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Screening Sandal Provenances for Spike Disease Resistance Using Molecular Markers

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Abstract of the Project Proposal

1. Project No. : KFRI 322/99

2. Title : Screening Sandal Provenances for Spike Disease Resistance Using Molecular Markers

3. Principal investigator : Dr. M. Balasundaran

4. Associate investigator : Nil

5. Research Fellows : Nil

6. Objectives :

i. To establish two provenance trial plots at Marayur and Veluppadam using seeds of eight provenances from India

ii. To establish two progeny trial plots at Marayur and Veluppadam using spike disease evaded trees and Candidate Plus trees (CPTs) from Marayur.

iii. RAPD analysis of sandal provenances, spike disease evaded trees, CPTs and their progenies

iv. Periodic observation on seedling growth and disease appearance

7. Date of commencement : April 1999

8. Duration : 3 Years

9. Funding Agency : Forest Development Fund

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Abstract

A preliminary field trial on spike disease resistance and adaptability of eight sandal provenances was carried out in one ha area at KFRI Field Research Centre at Veluppadam in Trichur District and 0.5 ha area of the spike disease affected region of Sandal Reserve 54 of Marayur Range under Munnar Forest Division in Idukki District. These provenances are Bangalore, Mandagadde (Shimoga) and Thangli (Chikkamangalur) in Karnataka, Javadis (Thirpathur) and Chitteri (Harur) in Tamil Nadu, Seoni in Madhya Pradesh, Koraput in Orissa and Marayur in Kerala. The survival of the provenance plants ranged from 1.6 per cent (Seoni and Koraput) to 17.5 per cent (Marayur) at Veluppadam plot and 2.5 per cent (Koraput) to 52.5 per cent (Marayur) at Marayur plot. None of the seedlings were affected by spike disease for the first two years. Marayur provenance is the most adapted for Kerala as seen from the comparative performance of the provenances.

Field experiments on induction of root sucker formation through trenching around 15 spike disease evaded trees, protected with chain link fencing, were carried out in Sandal Reserve 51 at Marayur in order to improve the stock of those trees presumed to be spike disease resistant. Root sucker induction experiments were also done on 15 trees at KFRI campus at Peechi. The percentage of sprouts from roots which developed into root suckers was 13 per cent at Marayur and 3.7 per cent at KFRI campus in Peechi at the end of two years. The mean number of root sucker produced per tree at Marayur was 0.9 while it was 0.5 at KFRI campus.

Random Amplified Polymorphic DNA (RAPD) finger printing of eight provenances using three Operon primers namely, OPA 07, OPA 09 and OPA 16 gave specific bands. Comparison of DNA fingerprints of spike disease evaded trees and diseased trees did not show any disease specific band to identify disease susceptibility/resistance. The provenance seedlings planted in the provenance trial plot at Marayur also did not show spike infection during the study period. Hence, it was not possible to confirm the resistance/susceptibility of the provenances to spike disease. Also, it was not possible to relate the DNA finger prints of provenances to spike disease resistance.

The genetic diversity studies of the eight provenances using RAPD finger prints showed that 91.67 per cent of the loci were polymorphic. Within single provenance, the proportion of polymorphic loci varied from 2.78 per cent (Seoni) to a maximum of 38.89 per cent (Marayur). Nei's gene diversity index varied from 0.01 (Seoni) to 0.14 (Marayur). The genetic superiority of Marayur provenance among all the provenances has also been revealed in the provenance trial. The UPGMA dendrogram showed the genetic relatedness of the eight provenances. Bangalore and Thangli were the genetically most similar provenances while Javadis and Seoni were the genetically most distant.

1. Introduction

The genus *Santalum* comprises 29 species distributed in India, Indonesia, Australia, New Guinea, New Caledonia and throughout the South Pacific (Hewson and George, 1984). The commercially important sandalwood, *Santalum album* L. occurs naturally in Southern India and in islands of Indonesia, notably Timor (Srimathi *et al.*, 1995). *S. album* is the only species naturally occurring in India. It is distributed in about 9600 km^2 mainly in Karnataka, Tamil Nadu and Kerala (Srimathi *et al.*, 1995). It is also found distributed in Andhra Pradesh, Madhya Pradesh and Orissa. Sandal is highly valued for its fragrant heartwood and oil. India exports approximately 2000 tonnes of sandalwood and 100 tonnes of oil annually to various countries. Both wood and oil are used in the preparation of incense, perfumes and medicines.

1.1. Spike disease of sandal

Sandal trees in the natural forests of all these states have been depleted considerably due to illicit felling and a serious disease known as spike disease caused by phytoplasma. Spike disease, the most serious disease of sandal, is characterized by extreme reduction in size of leaves accompanied by stiffening and reduction in length of internodes (Ghosh *et al.*, 1992); in advanced stage the whole shoot looks like a spike inflorescence (see PLATE 1).

Diseased trees die within 1-3 years after the appearance of the symptoms. The pathogen, a non-culturable phytoplasma, is found in the phloem of the infected trees. Ghosh *et al.* (1985) reported the occurrence of spike disease in Sandal Reserve Forests of Marayur Range in Munnar Forest Division, Kerala State. Extensive studies have been made on symptomatology, histopathology, epidemiology, insect vector and chemical control methods. Thomas and Balasundaran (1999; 2001) developed immunological and molecular techniques for early detection of phytoplasma before the external expression of disease symptoms. Balasundaran and Muralidharan (2004) attempted micropropagation through somatic embryogenesis of explants collected from spike disease-evaded sandal seedlings from Marayur. The disease-evaded trees

were more than 20 years old and they were found within the heavily infected patches of Marayur forest reserves. These trees had either by chance evaded the disease or they are disease resistant. However, these trees were not subjected to any detailed study with regard to spike disease resistance.

1.2. Sandal provenances

In India, eight sandal growing areas have been identified as potential provenances of sandal on the basis of population density, phenotypic characteristics, latitude, longitude and ecoclimate (Fig. 1 and Table 1) (Jain *et al.*, 1998). The provenances vary in climatic and edaphic preferences. Hence, there is a chance that some of these provenances may show genetic variation between them. However, they were not tested for spike disease resistance. Hence, in order to field test the provenances for spike disease resistance, these provenances should be planted in spike disease affected areas. The provenances can also be subjected to genetic diversity studies using molecular markers.

1.3. Molecular markers

Molecular markers are segments of genomic DNA, visualized as bands when such segments are separated on electrophoresis gel and stained with DNA specific dyes. Molecular markers can be used to assess genetic variation in plant populations. The genetic variation will be due to insertion or deletion of nucleotides in that particular DNA segment resulting in difference in length of such segments. Length variation can be detected through electrophoresis separation of DNA fragments.

DNA based markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and microsatellites have been applied for the detection of genetic variation in plants (Ahuja, 2001). RAPDs constitute a class of genetic markers, which produce arbitrary fragment length polymorphisms and utilize single, arbitrary decamer DNA oligonucleotide primers to amplify regions of the genome using the polymerase chain reaction (Williams *et al.*, 1990). Priming sites are thought to be randomly distributed throughout the genome and polymorphism in these regions

results in differing amplification products. RAPD analysis has been used successfully to identify and discriminate species, varieties, hybrids and cultivars, for QTL mapping and to analyze population genetic structure (Young *et al.*, 2000).

1.4. Identification of spike disease resistant plants

For a long-term solution to the problem of spike disease and for sustainable supply of superior sandalwood, development of spike disease resistant trees through breeding could be the best method (Venkatesh and Kedharnath, 1963; Srimathi *et al.*, 1980). The disease evaded/resistant trees found in the spike disease affected area of Marayur and the sandal provenances from different geographic areas could be ideal starting materials for this purpose. But before using such plants for tree improvement programmes, it has to be confirmed whether the plants from these sources are genetically resistant to spike disease.

Molecular markers provide a new alternative to conventional disease resistance screening. These markers allow us to identify resistance alleles without the use of pathogens and without the influence of environmental factors (Kuginuki *et al.*, 1997). In the present investigation RAPD analysis was carried out in spike disease evaded trees from Marayur and trees from the eight sandal provenances of India.

