CHAPTER - V
Introduction

Seeds are the fertilized ovules containing embryos surrounded by integuments and form the generative organ of plants. These seeds are distributed nationally and globally for their beneficial effect either as food, or raw material for industrial or agricultural products. About 90% of all food crops in the world are propagated by seeds (Schwinn, 1994). Various fungi, bacteria, viruses and nematodes are carried with, on and in seeds (Maude, 1996; Neergaard, 1977). The methods of detection of seed-borne microorganisms were explained by Baker (1972), Neergaard (1977), Agrawal and Sinclair (1987) and Khare (1996). Fox (1993) explained the principles supporting the general and specific diagnostic techniques used in plant pathology for the detection and identification of seed-borne fungi, bacteria and viruses. Review article of Neergaard (1977) on plant losses due to seed-borne pathogens of the major cereal, legume, oil, vegetable and fiber crops illustrated the magnitude of such problems and identified the need for research on seed-borne pathogens. Losses of US $ 5.6 million in the Pacific North-West due to smutness of wheat caused by Tilletia tritici and incidence of rice blast (Pyricularia oryzae) in Japan during 1930s and 1940s resulting in famine are examples of extreme effects of diseases arising from the use of infective seed material (Maude, 1996). McGee (1995) stressed the importance to study the seed-borne aspects of the pathogen in disease cycle.
Seed transmission of diseases occurs by the transfer of inoculum from infected seeds to the germinating seeds and seedlings (Maude, 1996). The vast number of fungi, bacteria and viruses were said to be seed transmitted (McGee, 1995; Mink, 1993; Phatak, 1980). The pathogens may be present on outer seed parts or enter deep into tissues forming non-systemic or systemic infection respectively.

With the advances of seed pathology and introduction of official seed testing stations in various countries, seeds of wide range of crops come under scrutiny for purity and quality. Quality is assessed by laboratory designed tests. It is well-known that seeds serve as the microcosm for a variety of microorganisms (Maude, 1996). The quality of seeds could be affected by the organisms present within or on the surface of seed tissues. Understanding of the microorganisms associated with seeds will contribute in a number of ways for developing effective strategies in crop improvement programmes. A knowledge of the fungi that are frequently associated with the seeds will help in evolving methods to prevent seed infections, to devise strategies for crop protection and to adopt required seed treatment.

Neem is propagated by both sexual and asexual methods. They can be regenerated by using seeds, seedlings, root suckers or tissue culture derived plantlets (National research council, 1992; Tewari, 1992). But it is generally grown from seeds either planted directly on the site or transplanted as seedlings from a nursery. Neem seeds are highly recalcitrant in nature and the viability of seeds falls off rapidly after a few weeks (Bhardwaj and
Chand, 1995). After 2-6 months in storage they will no longer germinate (Ezumah, 1986; National research council, 1992). However, Roederer and Bellefontaine (1989) reported that seeds stored in France showed 42% of germination even after more than five years of storage. Neem is found growing in about 72 countries worldwide and large number of countries import seeds (Figs. 34A and 34B) from either Asian or African countries for raising seedlings and pesticide preparation.

Neem seeds viability will be affected by both abiotic and biotic factors. Seed moisture content and storage temperature are the major abiotic or environmental factors affecting the viability of stored neem seeds (Justice and Bass, 1988). The dried neem seeds with moisture content of 46.8% when stored in cotton bags at 15°C, remain viable after four months. Such seeds have 62% germination (Chaisurisri et al., 1986). Bhardwaj and Chand (1995) reported that stored neem seeds at RT, 15°C and 5°C in sealed and perforated polythene bags, perforated cardboard boxes and over silica gel in desiccator showed varied germination. Conditions prevailing inside the sealed containers and low temperature (5°C) results in rapid loss of viability within four months. The reduced germination at low temperature was due to the chilling damage. The aerated containers at RT and 15°C showed viability even up to six months of storage. This is due to the fact that seeds with relatively high moisture content require high oxygen concentration for maximum retention of survival capacity (Maithani et al., 1989). This clearly indicated that neem seeds are highly recalcitrant. The biotic factors, mainly seed associated microorganisms, also have considerable effects on seed viability and quality (Dhingra and Sinclair, 1995; Maude, 1996).
Figure 34A. Neem seeds, a source for botanical pesticides

