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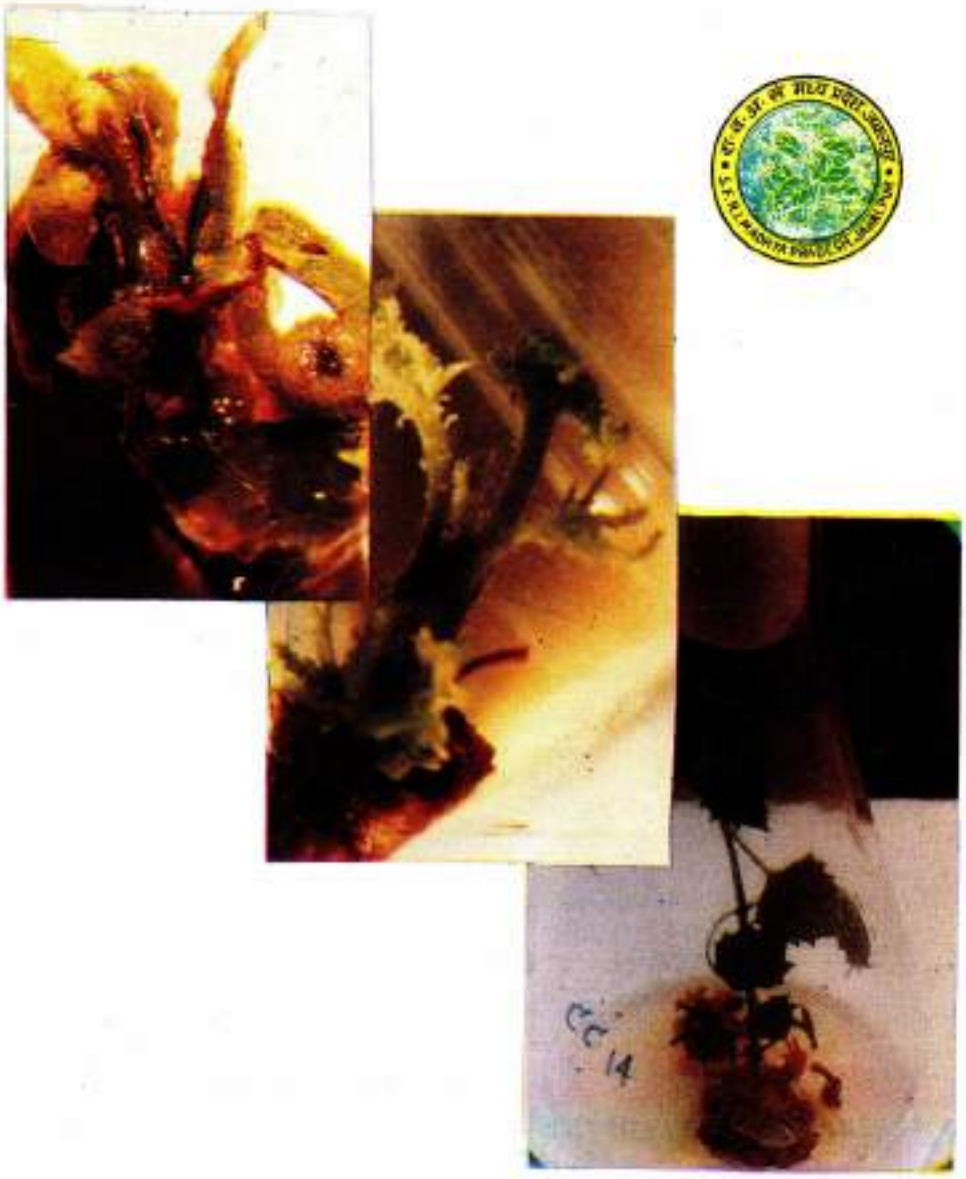
TISSUE CULTURE PROTOCOLS FOR TEAK (*TECTONA GRANDIS*), NEEM (*AZADIRACHTA INDICA*) AND KHAMER (*GMELINA ARBOREA*)



STATE FOREST RESEARCH INSTITUTE

Polipather, Jabalpur (M.P.) - 8

TISSUE CULTURE PROTOCOLS FOR TEAK *(TECTONA GRANDIS)*, NEEM *(AZADIRACHTA INDICA)* AND KHAMER *(GMELINA ARBOREA)*

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FOREWORD

Under Tree Improvement Programme (Govt. of Madhya Pradesh) it was proposed to develop a technology for mass multiplication of phenotypically superior planting stock of Teak. Accordingly, the State Forest Research Institute is engaged in tissue culture research since 1987. About 340 Plus Trees of Teak are available in M.P. The Plant Tissue Culture laboratory of this Institute has developed a protocol of its own and multiplied this species in mass, and for transfer of technology to the field.

Recently Govt. of Madhya Pradesh has declared this State as a "Neem State". The plant Tissue Culture laboratory of State Forest Research Institute, Jabalpur (M.P.) has taken up an inhouse project for development of suitable techniques for mass multiplication of Neem. The laboratory has developed a protocol of its own and has multiplied this species in mass, for large scale plantation in the field. Information regarding Neem Plus Trees are not available in Madhya Pradesh as well as other parts of the country. Only the preliminary work has been conducted for variability and Plus Trees selection for traits like general growth, medicinal, insecticidal and yield of fruits etc. Research in this direction is needed for further improvement of this species. The viability of Neem seed is very low. Several methods have been applied for viability elongation, but most of them are not successful on large scale.