1.5. Objectives of the study

The objectives of the present study were:

1. To establish two provenance trial plots at Marayur in Munnar Forest Division and Velupadam in Chalakkudy Forest Division using seeds of eight sandal provenances of India.
2. RAPD analysis of sandal provenances and spike disease evaded trees
3. Observations on survival, seedling growth and spike disease incidence in the provenance trial plots.

2. Materials and Methods

2.1. Establishment of provenance trial plots

2.1.1. Sample collection

Seeds were collected from eight provenances viz. Marayoor in Kerala, Bangalore, Mandagadde (Shimoga) and Thangli (Chikkamagalur) in Karnataka, Javadis (Thirpattur) and Chitteri (Harur) in Tamil Nadu, Seoni in Madhya Pradesh and Koraput in Orissa states of India (Table 1 and Fig. 1). Seed samples were collected randomly from 15-20 parent trees located in different places of the reserve forests

Table 1. Characteristics of the selected eight sandal provenances in India.

Provenance	Area (approx.) (ha)	Latitude/ Longitude	Altitude (approx.) (m)	Mean annual rainfall (mm)	Temp. Max. / Min.	Soil type
Marayoor	1497	10°1'N 77°1'E	1000	1450	36.0/ 10.0	Black clay
Bangalore	87350	12°58'N 77°38'E	1000	850	36.8/ 12.2	Red loam
Mandagadde	62529	13°9'N 75°40'E	650	2000	38.1/ 13.0	Acidic
Thangli	47251	13°40'N 76°00'E	766	1500	44.0/ 10.5	Alkaline
Javadis	16517	12°3'N 78°7'E	930	1200	38.0/ 12.4	Red loam
Chitteri	60000	12°0'N 78°7'E	1050	1000	35.2/ 8.2	Sandy loam
Seoni	3000	22°1'N 79°5'E	900	1600	40.0/ 5.0	Laterite
Koraput	2541	19°55'N 82°35'E	859	1525	38.0/ 4.5	Sandy loam

and the seeds were pooled together. Sufficient seeds were pre-treated with 0.05 % GA_3 solution overnight for uniform and fast germination. The treated seeds were sown in 15 x 10 cm polythene bags filled with potting mixture containing soil, sand and cow dung

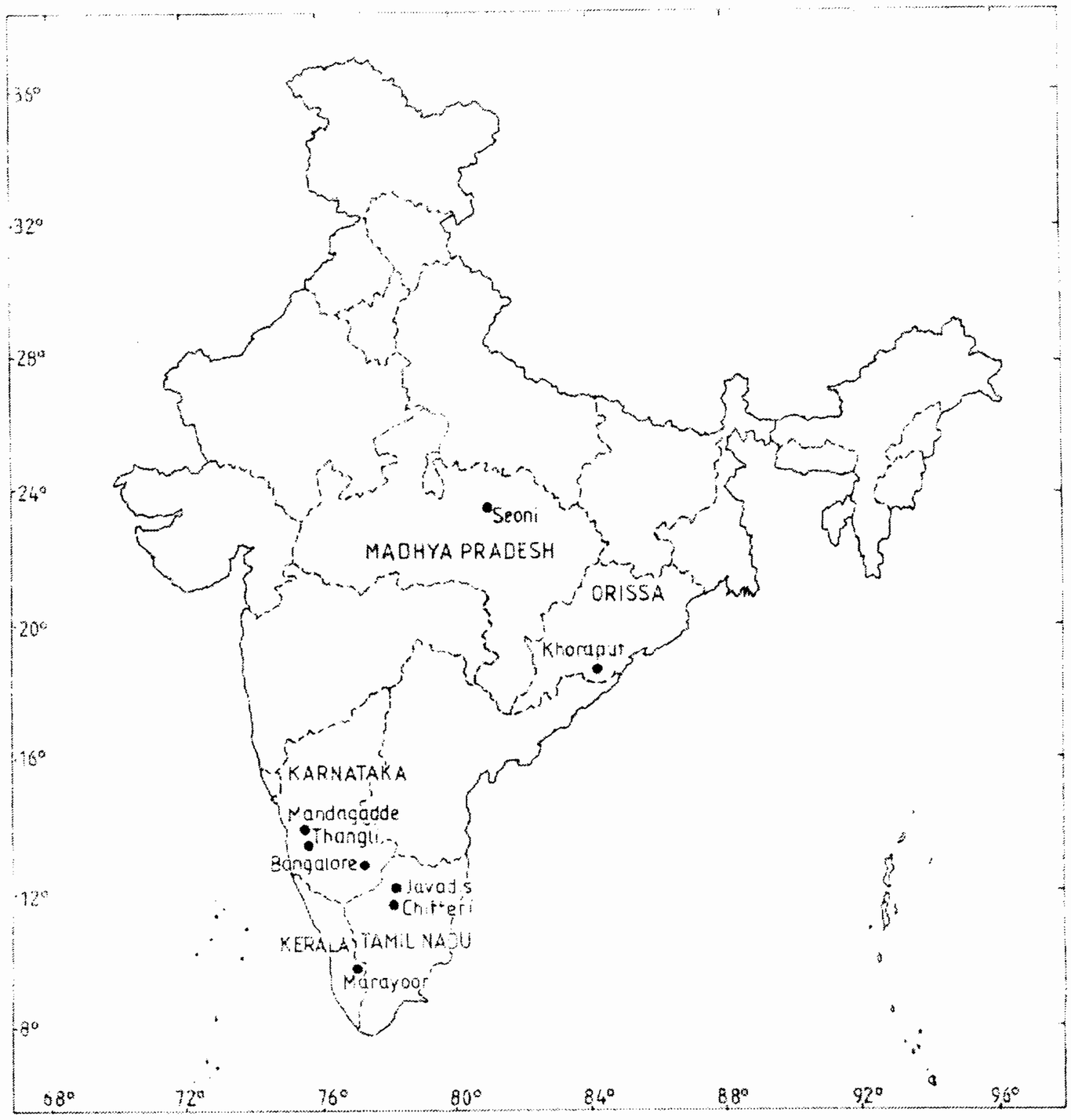


Fig. 1. Map showing the location of selected sandal provenances in India

In the ratio 2:1:1. The bags were kept in glass house, properly labeled and watered. The seedlings were kept outside the glass house after 6 months. Nine-month-old seedlings were planted in the provenance trial plots.

The provenances were planted approximately in one ha area in the KFRI field station at Veluppadam and in 0.5 ha in Sandal Reserve 54 at Marayur (PLATE I). Seven hundred and twenty seedlings comprising 120 seedlings each of Marayur, Bangalore, Thangli and Koraput provenances and 60 seedlings each of Mandagadde, Javadis, Chitteri and Seoni provenances were planted in 3 m x 3 m spacing at Veluppadam, Trichur District. The seedlings were replicated in two blocks. In each block, half (360 seedlings) the total number of seedlings were planted as line planting. Thirty plants were planted in each line. There were two lines each for Marayur,

Bangalore, Koraput and Thangli provenances. The intention of planting at Veluppadam was to raise a provenance trial plot out side the natural sandal area and observe the growth and adaptability of all the eight sandal provenances.

In Marayur range, each provenance was replicated in four blocks. Twenty seedlings of each provenance were planted in 2 m x 2 m spacing in a planting line and there were eight planting lines for 8 provenances. Thus the total number of seedlings for each provenance in the four blocks was 80. The provenance trial in Reserve 54 was chain link fenced on all sides. Since, Sandal Reserve 54 of Marayur range was highly affected by spike disease (PLATE I), the plot served as an ideal place not only for observing adaptability of provenances in a natural sandal area but also to screen the provenances for spike disease resistance. Observations on growth, survival and disease incidence were taken once in three months for first year of establishment and at the end of second year.

