CHAPTER 8

GENERAL SUMMARY AND CONCLUSIONS
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Neem (*Azadirachta indica* A. Juss.) is a native tree to India and commonly known as ‘Indian lilac’ or ‘Margosa’. The Persian name of neem is ‘*Azad-Darakth-E-Hind*’ which means “Free tree of India”. Presently it is found in about 72 countries of the world. India stands first in neem seed production. In India neem is regarded as a very valuable tree owing to its wide application. Both wood as well as non-wood products are used for varied purposes. In comparison to other tree species neem is well adapted to stress conditions. Plantation of neem thus helps in combating desertification, deforestation, reducing excessive global temperature, soil erosion and even helps in population control, because of which neem is referred to “Tree for solving global problems”.

Neem finds many applications in the fields of pest management, environment protection and medicine. Neem is a natural source of insecticides, pesticides and agrochemicals. About 135 and more active chemical compounds were isolated from various parts of neem. Neem products were reported to have antifungal, antibacterial, insecticidal and other versatile biological activities such as pharmacological activities viz., immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties. In Ayurvedic medicine, neem is used for more than 4000 years due to its medicinal properties. Owing to its medicinal value from simple disorders like common cough to most dreaded disease such as cancer and AIDS neem is considered as “Wonder tree of India”.

Though neem is known to have biological activity against many microorganisms it is not free from microbial diseases. Many bacterial and fungal diseases were reported
on neem. A new fungus *Phomopsis azadirachtae* Sateesh, Bhat and Devaki, a deuteromycetous fungus, was recorded on neem causing die-back. The symptoms include twig blight, inflorescence blight and even fruit rot resulting in almost 100% loss of fruit production and thus affecting the availability of seeds which are the major commercial product of neem. The fungus infects the neem trees irrespective of the age and size.

Review of literature on the die-back of neem revealed the absence of enough information regarding biodiversity of pathogen, role of toxin in pathogenicity and integrated disease management. Thus the present investigations were carried out to study the - cultural, morphological, biological and physiological variability among the isolates of *P. azadirachtae*, protein profile, molecular methodology for precise and rapid detection of the pathogen, phytotoxin production and its bioactivity. Studies on chemical, biological and integrated management strategies were also carried out.

*P. azadirachtae* was isolated aseptically from the die-back affected neem twig samples collected from different agroclimatic regions of Tamilnadu. Tamilnadu stands second with respect to the number of neem trees. This was accomplished through plating of surface-sterilized twig segments on sterile PDA amended with 100 ppm of chloramphenicol contained in sterile Petri dishes and incubating at 26 ± 2°C with 12 h photoperiod. The isolates obtained were designated as follows: TN 01 (Chennai), TN 02 (Coimbatore), TN 03 (Cuddalore), TN 04 (Dharmapuri), TN 05 (Erode), TN 06 (Kanniyakumari), TN 07 (Karur), TN 08 (Madurai), TN 09 (Palani), TN 10 (Perambalur), TN 11 (Ramanathapuram), TN 12 (Tiruchchirappalli), TN 13 (Tirunelveli), TN 14 (Tuticorin), TN 15 (Vellore), and TN 16 (Virudhunagar). The isolates were then compared for the cultural, morphological, biochemical and physiological variations.
Significant variability among the isolates was observed for the cultural, morphological and biochemical characteristics such as mycelial growth pattern, colour of the colony, production of pycnidia, mycelial protein content and mycelial total carbohydrate contents. Maximum mycelial protein content was observed in isolate TN 10 (150.00 mg / g) and minimum protein content in isolate TN 12 (68.67 mg / g). Isolate TN 06 (13.8 mg / g) had highest total carbohydrate whereas isolate TN 02 had least total carbohydrate content (7.47 mg / g).