Figure 34B. Closeup view of neem seed with hard endocarp (8X)
In the present work, the occurrence of *P. azadirachtae* and other mycoflora on neem seeds in various samples collected from different agroclimatic regions of Karnataka state, South India was determined and the presence of pathogenic *P. azadirachtae* in seed components, seed to seedling transmission and its significance were studied. Preliminary studies regarding the production of toxic metabolite in culture filtrate and its effect on the seed germination as well as seed vigour were carried out.

**MATERIALS AND METHODS**

**Collection of neem seed samples**

Fresh fruits were hand picked from neem trees showing die-back symptoms in a few branches. This was done in different agroclimatic regions of Karnataka viz., Mysore, Mandya, K.R. Pet and Bangalore. Later, pulp of the fruit was removed by rubbing against coarse surface. The seeds thus obtained were thoroughly washed with running tap water. These seeds were air dried for 24 h at RT. Seed collections were made during the first week of June to last week of August in the years 1995, 1996 and 1997. The seeds were stored in the brown paper bags at room temperature (25±2°C) until used for further study. All the seed samples were used within two weeks of their collection.
Detection of seed-borne *P. azadirachtae* and other fungi

Internally seed-borne *P. azadirachtae* and other fungi were detected on neem seeds by using standard agar plate method. The CDA or PDA media were used for this purpose (ISTA, 1993; Maude, 1996). Four hundred neem seeds from each sample were tested. The washed seeds were surface-sterilized by using 0.1% aqueous mercuric chloride (HgCl\(_2\)) for 10 min. These seeds were rinsed with sterile distilled water for five to seven times. For detection of externally seed-borne fungi, seeds were plated without surface sterilization. The seeds were plated on the media at the rate of ten seeds per Petri dish. The Petri dishes were incubated at 25±2°C for seven days under 12 h alternate light and dark period. The incubated seeds were observed under stereobinocular microscope. Fungi growing on the surface of the seeds were identified with the help of standard manuals considering the characters of colony, mycelium, conidiophores and the conidia. The fungal identity was further confirmed by observing the temporary mounts under compound microscope.

Data were recorded on the percentage seed germination, incidence of seed-borne *P. azadirachtae* and other mycoflora. *P. azadirachtae* was identified based on the description as in Chapter 2.

Location of *P. azadirachtae* within infected neem seeds

For determining the presence of the pathogen in infected seeds, component plating method (Dhingra and Sinclair, 1995; Maden, *et al*., 1975) was used. Seeds collected from Mandya and Mysore were selected for this purpose. The surface-sterilized seeds (0.1% HgCl\(_2\) for 10 min.) were soaked in sterile
distilled water for three hours. The seed components viz., seed coat, cotyledon and embryo portion were dissected aseptically on a blotter using sterilized needles. Each component was dipped separately in 1% sodium hypochlorite (NaOCl) for 30-60 seconds and was plated on agar medium (CDA / PDA) and incubated as described above. One hundred seeds were dissected in each sample and four replications were maintained. The associated fungi on each of the seed components were observed and identified using stereobinocular microscope. Fungal infection in different seed components was determined based on the appearance of the fungus on the medium and the percentage infection was calculated.

Seed to seedling transmission

The seed lots, which provided higher incidence of P. azadirachtae in agar plating method were selected for the investigations on seed to seedling transmission. One hundred seeds of each sample were used for determining the seed to seedling transmission. Surface-sterilized seeds (0.1% HgCl₂ for 10 min.) were placed in test tube on either agar medium or blotter stand dipped in water (Khare, 1996; Maude, 1996). The tubes were incubated at room temperature (25±2°C) for 15-20 days. After incubation seeds were observed for the percentage germination, infected seeds and appearance of lesions on the hypocotyl or cotyledons (ISTA, 1993).

