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DNA fingerprinting analysis of Eucalyptus species

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Abstract: *DNA fingerprinting is a straightforward approach for identifying variations at a locus. The term DNA-fingerprinting was introduced for the first time by Alec Jeffrey2 in 1985 to describe bar-code-like DNA fragment patterns generated by multilocus probes after electrophoretic separation of genomic DNA fragments. The emerging patterns make up an unique feature of the analyzed individual and are currently considered to be the ultimate tool for biological individualization. Recently, the term DNA fingerprinting/profiling is used to describe the combined use of several single locus detection systems and are being used as versatile tools for investigating various aspects of plant genomes. This technique used to detect the Cylindrocladium quinqueseptatum which infects Eucalyptus species, based on DNA based markers such as RAPD, PCR-RFLP, ITS. Eucalyptus is grown in many parts of the country it has become commercially very important as raw material for the pulp, paper and plywood industries of northern India. Some Eucalyptus species have attracted attention from global development researchers and environmentalists. Such species have desirable traits such as being fast-growing sources of wood, producing oil that can be used for cleaning and functions as a natural insecticide.*

Keywords: *DNA fingerprinting, Cylindrocladium quinqueseptatum, Eucalyptus.*

I. Introduction

Eucalyptus were introduce into India in the 18th century and since then over 100 species of eucalyptus have been planted in various states but only a few have been found suitable for large scale planting. It was first planted around 1790 by Tippu Sultan, the ruler of Mysore, in his palace garden on Nandi hills near Bangalore. According to one version he received seed from Australia and introduced about 16 species (Shyam Sundar, 1984).

Subsequent to the planting at Nandi Hills, the next significant introduction of Eucalyptus was in the Nilgiri hills, Tamil Nadu, in 1843, and later (1856) where regular plantations of E. globulus were raised to meet the demands for firewood, from 1856 (Wilson, 1972). It is reported during 1954-55 herbarium specimens of eucalypt trees grown at Nandi hills were sent to Australia and identified as E. camaldulensis, E. citriodora, E. crebra, E. major, E. intermedia, E. polyanthemos, E. robusta, E. tereticornis, E. tessellaris, a hybrid of E. robusta x E. tereticornis, and a hybrid of E. botryoides x E. tereticornis (Nanjundappa, 1957). The first Forest Department Eucalyptus plantation was in 1877 at Malabavi (Devarayanadurga), Tumkur District (Kadambi, 1944). Until the end of the 19th century small blocks of plantations were raised, often for experimental purposes.

In present situation of Eucalyptus plantations some 170 species, varieties and provenances of eucalypt were tried in India (Bhatia, 1984), out of which the most outstanding and favoured has been the E. hybrid, a form of E. tereticornis known as **Mysore gum**. The most important characteristics of E. hybrid contributing to its popularity under Indian conditions are: it is fast growing, capable of over topping weeds, coppices well, is fire hardy, browse resistant and it has the ability to adapt to a wide range of edaphoclimatic conditions (Kushalappa, 1984). Other species which are grown on plantation scale are E. grandis, E. citriodora, E. globulus, and E. camaldulensis.

Eucalyptus has become commercially very important as raw material for the pulp, paper and plywood industries of northern India. Some States have encouraged pulp and paper industries to raise their own captive plantations by leasing degraded forests and Government waste lands. The Government of India initially supported such ventures. With the new Forest Policy the industries should obtain their raw material from farm forest areas. The new Forest Policy has given a boost to farm forestry, and to eucalypts. The wood industries are encouraging farmers to raise trees in their lands by giving incentives. Some rich farmers have diverted good arable land with assured irrigation facilities, for growing eucalypt.

Large scale seedling mortality in Eucalyptus nurseries of North Indian states caused by Cylendrocladium quinueseptatum has posed serious threat to paper and pulp industries of this region fearing decline in production, as majority of industry-sponsored nurseries raised by local farmers for achieving the plantation targets have witnessed this disease in epipytotic proportions in the resent years.Worst affected North Indian states are Uttarakhand, Punjab and Haryana. Losses from Cylendrocladium quinqueseptatum seedling blight is due to out right killing of the entire seedling within a couple of weeks time during monsoon season and blightning of leaves in plantations thus adversely affecting the tree growth. Severe mortality has been recorded in nurseries and plantations in Uttarakhand, Punjab, Haryana and U.P. Cultural practice to avoid the disease

incidence is often ineffective as the pathogen over winters in soil and with the every passing year the inoculum increases in geomatrical proportions. The only option left with the nursery Manager is to shift the nursery to a newer area.

The objective of the present study was to find out the species specific conserved gene sequence by using DNA based markers such as RAPD, PCR-RFLP, ITS etc. and made to screen different microorganisms isolated/obtained from different sources against Cylindrocladium quinqueseptatum for their antagonistic effect. Specific aim was to identify Cylindrocladium quinqueseptatum from Eucalyptus foliar or twig tissues and soil. These fungi are most commonly found as the Cylindrocladium anamorph (asexual state) and those most commonly recorded often have very wide host ranges. For example C. reteaudii (as Clyndrocladium quinqueseptatum) has been recorded from many hosts in Northern Australia, South-East Asia and India.