Under the tree improvement programme modern technologies are being applied for production of genetically improved planting material for large scale plantation programme. *Gmelina arborea* was successfully multiplied through tissue culture technique at State Forest Research Institute Jabalpur (M.P). There are very few reports for mass propagation of this species through tissue culture technique in India. This species is very important from timber point of view. Plant tissue culture laboratory of this Institute has developed protocol of its own type and multiplied this species in mass, for transfer of technology to the field.

This bulletin deals with the technology evolved by the Scientists of SFRI and the information will be useful for further multiplication of these species for raising of Provenance Trials, Clonal Seed Orchards/Multiplication garden etc.

Contents

TISSUE CULTURE PROTOCOL FOR TEAK *(TECTONA GRANDIS)*

Introduction

Teak *(Tectona grandis)* (Fam. Verbenaceae) is an important forest tree, with a rounded crown, very variable in size. It is indigenous to Burma, Central and South India and Thailand. Teak has a worldwide reputation as a quality timber on account of its remarkable physical and mechanical properties.

Tectona grandis is generally propagated by seeds. Various methods of vegetative propagation have been used. For large scale plantation, method of planting of stumps from 30-40 cm. long seedlings and about one year old is often used. However, due to high cross-pollination incidence, the genetic variation is more pronounced among this species and plantations become uneconomical, by using inferior quality planting stock.

Clonal Multiplication Technique

The recent trend in genetic improvement of forest trees emphasizes the need for identification and multiplication of superior genotypes. These trees have certain desirable traits and are called 'elite' or 'plus trees'. This description deals with the micropropagation of such trees through tissue culture techniques. Direct or indirect morphogenesis has been observed. Direct cloning or shoot induction through explants technique was considered suitable because of high genetic stability.

Explants

Young juvenile explants, nodal and apical were collected from Seoni clones in the month of Feb-March 1993. The seedlings (Plus tree seeds) were also taken as a source of explants for preliminary study. The explants were cut into 1 to 2 cm with axillary buds. The collected explants were washed thoroughly by running tap water for 1 hr. and then washed 4 to 5 times with double distilled water. These explants were surface sterilized by 0.1% solution of Mercuric Chloride for 3 to 5 min., and were finally washed 4 to 5 times by sterile double distilled water.

Culture Media

Especially developed Murashige and Skoog's, (1962, MS) medium was used. (Table 1) This medium was supplemented with various combinations and concentrations of Auxins and Cytokinins. This medium was supplemented with 3% Sucrose and 0.8% Agar. The cultures were maintained at 25±2°C with illumination of 3000 lux. For each treatment 10 sets of cultures (with one set to act as control) were raised. pH of the medium was adjusted at 5.8 ±1.

Morphogenesis

The explants were cultured on MS medium supplemented with various concentrations of BAP and Kn either singly or in combination to establish shoots. Initially the medium turned black due to high Phenolic compound during first culturing. This was overcome by fast subculturing. Slight to moderate shoot bud formation was observed when medium was supplemented with Kn (0.5 to 2.0 mg/1) (Fig. No.1). Profused shoot induction was observed when medium was supplemented with Kn (4.0 to 5.0 mg/1). With this medium on an average 10 to 15 shoots were observed per explant. (Fig. No. 2). At higher level of Kn very poor performance in terms of shoot induction was observed.

Similarly BAP at (2.0 to 4.0 mg/1) in medium showed good morphogenetic response. On an average 20 to 25 shoot buds were observed per explant. IAA (2.0 mg/1) with combination of either BAP (4.0 mg/1) or Kn (3.0 mg/1) showed elongation of these shoot buds upto 2 to 4 cms. (Fig. No. 1A, 3 and 7). Higher level of IAA showed yellowing of *in vitro* shoots.

Profused callusing was observed when medium was supplemented with very low concentration of 2,4-D (0.2 to 0.5 mg/l). Indirect morphogenetic response was also observed, where a number of shoot buds were developed through callus. These shoot buds were excised from callus and transferred on BAP contained medium, for further multiplication. (Fig. No. 4, 5, 6)

In-Vitro And *Ex-Situ* Rooting

After establishment of shoots in culture, these shoots were excised and inoculated for *in-vitro* rooting as well as for direct rooting in Mist Chamber. Recovery of *in-vitro* grown plants were found better when they were directly transferred to mist chamber during Nov to Feb. at 27 to 30°C temperature and at 70 to 80% relative humidity on 1:1 ratio of sand +Soil. After 25 to 30 days the survival ratio of tissue culture plants under glass house conditions was found to be 70 to 80% (Fig. No.8).. This technique is very useful for plant establishment rather than *in-vitro* rooting technique as this reduces the cost of plants.

Field Trials

An experiment was taken up for field trials of tissue culture grown plants of teak at Jabalpur and Regional Research Centre at Seoni. These plants were planted with seed origin plants at an espacement of 4m x 4m. After 42 months, data showed that tissue culture grown plants showed uniformity in terms of growth. (Fig. No. 8A and 9).

FIG NO. 1

FIG NO. 1A

FIG NO. 2

FIG NO. 3

FIG NO. 4

FIG No. 5

FIG NO. 1	:	SHOOTBUD INDUCTION FROM EXPLANT
FIG NO. 1A	:	PROFUSED SHOOT MULTIPLICATION
FIG NO. 2	:	INITIAL STAGE OF SHOOT MULTIPLICATION
FIG NO. 3 & 7	:	YOUNG GREEN PROFUSED SHOOT
FIG NO. 4	:	MASSIVE GREEN CALLUS.