2.2. Establishment of progeny trial plots

Seeds were collected from 15 mature trees, apparently above the age of 20 years, located at different sites of the spike disease-affected Sandal Reserve 51 of Marayur Range. Though, most of the original 23,000 trees in 382 ha (Varghese, 1976) area of the reserve were destroyed mostly by spike disease, these 15 trees remained disease free. The seeds were germinated and the seedlings maintained as mentioned in para 2.1.1. Seeds were also collected from three Candidate Plus Trees (CPTs) identified in seed stand in Nachivayal I Reserve. Forty seedlings from five spike disease evaded trees and 24 seedlings from the CPTs were available for planting. These seedlings were planted in a separate block with in the same provenance trial plot at Marayur. Eight progenies from each parent tree were planted in this block with out any replication. Observations on growth, survival and spike disease incidence were taken once in three months for one year and at the end of the second year.

2.3. Induction of root suckers

Since, only very few seeds were available from the 15 trees selected in Reserve 51, an attempt was made to vegetatively multiply the selected trees through root sucker

induction. For this purpose, two experiments were initiated. One experiment was done in KFRI campus at Peechi to standardize the technique of vegetative multiplication of juvenile root suckers. The second experiment was carried out at Reserve 51, Marayur by injuring the roots of the selected 15 trees by digging 15 cm wide and 20 cm deep trenches, 1 m radius around the trees for inducing root sucker formation. All the trees were chain link fenced (3 m x 3 m) and protected from browsing. The number of root suckers formed was counted once in two months.

Root sucker formation was induced on roots of 5- to-15-year-old sandal of girth ranging from 10 to 63 cm in KFRI campus, Peechi. Trenches of 15 x 45 cm and depth of 15 cm were dug around each tree, 30 -100 cm away from the base of the trees depending upon the size of the trees and roots exposed. The circumference of the exposed roots ranged from 2.5 to 18 cm. Roots with circumference up to 12 cm only were cut to induce coppice shoot formation. On roots with more than 12 cm circumference, a superficial 'V' cut at about 1 cm deep was made so as to inflict root injury for induction of root sucker initiation. The number of root suckers formed was counted once in two weeks. The trees were watered during summer season.

2.4. RAPD analysis of sandal provenances, spike disease evaded trees and CPTs

RAPD analysis was carried out in all the eight provenances viz. Marayur, Bangalore, Mandagadde (Shimoga), Thangli (Chikkamagalur), Javadis (Tirupattur), Chitteri (Harur), Seoni and Koraput (Table 1). Seed samples were collected randomly from 15-20 parent trees located in different places of the reserve forests and the seeds were pooled together. From this bulk, 12 samples were drawn randomly for raising seedlings. Seedlings at four-leaf-stage (3-week-old) were used as the source material.

In the case of spike disease evaded trees, healthy leaf samples were available from 12 trees only because three trees were infected subsequently. But leaf samples were collected from diseased trees also in order to find out whether there is any variation between DNA profiles of healthy and diseased leaves. Samples were brought to laboratory either in liquid N_2 or by dipping the excised branches vertically in water and covering with wet polyethylene bags.

2.4.1. DNA extraction

Genomic DNA was extracted from tender leaf tissues following the modified method of Doyle and Doyle (1990). Samples were homogenated in hot (65° C) CTAB buffer containing 2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl and the sample homogenates were incubated at 60° C on a water bath for half an hour. The samples were extracted with chloroform/isoamyl alcohol (24:1) followed by low speed centrifugation (1600 g) for 5 minutes and the aqueous phase eluted out. This step was repeated again and to the aqueous layer, double the volume of cold (-20° C) absolute alcohol was added. After incubation at -20° C for 12 hours, the DNA precipitate was centrifuged for 10 minutes at low speed (1600 g). Ethanol (95%) was added to the DNA pellet and recentrifuged at the same speed. The supernatant was discarded and air-dried pellet dissolved in 100 µl double distilled water.

2.4.2. Polymerase chain reaction (PCR)

PCR-RAPD analysis was carried out using the following three primers viz. OPA 07 (GAAACGGGTG), OPA 09 (GGGTAACGCC) and OPA 16 (AGCCAGCGAA) selected from twenty primers of OPA series (Operon Technologies, Alameda, CA), based on the number and reproducibility of amplification products. In the case of disease evaded trees, one more primer namely, OPB 03 (CATCCCCCTG) from OPB series was also used. DNA was amplified in 25 µl reaction mixtures containing 100-125 ng of template DNA, 100 µM each of dATP, dTTP, dCTP and dGTP, 1.5 units of Taq polymerase, 1µl (250 ng) of each primer and 5 µl Taq buffer with 1.5 mM $MgCl_2$ (Genei, Bangalore, India). The incubation mixture was overlaid with one or two drops of mineral oil (Genei, Bangalore) and subjected to 45 cycles of amplification in PTC-150 Minicycler (MJ Research Inc., USA), each of 60 Seconds (S) denaturation (94° C), 60 S annealing (36° C) and 120 S extension (72° C). The last cycle was followed by incubation for 10 minutes at 72° C.

2.4.3. Separation and visualization of the amplification products

The PCR amplification products were electrophoresed in 1.5% horizontal agarose gel (Sigma, USA) in TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8.0). The gel, after the completion of electrophoresis was stained with ethidium bromide and bands were compared with a 100-bp DNA ladder (Genei). The gels were documented using Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak, USA).

2.4.4. Data analysis

The DNA fragment sizes were estimated comparing DNA size markers run on the same gel and the data scored for RAPD analysis. The bands were scored '1' for their presence and '0' for absence in each DNA sample to create binary data matrices. The data matrices were entered into the Popgene version 1.31 computer package and pair-wise comparison of provenances was made (Yeh *et al.*, 1999). Per cent of polymorphic loci (polymorphic bands per total bands) and Nei's G statistics (H_T, H_S *and* G_{ST}) (Nei, 1973) were determined. Nei's genetic distance (D) (Nei, 1978) was calculated and used to construct UPGMA dendrogram.

3. Results and Discussion

3.1. Survival and growth of sandal provenance seedlings

Seedling survival and growth were monitored for two years. Typical symptom of spike disease was not observed in any of the seedlings planted in spike diseased area of reserve 54 of Marayur range for the first two years. In general, the growth and survival of seedlings were extremely poor and lower for Veluppadam plot than for Marayur plot (Tables 2 and 3 and Figs. 2 and 3). At Veluppadam, the per cent of survival ranged from 1.6 for Seoni and Koraput to 17.5 for Marayur provenance followed by Bangalore (9.2%) at the end of second year. At Marayur, the lowest survival was for Koraput provenance (2.5%) and the highest for Marayur provenance (52.5%) followed by Bangalore (41.25%). Three patterns of seedling death were observed at both the places. Majority of the dead seedlings remained stunted before drying up; number of new leaves formed was very few. Such seedlings shed their leaves during dry season and gradually the seedlings dry up. A small group of seedlings shed their leaves during rainy season also, apparently due to fungal infection. Those seedlings rarely recovered after rainy season. In yet another group of seedlings, leaves turned papery white losing their chlorophyll with in a few days and they dried up rapidly. However, healthy seedlings, with comparatively thick, sturdy, brown stem and plenty of foliage grew up faster and remained healthy. Such seedlings were more in Marayur provenance.

Marayur provenance performed better than other provenances at both the locations indicating that the local provenance will be better for Kerala (Plate I). Probably, other provenances which performed very poorly could not adapt to the Kerala sites. The superiority of Marayur provenance with regard to seed sources had been recorded earlier also (Srinivasan *et al.*, 1992). However, in order to confirm the present findings with respect to other provenances, the field trial has to be repeated with more number of seedlings.