II. Materials and Methods

Infected materials: Infected leaf and twig samples of Eucalyptus were collected from the species from different nurseries and plantation to be examined and were washed and dried at room temperature. For isolating C.quinqueseptatum 5x10mm leaf pieces and 10 mm long twig pieces were aseptically excised from Eucalyptus plant samples showing disease symptoms, placed on potato dextrose agar (PDA), and incubate for seven days at 25°C. Meanwhile, the diseased plant samples collected were stored at 2° C. All the disease samples were transferred into a freezer at -20° C until DNA was extracted from them.

Fungal isolates: The samples were sterilized in alcohol (30 s), transfer to 2.5% sodium hypocloride (30 s) and wash in sterile water for 1 min. Isolation was perform on potato dextrose agar (PDA) plates containing streptomycin (0.1 mg/ml). Leaves were incubated at 25±1°C in the darkness for 4 days and the growing mycelia were transferred on PDA slants and then transferred after 10 days to a modified medium and incubated on lab bench at 24°C to induce sporulation. Isolates were single spore and subculture weekly on PDA. Cultures belonging to the genus Cylindrocladium were transferred to carnation leaf agar dishes until the formation of conida and vesicles to confirm the species. (Crous and Wingfild, 1994).

DNA extraction: DNA extraction from the mycelium was done according to the protocol described by Zolan and Pukkila (1986) . Approximately 10mg of lyophilized mycelium was ground in liquid nitrogen and mixed with 700 µl of extraction buffer (700 mM NaCl, 50 mM Tris-HCl (pH-8), 10 mM EDTA, 1% β 2 mercaptoethanol and 1% acetyl- trimethyl ammonium bromide).Then incubate at 65° C for 1 hour and extract by adding an equal volume of chloroform- isomyl alcohol (24:1), vortaxed, and centrifuged at 10,000g for 10 min. The aquas phase was washed with precipitated with cold isopropanol and centrifuge at 10,000g for 10 min. The pellets were washed with 70% ethanol , air dried, resuspended in 50 µl of TE buffer (10 mM Tris- HCl (pH-8), 1mM EDTA), and stored at -20°C until needed. DNA extractions directly from leaves and twigs were done by the method described by Saghai-Maroof et al. (1984).

Quantification of DNA: Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm are used as a measure of DNA purity. DNA absorbs UV light at 260 nm and 280 nm, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than1.8 . DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with a DNA marker of known concentration. Using the southern blot technique this quantified DNA can be isolated and examined further using PCR and RFLP analysis. These procedures allow differentiation of the repeat sequences within the genome. It is these techniques which forensic scientists use for comparison, identification and analysis.

RAPD-PCR analysis: RAPD stands for Random Amplification of Polymorphic DNA**.** It is a type of PCR reaction but the segment of DNA that are amplified are random. PCR was carried out in a final volume of 25 micro liter, containing 100mM of oligonucleotide primer (1 micro liter) , 25mM of each of the four deoxynucleotide triphosphates (.75 micro liter), 1 micro liter of Taq DNA Polymerase, 10X assay buffer with MgCl2 (3 micro liter). Amplification was carried out in a gradient thermal cycler with initial denaturation of 94°C for 4 min and 40 cycles of 94°C for 1 min, 34°C for 2 min and 72 °C for 2 min. PCR products were separated by gel electrophoresis on 1.3% agarose in TAE buffer and visualized by UV fluorescence.

Gel electrophoresis: It is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by the addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. Most agarose gel is made between 0.7-2%. 0.7% shows good separation of large DNA fragments of about 5 to 10 kb, whereas 2% shows good resolution for small fragments of about 0.2 to 1 kb.

ITS amplification and data analysis: ITS (for internal transcribed spacer) refers to a piece of non- functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. ITS-1, ITS-2 along with 5.8s rDNA were amplified using primers ITS1 and ITS4 (White et al., 1990). PCR reaction were

carried out in 25µl of reaction mixture containing 10 mM Tris-HCl (pH-8.3), 50mM MgCl2, 0.0001% gelatin, 400 µM dNTPs, 1 µM of each forward and reverse primers, 1 unit of Taq DNA polymerase and 1 µl of template DNA. The reaction were carried out on a gradient thermal cycler with an initial denaturation temperature 96°C for 3 min, followed by 30 cycles of 92°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 min. The reaction was completed by a final extension temperature at 72°C for 10 minutes.

Data analysis was done by Gene Profiler software. A dendogram was constructed using unweighted pair group method with arithematic averages. For further genetic analysis the RAPD data was treated haplotype comprising combinations of alleles at one of several loci. The data was tested for genetic diversity in intra-population isolates by analysis of genetic structure by Arlequin software.

