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MICROPROPAGATION OF IMPORTANT RARE AND ENDANGERED TREE SPECIES OF WESTERN GHATS

[Final Report of Project No. KFRI 253/96]

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ABSTRACT

Micropropagation of four species of rare endemic trees of the Western Ghats was attempted using standard tissue culture procedures. All types of suitable explants were used but the main ones were the nodal explants collected from seedlings or mature trees. Endogenous contamination was difficult to control in all the tree species when nodal explants were used. Seed and seedling derived explants were used to overcome this problem.

In *Syzygium travancoricum* sprouting of buds was obtained in 2 percent of the mature explants but no multiple shoots could be induced in the cultures. Nodal explants taken from seedlings derived from germinated seeds, gave rise to high frequency multiple shoot formation on Murashige and Skoog's (MS) basal medium supplemented with Benzyl Adenine (BAP) and Napthelene Acetic Acid (NAA). About 50-70 shoots developed in each subculture. Small clusters of shoots when transferred to a medium consisting of Kinetin (Kin) and NAA elongated and formed well-developed leaves and roots. Roots could be induced in excised shoots on a rooting medium consisting of MS with NAA and Indole Butyric Acid (IBA). Plantlets were transferred to a mixture of vermiculite and soil (1:1) and hardened in a mist propagation chamber for 4 weeks. More than 80 percent of the plantlets survived transfer to soil. Only 75 percent of the plantlets planted out in the original habitat of the species survived after eight months. Shoot cultures could be maintained at 22°C for up to 28 weeks. Somatic embryogenesis was obtained in cultures derived from mature leaf explants on Woddy Plant Medium (WPM) + 2,4-Dichlorophenoxyacetic acid (2,4-D) + BAP. On a hormone free medium somatic embryos developed further but failed to convert to plantlets.

In *Cinnamomum verum* nodal and shoot tip explants established and sprouted in culture but only in very low percentages. Induction of a highly embryogenic callus was obtained from leaf explants on a medium consisting of the minerals and vitamins of MS + BAP (3 mg/l) + Kin (1mg/l). Globular and cotyledonary stage somatic embryos were obtained on regeneration media but no plantlets were obtained.

Explants of *Gluta travancorica* and *Vateria macrocarpa* showed recalcitrance to a wide range of media and contanmination rates were also

high. Sparse development of callus from cotyledonary and leaf explants was obtained on auxin containing media, but no morphogenesis was obtained.

The results obtained in the study suggest that there is a need for additional and concerted efforts to ensure availability of suitable explants in sufficient numbers. Control of microbial contamination particularly of endophytes through prophylactic treatments of mother plants in field or under glasshouse and by use of systemic antibiotics *in vitro* is suggested. An understanding of the phenology of the species will also help in collection of immature stages of zygotic embryos that are more suitable explants for establishing cultures.

ABSTRACT OF THE PROJECT PROPOSAL

1.	Project No.	:	KFRI 253/96
2.	Title of the Project	:	Micropropagation of important rare and endangered tree species of Western Ghats
3.	Objectives	:	i. To develop tissue culture methods for mass propagation of the selected plant species. ii. To harden micropropagated plantlets and transfer them to soil. iii. To reintroduce the micropropagated plants into the natural habitat and monitor their performance.
4.	Date of commencement	:	April 1996
5.	Date of cimpletion	:	March 1999 (Extended to March 2000)
6.	Funding agency	:	Kerala Forest Department (Wildlife)
7.	Project Team		
	Principal Investigator	:	E.M. Muraleedharan
	Associate	:	
8.	Study area	:	
9.	Duration of study	:	4 years

Contents

Introduction

The Western Ghats are acknowledged as one of the hot spots of biodiversity in the world. The area is home to a large number of endemic plant species including several arborescent ones. A significant number of these plants are found in the area within Kerala State. Anthropogenic pressure in the form of developmental activities, encroachment into forest areas, overexploitation of forest resources etc. have resulted in damage to the habitat of several of the above species.

Efforts aimed at conservation of the rare, threatened or endangered plants fall into several categories. Protected areas like biosphere reserves and sanctuaries intended for conservation of total ecosystems or specific elements of fauna often result in the conservation of several of the plant species whose habitat lies within the area. *Ex situ* conservation is another accepted means of conservation where the species are maintained outside their natural habitat in the form of botanical gardens, germplasm collections, gene banks, seed collections etc.

In the case of plant species where natural regeneration is a limiting factor in ensuring survival of the species, artificial means of regeneration has to be resorted to for production of planting material either for the reintroduction into the natural habitat or for ex situ collections. A study of the reproductive biology of the species is often useful in identifying the reasons for poor regeneration. Improved germination rates can be obtained by resorting to simple procedures. If conventional means fail then the use of vegetative propagation methods have proven to be useful.

The use of *in vitro* techniques for mass clonal propagation i.e. micropropagation, is now a well established procedure for a wide range of economically important plant species particularly those of horticultural and agricultural importance. Several important forest trees are also in the list of species where successful micropropagation has been achieved.

