S.F.R. I. Technical Bulletin No. 37

TISSUE CULTURE PROTOCOLS FOR IMPORTANT MEDICINAL PLANTS OF MADHYA PRADESH

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PREFACE

A large number of medicinal and aromatic plants provide raw material for the pharmaceutical, perfumery, flavour and cosmetic industries and thus, help in earning foreign exchange. Natural forests, store house of the medicinal plants, are worsely disturbed due to over exploitation and biotic pressures. Deliberate attempts by government and non-governmental organizations aimed to conserve/ cultivate and multiply medicinal plants did not yield desirable success due to lack of technological interventions, unavailability of superior planting stock, inadequately developed nursery techniquies and lack of links between researchers and real users. State Forest Reasearch Institute, Jabalpur (M.P.) has already developed propagation and cultivation packages for several medicinal plants and also successfully extended information to growers.

Present attempt by Plant Tissue Culture Laboratory of Forest Genetics and Tree Improvement Division of this Institute is aimed at how best biotechnogical tools can be utilized for the conservation/ management of natural resources. Which has developed *in vitro* mass propagation protocols for the five important medicinal plants of this region. We hope that it will be useful to researchers and commercial propagators.

Authors

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1. *Rauwolfia serpentina* Benth ex Kurg

1.1. Introduction : *R. serpentina* (sarpagnadha : Family Apocynaceae) is a highly valued medicinal herb occurring in tropics and subtropics. The species grows one and half metre in height with lanceolate leaves arranged in whorls of 3-5 at the nodes of short rounded stem. It flowers during May-Oct. Extracts from roots contains alkaloids like reserpine, serpentine, ajmaline etc. Which have found use in the formulations to cure nervous disorders (psychosis, schizophrenia, insanity, insomnia, epilepsy) and intestinal troubles.

1.2. *In vitro* methodology:

1.2.1. Explant : Fresh shoots collected from morphologically superior individuals were cut in to 3-4 cm pieces having a single node and subjected to running tap water treatment for an hour. They were further washed with 1% Teepol solution for 3-5 min. Which was followed by several rinses in distilled water.

Cultures were raised on specially developed Murashige and Skoog medium (1962). The medium was prepared with macro and micro nutrients and supplemented with various combinations and concentrations of auxins and cytokinins. The pH of the medium was adjusted to 5.8 $\pm$ 0.1 and 3% sucrose was added as carbon source. Surface sterilized explants were aseptically placed on the medium in a controlled environmental conditions. The cultures were raised in 100 or 250 m1 Erlenmeyer flask.

1.2.2. Plant regeneration and multiplication : Cultures were maintained in culture room at 25 $\pm$ 2°C under light intensity of 3000 lux with cool fluorescent light. The explants showed morphgenetic response from slight to moderate level. Shoot induction from nodal explants was observed within 20 to 25 days, when medium was supplemented with BAP (0.5 to 3.0 mg l^{-1}). While profuse shoot induction was observed when medium was supplemented with 4.0 mg l^{-1} BAP.

1.2.3. *In vitro* rooting and plant establishment : *In vitro* grown plants were excised and 4.0 to 6.0 cm long shoots were transferred on to half strength MS medium for *in vitro* rooting. IBA supplementation at 1.0 mg l^{-1} showed good rooting within 25 to 30 days. Other concentrations of IBA showed poor response.

In vitro raised plants, after 5 to 6 weeks were thoroughly washed in running tap water and were transferred to pots having soil + vermiculite + sand mixture (1:1:1). Potted plants were placed in glass house conditions during Nov. to Feb. Initially plants were covered with polyethylene bags in order to avoid excessive transpiration. After three months of potting, plants were transferred to field.