FIG NO. 7

FIG NO. 8

FIG NO. 9

FIG NO. 8A

FIG NO. 5 and 6 : SHOOT BUD DIFFERENTATION FROM CALLUS
FIG NO. 8 : *IN VITRO* PLANTS UNDER HARDENING PHASE
FIG NO. 8A & 9 : FIELD TRIALS.

In-vitro techniques

Modern biotechnological approaches like Plant Tissue Culture are now-a-days applied for mass multiplication of forestry species. However, their recalcitrant behaviour as well as less totipotent nature of their tissues, their multiplication is still very difficult, especially when explants are collected from mature trees. This bulletin deals with *in-vitro* techniques for mass multiplication of "Neem". Both direct and indirect morphogenesis were observed.

Methodology

Juvenile nodal explants (with axillary buds) and apical meristem were collected from candidate plus trees near Rampur at MPEB Road, during Jan. to April. The explants were cut into 2 to 3 cm. long pieces and washed properly with running tap water for 2 hrs and then washed with double distilled water. These explants were treated with 0.1% solution of Mercuric Chloride solution for 2 to 4 min. for surface sterilization. Finally these explants were washed 4 to 5 times by sterile distilled water and were cut to 1 to 2 cm. long pieces.

Culture media

Especially developed Murashige and Skoog's (1962 MS) medium was used. (Table 1) This medium was supplemented with various combinations and concentrations of Auxins and Cytokinins. This medium was supplemented with 3% Sucrose and 0.8% Agar. The cultures were maintained at 25±2°C with illumination of 3000 lux. For each treatment, 10 sets of cultures (with one set to act as control) were raised.

In-vitro morphogenesis

The explants were aseptically inoculated on sterilized medium. This medium was supplemented with BAP singly or in combination with IAA. Profused shoot induction was observed when medium was supplemented with (3.0 to 5.0 mg/1) of BAP. (Fig. No.2 and 3). On an average 10 to 15 shoots were induced from each explants. Other group of Cytokinins (Kn) showed no morphogenetic response from lower to higher range. Shoot elongation was observed with Auxins and Cytokinins combinations. When medium was added with BAP (3.0 mg/l) and IAA (2.0 mg/l). About 4 to 5 cm shoot length was observed (Fig No. 1)

Indirect morphogenesis was also observed during study. Massive callus induction was observed when medium was supplemented with very low concentration of 2,4-D (0.5 mg/1) with IAA (1.0 mg/1) (Fig. No.4 and 6). Sometimes BAP at low level (1.0 to 1.5 mg/1) showed callus response. When this callus was subcultured on BAP (3.0 to 4.0 mg/1) containing medium, shoot differentiation was observed from callus. (Fig. No. 5).

The following protocol has been standardized for mass multiplication of *Tectona grandis*.

(i) MS + Kn (4.0 to 5.0 mg/1) better morphogenetic response.

(ii) MS + BAP (2.0 to 4.0 mg/1) Extensive shoot induction.

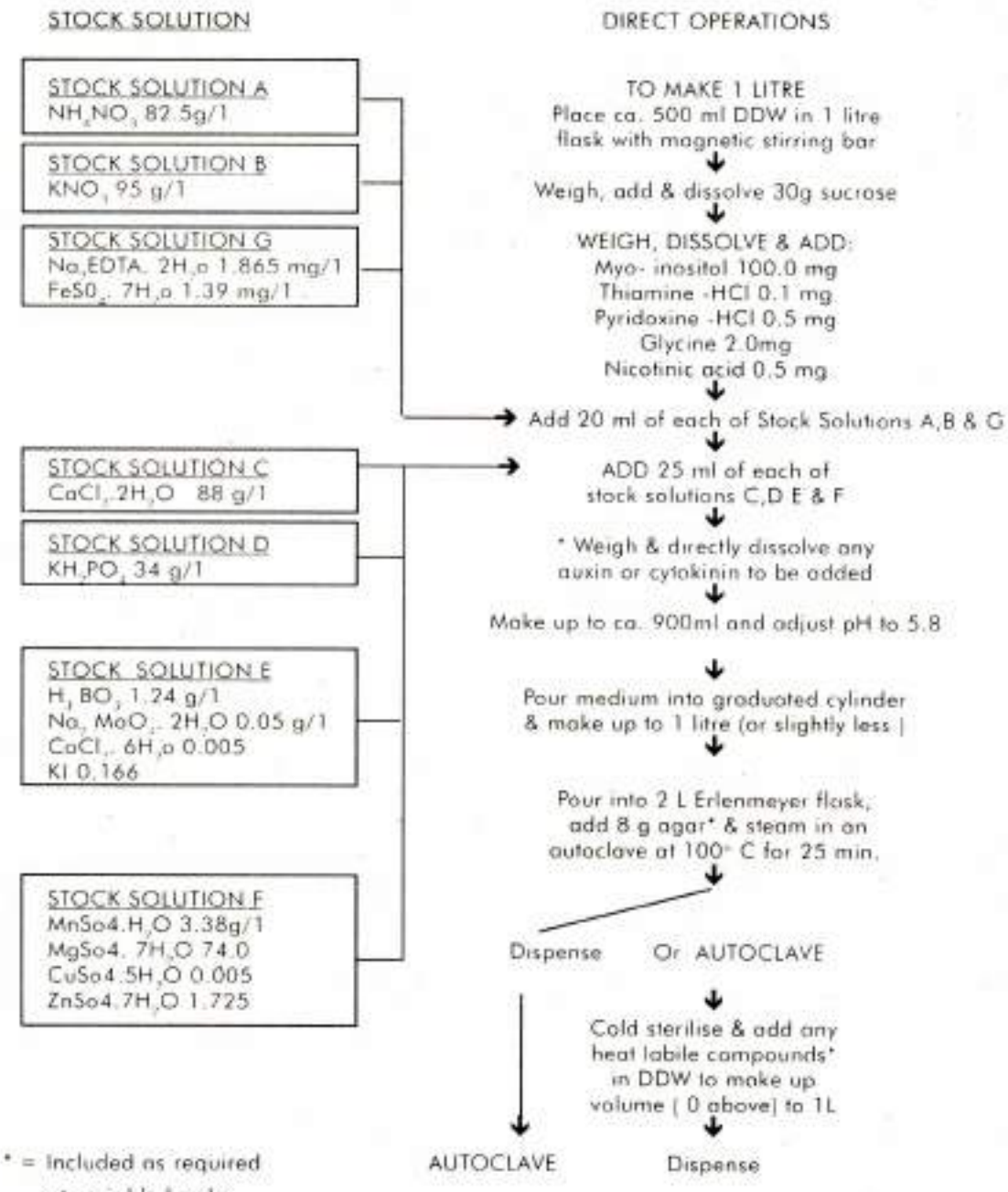
(iii(MS + BAP or Kn + IAA (3.0 mg/1) Elongation of shoots.