The advantages of micropropagation for mass propagation are manifold. The technique typically results in a high rate of multiplication compared to conventional vegetative propagation through rooted cuttings, layers or grafts. Starting with small plant parts cultures can be established and therefore non-availability of vegetative material is not a limitation unlike other methods. In the case of rare and endangered plants in vitro conditions offer a space saving repository free from the influence of vagaries of nature and from pests and diseases.

Another advantage of *in vitro* methods is the potential for long term storage of cultures under minimal growth conditions or for extremely long periods using cryopreservation. Minimal growth conditions are attained through use of growth retarding adjuvants to the culture media or by maintaining cultures under temperatures which are sub- optimal for growth. Thus the time period for which a particular plant tissue can be maintained in a culture container without transfer to fresh medium can be extended from a few weeks to several months. In cryopreservation the cultures are maintained, usually pretreated with cryoprotectants, at the temperature of liquid nitrogen (- 196^{0} C) in which tissues are in a state of

suspended animation and are potentially preserved for indefinitely long period. Plants can be regenerated after thawing through standard micropropagation procedures.

In this study the application of micropropagation for conservation of rare and endemic tree species of Western Ghats was explored. Four endemic tree species were selected. Conventional propagation methods were either not known or available and the potential of developing a mass propagation method was considered desirable.

Two of the species in the original list viz. *Diallium travancoricum* and *Diospyros assimilis* were not considered for the study because it was not found feasible to locate trees because of the extreme rarity of the species. Regular collection of plant material to facilitate experimentation would not have been feasible in the two species.

1. *Cinnamomum verum* (Lauraceae)

The species is often considered to be taxonomically identical with the cultivated species *Cinnamomum zeylanicum* the bark of which is the spice cinnamon. Large quantities of the bark are extracted from the wild in an unscientific manner, which is a threat to the survival of the species. Clonal propagation will be of immense benefit for *ex situ* conservation of germplasm and mass propagation of identified superior genotypes.

2. *Gluta travancorica* (Anarcardiaceae)

The species is distributed in the Thenmala Range and the Sendurny Wildlife Sanctuary. Natural regeneration does not take place in sufficient numbers in the locality probably because of human interference. The species is with good potential as a plantation tree since it produces a timber that is attractive and of much value. Clonal propagation of selected genotypes would be of benefit for tree improvement. *In vitro* techniques as a means of cloning as well as *ex situ* conservation is of great potential, considering the narrow natural distribution range of the species.

3. *Syzygium travancoricum* (Myrtaceae)

The species is reported to occur in several isolated patches in different part of Kerala. The habitat is described as `myristica swamp' forests. During the survey for locating trees for collection of plant material for culture, no seedlings or saplings of the trees were seen in any of the habitats, indicating absence of regeneration at least in the last 5-6 years but probably much longer. Fruits are found to be heavily infested with insect larvae but production of seedlings in sufficient numbers is still feasible if collection and sowing of the seeds is done at the right time.

Disturbance due to anthropogenic pressure is typical of all the habitats of the species visited during the study. *Ex situ* conservation is therefore of great importance for the species particularly because the existence of distinct populations throughout the length of Kerala. The ecological conditions of the natural habitat are however rather difficult to simulate in an *ex situ* collection and the application of *in vitro* techniques thus assumes some importance.

4. *Vateria macrocarpa* (Dipterocarpaceae*)*

The species is endemic to the Muthikulam range in Palakkad District. Trees grow to large sizes. In the recent years there appears to have been a very large decrease in the population since the number of fallen trees observed is high in the area. The number of trees in the smaller size classes is meager. Since part of the habitat of the species lie in private plantations conservation of the remaining populations in natural forests assumes importance.

Fruiting in the species appears to be once in three or more years. The large fruits are recalcitrant and often heavily damaged by pests. Seedlings do not survive in containers beyond a few months and thus *ex situ* conservation is difficult. Studies on soil microflora and soil chemistry might be of use in determining the reason for the difficulty in establishment of seedlings.

Being closely related to *V. indica* and having all the economically useful characters this species also could be exploited if the populations were stable and its range could be expanded artificially. The large cylindrical bole in the species , which is much larger than *V. indica*, would be a distinct advantage for commercial exploitation.

Materials and Methods

Plant Material

C*innamomum verum*

Young twigs were collected from the current year's growth from a young tree growing in KFRI campus. Unripe fruits were collected from the same tree.

Gluta travancorica

Trees were located in Rosemala in the Thenmala Division. Fresh sprouts from saplings and smaller trees were collected for culture. Fruits were collected and sown for germination in soil in earthen pots.