Physiological variability was studied by growing the isolates of pathogen at different temperature (20, 25, 30, 35, and 40 ± 1 °C) and pH conditions (4.0, 5.0, 7.0, and 8.0) in potato dextrose broth (PDB) and liquid basal medium (LBM) respectively. The ability of the isolates to utilize different carbon sources (Cellobiose, dextrose, sorbitol, starch, and sucrose), different nitrogen sources (Ammonium sulphate, L-asparagine, potassium nitrate, sodium nitrite and urea), and different media (Corn Meal Broth, Czapek-Dox Broth, Malt Extract Broth, Oat Meal Broth, and Potato Dextrose Broth) were also studied by growing the isolates of *P. azadirachtae* in LBM medium amended with different carbon, nitrogen sources, and in different media separately for 25 days and taking mycelial dry weight as the criterion. Temperature of 30°C and 35°C, pH of 6.0 were the best for the growth of all isolates. Starch (506.43 mg) and sucrose (485.29 mg) were the preferred carbon sources. Ammonium sulphate (510.57 mg) formed the good nitrogen source. Maximum mycelial dry weight of the pathogen was observed in Malt Extract Broth (542.81 mg) followed by Potato Dextrose Broth (499.17 mg) and Czapek Dox Broth (458.18 mg). The data showed significant effects of treatments and isolates on
growth as well as significant isolates – treatments interactions. All the isolates displayed considerable variations among themselves in their growth response to each treatment.

To determine the variability in toxicity of culture filtrate among the isolates of *P. azadirachtae*, the filtrate of each isolate was obtained from 25-day-old culture grown in potato dextrose broth. 400 healthy neem seeds were treated with culture filtrate of each isolate for overnight. Control was maintained having seeds treated with only distilled water and the medium. The seeds were incubated for germination at room temperature for 15 days following moist blotter and paper towel methods. Then the mean root length, mean shoot length, percentage germination and vigour index were calculated. Neem seeds treated with culture filtrate of isolates of *P. azadirachtae* showed reduction in their germination and seed vigour when compared to the seeds maintained as control. Culture filtrates of different isolates exhibited different extent of toxicity on the germination of neem seeds. The culture filtrate of TN 02 (Coimbatore) isolate was highly toxic and of the TN 11 (Ramanathapuram) isolate was least toxic. In all the test carried out the results obtained suggested the presence of variability among the isolates of *P. azadirachtae* and revealed its heterogeneous nature and genetic diversity.

Six Tamilnadu isolates (TN 02, TN 03, TN 04, TN 06, TN 08 and TN 12 collected from Coimbatore, Cuddalore, Dharmapuri, Kanniyakumari, Madurai and Tiruchchirappalli respectively) were subjected to Sodium Do-decyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The six isolates exhibited variability in their electrophoretic pattern of soluble mycelial proteins providing an additional proof for the heterogeneous nature and genetic diversity of the pathogen. Protein bands common to all the isolates and also bands specific to each isolate were observed. 42
bands of different molecular weights were present. The similarity index among the 
*P. azadirachtae* isolates ranged from 29.63% to 59.26%.

*P. azadirachtae* is identified by traditional methods of isolation and culturing. The observation of conidia is a must to confirm the identification but it is time consuming and requires about 20-25 days. PCR method with specific primers provides an easy way to detect fungal plant pathogens in both culture and plant tissues. PCR allows fast differentiation and identification of microorganisms. Primers, both forward and reverse, capable of producing 154 bp product were developed (Primer 3) by multiple sequence alignment of rDNA sequences (CLUSTAL W) and selecting conserved sequences of 179 bp. BLAST N revealed the homology of the selected sequence only with *Phomopsis* spp. and *Diaporthe* spp.

DNA was extracted from mycelium of *P. azadirachtae* and die-back affected neem tissues (twigs, seeds, embryos), and also from healthy neem explants (twigs, seeds, embryos), few other fungi such as *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Mycelia sterilia* and a bacterium isolated from neem. All the DNA samples were subjected to PCR using the primers developed and then the PCR products were subjected to gel electrophoresis. The primers amplified the DNA samples of *P. azadirachtae* and die-back affected neem tissues but not from other DNA samples such as DNA from healthy neem explants, other fungi and a bacterium. This shows the specificity of the primers towards *P. azadirachtae* and thus can be utilized for the precise and accurate detection of *P. azadirachtae* from die-back affected neem tissues. This will help to quarantine the neem seeds and prevent the spread of the disease.
The present investigations were focussed on isolation and partial characterization of the toxic metabolite from the culture filtrate of *P. azadirachtae* and the toxicity of that toxin on neem seed germination and neem callus growth *in vitro*. The pathogen was grown in potato dextrose broth for 25 days and the culture filtrate was collected. The crude extract of toxin was obtained by extracting the culture filtrate with methanol and chloroform. TLC of the crude extract of toxin revealed the presence of four bands *i.e.* four compounds. The chemical tests conducted such as Mayer’s test, Dragendorff’s test (for alkaloids), Ferric chloride test (for phenolics), Millon’s test, Biuret test and Ninhydrin test (for proteins) showed the presence of amino acids and proteins and the absence of alkaloids and phenolics in the crude toxin extract solution of *P. azadirachtae*.