(iv) MS + 2,4-D (0.5 mg/1) profused callusing.

(v) MS + BAP (3.0 mg/1) shoot differentiation from Callus.

(vi) MS + IBA (2.0 mg/1) *in-vitro* rooting.

TABLE I : ALTERNATIVE METHODS OF PREPARING MS MEDIUM: ACCORDING TO HELGESON (1979)

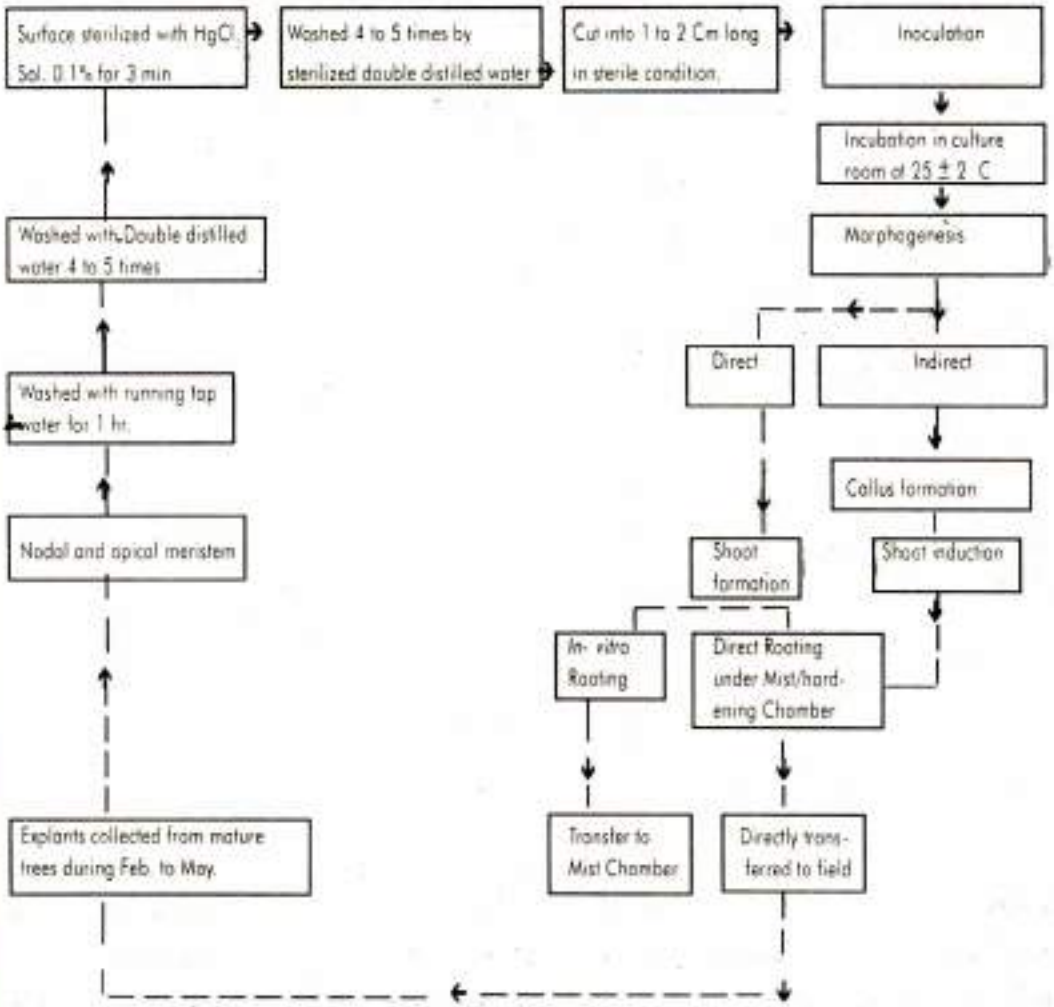


* = Included as required at variable levels.