III. Result and Discussion

The identification of pathogen was done by the key described by Crous and Wingfield (1994) and it was confirmed that all the All isolates under study were Cylindrocladium quinqueseptatum based on microscopic characters as described in Mycobank. Conidia were hyaline, cylindrical, straight having 5 to 6 septa and 560-1040 µm x 45-70 µm in length and diameter (Fig.1). In old cultures more then 6 septa also found. Conidiophore were hyaline, non septate or may be one septa arising from lateral stipe and stipe extension terminating with a clavate shaped vesicle. Phialides are hyaline, 2.5-3 µm wide.

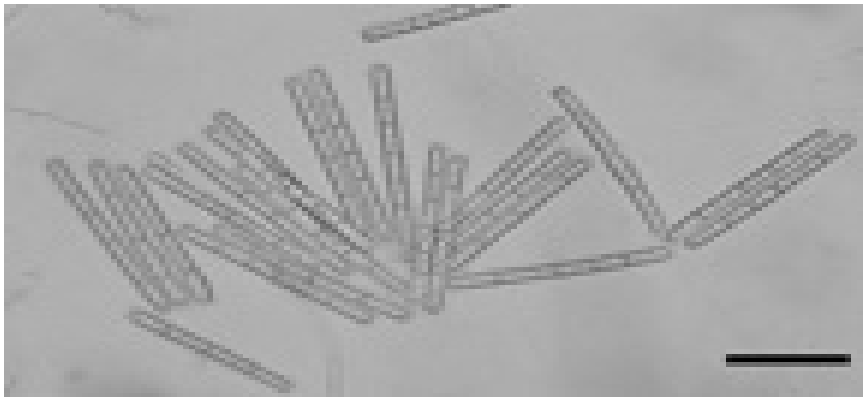

Figure 1. Macroconidia with, typically, 3–5 septa, bar = 32 µm

RAPD-PCR analysis is much simpler and faster to carry out. It is relatively an easy means to identifying fungi at the sub species level. RAPD analysis has been widely used for establishing phylogenetic relationship between isolates of fungal pathogens (Fig.2). Bending patterns for all primers was considered as monomorphic if a particular band was present in more than 90% of total isolates and polymorphic if the band was present in less than 90% of total isolates.

ITS region including 5.8S rDNA yielded characters which is similar to sequence submitted by different researchers to GeneBank. DNA template from diseased tissues and soil samples from Eucalyptus nurseries and plantations showed positive results for PCR assay. All the diseased tissue samples, soil samples and samples collected after 12 days of inoculation were subjected to isolation of the pathogen. Cylindrocladium quinqueseptatum was isolated from all these samples confirming its presence.

IV. Conclusion

With the increase threat posed by this pathogen in nurseries of North India, the RAPD-PCR provides a noble tool to detect Cylindrocladium quinqueseptatum in Eucalyptus planting stock with greater sensitivity. The detection of Cylindrocladium quinqueseptatum by PCR assay method may prove useful in determining the level of latent infection in planting materials. Hamelin et al. (1996) designed species specific primer for Cylindrocladium floridanum and Cylindrocarpon destructans using ITS region of rDNA. Similarly Henricot and Culham (2002) observed that the changes in the ITS sequences in all the isolates of Cylindrocladium buxicola in his study were consistent and can be used as one of the criteria to differentiate new species.

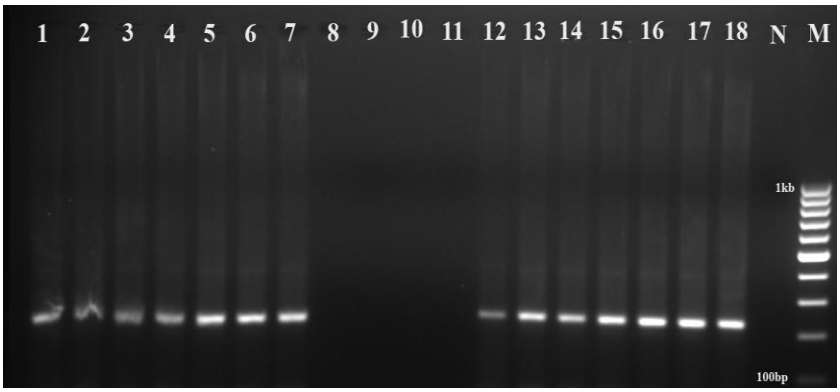

Figure 2: Amplification of ITS region by specific primer set designed for Cylindrocladium quinqueseptatum. Lane 1 and 2 represents the amplification of DNA isolated from post inoculation pre symptomatic seedlings, lane 3 to 7 show amplification from diseased tissue samples, lane 8 and 9 from healthy leaves, lane 10 and 11 from uncontaminated nursery soil samples, lane 13 to 18 from C. quinqueseptaum cultures of different isolates.

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