Syzygium travancoricum

Trees located in Asramam compound at Kollam, Andalurkavu a sacred grove near Thalassery and near Mananthavady, Wyanad were used as source of explants. Fresh sprouts were collected and brought to the laboratory in plastic bags. Layering was carried out in the field by bending down the lowermost branches of trees, covering the stem with soil and keeping it in place with a heavy stone. After 2-4 months the rooted branches were separated and transported to Peechi and planted in pots and maintained in the nursery. Plants were sprayed once a week with a solution of 0.2% (w/v) of Bavistin and 200 mg/l of gentamycin. Fresh leaves were collected from these plants routinely for experiments.

Seeds collected from trees growing in Wyanad were collected and sown in trays for germination. Nodal, shoot tip and leaf explants were taken from seedlings. Nodes and newly opened leaves collected from field were also used as explants.

Vateria macrocarpa

Young trees located in Mutthikulam forest Range were the source of explants. Stems from the current year's growth were collected and nodes and shoot tips excised for culture. Germinated seeds were also collected from the forest floor were brought to KFRI and maintained in pots in the mist propagation chamber.

Surface Sterilization

Excised plant parts were trimmed in the laboratory to convenient sizes and washed thoroughly with running tap water followed by a thorough wash with a dilute solution of detergent (Extran Neutral, Merck). After a final wash with water to remove the detergent the explants were given a pretreatment by soaking in a solution of Bavistin 0.2% + Gentamycin (80 mg/l) for 1 hr. This was followed by surface sterilisation of the explants by treatment with mercuric chloride in a sterile container in a laminar flow bench. Concentrations of 0.1, 0.5 and 1.0 % (w/v) were tested at various treatment durations of 10 and 15 min. to find out the optimum treatment.

Culture Media

Basal media: The mineral salts and organic components of Murashige and Skoog (1962) and the Woody Plant Medium (Smith and McCown, 1983) were tested as basal media for culture of various explants (Table 1). Other supplements were added as required for the different experiments.

Establishment media: The basal medium supplemented with Bavistin 0.2% and Gentamycin (80 mg/l) was used for culture of all explants taken from mature field growing trees to screen for contamination and to induce bud break.

Shoot initiation\ multiplication media were supplemented with the cytokinins BAP or Kinetin and with or without NAA or 2,4- D.

Callus induction was attempted in media supplemented with an auxin either singly or in combination with a cytokinin.

Regeneration media for organogenesis/Somatic embryogenesis: Explants cultured on a hormone containing media and callus induced on explants were subculture to media free of hormones or containing a lower level of the respective hormone or supplemented with 0.25 % (w/v) of activated charcoal, to induce morphogenesis. Such cultures were maintained in light. To induce normal development and maturation of somatic embryos, maltose (0.05, 0.1 and 0.2 M), mannitol (25- 800 mg/l) or ABA (0.05, 0.1, 0.5 and 1.0 mg/l) were added to the regeneration media.

Rooting media: MS or MS (½ strength) supplemented with either NAA (2 mg/l) or (IBA 2 mg/l) were used for *in vitro* induction of rooting in micro-shoots.

Formulation of culture media

Stock solutions of most of the media supplements were prepared and stored at room temperature or in the refrigerator. Appropriate aliquots of the stock solutions of basal media and other additives and sucrose were added to double glass distilled water and made up to slightly less than the final volume. The pH of the medium was adjusted with 0.1 or 1 N NaOH or HCl using a pH meter and water was added to make up the total volume. The solidifying agent was added wherever required. Media were heated in a microwave oven until the solidifying agent had melted and poured into the culture containers while still hot. Media to be poured into petridishes were autoclaved in test tubes and later poured after melting into the dishes under sterile conditions.

Culture vessels

Borosilicate glassware like test tubes (25 X 150 mm), petri dishes (45 X 15 and 85 X 15 cm) and conical flasks of 100ml and 250 ml were used in experiments involving smaller explants like embryos and single shoots. Test tubes and flasks had closures made of non-adsorbent cotton plugs. For multiplication and proliferation of shoots larger containers like Magenta (clear polycarbonate vessels with polypropylene (PP) closures; Sigma Co., USA) and Phytacon (Translucent polypropylene containers; Sigma Co. USA) and glass bottles with PP closures were used.

In the establishment stage, culture test tubes with 10 or 15 ml or petridishes with 20 ml of solid media were used for most cultures. When glass bottles, Phytacon or Magenta vessels were used, 30 ml of media was dispensed into each vessel.

Sterilization

Sterilization of empty glassware and culture media was done by autoclaving in a horizontal steam sterilizer or in a pressure cooker depending on the quantity of material to be sterilized. Empty glassware was autoclaved for 20 minutes and media for 15 min.

Culture conditions

Cultures were incubated in a culture room in which the temperature was maintained at 25° $\pm$ 5° C. Cultures were maintained on shelves either in dark or under fluorescent illumination with a photoperiod of 12 hrs dark/12 hrs light. The photon flux density in the PAR range in the culture room at the level of the culture vessels was 25 μ E m^{-2} s^{-2}.