Parts of the seedling that showed necrotic lesions were cut along with the adjacent healthy portion and surface-sterilized with 1% NaOCl for 60 seconds. They were separately plated on to CDA medium and incubated as described above for reconfirming the presence of the pathogen.
Effects of *P. azadirachtae* on germination of neem seed and seedling growth

*P. azadirachtae* was grown on PDA medium by inoculating five mm mycelial agar disc and incubated at 25°C for 12 days under continuous illumination (Uecker and Johnson, 1991). The culture which produced good conidial ooze were used for seed treatment. Four sets of 50 neem seeds were used for observing the effect of the fungal inoculation. The seeds were surface-sterilized using 0.1% HgCl₂ for 10 min. and washed seven times by using sterile distilled water. Seeds were rolled on surface of the cultures until they were covered with conidia and mycelium of the pathogen (Ellis *et al.*, 1974; Krishnamurthy, 1996). These seeds were sown on the three layers of moist blotter sheets and rolled (ISTA, 1993). These seed-sown rolls were placed in plastic trays containing sterile water at bottom and covered by polythene bags sprayed with water. They were incubated for 15 days at 25±2°C. Seeds and seedlings were observed for the disease symptoms on their parts. Untreated healthy seeds served as control. Root and shoot lengths of the germinated seedlings from each treatment were recorded. Vigour index was calculated by using the formula as per Abdul-Baki and Anderson, (1973):

\[(\text{Mean shoot length} + \text{Mean shoot length}) \times \text{per cent seed germination}\]

Effects of *P. azadirachtae* culture filtrate on germination of neem seed

To determine the effect of toxic metabolite of the pathogen on host, *P. azadirachtae* isolated from the seeds was used. The filtrate was obtained
from the 25-day-old culture grown on CD / PD broth. Four hundred healthy surface-sterilized seeds were treated with the culture filtrate by incubating in a beaker for 24 h. The control treatment included medium alone and sterile distilled water. Seeds were plated on sterile moist blotter in Petri dishes or on standard paper towels or in sterile soil (ISTA, 1993) and incubated at room temperature. After 10 days shoot length, root length and percentage germination were recorded. Vigour index was calculated using the formula of Abdul-Baki and Anderson (1973).

RESULTS

Detection of seed-borne *P. azadirachtae* and other fungi
Sporulation of *P. azadirachtae* occurred after 10-15 days on naked neem seeds (Fig. 35) whereas on the intact seeds it took 20 days. Occurrence of *P. azadirachtae* was 38% on naked seeds and 26% on intact seeds (Table 6). Seeds without outer hard seed coat gave better sporulation of *P. azadirachtae* on medium wherein intact seeds sporulated well on blotter stand dipped in water (Fig. 36). The incidence of *P. azadirachtae* ranged between 0 to 60% in tested seed samples. Individual seed sample also exhibited variation in infection range when seeds were collected at various time intervals. Large number of seeds showed rotting and failed to germinate (Fig.37). Incidence of other fungi is recorded in the Table 6.
Figure 35. Neem seed rot caused by seed-borne *Phomopsis azadirachtae*

Figure 36. Sporulation of *Phomopsis azadirachtae* on neem seed plated on blotter stand dipped in water
Figure 37. Growth of *Phomopsis azadirachtae* from surface sterilized neem seeds plated on potato dextrose agar medium. Seeds collected from:

a) Mysore

b) K.R. Pet

c) Mandya
Table 6. Occurrence of *Phomopsis azadirachtae* and other fungi on neem seeds

<table>
<thead>
<tr>
<th>FUNGUS</th>
<th>PER CENT INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
</tr>
<tr>
<td><em>Phomopsis azadirachtae</em></td>
<td>26</td>
</tr>
<tr>
<td><em>Aspergillus ochraceus</em></td>
<td>10</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>10</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>08</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>07</td>
</tr>
<tr>
<td><em>Penicillium species</em></td>
<td>03</td>
</tr>
<tr>
<td><em>Mycelia sterilia</em></td>
<td>04</td>
</tr>
<tr>
<td>Unidentified <em>species</em></td>
<td>04</td>
</tr>
</tbody>
</table>

Location of *P. azadirachtae* within infected neem seeds

Mycelium of *P. azadirachtae* was obtained from all the seed components (Figs. 38a, 38b and 38c). Percentage infections of various seed components are shown in Table 7. The pycnidia were formed on the surface of all the seed components. Observations on the seed components of different samples revealed that higher number of embryo infection was seen in the seeds collected in the later part of rainy season.