Table 2. Survival of sandal seedling in the provenance trial plot at Veluppadam

Sl.No.	Provenance name	No. of seedlings planted	Survival of seedlings				
			After 3 months	After 6 months	After 9 months	After 12 months	After 2 years
1	Marayur	120	108	84	48	25	21(17.5)*
2	Bangalore	120	96	66	43	19	11(9.2)
3	Mandagadde	60	27	26	20	5	3(5.0)
4	Thangli	120	92	51	49	13	7(5.8)
5	Javadis	60	39	29	12	5	2(3.3)
6	Chitteri	60	48	27	15	4	3(5.0)
7	Seoni	60	36	27	10	2	1(1.6)
8	Koraput	120	107	58	19	7	2(1.6)

*Percentage of survival after 2 years

Fig. 2. Graph showing seedling survival at Veluppadam

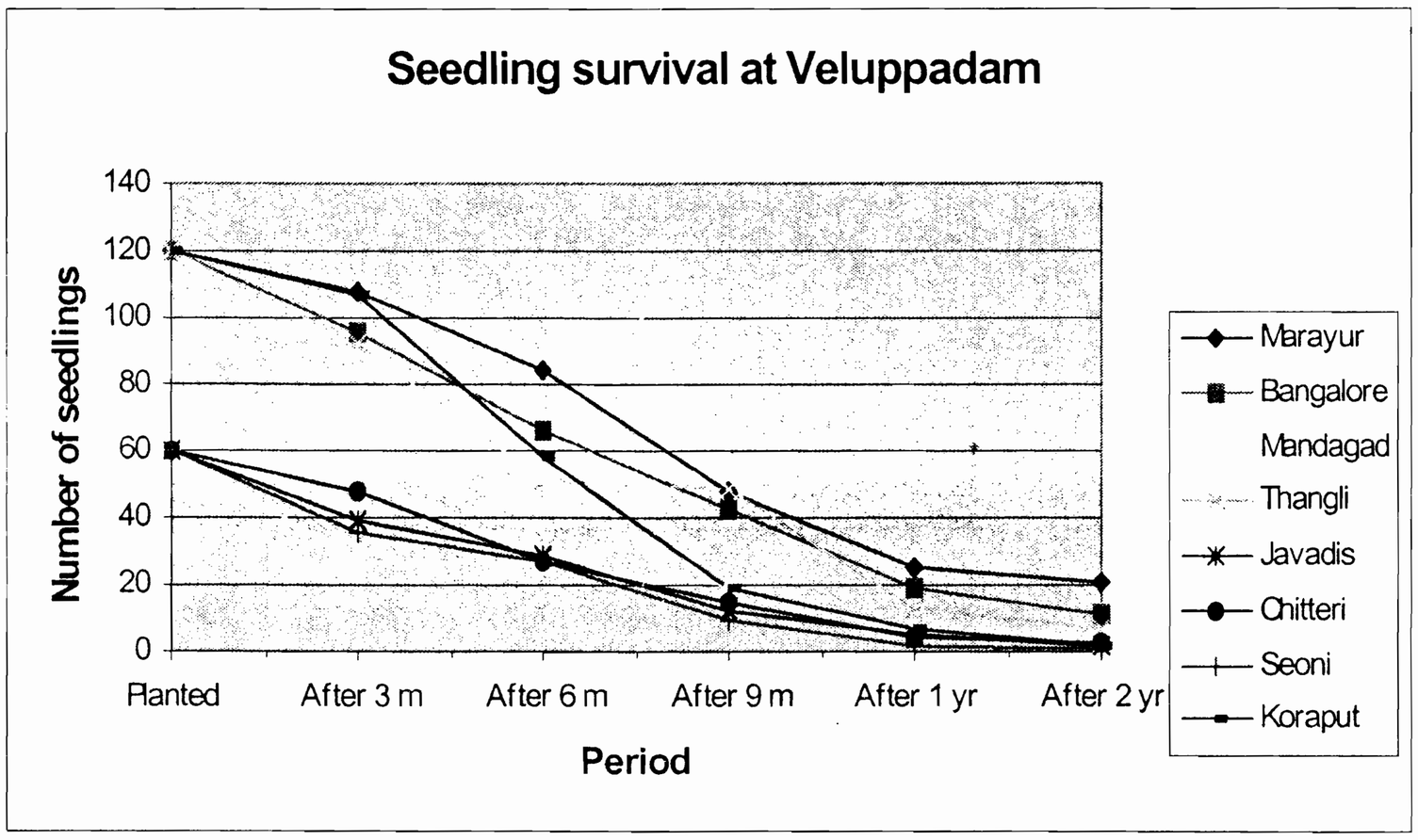


Table 3. Survival of sandal seedling in the provenance trial plot at sandal reserve 54 at Marayur

Sl. No.	Provenance name	No. of seedlings planted and mean height (cm)	Survival of seedlings and mean height in parenthesis (cm)					% survival at the end of 2 years
			After 3 months	After 6 months	After 9 months	After 12 months	After 2 years	
1	Marayur	80 (32.5)	75 (36.2)	62 (48.6)	55 (66.4)	48 (82.4)	42 (101.5)	52.5
2	Bangalore	80 (33.6)	76 (36.7)	56 (42.3)	40 (60.5)	38 (70.3)	33 (86.4))	41.25
3	Mandagadde	80 (23.1)	55 (24.8)	39 (26.4)	16 (30.8)	15 (43.7)	4 (54.2)	5.0
4	Thangli	80 (26.0)	47 (26.3)	32 (27.4)	27 (29.3)	21 (57.4)	9 (69.6)	11.25
5	Javadis	80 (30.1)	33 (30.9)	26 (32.3)	19 (34.4)	14 (39.3)	6 (42.3)	7.5
6	Chitteri	80 (28.9)	47 (30.0)	41 (30.4)	31 (34.6)	28 (44.6)	12 (50.2)	15.0
7	Seoni	80 (20.0)	21 (20.6)	18 (21.9)	15 (23.6)	9 (25.1)	3 (35.8)	3.75
8	Koraput	80 (23.7)	24 (24.1)	21 (25)	17 (29.3)	6 (34.6)	2 (43.4)	2.5