Cultures of S. *travancoricum* which proliferated well under such conditions were also incubated in one experiment at a temperature of 22°C to test the effect of lower temperatures on long term maintenance of cultures.

Hardening

Plantlets with well developed roots growing in vitro were washed in water to remove traces of culture media, given a treatment with a solution of 0.2 % (w/v) Bavistin for 1 hr. and planted in plastic cups or polyethylene bags containing a vermiculite-sand mixture (1:1). Plantlets were either placed in a mist-propagation unit or in the greenhouse covered with a inverted polyethylene bag. Intermittent misting was provided to maintain humidity in the mist-propagation unit. Plants in the green house were watered every alternate day.Plantlets that survived after 4-6 weeks of hardening were transferred to larger polyethylene bags containing soil and kept in the nursery until filed planting.

Results

1. ***Cinnamomum verum***

Nodal explants of C. *verum* showed high rates of fungal and bacterial contamination. Up to 80, 78, and 62 % of the explants showed contamination in the surface sterilization treatments for 10 min. with 0.1, 0.5 and 1.0 % $HgCl_2$ respectively. When the treatment duration was increased to 15 min. the explants showed signs of damage in the form of rapid browning in 2-5 days although bacterial contamination was reduced notably. The fungal contamination was in most cases seen from the cut ends of the explants indicating that endophytes (endogenous microbes) were the cause.

Explants that escaped contamination were slow in response in terms of sprouting of axillary buds. Upto 3-4 small shoots with minute leaves were obtained from one explant on MS+ Kin(0.1mg/l) but the cultures turned brown in one week after subculture.

Leaf explants taken from mature trees and seedlings gave rise to callusing on MS media supplemented with auxin as well as on cytokinin containing media. Callusing was mostly seen forming in 1-2 weeks from cut edges of the explants and on the midrib wherever present. On NAA containing media (1 to 5 mg/l) sparse dark brown calli were induced which was slow growing. Callus was yellow and fast growing on media containing 2,4-D (0.5 to 5 mg/l). On MS + 2,4-D (3 mg/l) supplemented with either BAP (1 mg/l) or Kin (1 mg/l) a fast growing friable and granular callus was obtained from seedling leaf explants. An embryogenic fast growing yellow coloured callus (Fig. 1) maintained on media supplemented with BAP (3 mg/l) + Kin (1 mg/l).

On the regeneration media consisting of MS with low levels of auxin and cytokinins or free of hormones, with or without activated charcoal, the somatic embryos developed asynchronously up to the cotyledonary stage and sometimes turned green but failed to convert to plantlets. Shoots developed in some of the somatic embryos but no further development was obtained.

To induce normal development the effect of maltose , mannitol and ABA were tested separately in a regeneration media consisting of MS + 2,4D (0.5 mg/l) + BAP (2 mg/l) The callus growth slowed down in the above media and turned brownish in colour. There was no appreciable difference in the quality of the somatic embryos on these combinations and further development was not observed.

Leaf explants cultured on the hormone containing media were also subcultured after 4-7 days of inoculation to hormone free medium to induce organogenesis without callus induction. Only rhizogenesis was observed in some of the explants.

2. ***Gluta travancorica***

In *Gluta travancorica* embryos excised from seeds germinated *in vitro* on a simple medium consisting of MS + 2% sucrose. Germinated embryos were transferred to media containing cytokinins BAP or Kin (3 mg/l) but no multiple shoot formation was obtained except in a few cultures grown on BAP (Fig. 2) where two shoots were produced. Probably this was the result of the development of the two cotyledonary nodes. Only one explant produced eight shoots. These shoots however had only

rudimentary leaves and did not develop further. Cotyledonary explants cultured on MS + 2,4- D at various concentrations (1.0, 3.0 , 6.0 mg/l) developed a brown callus which however did not develop further on subculture.

Explants taken from mature trees produced heavy leaching of phenolics into the medium. Treatment with 0.25 % polyvinylpyrollidone (PVP) for 30 min. resulted in some reduction of browning of the media but did not prevent the gradual browning of the explants. No sprouting of buds was observed in any of the media tested.

Nodal explants and leaf explants taken from seedlings of *Gluta* grown in the nursery also failed to establish in culture beyond 4 weeks. Browning of the explants followed by gradual death was observed. Leaf explants developed a dark brown callus on cut edges particularly on the mid-rib, but on sub-culture no further growth was noticed.

3. Syzygium travancoricum

Mature tree nodal and shoot tip explants showed very high rates of contamination in all the treatments tested. After 4 weeks of culturing, fungal contamination appeared in more than 98 % of the explants. In a majority of the explants the fungal growth appeared when the bracts covering the buds opened after a few days in culture. Sprouted buds obtained in 2 % of the explants remained without elongating in spite of two subcultures into fresh media at the intervals of 3-4 weeks each.

