1. INTRODUCTION

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Sandal (*Santalum album* L.) (Family: Santalaceae), the xylem tapping root hemi-parasitic tree is the source of the aromatic East Indian sandalwood and oil. Sandalwood oil, formed in the heartwood of the tree has a characteristic sweet, woody odour. The oil is widely employed in the fragrance industry, particularly in the higher priced perfumes. Both the wood and oil are used in incense and medicine; besides, the wood is used in carving (Srinivasan *et al.*, 1992; Coppen, 1995).

1.1. DISTRIBUTION OF SANDAL

The genus, *Santalum* consists of 25 species distributed between 30^0N and 40^0S, from India in the West to Juan Fernandez Islands in the East and from Hawaiian Archipelago in the North to New Zealand in the South (George, 1984; Srinivasan *et al.*, 1992). *Santalum* species are characterised by two main features - obligate hemi-parasitism and aromatic heartwood. They vary greatly in habit from small shrubs to large trees (Radomiljac, 1994). The commercially valuable sandalwood, *Santalum album* L. occurs naturally in Southern India and in the islands of Eastern Indonesia, notably Timor and both the countries are the major producers and exporters of East Indian sandalwood and oil (Fox *et al.*, 1994; Coppen, 1995).

In India, sandal is found mainly in the Deccan Plateau and its extension, and in small numbers in almost all regions except the Himalayas. Large natural stand of sandal occurs in Karnataka (5,245 km^2) and Tamil Nadu (3,040 km^2) accounting for nearly 90% of sandal in India (Venkatesan, 1981). Sandal forests in Kerala are chiefly distributed in the Anjanad Valley in the eastern side of Western Ghats falling in Marayoor forest range of Munnar forest division with an extent of 15.42 km^2 in reserved forests and 47.26 km^2 in revenue lands (Mathew, 1995). Limited distribution of sandal is also seen in the reserves of Arienkavu and Kasargod forest ranges (Chand Basha, 1977). Production of sandalwood has plummeted from around 3000 tonnes per annum during 1985 to around 1000 tonnes in 1997; similarly oil production also declined from 140 tonnes in 1985 to 40 tonnes in 1997 (Jain *et al.*, 1999).

1.2. DISEASES OF SANDAL

Diseases of sandal include seedling diseases caused by *Phytophthora* sp. and *Fusarium oxysporum* and leaf spot diseases caused by different fungal pathogens like *Ascochyta santali*, *Macrophomina phaseoli*, *Asterina congesta* and *Sphaceloma santali*. *Ganoderma applanatum* causes white mottled rot, whereas, *Ganoderma lucidum* causes white spongy rot, attacking roots and spreading to the basal part of the stem. Another disease, though not of much economical importance is the leaf curl disease caused by a virus. But the major disease of sandal is the spike disease (Mukerji and Bhasin, 1986; Ghosh *et al.*, 1992; Srinivasan *et al.*, 1992).