DDW= Double distilled water.

Source: Gorge & Sherrington (1984)

IN-VITRO TECHNIQUES USED DURING STUDY



IN-VITRO PROTOCOL FOR NEEM *(AZADIRACHTA INDICA)*

Introduction

Neem is a large evergreen tree belonging to family *Meliaceae*. This tree has a straight stout trunk and branches which can grow without irrigation in arid and semi-arid regions with annual rainfall of 500mm or less. Normally, it attains a height of 12-15 m and a girth of 1.8 to 2.5 m. Neem is a good coppicer. It flowers during March to May. Commonly a mature tree produces 30 to 35 Kg of fruit per year. Neem seeds are dispersed by birds under natural conditions, but the seed viability is very poor, about 1 to 2 weeks.

Anciently, Neem is well known in India for its medicinal and insecticidal properties and has been considered as one of the most valuable tree species. Neem tree is important culturally as it is grown on holy places and nearer to the habitation for purifying the environment. Medicinally it is amoebicidial, antiallergic, antidermatic, anti-inflammatory, anti-peridontitic, anti-pyerrhoeic, anti-scabic, piscidal and its derivatives have other biological properties.

Neem can be artificially reproduced by either direct sowing of seeds and transplanting or by stump planting. Due to its low seed viability other biotechnological tool, such as tissue culture and vegetative propagation techniques have a potential for its mass multiplication.

FIG No.1

FIG NO. 2

FIG NO. 3

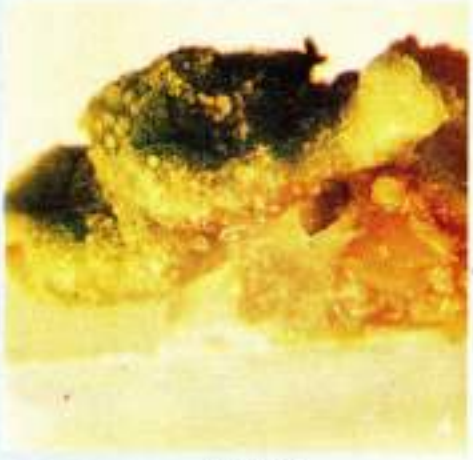


FIG NO. 4

FIG No. 1 : SHOOT ELONGATION

FIG NO. 2 & 3 : PROFUSED SHOOT INDUCTION AND PROFUSED SHOOT MULTIPLICATION,

FIG NO. 4 : GREEN MASSIVE CALLUS STAGE FOR SHOOT DIFFERENTATION.

FIG No. 5

FIG NO. 6

FIG NO. 7

FIG NO. 9

FIG NO. 8

FIG No. 5 : YOUNG SHOOT DIFFERENTATION FROM MASSIVE CALLUS
FIG NO. 6 : 3 MONTHS OLD CALLUS
FIG NO. 7 : *IN VITRO* PLANT ESTABLISHMENT IN HARDENING CHAMBER
FIG NO. 8 & 9 : FIELD TRIALS OF *IN VITRO* GROWN PLANTS

Plant establishment

.The *in-vitro* regenerated shoos nbout 4 to 5 cm long were excised from bunch and directly transfered to mist chamber during Nov. to Feb. at 27 to 30 ± 2 C temp. These shoots were transfered on soil and sand with 1:1 ratio. Within 20 to 25 days profused rooting was observed, and these shoots were treated as miniature cuttings. Recovery of plantlets of Neem by this technique for plant-establishment has been found to be most suitable rather than *in-vitro* rooting, and then hardening. (Fig. No.7)

Field trials

An experiment was taken up for field trials of tissue culture grown plants of Neem at Jabalpur. These plants were planted with seed origin plants at an espacement of 4m x 4m. After 6 months data showed that tissue culture grown plants showed uniformity in terms of growth. (Fig. No.8 and 9).

The following protocol has been standardized for mass multiplication of Neem.

Standardized protocol

(i) MS + BAP (3.0 to 5.0 mg/1) Profused shooting. (Fig. 2 & 4).