Sprouting of nodal explants (Fig. 5) taken from seedlings was obtained in almost all the cultures on media containing cytokinins and cytokinins + auxins. Very high rates of shoot multiplication was obtained on WPM + 18 μM BAP and 0.5 μM NAA (Fig. 6). Shoots were thin and with small unexpanded leaves. When seperated into clumps of shoots and shifted to WPM + 18 μM Kin and 0.5 μM NAA, elongation of shoots and development of large expanded leaves was observed (Fig. 7). Rooting occurred spontaneously in several of such elongated shoots. Rooting was induced in excised shoots transferred to a liquid medium consisting of WPM + NAA (5 μM) or WPM + IBA (5μM) with filter paper supports. About 2-6 well developed roots developed at the base of the shoots in about 2 weeks. Sometimes the rooting was accompanied with some callus formation.

Rooted plantlets when transferred to the sterile soil vermiculite medium and maintained in the hardening chamber established well and developed further. More than 80 % of the plantlets survived after two weeks of transfer (Fig. 8).

Hardened plantlets obtained from seedling explants were maintained in polybags and kept in a nursery with intermittent watering until planting out to the field. Plantlets have been planted out in the natural habitat in Wyanad (10 Nos.) and Thalassery (15 Nos.) to assess the survival and field performance. Plantlets growing in polybags were planted in the month of June in pits of approximately 30 X 30 X 30 cm in a spot in the forest offering some protection from damage by cattle or human interference. All plantlets survived after two months of planting out. After eight months in field only 75 % of the plantlets survived but the reason for mortality could not be ascertained. Growth of plantlets was slow and except for the addition of one or two leaves no development was visible.

Cultures maintained in multiplication medium showed no visible sign of deterioration even when transfer to fresh media was delayed for 20 weeks. Maintenance of cultures at lower incubation temperatures was tested only up to 22°C and no deleterious effects were observed. Shoots grew slower and thus subcultures could be postponed even beyond 28 weeks

Leaf explants taken from mature tree layers and maintained in pots, when cultured on WPM + 2,4-D + BAP at various concentrations gave rise to a yellowish white friable callus in 2 weeks . The callus was maintained by subculture to fresh media every 5 weeks. On transfer to a regeneration medium free of hormones or with low levels of the hormones, the callus developed organised structures with pink colour (Fig. 3). As in *Cinnamomum*, the somatic embryos occasionally turned green with a rudimentary shoot pole, but conversion to planlets were not obtained even with media supplemented with maltose, mannitol or ABA.

5. Vateria macrocarpa

Nodes and shoot tip explants taken from young trees of Vateria macrocarpa failed to respond on all the media tested. As in other species studied fungal contamination due to endophytes was very high. Sterile explants were obtained by treatment with 1.0 % $HgCl^2$ for 10 min. Explants remain green for upto 5 weeks but failed to show signs of sprouting of buds.

Zygotic embryos were difficult to bring into culture because of their large sizes. Sterile seedlings were obtained by germination of embryos in Magenta vessels. After excising the roots and cotyledons the embryo axes were transferred to media containing BAP. Cotyledonary nodes developed in embryo axes in which the plumule was removed but no further development was observed although the tissues survived in culture for more than 10 weeks.

Mature leaf explants cultured on 2,4-D containing media gave rise to a soft white callus (Fig. 4) in about 10 % of the cultures. This callus could not be maintained through subsultures and also failed to show any morphogenesis on transfer to regeneration media.

Discussion

Trees, particularly tropical forest trees species growing in the wild, are difficult candidates for tissue culture and micropropagation. In many of the tree species which are not domesticated, carrying out experimentation for standardization of *in vitro* culture is difficult due to limitations of obtaining sufficient plant material. In the present study, the selected species being rare species, collection of plant material routinely for culture was not possible at frequent intervals. Since the phenology of the species is not understood well, seasonal variations which can influence the response of tissues in culture cannot be taken into consideration and collection of young developing fruits is not often possible.

The recalcitrance of explants taken from mature trees to response in culture vis-à-vis juvenile tissues is well known. This recalcitrance is similar and probably related to the similar phenomenon observed in vegetative propagation, especially in rooting of stem cuttings. Some degree of rejuvenation can be achieved by repeated pruning and induction of new flushes of shoots as has been attained in several tree species for obtaining cuttings that root well.

In all the four tree species studied here, a very high percentage of the explants taken from trees growing in the field had to be finally discarded due to fungal and bacterial contamination. Microbial contamination is a serious problem that limits micropropagation of tropical tree species. This problem is severe in the moist tropical regions and the monsoon season is particularly unsuitable for collection of plant tissues since the conditions are conducive to proliferation of microbes. Use of an appropriate combination of surface sterilizing agents and treatment duration is the standard means of arriving at a procedure that controls contamination within acceptable levels so that sufficient sterile cultures are available for further culture. However the presence of endophytes (endogenous microbial species) within the tissues of most tree species makes the problem more complicated. If feasible prophylactic spraying of plants or branch cuttings with systemic antimicrobials could lead to a solution. Under field conditions this strategy was not feasible since the trees species grew in remote forest areas. Only in the case of *S. travancoricum*, branch cuttings /layers could be transported to nursery and maintained in pots. These plants were sprayed with Bavistin and Gentamycin solutions once in a week and sterile cultures from leaf explants were possible. Since the potted plants were small in size it was not feasible to take node or shoot tips explants for culture. In none of the other species it was possible to induce layering or establish stem cuttings.