*Mycelia sterilia* was detected in 2% of seed coats and it was also found in 1% of the cotyledons, but it was absent in the embryos. *Aspergillus ochraceus*, *A. niger*, *A. flavus*, *F. oxysporum* and *Penicillium* species were
Figure 38. Component plating of neem seeds on potato dextrose agar medium showing outgrowth of *Phomopsis azadirachtae* from

a) Seed coat

b) Cotyledons and

c) Embryo
found in 17%, 8%, 2%, 1% and 3% of seed coat respectively, but were not detected in either embryo or cotyledon (Table 7).

Table 7. Occurrence of fungi in different components of neem seed

<table>
<thead>
<tr>
<th>FUNGUS</th>
<th>PER CENT INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seed coat</td>
</tr>
<tr>
<td>Phomopsis azadiractae</td>
<td>40</td>
</tr>
<tr>
<td>Aspergillus ochraceus</td>
<td>17</td>
</tr>
<tr>
<td>A. niger</td>
<td>08</td>
</tr>
<tr>
<td>A. flavus</td>
<td>02</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>10</td>
</tr>
<tr>
<td>Penicillium species</td>
<td>03</td>
</tr>
<tr>
<td>Mycelia sterilia</td>
<td>02</td>
</tr>
<tr>
<td>Unidentified species</td>
<td>03</td>
</tr>
</tbody>
</table>

Seed to seedling transmission

As the seeds germinated through seed coat, the emerging radicle was covered with mycelium growing from seed coat. The hypocotyl and cotyledons also got infected from seed coat and cotyledon inoculum. The symptoms included brown necrotic spot on the cotyledon and hypocotyl.
The other symptoms included seed-rot, seedling-rot, weak seedlings, seedlings with fibrous root system and seedlings without root system (Figs. 39a-e).

**Effects of *P. azadirachtae* on germination of neem seed and seedling growth**

The conidial treatment resulted in reduced germination percentage, vigour of the seedling and produced the characteristic symptoms on seeds and seedlings. The major symptoms included seed rot, yellowing of cotyledons, shoot blight and seedling with lack of root (Fig. 40). Seed rot was the most pronounced symptom on the seeds. Seedlings also showed delayed root formation and root rot.

**Effects of *P. azadirachtae* culture filtrate on germination of neem seed**

Culture filtrate-treated seeds showed reduction in their germination and seed vigour when compared to control seeds, both in Petri dish (Fig. 41) and in soil (Fig. 42). Seedling quality was significantly affected by culture filtrate of *P. azadirachtae*, whereas percentage germination was not much affected (Table 8).
Figure 39. Seed to seedling transmission of *Phomopsis azadirachtae* and its effects on seeds and germinated seedlings

a) Seed rot

b) Unopened cotyledons

c) Weak Seedling without root

d) Seedling blight

e) Fibrous root

f) Control
Figure 40. Various symptoms produced by artificially inoculated *Phomopsis azadirachtae* on germinating neem seed

a) Seed rot

b) Yellowing of cotyledons

c) Shoot blight

d) Seedlings with lack of root and

e) Control
Figure 41. Effect of *Phomopsis azadirachtae* culture filtrate on germination of neem seeds plated in Petri dish on blotter disc

a) Untreated    b) Treated

Figure 42. Effect of *Phomopsis azadirachtae* culture filtrate on germination of neem seeds sown in soil

a) Untreated    b) Treated
Table 8. Effect of culture filtrate of *Phomopsis azadirachtae* on germination of neem seeds