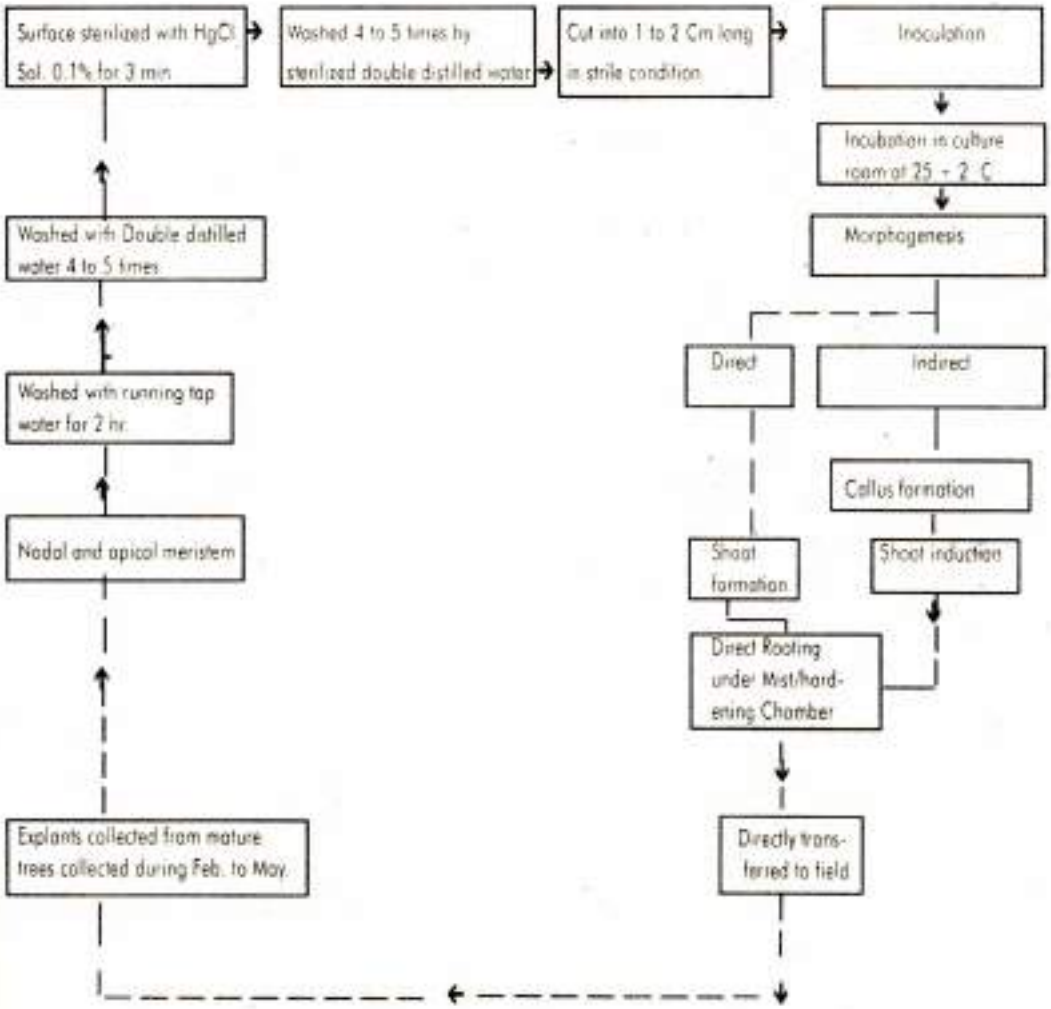
(ii) MS + BAP (3.0 mg/1) + IAA (2.0 mg/1) Shoot elongation plant establishment. (Fig. 3 & 5).

(iii) MS + 2,4-D (0.5 mg/1) +IAA (2.0 mg/1) Massive callus.

(iv) MS + Callus + BAP (3.0 mg/1) Shoot differentiation from callus (Fig. 6,7 and 8).

Sand + Soil (1:1) plant recovery 70 to 80%.

IN-VITRO TECHNIQUES USED DURING STUDY



IN-VITRO PROTOCOL FOR KHAMER *(GMELINA ARBOREA)*

Introduction

In recent years plant tissue and organ culture techniques have developed into multiplication of many important tree species. The advantage of adopting plant tissue culture for *ex-situ* conservation include the maintenance of genetic stability free from natural hazards feasibility for storage and quick recovery of large scale population in lesser space and time and in case of international exchange of germplasm avoiding quarantine controls.

Gmelina arborea Roxb. (Verbenaceae) is a tall popular forest tree. Its timber is excellent, durable and highly valuable economically. The species is next to Teak. The plant is found to grow in India, Ceylon, Malaya and Phillipines.

The traditional practice for raising the planting stock of this species is from seed, but poor seed germination and viability and paucity of good quality seed in adequate quantity pose difficulties for raising large scale planting stock of this species. In order to overcome these problems micropropagation using tissue culture techniques is very useful for raising genetically superior planting stock.

In-Vitro Techniques

Micropropagation using tissue culture techniques is still very difficult for forest trees when, they are propagated from mature or superior phenotype. In recent years it has become possible to develop biological processes which manipulate living organisms. These processes may be entirely controlled by man. The most notable example of such process occurs in the artificial modification as a biotechnological tool such as tissue culture.

Media Used

Especially developed Murashige and Skoog's (1962 MS) medium was used (Table I). The medium was supplemented with 3.0% sucrose as a carbon source and 0.8% agar. Various combinations and concentrations of auxins and cytokinins were also added to the medium. The culture were maintained at 25 ± 2 C with illumination of 3000 lux. pH of the medium was adjusted at 5.8 ± 0.1.

Methodology

Juvenile shoots and nodal explants were collected from five year old plants at SFRI nursery during Feb. to May 1992. Explants were thoroughly washed with running tap water and surface sterilized with 0.5% aqueous Mercuric Chloride ($HgCl_2$) solution for 3 to 5 minutes and finally washed with sterilized distilled water for 3 to 4 times till the sterillent was completely removed. These explants were aseptically inoculated on especially developed culture medium. For each treatment 10 sets of cultures (with one set to act as control) were raised.

Morphogenesis

Initially, the medium turned black due to high Phenolic compound in explants. This was overcome by adding Polyvinly Pyrolidone (PVP), when supplemented in medium by 0.5%. Slight to moderate morphogenetic response (Shoot bud induction) was observed when medium was supplemented with Kn (2.0 to 5.0 mg/1) within 15 days. Extensive shoot bud induction was observed when medium was either supplemented with Kn (5.0 mg/1) or BAP (5.0 mg/1). Cytokinin and Auxin in combination showed very good morphogenetic response. The combination was BAP and IAA (5.0 mg/1+ 3.0 mg/1) (Fig No 1, 2, 3 & 4). Indirect morphogenesis was also observed through callusing. Shoot differentiation was observed from callus when medium was supplemented with 2,4-D (0.5 mg/1) and BAP (3.0 mg/1) (Fig. No.5 and 6.)