In the present study attempts were made to use nodal and shoot explants taken from mature trees of the so that if successful the method could be used for cloning of specific individuals (genotypes). Since in the preliminary experiments itself the a high percentage of cultures were lost due to contamination the emphasis was shifted to use of juvenile explants like seedlings, embryos and potted branch cuttings so that sterile cultures could be established.

Micropropagation through multiple shoot formation from shoot tips and axillary bud followed by rooting is recognized as the most suitable method to ensure minimum of culture induced variability in the regenerated plantlets. Micropropagation procedures involving a callus phase carry a risk of genetic variability in the form of somaclonal variation (Skirvin *et al*., 1994)

Tissue culture of *C. verum* was earlier attempted by Mathai *et al.* (1997) who report the formation of multiple shoots from shoot tips and nodal explants taken 7-8 year old plants. More than 6 multiple shoots could be induced on WPM medium supplemented with BAP (3 mg/l) and Kin. (1 mg/l). Rooting was also induced on WPM supplemented with 0.2% AC and plantlets could be established with 80% success. Their success could be attributed to the fact that they were working with cultivated plants maintained in the garden and in our study the plants were wild.

Rai and Jagadish Chandra (1987) have reported the formation of multiple shoots form nodes of seedlings of *C. zeylanicum*, on media containing BAP and Kinetin. However the maximum number of multiple shoots were limited to just 4 which is insufficient for a mass clonal propagation method. Such low levels of multiplication is not uncommon in seedling cultures were cotyledonary nodes are used as explants.

Mathai *et al.* (1997) also obtained somatic embryogenesis in cultures derived from seeds of *C. verum* but report that conversion to plantlets was possible in only 5 % of somatic embryos. Our studies also indicate that a wider spectrum of culture conditions including media combinations are required to be tested to obtain normal development of somatic embryos and regeneration of plantlets.

In *Gluta travancorica,* the inhibition of growth response in nodal and shoot tip explants appears to be partly due to the effect of phenolic compounds. Plants of the family Anarcardiaceae to which several economically important tree species like mango and cashew belong, have been recalcitrant to micropropagation. In spite of attempts by several workers, establishment in culture of mature tree explants have been difficult (Husain Ara *et al.*, 1998; Litz *et al.*, 1984b). The presence of high levels of phenolic compounds in the tissues particularly in the mature trees is one of the major hurdles and the presence of endophytic fungi in the tissues also results in a loss of cultures in the initial stage. Even in explants that escape contamination, shoot growth is rarely observed beyond the initial expansion and sprouting of the buds as seen in the present study. Activate charcoal is added to tissue culture media to remove compounds inhibitory to morphogenesis through adsorption (Fridborg *et al.*, 1978). Gupta *et al.(*1980) have used polyvinylpyrollidone (PVP) to remove the inhibitory effects of phenolic compounds in cultures initiated from mature teak buds.

Better results can be expected through induction of fresh sprouts by repeated pruning of branches together with spraying with systemic antibiotics before collection of explants. For practical reasons the use of seedling or young saplings maintained in pots under controlled conditions, as source of explants appears to be more reasonable. The approach used in mango and many other tropical tree species (Litz 1984a, b), viz. the use of nucellar explants for induction of somatic embryogenesis also has to attempted in *Gluta.* This would however require an understanding of the phenology of the species and the selection of the immature stages of zygotic embryo, which is known to be appropriate for induction of somatic embryogenesis.

As seen in the present study with mature shoot explants of *S. travancoricum*, very high rates of contamination (95%) was seen in cultures of clove (*S. aromaticum*) by Rao *et al*. (1997) . They report that even cultures that escape contamination showed poor response with regard to multiple shoot formation.

Observation in the field indicates that trees of *S. travancoricum* show seasonal flushes of shoot growth. Perhaps better response to culture can be obtained by testing the seasonal behaviour of nodal and shoot tip explants. As in the other species the success depends greatly on obtaining a reasonable percentage of sterile cultures. Since establishment of potted plants through layering is possible the chances of getting sterile cultures through pretreatment with systemic antibiotics is high. The maintenance of a large stock of such potted plants will however be required so that sufficient explants can be taken regularly. This was clearly a limitation in the present studies.

Very high rates of multiplication were obtained in shoot cultures derived from seedling nodes in *S. travancoricum* in media containing cytokinins. Shoots were thin and undeveloped on BAP containing medium and required a transfer to Kin. containing medium for expansion of leaves and elongation. Yadav *et al.* (1990) had reported similar results in the micropropagation of *S. cuminii* using shoot tips and nodal explants taken form 10-15 day old seedlings. Highest multiple shoot formation was obtained on media containing BAP either singly or in combination with IBA or NAA. Rooting was induced in the species on a medium containing NAA or IBA followed by transfer to a hormone free medium.