1.3. Protocol summary (Re-cap)

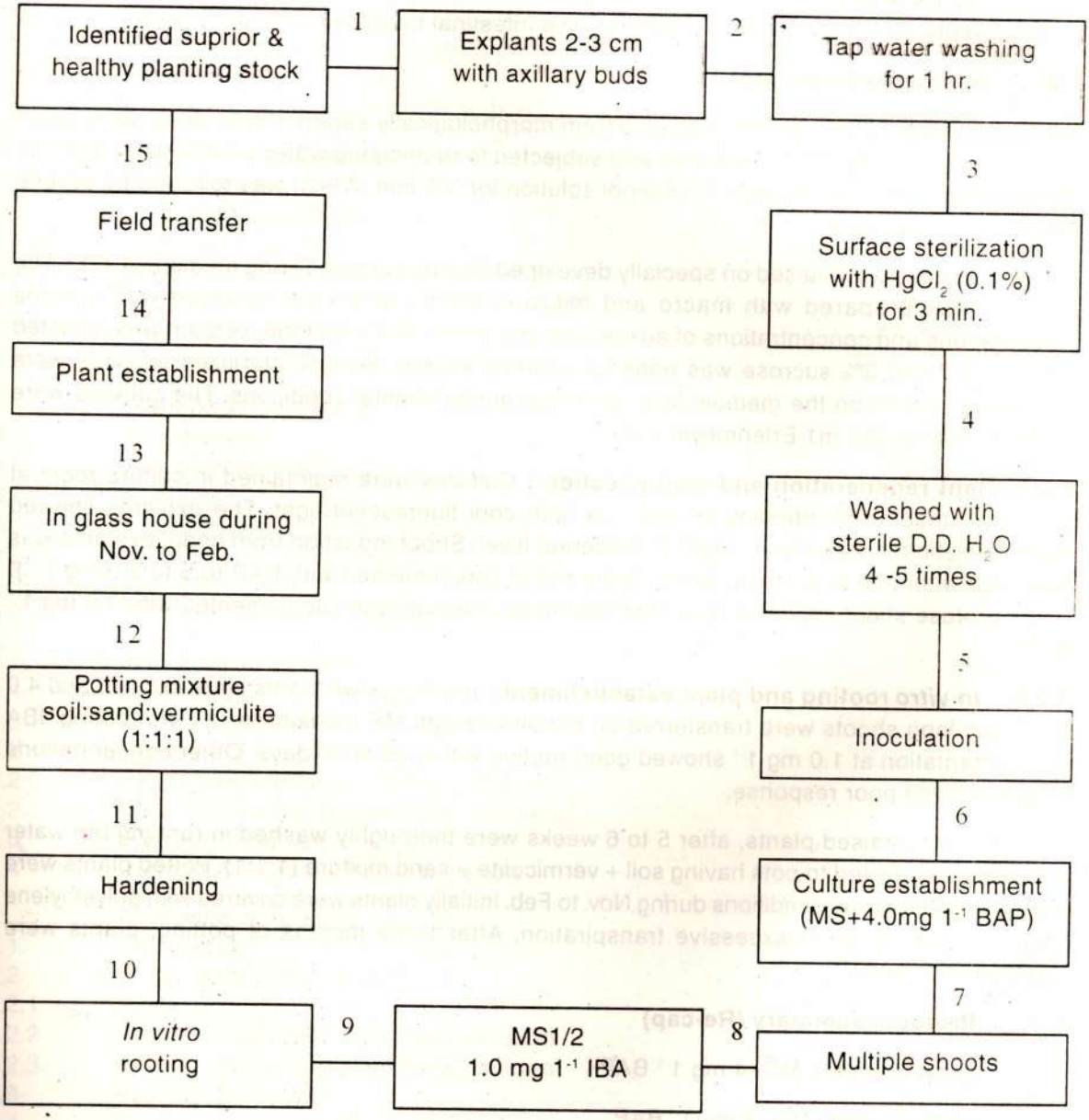
Establishment: MS+4 mg l^{-1} BAP

Multiplication: MS+4 mg l^{-1} BAP

In vitro rooting: 1/2MS+ 1.0 mg l^{-1} IBA

Potting mixture: soil: vermiculite: sand (1:1:1)

1.4. Protocol Schematic Representation :



2. *Curcuma angustifolia* Dalz.