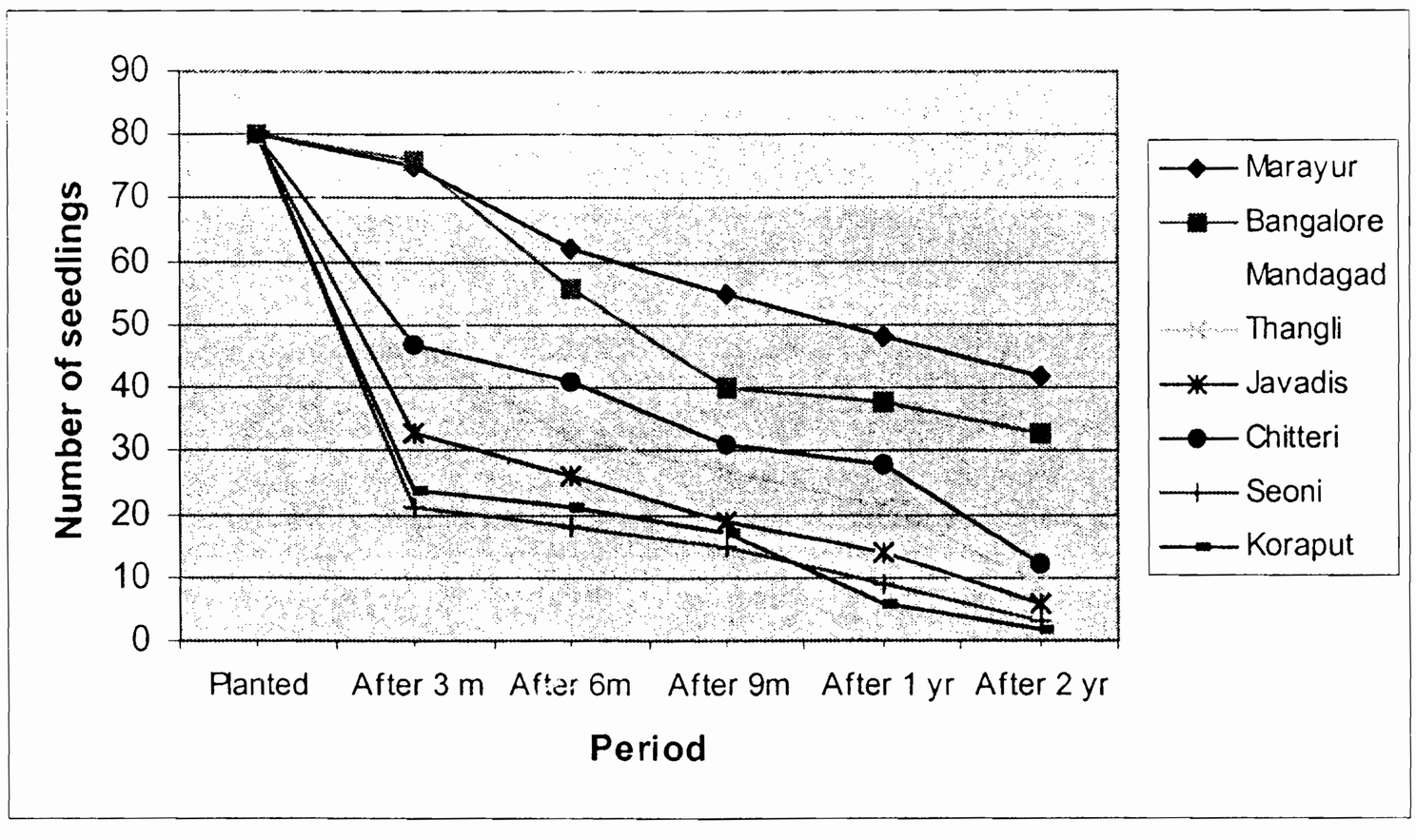


Fig. 3. Seedling survival at Marayur

3.2. Root sucker induction

Field experiments were conducted at KFRI campus and Sandal Reserve 51 on induction of root sucker formation through trenching around the trees after the monsoon. Fifteen trees were selected at both the places. Sandal trees planted in KFRI campus and the spike disease evaded trees in Reserve 51 at Marayur were used for the experiment. Marayur trees were protected with chain link fence (Plate II). Survival of the root suckers were more at Marayur than in KFRI campus, even though the sprouts were watered in KFRI campus during summer. Only injured roots produced root suckers. Even though the number of sprouts arising from a single site of root injury was as high as 43 (Plate II), only one or two sprouts survived to develop into healthy root suckers. Rest of the root suckers perished either during summer or during rainy season (Tables 4 and 5 and Figs. 4 and 5).

Table 4. Root sucker induction through trenching in KFRI campus, Peechi

Tree No.	GBH of trees (cm)	Exposed roots (number)			Survival of spouts as root sucker (number)				
		Total number of roots exposed	Sprouts from uninjured roots (number)	Sprouts from injured roots (number)	After 3 months	After 6 months	After 9 months	After one year	After 2 years
1	42.2	4	Nil	15	9	2	Nil	Nil	Nil
2	49.0	2	Nil	6	3	1	Nil	Nil	Nil
3	24.1	2	Nil	Nil	Nil	Nil	Nil	Nil	Nil
4	23.0	4	Nil	3	2	Nil	Nil	Nil	Nil
5	53.3	3	Nil	12	5	1	1	1	1
6	44.0	7	Nil	7	4	2	Nil	Nil	Nil
7	43.0	9	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8	16.9	6	Nil	6	3	1	Nil	Nil	Nil
9	19.2	4	Nil	10	6	2	Nil	Nil	Nil
10	20.1	7	Nil	21	13	2	1	Nil	Nil
11	13.4	4	Nil	Nil	Nil	Nil	Nil	Nil	Nil
12	32.3	4	Nil	41	12	3	2	2	2
13	16.0	5	Nil	31	15	6	3	3	3
14	9.5	5	Nil	19	7	2	1	Nil	Nil
15	12.5	2	Nil	43	17	6	2	2	2

Table 5. Root sucker induction around spike disease evaded trees in Sandal Reserve 51 of Marayur Range

Tree No.	GBH (cm)	Exposed roots (number)			Survival of spouts as root sucker (number)				
		Total number of roots exposed	Sprouts from uninjured roots (number)	Sprouts from injured roots (number)	After 3 months	After 6 months	After 9 months	After one year	After 2 year
1	34.4	3	Nil	12	3	2	2	2	2
2	44.8	3	Nil	Nil	Nil	2	2	0	0
3	30.2	2	Nil	Nil	1	1	1	2*	2
4	32.6	3	Nil	5	2	2	1	2*	2
5	38.1	4	Nil	Nil	Nil	1	1	1	1
6	42.4	2	Nil	7	2	2	0	0	0
7	53.8	5	Nil	Nil	1	1	1	2*	0
8	54.4	6	Nil	2	3	3	3	2	0(Spiked)
9	53.9	6	Nil	23	9	3	3	2	0(Spiked)
10	42.3	4	Nil	16	3	3	1	1	1
11	41.2	5	Nil	11	2	2	2	2	1
12	36.3	2	Nil	3	2	1	1	1	1
13	54.6	4	Nil	Nil	15	2	2	2	2
14	42.8	5	Nil	2	1	1	1	1	1
15	52.7	3	Nil	Nil	Nil	0	0	0	0

*New root sucker produced after one year

The average number of healthy root suckers developed per sandal tree was only 0.5 in KFRI campus and 0.9 in Reserve 51 at Marayur. While 13 per cent of the initial sprouts developed into root suckers at Marayur, only 3.7 per cent of the sprouts developed into root suckers at KFRI campus. Irrigation provided might be the reason for increased production of sprouts initially in KFRI campus.

Even though sandal stock can be improved in the reserve forests through root sucker induction, this practice has got disadvantages. Only some of the injured roots produced root sprouts; more over, only a fraction of them (3.7%) established as root suckers. The injury caused to the root system affects the efficiency of the root system which in turn affects the health of the tree. Pathogens and pests can enter through the injury causing damage to the root system and decay fungi can cause rotting of heartwood. The root suckers formed will be exactly of the same genetic make up of the mother tree and they remain connected through root system. Hence, there is increased chance of rapid spread of systemic diseases such as spike disease. The parasitic nature