A highly embryogenic callus was obtained from leaf explants taken from plants maintained in pots (mature tree layers). The pink colour was characteristic of the somatic embryos. The formation of anthocyanin pigmentation has been reported in embryogenic cultures of *Eucalytpus citriodora* (Muralidharan and Mascarenhas, 1989). Normal morphology of somatic embryos and synchronization in their development was lacking in the cultures as is commonly seen in many tree species where somatic embryo induction has been achieved (Mathai *et al.,* 1997:). Further experimentation is required to modulate the maturation of somatic embryos and obtain high rates of conversion to plantlets. This would involve modifying the media on which early embryo development is carried out and inducing synchronous development .

Shoot cultures of *S. travancoricum* maintained in multiplication medium showed no visible sign of deterioration even when transfer to fresh media was delayed for 20 weeks. The points to the possibility of long-term storage of cultures through maintenance under minimal growth conditions. Maintenance of cultures at lower incubation temperatures was tested up to 22°C and no deleterious effects were observed for upto 28 weeks. By testing a range of lower incubation temperatures and also testing other minimal growth conditions such as providing a high osmoticum in the medium or deletion of a component of the medium, it should be possible to devise a medium- term conservation method for this species.

Dipterocarps are among the most important of commercially important timber species, but tissue culture has been relatively unsuccessful in this family. In the early work by Smits and Struycken (1983) most of the explants of *Dipterocarpus grandiflorus* were contaminated. Scott *et al.* (1995) obtained development of axillary shoots on cotyledonary nodes and stem nodes of embryos of *Hopea odorata* germinated on MS+ BAP (8.9 μM). Excised axillary shoot s formed few buds on BAP containing medium. Linington (1991) has reported *in vitro* propagation of *Dipterocarpus alatus* and *D. intricatus* where aqseptically grown seedlings were used. On WPM supplemented with the cytokinins BAP or 2-isopentyl adenine axillary

shoots developed from excised cotyledonary nodes. Rapid multiplication was obtained in *D. intricatus* and rooting obtained on an IBA containing medium. Both these dipterocarps produce orthodox seeds unlike most species of this family where seeds are recalcitrant and hence pose problems for long-term survival. In *Shorea contorta*, callus formation was the only response in explants taken from mature dipterocarp trees although upto 2-3 shoots were obtained from axillary buds (Pollisco, 1996). In *Anisoptera thurifera*, dome like structures were produced in callus cultures and single shoots from axillary buds (Pollisco, 1994).

Further experiments in the *in vitro* culture of this species is necessary since the frequent visits to the forest was not feasible during the period of the present study. Explants can be taken from fresh shoots induced by pruning of branches. This will be a way of overcoming any seasonal or dormancy affect that the trees might have. Through proper monitoring of the flowering season and collection of developing young fruits, establishment of cultures of early stages of zygotic embryos can be done which offers better prospects of inducing somatic embryogenesis and multiple shoot formation.

Conclusions

Establishment of sterile cultures in the four selected species was difficult when explants were taken from mature field growing trees due to the rarity of the species, remoteness of the habitat, lack of knowledge of the phenology and the presence of endophytes. Maintenance of branch cuttings, layers or seedlings in nursery or garden should be attempted to solve some of these problems so that large scale experiments can be undertaken to standardize culture procedures and media. The rejuvenation of mature tree tissues can be achieved to some extent by repeated pruning and induction of fresh sprouts on a continuing basis in the field or in plants grown in pots.

Since standard surface sterilization methods have failed to produce an acceptable level of sterile cultures a judicious use of systemic antimicrobials for pretreatment of mother plants or explants as well as addition to the culture media appears to be an option that should be pursued.

The application of micropropagation for mass clonal multiplication of *Syzygium travancoricum* was found to be feasible. The survival of plantlets that were planted out was however influenced by the disturbances in the habitat. The conservation of the species under *in vitro* conditions is feasible through normal subcultures of about 3-5 months but maintaining under minimal growth conditions like low incubation temperatures can prolong the interval to more than seven months. Plantlet regeneration from somatic embryos in the species would require further experimentation with physical and chemical parameters, particularly those known to improve somatic embryo development and conversion to plantlets. This could also form an excellent means of long-term storage and offer better scope for conservation through cryopreservation.

Somatic embryogenesis was also induced in *Cinnamomum verum* cultures but synchronization of embryo development and conversion to plantlets require further experimentation as in the case of *S. travancoricum*. If plants could be maintained under more controlled conditions the feasibility of micropropagation through multiple shoot formation could be improved by control of endophytes and harvest of buds from flushes of shoots induced by pruning.