Neem seeds were treated with 500 ppm and 1000 ppm of crude toxin extract. 400 healthy neem seeds were treated for overnight. Control was maintained having seeds treated with only 5% methanolic solution. The seeds were incubated for germination at room temperature for 15 days following moist blotter and paper towel methods. Then the mean root length, mean shoot length, percentage germination and vigour index were calculated. Neem seed germination was completely inhibited at both the concentrations of the toxin.

Neem callus was grown on the MS medium with 6-benzylaminopurine (one ppm) and Kinetin (one ppm) for 30 days using neem cotyledons as host explants. The calli were then grown on MS medium amended with different concentrations of *P. azadirachtae* crude toxin (10, 100, 250, 500 and 1000 ppm) for 30 days. Progressive inhibition of neem callus growth was observed, and necrosis was observed at 250 ppm
and above. These studies revealed the ability of the pathogen to produce phytotoxin and the role of this toxin in the pathogenesis.

Six systemic fungicides were screened against *P. azadirachtae* such as Carbendazim, Hexaconazole, Metalaxyl, Isoprothiolane, Tricyclazole, and Thiophanate-methyl. These fungicides were tested against the pathogen using poison-food technique. Initial screening included 10, 100, 250, 500, 1000 ppm concentrations of all the fungicides. Carbendazim, hexaconazole and thiophanate-methyl inhibited the growth of *P. azadirachtae* at 10 ppm. Thus these fungicides were screened at lower concentrations (1.0, 2.0, 4.0, 6.0, 8.0, 10.0 ppm).

Carbendazim and thiophanate-methyl completely suppressed the pathogen growth at 1.0 ppm and these two fungicides were selected for further screening at still lower concentrations (0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75 and 1.0 ppm). The parameters studied included colony diameter, mycelial dry weight, pycnidial formation and the germ tube growth. Carbendazim completely inhibited the growth of pathogen at 0.25 ppm and thiophanate-methyl at 0.75 ppm. A statistically significant difference was observed between the treatments on the growth of pathogen (*P ≤ 0.000*).

Biocontrol of plant diseases using microorganisms provides a possible alternative to decrease the input of agrochemicals in agriculture. In the present study, four bacterial and two fungal antagonists were screened against *P. azadirachtae in vitro*. The antagonistic microbes were obtained from MTCC, IMTECH, Chandigarh, India and included *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 619), *Pseudomonas aeruginosa* (MTCC 2581), *Pseudomonas oleovorans* (MTCC 617), *Trichoderma harzianum* (MTCC 792) and *Trichoderma viride* (MTCC 800). The bacteria were grown
in nutrient broth for 72 h and the culture filtrate of each bacterium was collected separately and subjected to ethyl acetate extraction to obtain crude extract. Similarly fungi were grown in potato dextrose broth for 25 days and the culture filtrate of each fungus was collected separately and subjected to ethyl acetate extraction to obtain crude extract. These ethyl acetate fractions were tested against the pathogen using poison-food technique.

Initially all the ethyl acetate extracts were screened at 25, 50, 100, 250, and 500 ppm. The ethyl acetate fractions of culture filtrates of *B. subtilis* and *Ps. aeruginosa* suppressed the mycelial growth of *P. azadirachtae* at lower concentration (25 ppm) in comparison with the ethyl acetate fractions of the other antagonistic microorganisms. Thus these two ethyl acetate fractions were screened at much lower concentrations viz., 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 ppm. The parameters studied included colony diameter, mycelial dry weight, pycnidial formation and the germ tube growth. Ethyl acetate extracts of both these bacteria inhibited the growth of *P. azadirachtae* at 25 ppm concentration. A statistically significant difference was observed between the treatments on the growth of pathogen (*P ≤ 0.000*).