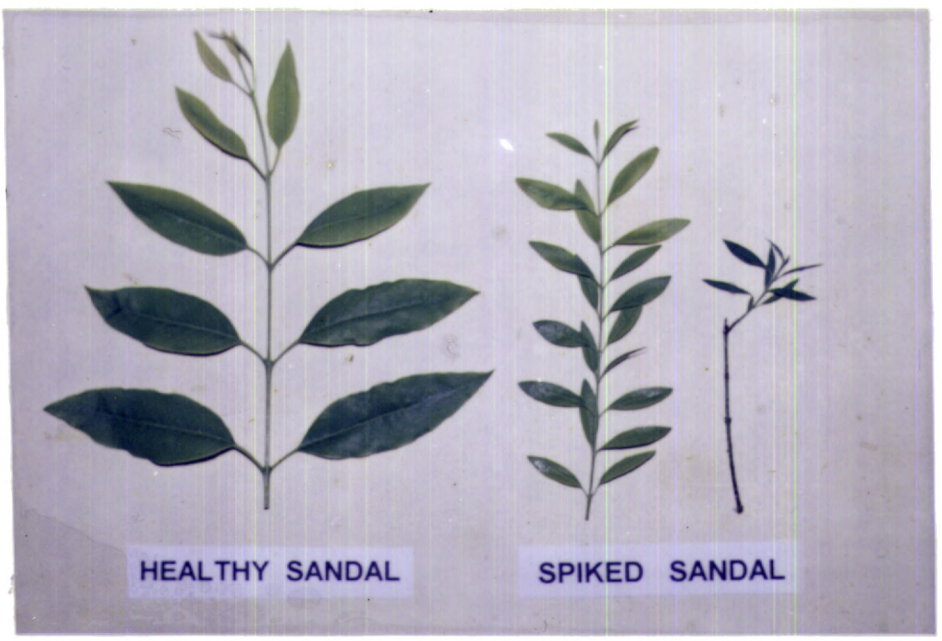
1.2.1. Spike disease

Spike disease, the most serious disease of *S. album* is characterised by extreme reduction in the size of leaves and internodes accompanied by stiffening of the leaves. In advanced stage, owing to the progressive reduction in leaf size and internodes, the whole shoot looks like a `spike inflorescence' (Figs.1.1, 1.2). Spiked plants do not bear flowers or fruits; occasionally abortive flowers are developed. Spiked trees usually die within one-to-two years after the appearance of the symptoms. In Kerala, although the production of sandalwood has not declined markedly because of the extraction of dead trees (which increased in number as a result of spike disease), the stock in the forest is depleted considerably (Ghosh *et al.*, 1992); whereas, in Karnataka, the growing stock has been reduced to 25 percent of its initial level in the last two decades (Swaminathan *et al.*, 1998). The disease is not known in Timor and does not affect other species of *Santalum* (Fox and Barrett, 1994).

Although, spike disease was first observed in Coorg by McCarthy in 1899 (McCarthy, 1899; Barber, 1903), subsequent investigations showed that the disease had made its appearance in Coorg several years before McCarthy noticed it. The disease was observed in North Coimbatore in 1903, in Salem in 1913, and in Tirupathur Javadis in 1917 (Srinivasan *et al.*, 1992). In Kerala, the disease was first noticed at Marayoor in 1980 (Ghosh *et al.*, 1985).

Fig.1.1. Sandal tree infected with spike disease.

Fig. 1.2. Comparison of healthy and spike disease affected sandal twig.



1.2.2. The pathogen

Sandal spike disease was thought to be caused by a virus (Coleman, 1923; Parthasarathi *et al.*, 1966) until 1969 when three independent groups by electron microscopy confirmed that the disease was caused by a phytoplasma (Dijkstra and Ie, 1969; Hull *et al.*, 1969; Verma *et al.*, 1969).

Phytoplasmas were first reported by Japanese workers in 1967 (Doi *et al.*, 1967). The pathogens are seen exclusively in the sieve tubes of phloem tissues of leaves, petioles, stem and root causing symptoms such as leaf yellows, little leaf, phyllody, witches' broom, etc. Phytoplasmas has been implicated as pathogens in more than 300 plant diseases world-wide (McCoy *et al.*, 1989). The disease caused by the pathogens include peanut witches' broom, lilac witches' broom, ash yellows, chrysanthemum yellows, lethal yellowing of palms, blue berry stunt, rice yellow dwarf, X disease of *Prunus*, sugarcane white leaf, apple chlorotic leaf roll, etc. (Sinclair *et al.*, 1996).

Morphologically, phytoplasmas resemble animal or human mycoplasmas (Class: Mollicutes) and share several characteristics with mycoplasmas. These include unicellular and pleomorphic nature and absence of cell wall, the cells being delimited only by a membrane, passage through bacteriological filters and resistance to antibiotics that interfere with cell wall formation (Neimark and Kirkpatrick, 1993). The change in terminology from mycoplasma-like organism (MLO) to phytoplasma in 1994 reflected new knowledge about the plant inhabiting mollicutes (Sears and Kirkpatrick, 1994).

Phytoplasmas has remained uncultured despite extensive efforts over many years. The inability to grow these agents *in vitro* has severely hindered their study. As a result, phytoplasmas are among the most poorly characterised groups of plant pathogens. Until recently, phytoplasmas could be studied only on the basis of biological properties such as disease symptoms, plant host range, and insect vector specificity. Now, the pathogen could be visualised by electron microscopy and their presence in phloem tissues demonstrated by fluorochromic DNA stains; but these methods do not discriminate among different groups of phytoplasmas (Clark *et al.*, 1989; Neimark and Kirkpatrick, 1993).