A critical perusal of *in vitro* research in species belonging to the families of Anarcardiaceae and Dipterocarpaceae will be of help in designing a strategy for standardization of culture conditions for *Gluta travancorica* and *Vateria macrocarpa.*

If concerted efforts are undertaken to locate plants of *Diallium travancoricum* and *Diospyros assimilis* and the phenology studied, further studies on micropropagation could be undertaken. The application of micropropagation for the conservation of these two rare species assumes much importance.

Recommendations

The micropropagation techniques developed through this study can be applied to devise a strategy for *ex situ* conservation of *Syzygium travancoricum* as well as to aid regeneration in the natural habitats. Planting of micropropagated plants should be taken up at different localities after providing for adequate protection. Storage of shoot cultures at low temperatures can be undertaken at a suitable *in vitro* germplasm collection. Since the species is found in several localities in Kerala, accessions from different populations might be more appropriate to conserve a wider spectrum of the genetic diversity.

Further research is warranted in developing suitable micropropagation techniques for all the other species especially *Gluta travancoricum, Vateria macrocarpa, Diallium travancoricum and Diospyros assimilis*. Studies need to be taken up to assess the status of these species in the field so that a strategy can be evolved to facilitate research including standardizing tissue culture methods. Conservation of the germplasm of *Cinnamomum verum* is particularly important since it has commercially importance.

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Table 1: Composition of Basal Media used in the study

Compound	Concentration in mg/l	
Mineral salts	**MS**	**WPM**
KNO_3	1900	-
NH_4NO_3	1650	400
$Ca(NO_3)_2 4H_2O$	-	556
$CaCl_2. 2H_2O$	440	96
$MgSO_4. 7H_2O$	370	370
KH_2PO_4	170	170
K_2SO_4	-	990
$MnSO_4. 4 H_2O$	22.3	22.3
$ZnSO_4. 7 H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0. 83	0.83
$CuSO_4. 5H_2O$	0.025	0.25
$NaMoO_4. 2H_2O$	0.25	0.25
$CoCl_2. 6 H_2O$	0.025	-
$FeSO_4.7H_2O$	27.8	27.8
$Na_2FeEDTA. 2H_2O$	37.3	37.3
Organic Supplements		
Thiamine-HCL	0.1	1.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Inositol	100.00	100.00
	2.0	-

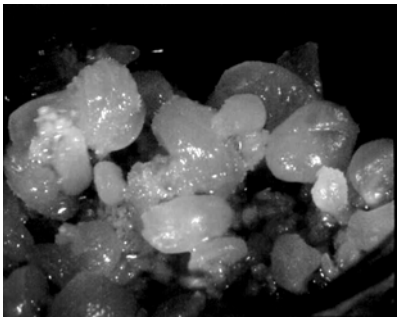

Fig. 1 Embryogenic callus of *C. verum*

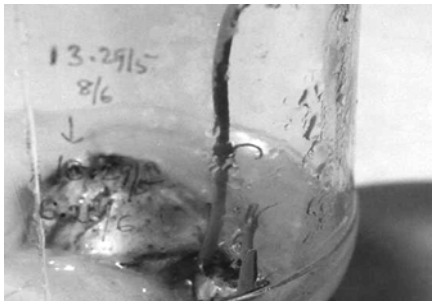

Fig. 2. Two shoots developing from embryo explants of *G. travancorica*

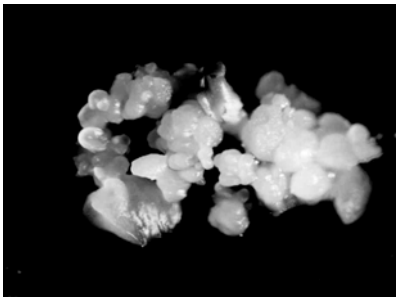

Fig. 3. Embryogenic callus of *S. travancoricum*

Fig. 4. Callus induced on leaf explants of *V. macrocarpa.*

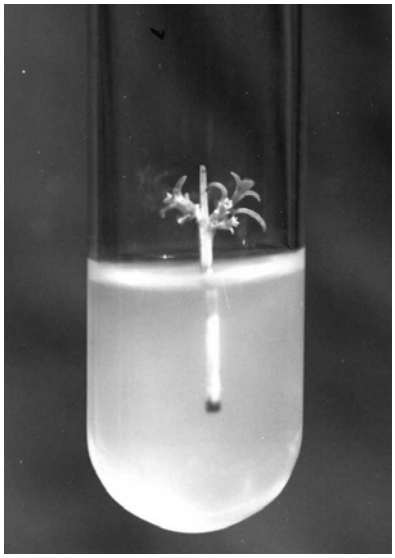

Fig. 5. Sprouting of axillary buds in nodal explants taken from S. travancoricum

Fig. 6. Multiple shoot formation in cultures derived from seedling nodes of S. travancoricum

Fig. 7. Elongated shoots of S. travancoricum

Fig. 8. 3 month old hardened plantlets of *S. travancoricum* growing in soil-vermiculite mixture.