CHAPTER 2

REVIEW OF LITERATURE
Die-back disease of neem (*Azadirachta indica* A. Juss.) is caused by *Phomopsis azadirachtae*. Presently literature available on the pathogen causing die-back disease of neem is very limited. The occurrence of die-back disease of neem was first reported from new forests of Dehra Dun, North India (Khan, 1992). Sateesh *et al.* (1997) first identified and reported the pathogen causing die-back of neem as *Phomopsis azadirachtae*. The disease symptoms include twig blight, inflorescence blight and fruit rot. At present it is the major, devastating disease of neem (Bhat *et al.*, 1998).

### 2.1 Disease symptoms

The die-back has been noticed in neem trees irrespective of age, size and height. The disease is more pronounced during August to December, though can be observed throughout year. Appearance of symptoms starts with the on-set of rainy season and becomes progressively severe in later part of the rainy season and early winter season. The terminal branches are mainly affected. The disease results in the progressive death of the tree, year after year (Sateesh, 1998). Twig blight is the major symptom (Fig. 3). The disease also results in inflorescence blight and fruit rot resulting in almost 100% yield loss (Bhat *et al.*, 1998). It spreads through conidia that are disseminated by rain droplets and insects (Sateesh, 1998). The pathogen is seed-borne and seed-transmitted (Sateesh and Bhat, 1999).
A. Healthy

B. Die-back affected

Fig. 3. Neem tree
2.2 The pathogen

*P. azadirachtae*, the incitant of die-back on neem is a deuteromycetous fungus (Fig. 4). The pathogen was successfully isolated from all the twig explants collected from the diseased neem trees on potato dextrose agar (Fig. 7A). Though many species of *Phomopsis* have been reported to have teliomorph (*Diaporthe*), it was not identified with *P. azadirachtae*. It was not possible to induce teliomorphic or sexual phase in *P. azadirachtae*, in spite of cultivating the pathogen on specific media under specific conditions required to induce sexual phase. No collateral host has been identified. The pathogen produces two types of spores: Alpha (α) – conidia and Beta (β) - conidia (Fig. 5 and 6). α – conidia are fertile and germinate readily but germination of β- conidia has not been observed. The ergosterol estimation study confirmed the presence of pathogen in neem tissues (Sateesh, 1998).

The description of pathogen is as follows:

“Mycelium immersed, branched, septate, profuse, colourless, becomes pale brown later. Conidiomata pycnidial, solitary or aggregate, half-immersed, pale brown to dark brown or black, ampuliform or subglobose, unilocular, thick-walled, textura annularis, uniform throughout with the endogenous basal swelling cone with lumina of bigger cells, outer layers melanised, 300-500 µm high, up to 900 µm, wide in sections, very short basal clypei, ostiole single, unilocular, circular, papillate. Conidiophores simple or branched, short or elongate, septate, filiform, hyaline, line the inner layer of locule, 12-20 X 1.6-2.0 µm conidiogenous cells phialidic, subulate or filiform, integrated or discrete, channel and collarette minute, hyaline, periclinal thickenings of variable thickness, 5-8 X 1.6-3 µm, produce both alpha-conidia and beta-conidia, conidia acropleurogenous. Conidia of two
types, in a cream to dark yellow coloured slimy cirri: alpha-conidia hyaline, fusiform, straight, 2-4 guttulate, smooth, aseptate, 4.8-11 X 1.6-3.2 μm, germinate readily, beta-conidia hyaline, filiform, hamate, eguttulate, aseptate, 16-25.6 X 1.6-2.0 μm germination unknown” (Sateesh et al., 1997).

2.3 Cultural conditions

Light was found to have effect on sporulation, but not on mycelial growth. Sporulation requires proper light of about 8 - 12 h per day along with high relative humidity. The optimum temperature for vegetative growth of P. azadirachtae is in the range of 26 - 28°C and the pathogen can grow in a wide range of 10 - 35°C. Optimum pH was found to be 6 (range 4 - 9). Out of eleven carbon sources viz., cellobiose, cellulose, fructose, galactose, glucose, maltose, mannitol, lactose, sorbitol, starch, and sucrose; sucrose and starch were the best carbon sources. The pathogen also utilized cellobiose. Among the different nitrogen sources such as ammonium sulphate, asparagine, glycine, potassium nitrate, sodium nitrite and urea; ammonium sulphate and potassium nitrite were opted by the pathogen. Among the various vitamins supplied such as thiamine, pyridoxine, riboflavin, nicotinic acid, biotin and inositol, P. azadirachtae grew well on thiamine, riboflavin, nicotinic acid and pyridoxine amended media. The pathogen requires about 7 - 8 days for complete growth of mycelial mat in a 90mm diam. Petri dish and sporulation occurs within 12 days (Sateesh, 1998).
Fig. 4. Culture of *Phomopsis azadirachtae* on potato dextrose agar (10-day-old)

Fig. 5. Alpha conidia of *Phomopsis azadirachtae* (1000X)

Fig. 6. Beta conidia of *Phomopsis azadirachtae* (1000X)
2.4 Pathogenicity studies

Conidial inoculation, mycelial inoculation and tooth-pick inoculation methods were tried. Conidial inoculation method proved to be suitable to establish the pathogen. The same fungus was isolated from the twigs of all the neem plants inoculated with conidia (Sateesh, 1998).

2.5 Viability of mycelia and conidia

At room temperature, aerial mycelia remained viable for about 18 months on all types of media tested. Viability of mycelium varied at refrigerated conditions with respect to different media ranging from 21 - 36 months (Sateesh, 1998). The viability and germination of conidia also varied depending on the type of media and storage conditions. Conidia could be maintained viable up to 24 months at room temperature and up to 36 months at refrigerated conditions especially on potato dextrose agar and malt extract agar (Sateesh, 1998).