1.2.3. Disease diagnosis and detection

Accurate diagnosis is a necessary prelude to any successful disease control. However, diagnosis of plant mollicute diseases has often been one of the difficult aspects of the study of these diseases. This is principally due to the lack of methodologies for detection of pathogens in the field or in quarantine material. In addition, the relatedness among various phytoplasma groups and the epidemiology of many of the phytoplasma diseases could not be studied until recently. Fortunately, development and exploitation of new approaches to pathogen detection now promise to overcome many of the problems that hinder correct diagnosis and identification (Davis and Lee, 1988).

It has long been known that most plant pathogens possess, as part of their structure, specific antigenic determinant in the form of proteins or other antigenic moieties. Recognition of the diagnostic potential of such determinants for both experimental and applied investigations in plant pathology has resulted in an array of techniques, collectively referred to as immunoassays. Immunoassays are used in plant pathology for identification, diagnosis and quantitation of plant pathogens (Barbara and Clark, 1986). The development of procedures to obtain phytoplasma enriched preparations from infected plants has permitted the production of phytoplasma specific polyclonal and monoclonal antibodies and has been routinely used to detect the pathogen in a wide variety of plants (Hobbs *et al.*, 1987; Clark *et al.*, 1989; Saeed *et al.*, 1993).

Detection and identification of phytoplasma using molecular techniques like polymerase chain reaction (PCR) has become popular because of the high sensitivity of the test. PCR is preferred in situations where the concentration of phytoplasma may be very low. Thus PCR followed by restriction fragment length polymorphism (RFLP) is employed in the detection and identification of the pathogen (Saeed and Cousin, 1995). Seemuller *et al.* (1998) has classified phytoplasmas into twenty groups based on the RFLP analysis of 16S rDNA (ribosomal DNA).

1.3. OBJECTIVES OF THE WORK

Spike disease in sandal is generally diagnosed by the manifestation of external symptoms. Attempts have been made to detect the diseased plants by determining the length/breadth ratio of leaves (Iyengar, 1961) and histochemical tests using Mann's stain (Parthasarathi *et al.*, 1966), Dienes' stain (Ananthapadmanabha *et al.*, 1973) aniline blue and Hoechst 33258 (Ghosh *et al.*, 1985, Rangaswamy, 1995). But most of these techniques are insensitive, indirect detection methods leading to misinterpretation of results. Moreover, to identify disease resistant sandal trees, highly sensitive techniques are needed to detect the presence of the pathogen. In sandal forests, several host plants of sandal like *Zizyphus oenoplea* (Fig. 1.3) also exhibit the yellows type disease symptoms. Immunological and molecular assays have to be developed to confirm the presence of sandal spike phytoplasma in such hosts. The major objectives of the present work includes:

1. *In situ* detection of sandal spike phytoplasma by epifluorescence microscopy and scanning electron microscopy.
2. Purification of sandal spike phytoplasma and production of polyclonal antibodies.
3. Amino acid and total protein estimation of sandal spike phytoplasma.
4. Immunological detection of sandal spike phytoplasma.
5. Molecular detection of sandal spike phytoplasma.
6. Screening for phytoplasma in host plants of spike disease affected sandal using immunological and molecular techniques.

Fig.1.3. Comparison of healthy (left) and witches' broom disease affected (right) *Zizyphus oenoplea.*

1.4. ORGANISATION OF THE THESIS

The thesis is organised into eight chapters. The first chapter introduces the topic of research and the objectives of the work. The second chapter reviews the work done so far in sandal spike disease and also describes the immunological and molecular techniques used to detect phytoplasma affecting other plant species. The third chapter describes several *in situ* detection techniques to detect the pathogen. The method used to purify sandal spike phytoplasma is explained in the fourth chapter. The chapter also brings out the results of protein and amino acid studies of the pathogen. The fifth chapter explains different immunological tests used to detect sandal spike phytoplasma, while the sixth chapter deals with the molecular detection techniques. Commercial exploitation of techniques developed to detect the pathogen is discussed in the seventh chapter and the major results are summarised in the last chapter. There is a common bibliography for all the chapters under the title `References'