2.1. Introduction

C. angustifolia (Tikhur ; Family : Zingeberaceae) is a rare herb distributed in tropical deciduous forests. The rhizome is sweetish and fragrant. The rhizomatous extract is found effective to cure leprosy, asthma, jaundice, leucoderma, stones in urinary bladder, wining discharges, peptic ulcer etc. The use of this herb in several aurvedic medicines underlines need for raising commercial plantation. The plant is conventionally propagated through rhizomes. Which is slow method and incapable of producing large number of plants. Seedsetting rarely occurs and mostly bears non-viable seeds. Biotechnological methods can effectively be utilized to obtain large number of plants in short time span and can be propagated throughout the year.

2.2. *In vitro* methodology

2.2.1. Explant : Plant material for the *in vitro* propagation was collected from natural population in Amarkantak area during Sept- Oct. Rhizomes were planted in mist chamber. Upon the fresh growth of aerial stem, shoot explants were collected.

Rhizomatous buds as well as portion of rhizomes excised and subjected for running tap water (15 min) and 1% teepol solution for 5 min. Which was followed by distilled water rinse. Explants were then surface sterilized by using 5% sodium hypochlorite solution for 5 min followed by 4-5 rinses in sterile distilled water.

2.2.2. Plant regeneration & multiplication : Rhizomatous buds were aseptically excised and placed on establishment medium. MS medium supplemented with BAP was tested. Medium supplemented with 8.0 mg 1^{-1} BAP showed best explant response and produced fresh sprouts. New shoot buds were originated from the basal nodes of the explants after 20 days of culture. *In vitro* developed shoots elongated into 3-4 cm in length within 4 weeks. Explants placed on hormone free medium (control set) failed to produce new shoots.

After the harvest of new shoots original explant was subjected to recycling on MS medium containing 8.0 mg 1^{-1} BAP. The practice was continued over eight times thus efficiently produced large number of shoots for rooting.

2.2.3. *In vitro* rooting and plant establishment : To induce adventitious roots *in vitro* raised shoots were planted on 1/2 MS medium incorporated with various concentrations of auxins (IBA, IAA or NAA). Half- strength, agar-gelled MS medium supplemented with 0.5 mg 1^{-1} IBA found to be sufficient for rooting. Root formation occurred within 10-13 days after planting. IBA or NAA addition at 0.5 mg1^{-1} improved plant growth. Shoots planted on 0.5 mg 1^{-1} IBA contained medium produced on an average 5 roots/ shoot within 13 days. IAA contained medium and control shoots failed to produce roots. After 6 weeks, plants were washed thoroughly in running tap water and dipped in 0.1% Bavistin solution for 5 min. They were potted experimentally in various potting mixtures and incubated in mist chamber and glass house. Maximum survival was observed when plants potted in soil:sand: vermiculite (40:30:30). The entire process of plant hardening took 3 months. Thus, fully acclimatized plants obtained were transferred to experimental plots for growth performance/evaluation.