For root induction *in-vitro* regenerated shoots were excised and inoculated on rooting medium, extensive root induction was observed when medium was supplemented with either IBA or NAA (2.0 mg/1) (Figure No. 5)

Plant Establishment

In-Vitro grown plants were transferred to Mist Chamber during Nov. to Feb. on soil and sand at 1:1 ratio. Recovery of plantlets under this condition was found 60 to 70%.

Field Trials

An experiment was taken up for field trials of tissue culture grown plants at Regional Research Centre Seoni in July 1996. The survival percent of tissue culture grown plants was found 80 to 90%. (Fig. No. 6,7.)

FIG No. 1

FIG NO. 2

FIG NO. 3

FIG NO. 4

FIG No.1 : SHOOT INDUCTION FROM EXPLANTS
FIG NO. 2 & 3 : SHOOT MULTIPLICATION
FIG NO. 4 : SHOOT DIFFERENTATION

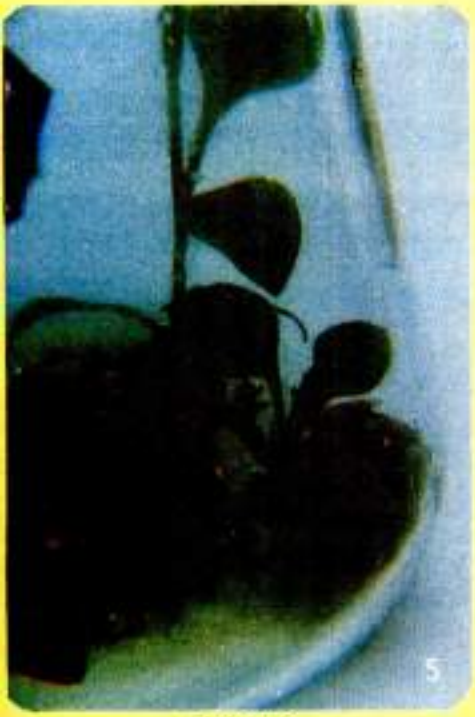


FIG NO. 5

FIG NO. 6

FIG NO. 7

FIG No. 5 SHOOT DIFFERENTATION FROM MASSIVE CALLUS
FIG NO 6 MASSIVE ROOT INDUCTION FROM *IN VITRO* REGENERATED SHOOTS
FIG NO 7 PLANTS UNDER HARDENING CONDITIONS

Standardized Protocol

Kn (5.0 mg/1) for Shoot bud induction.

BAP (5.0 mg/1) for Shoot bud induction.

IAA (1.0 to 5.0 mg/1) for Poor Shoot induction.

BAP + IAA (5.0 mg/1 + 3.0 mg/1) Shoot proliferation and Shoot elongation.

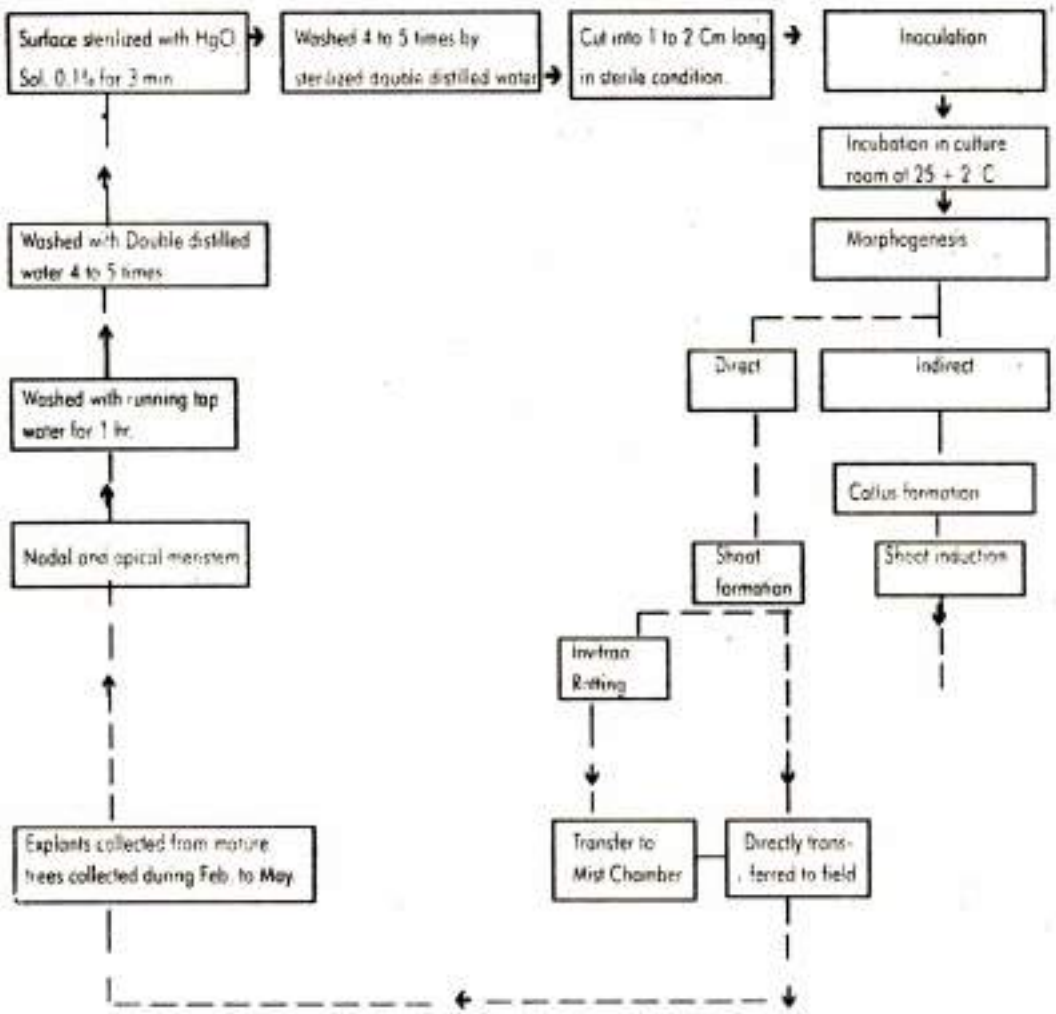
2,4-D (0.5 mg/1) Callus induction.

