7. COMMERCIAL EXPLOITATION OF TECHNIQUES DEVELOPED TO DETECT SANDAL SPIKE PHYTOPLASMA

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Immunological and molecular techniques have been developed to detect sandal spike phytoplasma. The reagents developed for diagnosis can be marketed as kits, which may be useful for the sandalwood industry. The possible commercial exploitation of the methods developed is explained in this chapter.

7.1. COMMERCIAL EXPLOITATION OF PLANT DIAGNOSTICS

Both wild and cultivated plant species are subjected to diseases to such an extent that about 80,000 diseases of plants have been recorded throughout the world. Microorganisms causing plant diseases can be classified into six major groups viruses, viroids, fastidious prokaryotes (which include phytoplasmas and spiroplasmas), bacteria, fungi and protozoa (Agrios, 1997). The control of plant diseases depends on accurate and rapid detection and identification of the pathogens. In this context, detection is the process of testing for the presence of pathogens, while identification is defined as specific grouping of the causal agent (Chu *et al.*, 1989).

Traditionally, diagnosis of plant diseases has been based on recognising characteristic symptoms expressed by diseased plants and

looking for the presence of pathogens on the surface of diseased material (McIntyr and Sands, 1977). This together with other observations and evaluation of the environmental conditions, generally allow the causative agent to be identified. While considering pathogen detection, problems can arise due to the multiplication of pathogens in internal tissues, ability of the pathogens to survive as saprophytes, obscure and seasonal expression of disease symptoms etc.

In such cases, special methods are required to isolate or detect pathogens amidst the cell components and other saprophytes. This usually involves performing a series of diagnostic tests. During the past few years, progress in molecular biology, biochemistry and immunology has promoted the development of many new methods of pathogen detection and disease diagnosis (Miller and Martin, 1988). Diagnostics is considered as one of the three major applications in agricultural biotechnology, the other two being biopesticides and transgenic plants (Mannion, 1998).

7.1.1. Diagnostics

Diagnostics can be viewed as a discipline in its own right, combining a wide range of techniques in developing simple, fast and reproducible procedures that measure a feature of the biological material in hand in a way that is easily interpretable. Improved diagnostics are useful in epidemiological

studies to determine the distribution and abundance of pests and pathogens (Skerritt and Appels, 1995).

Biotechnology offers forestry the possibilities of an array of new procedures for overcoming major constraints in woody plant improvement, protection and utilisation (Krugman, 1990). It also provides an array of new methods for the early detection and identification of pathogens of woody plants. Immunoassays have been developed and are commercially available for the identification of plant pathogens, mycotoxins, pesticides and plant hormones. Polyclonal antibodies are extremely useful since their broader spectrum can sometimes be more useful than the highly specific monoclonal antibodies (Miller and Williams, 1990).

Nucleic acid probes have been developed for detection of many plant pathogens. The availability of nucleotide sequences has made possible the development of PCR assays for the detection and diagnosis of several viroids, viruses and other pathogens. Because of its great sensitivity, the PCR provides a good alternative to other diagnostic methods and can speed up diagnosis, reduce the sample size required, and often eliminate the need for radioactive probes (Hadidi *et al.*, 1995).

7.1.2. Comparison of nucleic acid probes and antibody assays

In some cases, a decision will have to be made as to whether it is more appropriate to pursue an antibody or nucleic acid probe in the development

of a diagnostic test. Clearly, small molecules such as lipids, mycotoxins or agrochemicals are amenable only to antibody based detection, but for macromolecules and plant breeding applications, the choice is not so clear cut. Nucleic acid probes are generally better for genotype characterization, while antibodies could provide a better test for phenotype - especially if the target is a product of a gene whose expression is influenced by environment (Skerritt and Appels, 1995).

7.2. COMMERCIAL EXPLOITATION OF THE TECHNIQUES BASED ON THE PRESENT WORK

The techniques used in the present study for the purification of sandal spike phytoplasma using the differential filtration method was rapid and economical. The purity of the phytoplasma cells thus generated was found to be very high. The purified phytoplasmas elicited immune response in rabbits to produce highly sensitive polyclonal antibodies, which could be used in different immunological techniques. Thus the immunological tests like Ouchterlony double diffusion test, direct and indirect ELISA, direct and indirect DIBA and various immuno microscopic techniques could be standardised for pathogen detection. Since one hour of duration was found to be optimum for both coating and incubation, direct ELISA could be completed within three hours and indirect ELISA within seven hours. Eventhough, the number of steps were higher in indirect ELISA, the sensitivity of the test was high when biotin-streptavidin system was used.