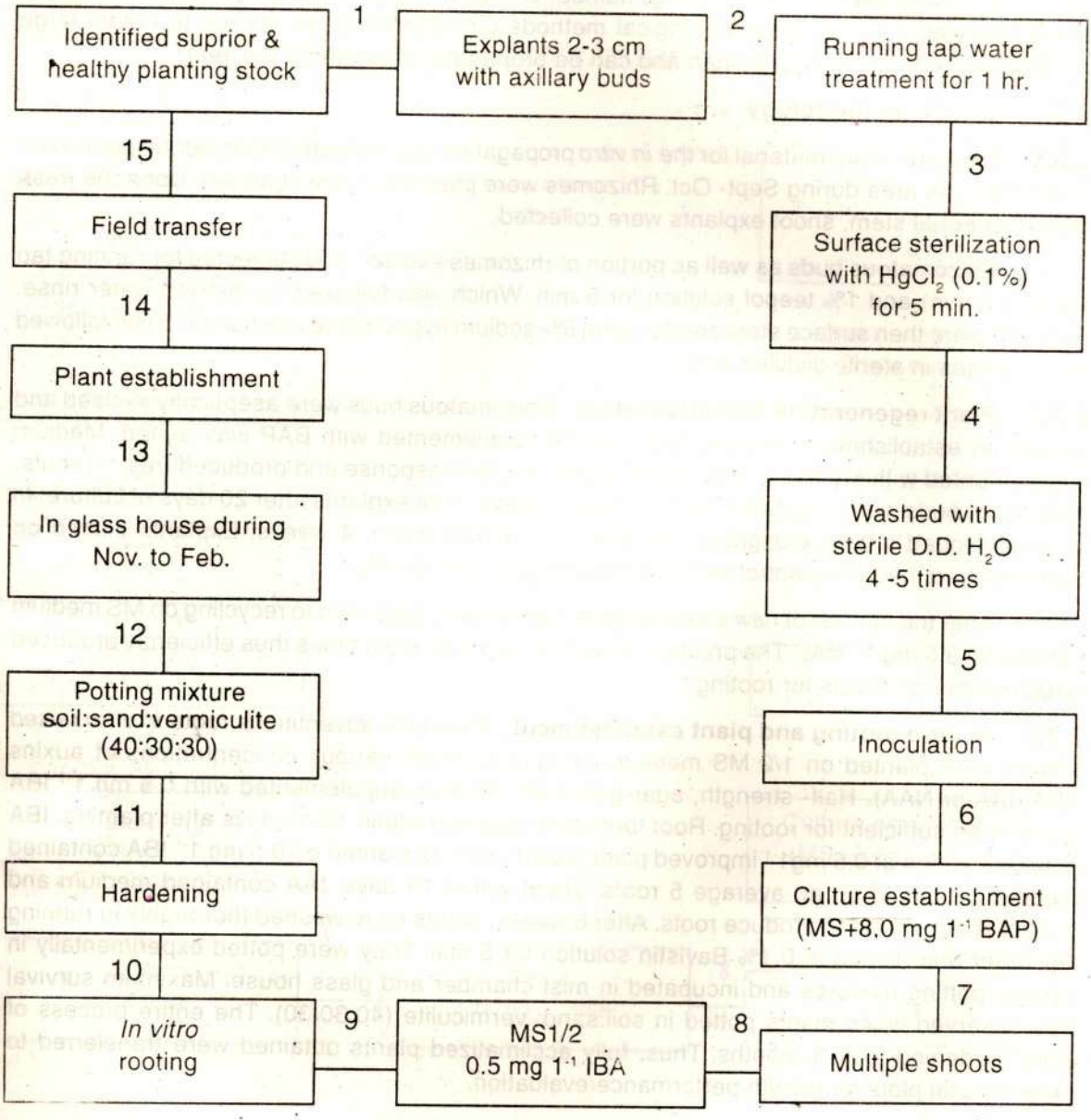
2.3. Protocol summary (Re-cap)

Establishment and multiplication: MS+8.0 mg 1^{-1} BAP

In vitro rooting: 1/2 MS + 0.5 mg 1^{-1} IBA

Potting mixture: soil:sand:vermiculite (40:30:30)

2.4. Protocol : Schematic representation



3. *Plumbago zeylanica* Linn.

3.1. Introduction : ***P. zeylanica*** (Chitrak Family : Plumbaginaceae) is a small perennial herb growing in Central India. The species is an important source an acrid crystalline alkaloid, plumbagin in the form of yellow needles. Plumbagin is widely used in medicines against irritation on skin. Root is highly poisonous and used for formulation of medicines given to pregnants. The paste of root is also applicable to leprosy and skin diseases. Poor regeneration in forests and low germination capacity of this species underlines need for artificial regeneration. Conventional vegetative propagation technique is less applicable due to limited success and season specific response. Expected outcome of *in vitro* experiments therefore, will be useful to over come these impediments.

