mixed with attractant could increases the transmission of fungal infection within the termite population.

6.2.6 Toxicity effects of crude soluble protein extract of *M. anisopliae*

The entomopathogenic fungus *M. anisopliae* produces several extracellular metabolites which are toxic to insects. In the present investigation, the toxicity effects of the crude soluble protein extract of ten most virulent isolates were studied against *H. robusta* and *O. obesus*. The protein extract of the isolate IWST-Ma7 showed the lowest LD$_{50}$ of 2.6 mg/ml against *H. robusta* while protein extract from IWST-Ma13 showed LD$_{50}$ of 0.24 mg/ml against *O. obesus*.

6.2.7 Field efficacy of effective *M. anisopliae* isolates against *H. robusta*

Field efficacy of the potent isolates against the shoot borer, *H. robusta* infesting the saplings of mahogany showed that the pest infestation was reduced to 23 and 13% after five months of foliar spray of the isolates IWST-Ma7 and ARSEF 2596. In the case of shoot injection, the larval mortality and mycosis were increased to 23 and 72% respectively. When different application methods were compared, the relative potency index based on mortality and mycosis was lowest (0.64) for the shoot injection method. Though the relative potency indices were superior when the spores were injected into the shoot, topical spray may still be the best for effective control since the first and second
stage of larvae feed on the external plant parts. The prophylactic sprays of *M. anisopliae* in nurseries against *H. robusta* larvae need to be repeated frequently on rapidly growing shoots. Spraying may provide longer protection since the fungal spores are borne by wind, which facilitate quick transmission among the low-density larval population.

### 6.2.8 Field efficacy of effective *M. anisopliae* isolates against *O. obesus*

A major limiting factor in the control of termites in forest plantations was whether the success of the treatments relies on the elimination/suppression of the colony or avoidance of treated areas by the termites. To address these, four experimental approaches were followed, *viz.*, stake treatment, tree treatment, bait application and mound treatment. When the stakes were treated with conidia of the two isolates IWST-Ma13 and ARSEF 7413 mixed with attractant, the weight loss of the stakes was found to be comparatively low. The mortality and mycosis of *O. obesus* were highest in IWST-Ma13 (49.7 and 39.5 %) at the end of five months. It was also found that the control stakes were completely damaged which indicated the termites did not completely avoid the treated area, rather the colonies were suppressed.

In the trees treated with dry conidia and conidia mixed with attractant, the width of mud galleries covered was much less (16 and 19 cm) when the conidia were mixed with attractants. Highest mortality of 38 and 29% and mycosis of 89 and 78% were recorded respectively for IWST-Ma13 and ARSEF7413 after 98 days of treatment, when conidia were mixed with attractants. When the conidia mixed bait (mixture of sugarcane bagasse, cardboard powder and sawdust) was applied in the soil around the infected trees, the suppression of the termite population was more apparent than when dry conidia
alone was applied. The isolate, IWST-Ma13 showed highest mortality and mycosis of 53 and 67% when the conidia were mixed with bait. Though the mortality caused by the most virulent strain IWST-Ma13 was about 39% when applied as dry conidia, the mycosis was comparatively less. The baits buried inside the soils were completely eaten by the termites. This could suggest that the pathogen alarm behavior exhibited by termites was significantly masked by baiting. As the termites move for a long distance inside the galleries, the groomed termites could horizontally transfer the fungal infections which could suppress the population.

Mound treatments were done by direct dusting of large quantities of conidia mixed with attractants into the galleries to specifically eliminate the colonies. The activity of the termites reduced gradually over a five month period compared to that of control. The ‘head banging sound’ emitted by the termites inside the mound was measured as Acoustic emission signal voltage. As this signal constantly exceeds the threshold voltage level inside the control mound, the relative magnitude signal expressed as root mean square (RMS) remains maximum at the end of five months. In the fungus dusted mound the RMS successively decreased over a period of five months. The ratio plotted for Max RMS to Avg RMS and Max RMS to Min RMS peaks are low indicating that the ratio of the termite population (soldiers and workers) was less after five months of treatments.

6.2.9 Morphology and cultural characters of *M. anisopliae* isolates

The morphological features like color, size, shape of conidia and media pigmentation of the isolates showed that, the conidia were cylindrical to oval in shape.
and generally light green in color. The length/width ratio of the conidia varied from ≤ 2.58 µm (low ratio group), 2.79 to 2.94 µm (medium ratio group) and ≥ 3.00 µm (high ratio group). The temperature requirement for all the isolates ranged from 23 to 27.5 ºC with highest growth observed at 25.2 ºC for majority of the isolates. Highest growth was observed between RH 94.7 to 97.4 % and pH 5.2 to 5.8. The mean growth rate and biomass (dry weight) was highest for IWST-Ma7 (6.95mm and 1.45g) and IWST-Ma13 (6.89 mm and 1.36 g).