2.6 Seed-borne nature of \( P. \) azadirachtae in neem

\( P. \) azadirachtae is seed-borne and seed transmitted (Fig. 7B). Thus it gets transmitted from seed to seedling and might result in wide spread of the disease. Studies on the seed-borne nature of the pathogen revealed that \( P. \) azadirachtae was deep seated. The pathogen was present in seed coat, cotyledons and embryo. Many other fungi were isolated from seeds, except embryo, such as \( Aspergillus \) ochraceus, \( A. \) niger, \( A. \) flavus, \( Fusarium \) oxysporum, \( Penicillium \) sp. and \( Mycelia \) sterilia. Only \( P. \) azadirachtae was isolated from embryo (Fig. 7C). Seed to seedling transfer studies resulted in seed rot,
seedling rot, formation of weak seedlings, seedlings with fibrous root system and without root system (Sateesh, 1998; Sateesh and Bhat, 1999).

2.7 Biological studies

Variations among the P. azadirachtae isolates collected from different districts of Karnataka were studied. P. azadirachtae isolates showed cultural, morphological, pathogenic and biochemical variation (Fathima, 2004; Fathima et al., 2004 a).

Histopathological studies of naturally infected neem tree explants and seeds showed the presence of the pathogen in diseased neem tissues. The pathogen was found to be both intra as well as intercellular. The pathogen presence was also observed in vascular tissues revealing its systemic nature (Fathima, 2004; Fathima et al., 2004 b).

Fluorescence microscopy approach to evaluate the viability of conidia showed that α- conidia are more fertile than β- conidia. It was difficult to study viability of β- conidia using natural autofluorescence method as β- conidia didn’t germinate readily. The percentage germination data of α- conidia didn’t match the percentage non-fluorescing conidia data, and this might be because of presence of dormant α- conidia (Fathima, 2004; Fathima and Bhat, 2004).

P. azadirachtae produces phytotoxins. Culture filtrate of P. azadirachtae inhibited the germination of neem seeds (Sateesh, 1998). The isolates of P. azadirachtae collected from different regions of Karnataka, South India varied in their phytotoxic effect on the neem seed germination (Fathima, 2004).
A. From neem twigs

B. From neem seeds

C. From neem embryo

Fig. 7. Culture of *Phomopsis azadirachtae* from die-back affected neem tissues
2.8 Management studies

Six different systemic fungicides viz., Bavistin 50% W.P., Bayleton 25% W.P., Baynate 75% W.P., Calixin 80% E.C. and Kitazin 48% E.C., were tried. Bavistin was most effective. Bavistin completely suppressed mycelial growth, sporulation and conidial germination at 0.3 ppm. Seed treatment of neem seeds with bavistin resulted in the death of the seed-borne pathogen. Germination of neem seeds was not affected by bavistin even at higher concentrations (up to 2000 ppm) (Sateesh, 1998).

Effect of bavistin on neem callus cultures was studied. Cotyledonary explants produced good callus on MS medium amended with 1 ppm 6-Benzylaminopurine (BAP) and 1 ppm Kinetin. Exposure of neem calluses to bavistin at 200 ppm and above resulted in reduced growth and necrosis was observed on exposure to bavistin at 500 ppm and above concentrations (Sateesh, 1998).

Bacterial antagonists, Bacillus cereus, B. subtilis, Enterobacter aerogenes, Pseudomonas fluorescens and fungal antagonists, Trichoderma harzianum, T. viride, Gliocladium virens, Aspergillus niger, A. oryzae, Penicillium chrysogenum, Chaetomium globosum were tested against P. azadirachtae by dual culture method. Bacillus subtilis showed significant inhibitory effect on P. azadirachtae. Ten times concentrated culture filtrate of B. subtilis completely inhibited the growth of P. azadirachtae. Volatile compounds of B. subtilis had no effect on the growth of pathogen. B. subtilis produced both heat labile and heat stable antibiotics. Heat labile antibiotics were found to be highly potent against P. azadirachtae (Sateesh, 1998).

Effect of 24 botanical pesticides was studied. Only five plant species produced good results i.e., Lawsonia inermis, Asparagus officinalis, Bambusa arundinacea,
**Lantana camera** and **Macrosolen parasiticus**. *L. inermis* produced greater inhibitory effect at lower concentration in comparison with the other four plant species. Ethanol, methanol and aqueous extracts of *L. inermis* were tried and aqueous extracts were highly effective against *P. azadirachtae*. Thus *L. inermis* could be considered as potent botanical pesticide against *P. azadirachtae* (Sateesh, 1998).

Five essential oils – eucalyptus oil, pepper oil, nutmeg oil, coriander oil, fennel oil and two oleoresins – turmeric oleoresin and capsicum oleoresin were screened against *P. azadirachtae*. High activity was observed with nutmeg oil which completely inhibited the growth of *P. azadirachtae* at 2000 ppm (Fathima, 2004).

Critical review of literature indicated that few gaps exist in understanding the biology of the pathogen. Many more management strategies have to be considered and integrated management package has to be developed. Thus the present investigations were undertaken in the following areas:

- Screening of the diseased neem explants from different geographical regions of Tamilnadu and isolation of *Phomopsis azadirachtae*.
- Studies on the variability among the isolates of *P. azadirachtae* collected from different regions of Tamilnadu state, to understand the biodiversity of the pathogen.
- Identification and detection of presence of the pathogen in neem tissues using molecular tools (PCR).
- Isolation and characterization of phytotoxin and study of its role in pathogenesis.
- Chemical, biological and integrated management of *P. azadirachtae*. 