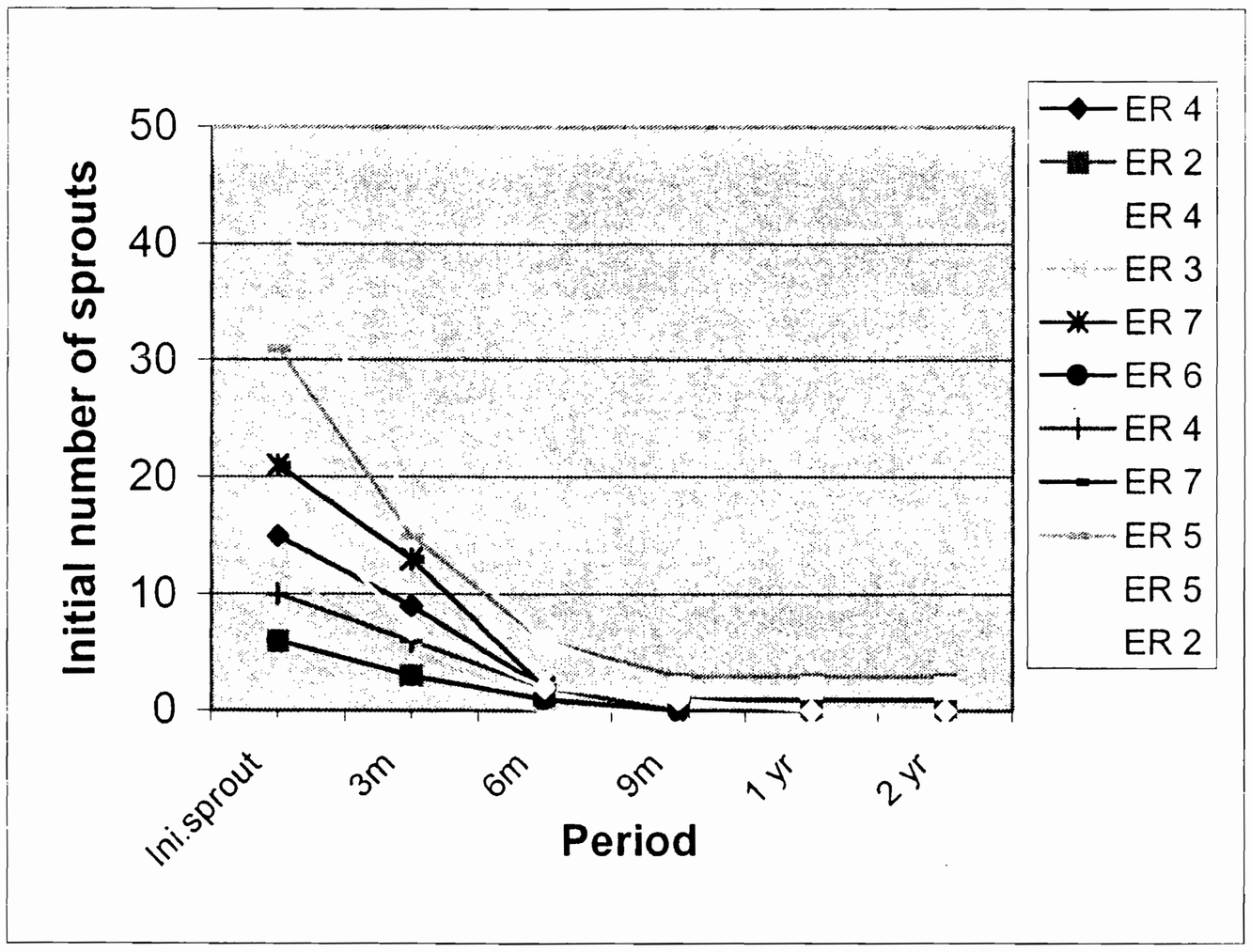


Fig. 4. Root sucker induction at KFRI campus showing very low survival of suckers

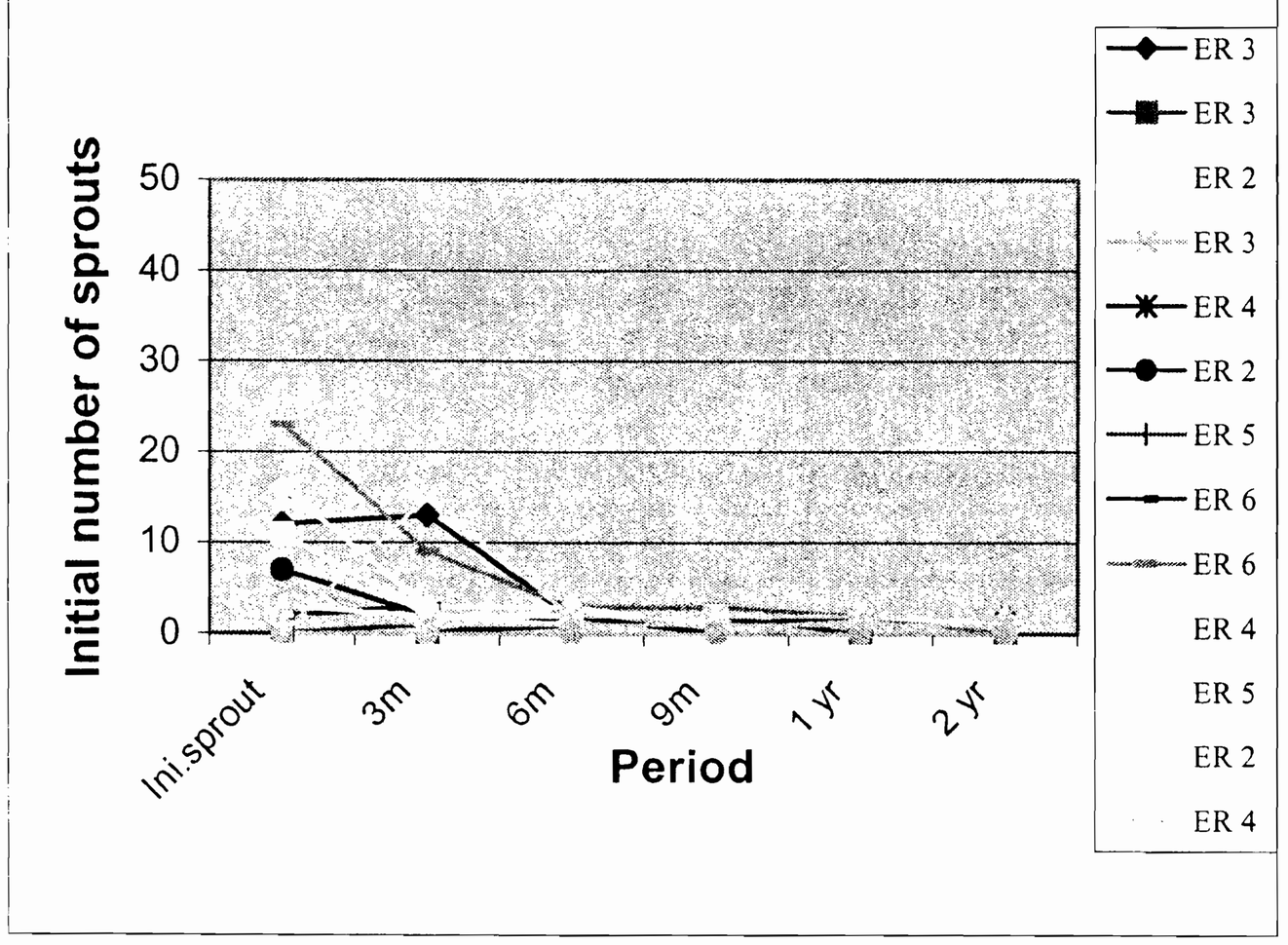


Fig. 5. Root sucker induction at KFRI campus showing very low survival of suckers

of the tree aggravates this problem due to root connections with host plants. Even though seedling mortality is almost 50 per cent, restocking sandal forests through planting superior seedlings will be a better option than root sucker induction for genetic conservation of this precious tree species.

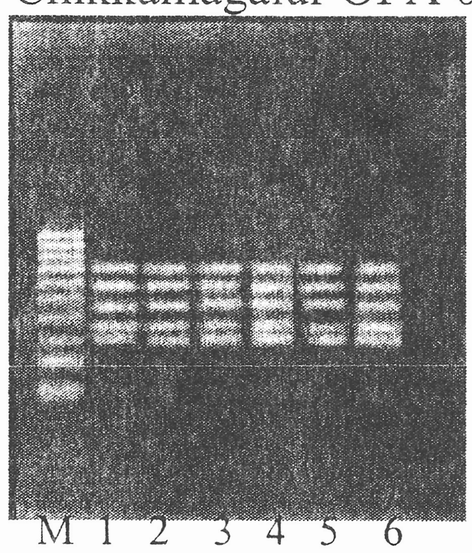
3.3. DNA finger printing of provenances and their genetic diversity assessment

Three primers from OPA series (OPA 07, OPA 09 and OPA 16) were chosen, out of the 20 OPA series primers tested, on the basis of number and reproducibility of amplified products. Twelve individuals from each of the eight provenances were screened for genetic variation using the three selected primers. Thirty six RAPD bands (amplified DNA fragments) were scored for their presence or absence. The molecular size of the amplified DNA fragments ranged between 100-1000 base pairs. The RAPD fingerprints of the provenances using the above primers and provenance specific bands are provided in Plate III. Assuming that each RAPD product represented a single locus, 91.67 % (33 loci) were found to be polymorphic at least in one of the individuals analyzed.