Extensive utilization of fungicides has resulted in many problems including the risk of development of fungicide resistance by the pathogen. Biocontrol agents may not function well under certain conditions. These problems with extensive fungicide application and inefficiency of biocontrol agent can be overcome by ‘Integrated Disease Management (IDM)’ strategy. Combination of chemicals and antagonistic microorganisms has received major preference. During the present investigations carbendazim and thiophanate-methyl were combined with the ethyl acetate extracts of
culture filtrates of *B. subtilis* and *Ps. aeruginosa* and the effect of these combinations on the growth of *P. azadirachtae* was studied. The effect of these combinations on neem seed germination and growth of seed-borne pathogen was also studied.

Each fungicide was combined with ethyl acetate extract of each bacterium separately to obtain different concentrations viz., 100F: 0E, 80F: 20E, 60F: 40E, 50F: 50E, 40F: 60E, 20F: 80E, 0F: 100E. Based on the results of the previous chapters the 0.25 ppm and 0.75 ppm concentrations of carbendazim and thiophanate-methyl respectively were taken as 100%. Similarly 25 ppm concentration was considered as 100% for ethyl acetate extracts of both *B. subtilis* and *Ps. aeruginosa*. The tests were carried out using poison-food technique. The parameters studied included colony diameter, mycelial dry weight, pycnidial formation and the germ tube growth. These combinations in all concentrations tested, except 20F: 80E completely inhibited the vegetative growth on agar medium, sporulation and germination of spores of the pathogen. At 40F: 60E concentrations of combinations of thiophanate methyl and ethyl acetate extracts of *B. subtilis* and *Ps. aeruginosa* mycelial growth on solid media was observed. Even the 20F: 80E and 40F: 60E concentrations of all the combinations completely checked the growth of *P. azadirachtae* in broth medium. A statistically significant difference was observed between the treatments on the growth of pathogen (*P* ≤ 0.000).

Based on these results 50F: 50E concentration of each combination of fungicides and ethyl acetate extracts and its multiple concentrations viz., 50F: 50E X 10, 50F: 50E X 50, 50F: 50E X 100, 50F: 50E X 500 were tested for possible toxicity against neem seed germination and its effect on seed-borne pathogen. 400 healthy neem seeds were treated
for overnight. Control was maintained having seeds treated with only distilled water. The seeds were incubated for germination at room temperature for 15 days following moist blotter and paper towel method. Then the mean root length, mean shoot length, percentage germination and vigour index were calculated. For studying its effect on seed-borne pathogen, 400 diseased neem seeds were treated for overnight. Control was maintained having seeds treated with only distilled water. The seeds were incubated at room temperature for seven days on potato dextrose agar. While the 50E: 50E concentration and its multiple concentrations of all the combinations tested completely inhibited the pathogen growth, they had no significant inhibitory effect on germination of neem seeds. Thus the 50:50 concentrations of all the combinations of systemic fungicides and ethyl acetate extracts of bacteria can be considered as potential integrated control measure against *P. azadirachtae*.

To conclude, the work presented in this thesis furnishes an insight into the variability of *P. azadirachtae*. The knowledge gained regarding the cultural, morphological, biochemical and physiological variations among the isolates of *P. azadirachtae* can be applied for effective management of the pathogen. Results of present research work disclosing the presence of intraspecific variability among the *P. azadirachtae* isolates could be utilized for the characterization of the pathogen into sub-specific taxa. The primers that are developed can be employed for rapid detection and identification of *P. azadirachtae* in neem tissues affected with die-back. PCR based detection method would be most useful for seed health testing and with proper certification infected seeds reaching the growers can be avoided.
The ability of the pathogen to produce toxin, its chemistry and its role in
pathogenicity has been understood to some extent. The knowledge gained during the
present studies will provide a foundation for further research work on the phytotoxin of
*P. azadirachtae*. The results of the present investigations regarding management of the
die-back pathogen would be helpful in designing eco-friendly integrated strategies for the
effective management of the die-back of neem. The related literature from different
sources compiled in the bibliography would form a valuable data base to carry out further
research work on die-back of neem.