BAP + IAA (3.0 mg/1 + 2.0 mg/1) + Callus - Shoot differentiation from Callus.

Regenerated shoots + IBA or NAA (2.0 mg/1) Root induction.

Soil + Sand (1:1) Plant establishment during Nov. to Feb.

IN-VITRO TECHNIQUES USED DURING STUDY



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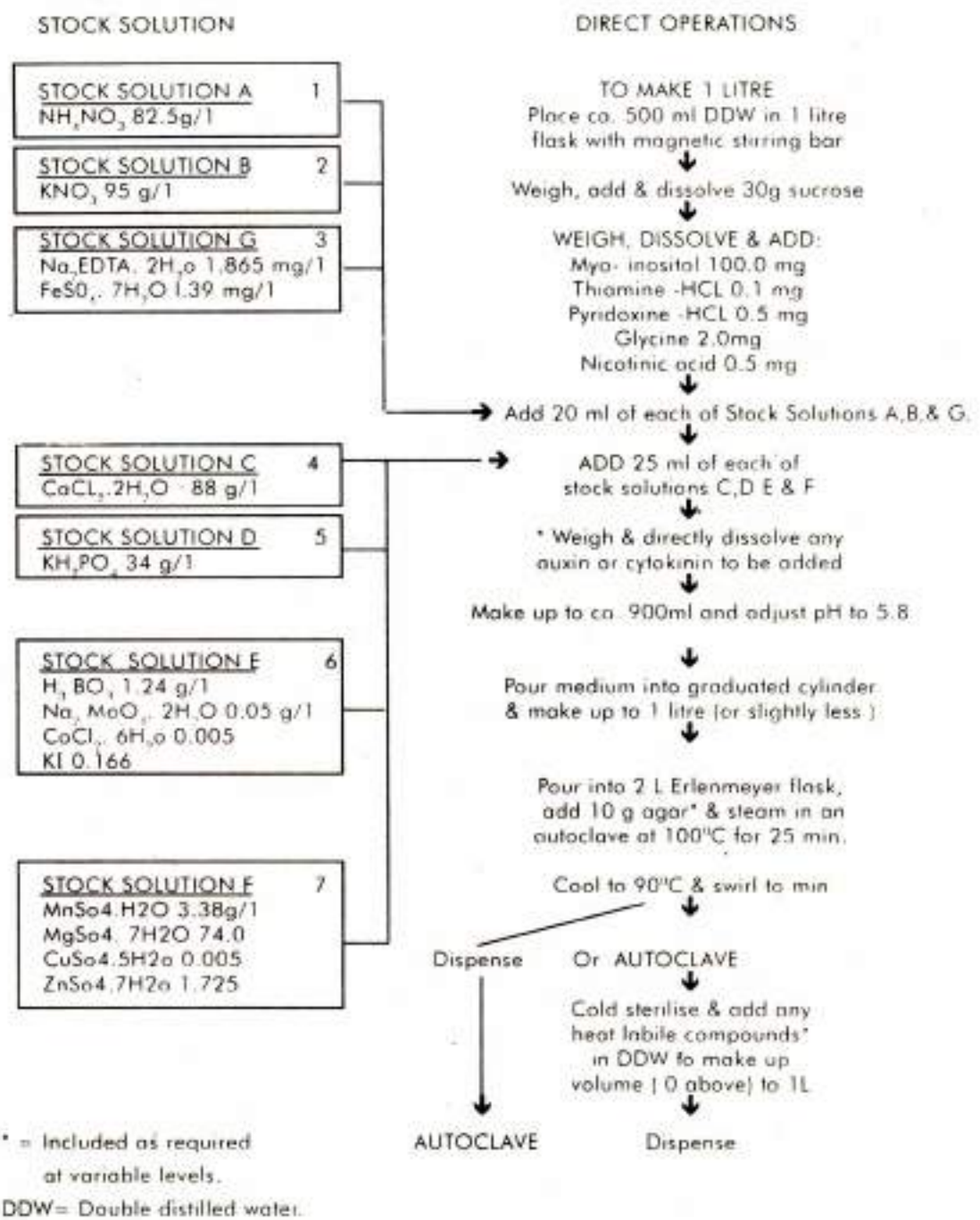

* = Included as required at variable levels.

DDW= Double distilled water.

Source: Gorge & Sherrington (1984)

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