3.2. *In vitro* methodology

3.2.1. Explant : Nodal explants (3-4 cm) with axillary buds were collected from, medicinal plant nursery of SFRI, Jabalpur. Collected explants were washed in running tap water and then in 1-2% Tween-20 solution for 15 min.

3.2.2. Plant Regeneration And Multiplication : Explants were surface sterilized in 0.5% $HgCl_2$ solution containing few drops of Tween-20 for 3min. After trimming both ends, explants were aseptically placed in culture medium. Effect of semi-solid MS medium (pH 5.8, sucrose 3%, agar 0.8%) supplemented with cytokinins (BAP and Kin) and auxins (IBA, IAA and NAA) combined or alone was tested at 0.5-5.0 mg l^{-1} concentration. Shoot Proliferation was occurred after 2 weeks of culture. Axillary buds starts to produce new shoots and elongated into 3-4 cm in length. Medium containing 5 mg l^{-1} BAP resulted in the production of extensive lateral shoots from the axis of primary shoot. These shoots start to produce tertiary buds also. On this medium on an average 20-25 shoots were produced perculture. Kinetin (5.0 mg l^{-1}) also gave comparable result. Addition of kinetin reduced formation of callus at the base. Combination of BAP or Kin. with auxin (IAA) gave slight to moderate shoot proliferation. IBA or NAA at 0.5 - 5.0 mg l^{-1} concentration alongwith cytokinins did not improve morphogenesis. The shoots obtained were subcultured on MS medium containing 5.0 mg l^{-1} BAP for mass production of shoots.

3.2.3. *In-Vitro* rooting and plant Establishment : *In vitro* raised shoots were induced rooting by culturing on 1/2 MS medium (pH 5.8, sucrose 3%, agar 0.7%) supplemented with or without auxin (0.0-5.0 mg l^{-1} NAA and IBA). Shoots planted on 1/2 MS medium supplemented with 1.0 mg l^{-1} NAA resulted maximum percentage rooting. Developed roots elongated further into 2-3 cm within 4 weeks. IBA incorporation in rooting medium caused extensive callusing. Likewise, auxins (IBA or NAA) at supra-optimal level also caused extensive callus formation.

Six weeks after rooting, plants were transferred to potting mixture. Plants transferred to soil:sand:vermiculite (1:1:1:) and incubated at mist chamber conditions resulted in optimum survival. Plants were initially watered with Hoglands solution in every alternate days. Fully acclimatized plants were later moved to nursery beds.

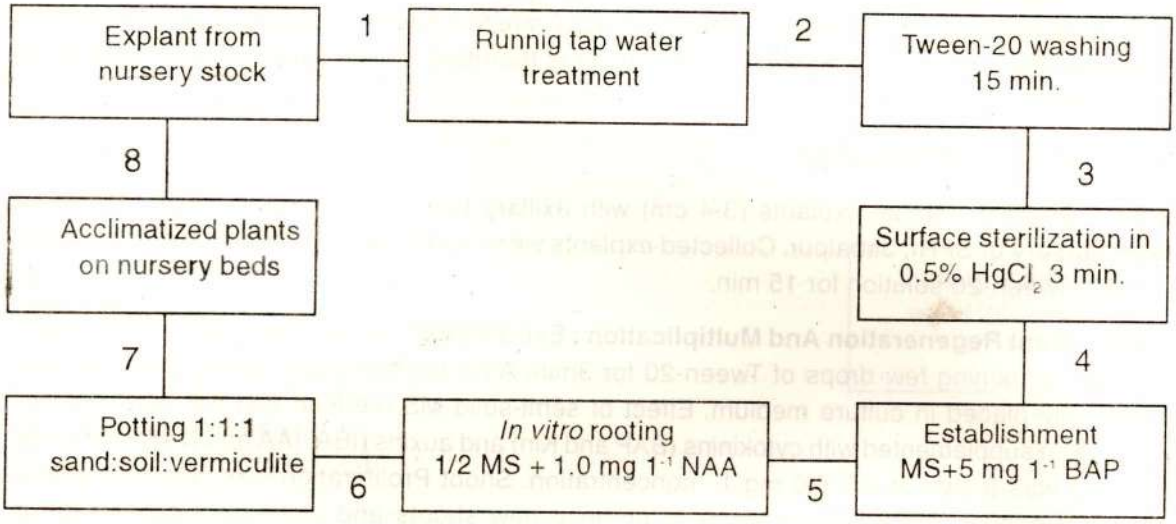
3.3. Protocol Summary (Recap)