Within single provenance, the proportion of polymorphic loci (ppl) varied from a minimum of 2.78 % (Seoni) to a maximum of 38.89 % (Marayur) (Table 6). Nei's (1973) gene diversity index (H_S) varied from 0.01 (Seoni) to 0.14 (Marayur). According to Nei's (1973) G-statistics, the mean total genetic diversity (H_T) is 0.29. The relative magnitude of genetic differentiation among subpopulations (G_{ST}) is 0.78 indicating that 78 % of the total diversity was between provenances while the rest (22%) of the total variations occurred within provenances. The gene flow estimated from G_{ST} value was found to be very low, 0.14. The estimates of standard genetic distance (D) unbiased for sample size (Nei, 1978) for all pair wise provenance comparisons were calculated (Table 7). The genetic distance varied from 0.12 (between Bangalore and Thangli) to 0.57 (between Javadis and Seoni). An Unweighted Pair Group Method with Arithmetic means (UPGMA) dendrogram was constructed to estimate the differentiation of the eight provenances using Nei's genetic distance (1978). Two main clusters with clear separation were observable in the dendrogram (Fig. 6). The first main cluster consisted of five provenances, of which Marayur, Bangalore and Thangli formed one sub cluster, and Mandagadde and Javadis formed

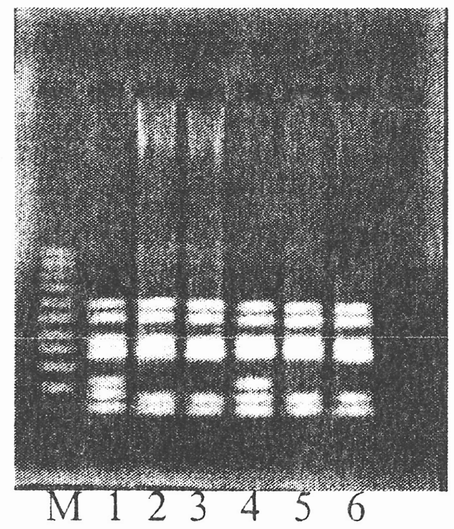
PLATE III - Provenance specific RAPD markers

Chikkamagalur OPA 07



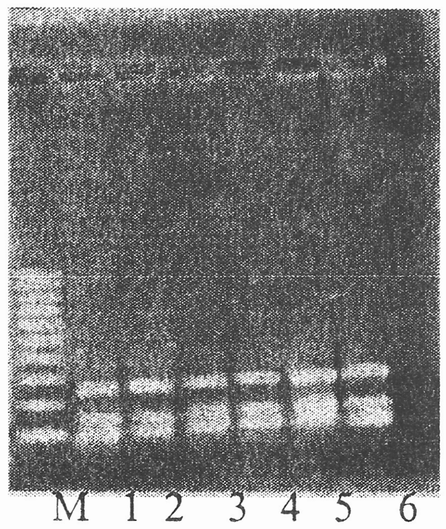
M 1 2 3 4 5 6

Shimoga OPA 07



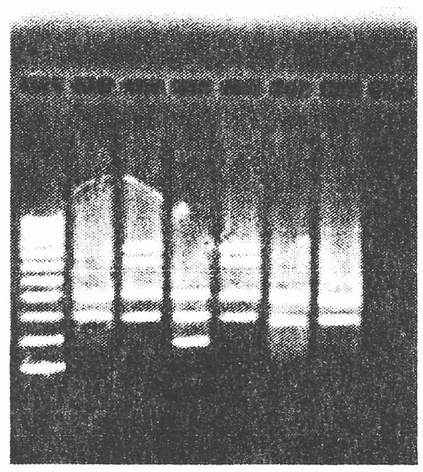
M 1 2 3 4 5 6

Thirupattur OPA 07



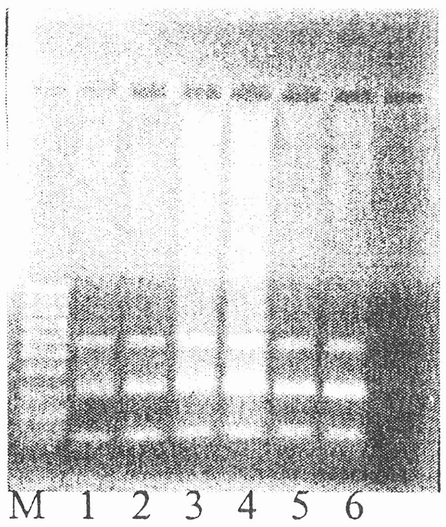
M 1 2 3 4 5 6

Chikkamagalur OPA 09



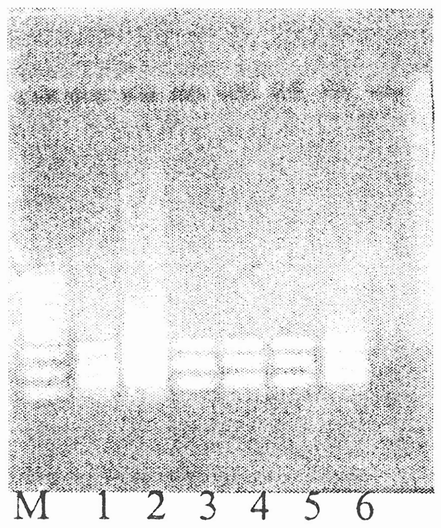
M 1 2 3 4 5 6

Koraput OPA 09



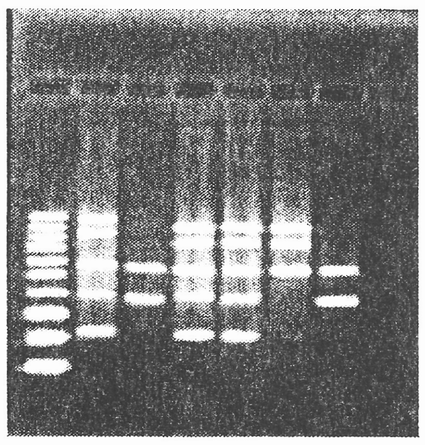
M 1 2 3 4 5 6

Harur OPA 09



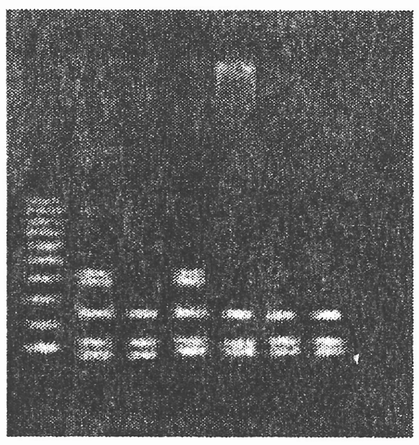
M 1 2 3 4 5 6

Marayoor OPA 16



M 1 2 3 4 5 6

Shimoga OPA 16



M 1 2 3 4 5 6

M = DNA marker (100bp DNA ladder); Lane 1-6 lanes = RAPD profiles of sandal trees

another sub cluster. The second main cluster comprised of three provenances, namely Chitteri, Koraput and Seoni.