6.2.10 Enzymatic characterization of M. anisopliae isolates

Entomopathogenic fungi invade insects by a combination of mechanical pressure and enzymatic degradation of the cuticle. Proteases play a major role in cuticle degradation by M. anisopliae, as chitinolytic enzymes appear after the enzymes of the proteolytic complex. The enzymatic activity of crude soluble protein extract of ten M. anisopliae isolates showed that chitinase and chitin deacetylase activity were high (0.69 to 1.90 U/ml and 0.45 to 1.80 U/ml) for all the isolates tested. Activity of all the four enzymes remained highest for IWST-Ma7. The protein band pattern in the SDS-PAGE showed that the molecular weight of the crude soluble extract of ten virulent isolates varied form 66 kDa to 14.2 kDa.

6.2.11 Phylogenetic analysis of M. anisopliae isolates

The 5.8S intergenic regions of 16S rDNA of eighteen M. anisopliae isolates were amplified using primers ITS-1 and ITS-4 which showed that these isolates could be grouped under five major clades A, B, C, D and E. Among the isolates, the intergenic region of the three isolates IWST-Ma1, IWST-Ma14 and IWST-Ma3 were closely related.
to the reference strain *M. anisopliae* var *anisopliae*, ARSEF442. Fourteen isolates were grouped with the reference strains of *M. anisopliae*, ART2455, ARSEF9612, ARSEF3145, ARSEF794 and KS0806. The intergenic region of the rDNA of IWST-Ma2 was distinct and formed a separate clade (clade E). It was also observed that none of our isolates showed sequence similarities with *M. anisopliae* var *acridum* and *M. flavoviride*.

### 6.3 Salient findings

a. In the present study, eighteen *M. anisopliae* isolates were recovered and purified from cadavers of insects belonging to the orders Lepidoptera, Coleoptera, Homoptera, Isoptera and Orthoptera.

b. Infectivity of these isolates and four ARSEF isolates were tested against *H. robusta* and *O. obesus*. IWST-Ma7 was found to be the most effective isolate against the mahogany borer and IWST-Ma13 against *Odontotermes* sp. based on mortality, mycosis, LC\(_{50}\), AST, horizontal transmission, repellency tests and toxicity effects.

c. The field potency of these isolates was evaluated against *H. robusta* and *O. obesus* in the nurseries and plantation of mahogany, teak and eucalyptus.

d. The infestation levels of *H. robusta* was reduced to 23% when prophylactic spray of IWST-Ma7 was given to the saplings of *S. mahagoni* during the period of pest infestation.

e. Field potency of IWST-Ma13 against *O. obesus* was more when conidia were mixed with the bait (sugarcane bagasse, saw dust and cardboard powder) and applied at the base of the trees.
f. Direct dusting of conidial mixture into the mound galleries could successively eliminate *O. obesus* after five months of treatment.

g. The morphological features of the twenty two *M. anisopliae* isolates showed that the conidia were cylindrical to oval in shape and generally light green in color.

h. The enzymatic activity of crude soluble protein extract of ten *M. anisopliae* isolates suggested that the chitinase and chitin deacetylase activity were high.

i. The protease, lipase, chitinase and chitin deacetylase activity of IWST-Ma7 remained highest among the isolates tested.

j. The 5.8S intergenic regions of 16S rDNA of eighteen *M. anisopliae* showed that these isolates were grouped under five major clades, A, B, C, D and E.

k. The sequences of the ITS region of the eighteen isolates were deposited in GenBank and got published along with accession numbers.

### 6.4 Conclusion

*M. anisopliae* isolates could not completely eliminate *H. robusta*; however, it reduced damage to a tolerable level. Prophylactic sprays of *M. anisopliae* in nurseries of mahogany against *H. robusta* larvae would have to be repeated frequently during the peak periods of plant susceptibility to the pest attack. Though the larvae are internal feeders, since the initial larval stages feed on tender leaves, topical spray will still be effective for control of the shoot borer. Application of fungal conidia may provide longer protection since they are borne by wind which also increases transmission in low density larval populations like *H. robusta*.

Grooming and social interactions of termites are seen to have the potential to spread the fungus through the colony. A long term control measure for termites could be
by direct dusting of termite nests. However factors such as avoidance of the fungus conidia, removal and burial of fungus killed termites, together with defensive secretion and inhibitory components in frass and possiblity of humoral resistance may limit the spread of the diseases in the colony. This can be overcome by mixing the conidia with attractants like sugarcane bagasse, sawdust and cardboard powder which make them non-repellent. Even if *Metarhizium anisopliae* cannot routinely eliminate termite colonies in forest areas, there will still be a place for products based on theses fungi as termite control is becoming more focused on suppression of colonies.