i. Establishment and multiplication: MS +5mg 1^{-1} BAP

ii. Root induction: 1/2 MS +1 mg 1^{-1} NAA

iii. Potting mixture : Soil: sand: vermiculite (1:1:1)

3.4. Protocol Schematic Representation



4. *Withania somnifera* (L.) Dunal

4.1. Introduction :

W. somnifera (Ashwagandha, Family ; Solanaceae) is an erect shrub which grows to 0.3 to 1.5 m in height and commonly distributed in Central India. Leaf extract is used in aurvedic formulations to cure asthma, ulcer, leucoderma, bronchitis. Tuber of Ashwagandha has bitter sharp acrid taste useful for the preparation of aphrodisiac tonic to cure inflammation. *W. somnifera* naturally regenerated through seeds. Germination is easy and comparatively cost effective, but can result in the production of genetically heterogeneous population. Conventional techniques found to be less applicable to this species. Micropropagation trials on this species therefore, envisaged to address these problems effectively.

4.2. *In vitro* culture methodology :

4.2.1. Explant : Nodal segments were collected from 3-4 year old plants growing in SFRI, Jabalpur Nursery. Collected explants were processed into 1-2 cm long pieces and were washed in running tap water for an hour and in detergent solution (Tween-20, 1% v/v) for 15 min. They were then surface sterilized by 0.5% $HgCl_2$ solution containing few drops of Tween-20 for 3 min. and were inoculated in establishment medium.

4.2.2. Plant regeneration and multiplication : Response of MS medium supplemented with different levels of cytokinin (BAP and Kin.) was tested. MS medium supplemented with BAP (0.5 mg 1^{-1}) alongwith 0.1 mg1^{-1} IAA showed an early bud break. Shoots proliferated on this medium rapidly multiplied at the rate of 15-20 shoots per culture. New shoots subsequently elongated adequately (2-3 cm) within 4 weeks of culture. Newly formed shoots were re-multiplied on MS medium supplied with 0.5 mg 1^{-1} BAP and 0.1 mg 1^{-1} IAA. Original explant also recycled on above said medium.

4.2.3. *In vitro* rooting and plant establishment : Shoots induced roots by auxin supplied half-strength MS medium. Comparatively low concentration of auxin gave good rooting response. Shoots cultured on half-strength MS medium containing 0.25 mg 1^{-1} IBA resulted maximum (80%) rooting. Shoots produced an average 5-6 roots and were elongated to 2.5 cm within 4 weeks. Auxin (IBA or NAA) at high concentration caused callus at the base. Controlset planted on hormone free half-strength agar gelled MS medium gave 20% rooting.

Six weeks old plants were transferred to potting mixture (soil:vermiculite). Prior to potting plants were thoroughly washed in tap water and diped in 0.1% Bavistin suspension. Potted plants were covered initially with polyethylene bags for two weeks and incubated at glass house conditions. Covering was removed after two weeks of potting. Upon the development of new leaves plants were moved to nursery beds.