The indirect DIBA could be completed within three hours. The *ex situ* detection of phytoplasma by immunoelectron microscopy was very rapid since the whole test could be completed within four hours.

For molecular studies the DNA could be isolated using a modified CTAB method which used minimum amount of chemicals and was found to be very rapid. The specific primers could easily detect the presence of phytoplasma using polymerase chain reaction within four hours. The identity of the organism could be proved by restriction fragment length polymorphism analysis. The reagents used in these techniques could be marketed as kits as shown in figure 7 1.

Thus three major kits could be developed based on the present studies viz., sandal spike phytoplasma purification kit, immuno detection kit and molecular detection kit. The reagents that can be supplied are listed in table 7 1.

For purification of sandal spike phytoplasma the major instruments needed includes filtration apparatus and ultracentrifuge. An ELISA reader is the only instrument needed for detecting the pathogen when the ELISA technique is employed, whereas, a thermal cycler and horizontal electrophoresis system are the instruments needed for molecular detection of the pathogen. Fluorescence microscope and electron microscope are essential for visual confirmation of the presence of the specific pathogen when the immunomicroscopy technique is employed.

Table 7.1. Kits and reagents developed to detect sandal spike phytoplasma.

KIT	REAGENTS
SANDAL SPIKE PHYTOPLASMA PURIFICATION KIT	Healthy sandal antibody, Glycine buffer.
IMMUNO DETECTION KITS **1. Double diffusion kit** **2. ELISA kit** **3. DIBA kit** 4. **Immuno microscopic kit**	Phytoplasma specific antibody, Phytoplasma specific antibody-HRP, Anti-rabbit antibody-HRP, Anti-rabbit antibody biotin, Streptavidin-HRP, Anti-rabbit FITC, Protein-A gold, Agarose, Phosphate buffered saline, Positive control (phytoplasma).
MOLECULAR DETECTION KIT	16S rDNA primer, Alu I, Taq polymerase, Taq buffer, dNTP solution, Positive control DNA.

Eventhough, India has a monopoly in the production and export of sandal and its products, the marketing of the species is primarily restricted exclusively to government agencies leaving very little scope for private agencies. So the kits developed *per se* has limited chance for commercial exploitation in the open market in the present scenario. But, since, the techniques developed are highly specific and sensitive, the same can be used in tree improvement projects of sandal, particularly for screening disease resistant trees. Many states are planning to remove restrictions on possession and trade of sandal. In such a scenario, most of the farmers will be very enthusiastic to cultivate the species due to the high price fetched by sandalwood. In that situation, the kits can be used for ensuring disease-free planting stock for introduction to non-sandal area. Any farmer can easily detect the pathogen using double diffusion test or dot immunobinding assay; so the reagents for these techniques can become popular among the farming community. All the other techniques require sophisticated instruments and technicians and may not be popular since only a few laboratories have the necessary infrastructure. Nevertheless, the reagents will be of much use to quarantine laboratories for screening sandal, especially seedlings, for the presence of pathogen using ELISA and molecular techniques.

Most of the companies supply antibodies either as freeze dried powder or with preservatives which has a shelf life of about 12 months at 4^0C or for longer period at -20^0C (Adgen, 1998). The positive and negative

control samples are supplied as freeze-dried sap extract which should be reconstituted with distilled water or buffer before use. Adgen sells most phytoplasma kits of 1000u for around US$600. These kits contain reagents for ELISA only. For molecular detection, the company charges around US$ 120 per sample (sample size: 30 stems/100g grain). The reagents of sandal spike phytoplasma detection kit (both immunological and molecular kits), when produced in large scale, could be supplied at a lower price due to lower labour and other input costs in India.

Fig.7.1. Kits developed for purifying and detecting sandal spike phytoplasma.