<table>
<thead>
<tr>
<th></th>
<th>Mean shoot Length (in cm)</th>
<th>Mean root Length (in cm)</th>
<th>Percentage germination</th>
<th>Vigour index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.31 ± 0.17</td>
<td>7.52 ± 0.30</td>
<td>80 ± 1.15</td>
<td>866.4 ± 25.83</td>
</tr>
<tr>
<td>Treated</td>
<td>0.79 ± 0.10</td>
<td>3.26 ± 0.15</td>
<td>70 ± 1.73</td>
<td>283.5 ± 11.18</td>
</tr>
</tbody>
</table>

Values are means of three experiments and each with five replications ± S.E.

**DISCUSSION**

The present study revealed that *P. azadirachtae*, the pathogenic fungus causing die-back disease of neem is seed-borne and seed transmitted in nature. This is the first report regarding seed-borne and seed to seedling transmission of *P. azadirachtae* in neem seeds. This may be the cause for the wide spread die-back disease in Karnataka and other states of India. Several species of *Phomopsis* are known to be seed-borne and seed transmitted in crop plants and tree species (Richardson, 1990). A few *Phomopsis* species are well-known to cause seed rot and seed decay in various crop plants (McDonald and Copeland, 1989; Sinclair, 1988). Other pathogenic species *Fusarium oxysporum* (Tewari, 1992) is present only in seed coat. *Fusarium* species has been reported as seed-borne in neem seeds (Uniyal and Uniyal, 1996).
Owing to biohazardous effects of synthetic pesticides more emphasis is given to utilize biopesticides in agricultural crops and maintain ecosystem in balance (Heywood, 1995). Neem-based pesticides are effective, safe, chemically stable, biodegradable and low cost (Rembold, 1996). Hence lot of importance is given to utilize this in agriculture and neem-cake is also used as organic manure. As neem seeds are viable for only a short time, maximum healthy seedlings should be obtained from them within a short period of time. Thus, it is very much necessary to know the seed mycoflora and seed-borne pathogens of neem. Detection of *P. azadirachtae* in neem seeds is highly significant due to its destructive nature on neem trees.

The standard agar plating of seeds provides an indication of the viable inoculum present in an infected seed sample (ISTA, 1993). Surface-sterilization of seeds was done to evaluate the deep-seated microflora (Neergaard, 1977). In the present experiments, agar plating and surface-sterilization was followed for detection of *P. azadirachtae*. The results indicate that *P. azadirachtae* is a deep-seated in the seed tissue because it emerged from the seeds even after surface-sterilization was done with 0.1% HgCl₂, a highly toxic chemical.

Component plating of neem seeds revealed that the pathogenic *P. azadirachtae* is present in seed coat, cotyledons and embryo with highest percentage in seed coats. Thus infected seeds become a major disseminating agent for the pathogen. This spread may be within the country or of wide geographical distance as now neem seed has become an export commodity because of its biopesticidal active ingredients.
Transmission studies revealed that \textit{P. azadirachtae} can show seed to seedling transmission with greater percentage of seed rot symptoms. It clearly indicated that if \textit{P. azadirachtae} is embryo-borne then it becomes very destructive and resulting in complete mortality of the seeds and seedlings. The artificial inoculation to seeds also showed similar type of results and confirms that \textit{P. azadirachtae} is pathogenic to neem. It is reported that various species of \textit{Phomopsis} were known to be seed-borne and seed-transmitted in different crop and forest plants and causing significant loss of seed quality and seedling vigour (Dhingra and Sinclair, 1995; Richardson, 1990).

Effect of the culture filtrates on neem seeds clearly indicates that \textit{P. azadirachtae} produces a toxic secondary metabolite into medium, which reduces the seed vigour and seed quality. Various species of Deuteromycetes are known to release toxic secondary metabolites into media (Agrios, 1997; Castor and Frederikson, 1980; Hilty and Goodfellow, 1988; Maude, 1996).