Table 6. Comparison of provenances for various genetic diversity measures

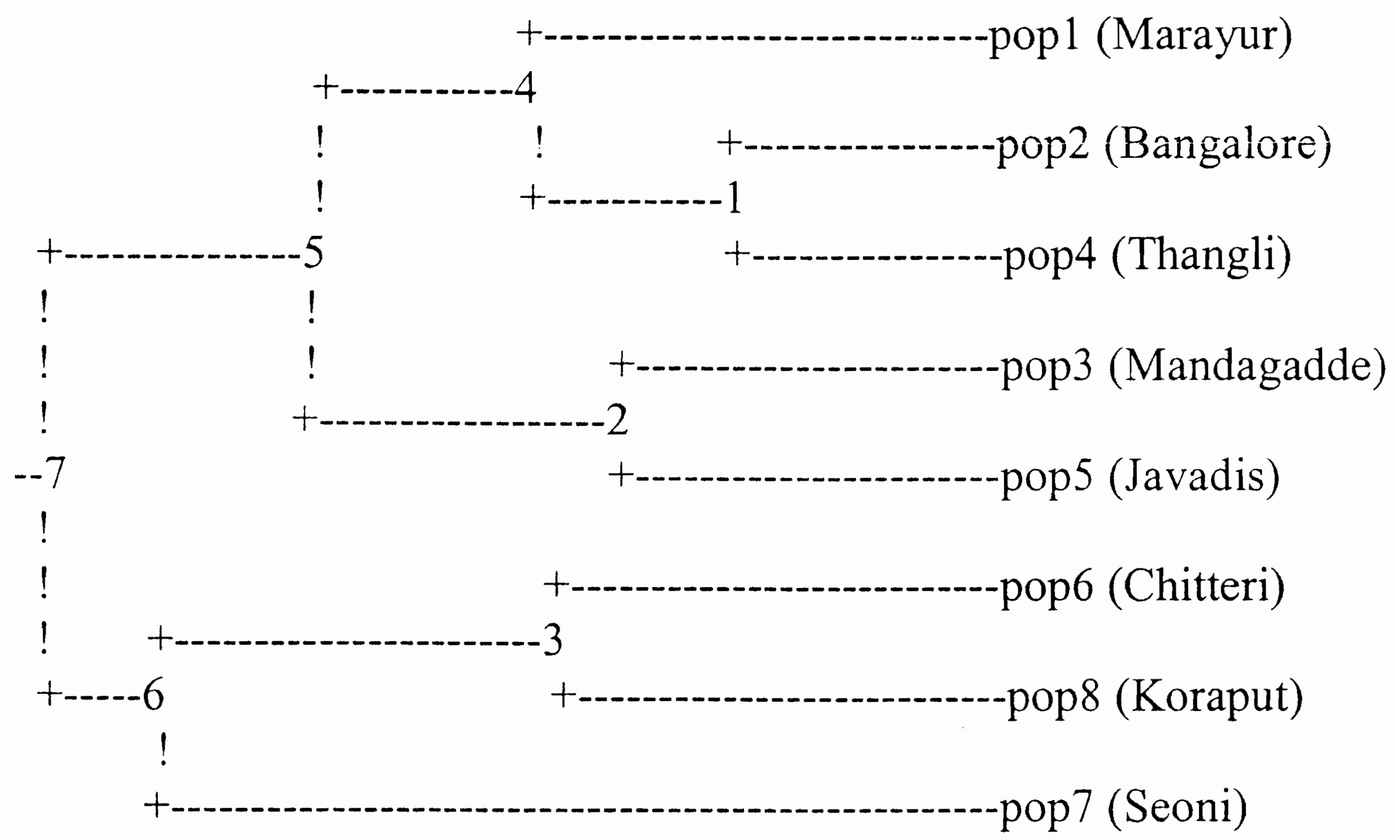
Provenances	Nei's (1973) gene diversity (h)	Per cent of polymorphic loci ppl (%)
Marayur	0.14	38. 89
Bangalore	0.10	27. 78
Mandagadde	0.03	16. 67
Thangli	0.11	33. 33
Javadis	0.04	13. 89
Chitteri	0.08	22.22
Seoni	0.01	2.78
Koraput	0.02	5.56

Table 7. Nei's (1978) measures of genetic distance

(Provenance 1 - Marayur, 2 - Bangalore, 3 - Mandagadde, 4 - Thangli, 5 - Javadis, 6 - Chitteri, 7 - Seoni, 8 - Koraput)

Proven-ances	1	2	3	4	5	6	7	8
1	****							
2	0.2317	****						
3	0.3503	0.2355	****					
4	0.1701	**0.1202**	0.3493	****				
5	0.2924	0.1442	0.1520	0.3167	****			
6	0.2988	0.2610	0.2087	0.3213	0.2544	****		
7	0.3677	0.3800	0.4539	0.4673	**0.5671**	0.3496	****	
8	0.4554	0.5041	0.3968	0.4404	0.5465	0.1878	0.3579	****

Fig. 6. Dendrogram based on Nei's (1978) genetic distance: Method = UPGMA



In the present study, genetic diversity among provenances had a high value (G_{ST} = 0.78). High value of G_{ST} was reported to happen when continuously distributed population is fragmented into small individual breeding units as a result of human disturbances, random genetic drift and differential natural selection and consequent reduction in the rate of gene flow. The result of the present study is in contrast to earlier findings of other tree species in which most of the genetic diversity resides within rather than between populations such as in *Azadirachta indica* (Kundu, 1999) and *Eucalyptus grandis* (Grattappalia *et al.*, 1997).

The eight provenances under study covered a broad geographic range differing in rainfall (850-2000 mm) and altitude (650-1050 m) (Fig.1 and Table1). The UPGMA dendrogram revealed the genetic relatedness of the eight provenances. The genetic separation of the provenances is partly in agreement with geographic isolation. The most genetically similar provenances, Bangalore and Thangli are geographically separated by a distance of only about 210 km (approx.) while the genetically distant provenances, Javadis and Seoni are separated by about 1050 km (approx.). The lack of correlation between geographic and genetic distance in some cases might be due to the geographic isolation of the area and the evolutionary forces like mutation, migration and natural selection operating in the area.

The results of the present study offer information regarding the genetic diversity structure of the sandal resources in India. A few provenances such as Marayur, have been severely disturbed recently, by spike disease and indiscriminate felling of superior trees. In such cases, out-planting of seedlings raised from seeds, back to the disturbed sites would help maintain an effective population size and the evolutionary forces naturally operating would restore the genetic diversity.

3.4. RAPD evaluation of disease evaded trees

A total of 17 discrete PCR amplified products ranging in size form 100bp to 600bp were obtained i.e. six bands for OPA 07, three bands for OPA 09, three bands for OPA 16 and five bands for OPB 03. Of these, OPA 09 and OPB O3 gave two types of banding patterns due to genotypic polymorphism. The majority of these RAPD products were identical both in the disease evaded and infected trees. The genotypic differences in banding patterns amplified by OPA 09 and OPB 03 primers were present in both diseased and disease evaded trees.

In the present study, no specific locus linked to disease resistance could be detected in the RAPD products of disease evaded/infected trees analysed with the four primers. In order to use RAPD markers for resistant breeding, at least a single locus linked to spike disease resistance must be identified unequivocally. The minor differences observed in RAPD profiles were present in both disease evaded and infected trees and hence cannot be attributed to disease resistance. The polymorphism might be due to genotypic differences unrelated to spike disease. It had been reported earlier that there was no natural resistance to spike disease (Iyengar, 1955; Nayar, 1986). However, with the present study, it was not possible to prove the absence of genetic resistance against spike disease conclusively. Adequate number of disease evaded mature trees were not available to have a detailed study. Further conclusive evidence will be possible only through a wider study of disease evaded parents and their progenies showing co-segregating markers linked to disease resistance.

Breeding for disease resistance and restocking the forest with resistant varieties is an effective approach to minimize the depletion of forest stock due to illicit felling

and disease problems. Utilizing spike disease evaded trees located in highly diseased tract for resistance breeding has been suggested by Venkatesh (1978). If the disease free trees at Reserve 51 at Marayur could be confirmed as disease resistant, those trees could be used for mass propagation vegetatively or through tissue culture technique, and the plantlets used for afforestation of the spike diseased areas.

PLATE 1

6-month-old sandal seedlings

Seedlings being loaded for transporting to planting site

Seedlings planted in provenance trial

Seedlings protected from sun scorch

2-year-old Marayur seedlings in provenance trial plot

A spike diseased tree in reserve 54

PLATE II

A healthy sandal in Reserve 51 protected with chain link fence. Note the trenches dug for inducing root sucker formation (arrow)

A healthy root sucker from injured portion

A large number of root suckers from severed root

4. Conclusions and Recommendations

In the provenance trial plot at Marayur, the provenance seedlings had not started to show the symptoms of spike disease and hence it was not possible to identify spike disease resistant provenances during the project period. Therefore, the occurrence of spike disease has to be monitored periodically in future and the resistant provenances identified.

The project work has resulted in the establishment of two sandal provenance trial plots for future studies. The genetic diversity studies using RAPD markers have shown that Marayur provenance is better than all other provenances with regard to genetic diversity. Hence, Marayur seed source will be better than other sources for raising sandal plantation.

The study has also revealed that augmenting the sandal population through development of root suckers is possible only to a very limited scale. Seedlings are better planting stock for afforestation. Moreover, the Marayur seedlings have shown 52.5 per cent survival in the provenance trial plot at Marayur. The advantages of seedlings are that seedling stock will increase the heterozygosity in the population while the root suckers will increase homozygosity, because the root suckers formed will be genetically identical to the mother tree. Homozygosity will increase the risk of disease susceptibility.

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