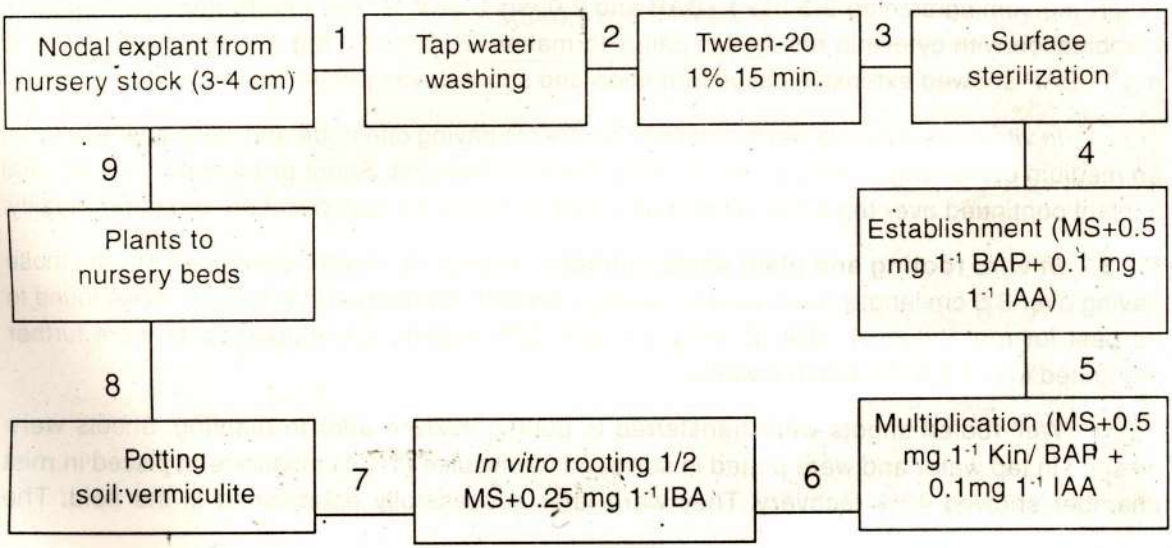
4.3. Protocol summary (Re-cap)

Establishment: MS + 0.5mg 1^{-1} BAP + 0.1 mg 1^{-1}IAA

Multiplication: MS+ 0.5 mg 1^{-1} BAP/Kin + 0.1 mg 1^{-1} IBA

Potting:soil:vermiculite (1:1)

4.4. Protocol: Schematic representation



5. *Psoralia corylifolia* Linn.

5.1. Introduction : *P. corylifolia* (Bavachi, Leguminosae) is a small woody shrub of tropical deciduous forests with spreading gland dotted leaves, grooved stem and branches. It is valued for its seeds containing essential oil psorolin, which is used in the aurvedic medicines and perfumed oils. Seeds are being used in bilious affections. Due to over exploitation and poor regeneration, this species is vanishing rapidly from natural stands. Limited applicability of conventional propagation techniques calls for *in vitro* propagation which seems an efficient alternative to address regeneration of this species.

5.2. *In vitro* methodology

5.2.1. Explant : Fresh shoots were collected from individuals growing in SFRI nursery. Collected shoot were washed primarily in running tap water for an hour followed by washing with Teepol solution and then 2-3 rinses in distilled water.

5.2.2. Plant regeneration and Multiplication : The processed explants were surface sterilized in 0.5% mercuric chloride solution containing few drops of Tween-20. This was followed by five rinses in sterile distilled water. Both exposed end of the explant were trimmed and placed aseptically in 25X150 mm test tubes containing initiation medium. MS medium supplemented with 3% sucrose and varying levels of auxins and cytokinins were tested. The medium was solidified with 0.7% agar and adjusted to pH 5.8 before autoclaving at 108 kPa for 20 min.

MS medium containing 5.0 mg l^{-1} BAP and 0.5 mg l^{-1} IAA gave maximum axillary bud proliferation. *In vitro* proliferated shoots grew into 3-3.5 cm size within 4 weeks. These shoots have short internodes which produce lateral shoots after 3 weeks of culture. Response to BAP and IAA at high and low proportions are also comparable. However, BAP addition (1.0 mg l^{-1}) caused suppression of shoot growth. Shoot elongation was occurred when cultures transferred to MS medium containing 3-5 mg l^{-1} BAP and 2.0 mg l^{-1} IAA. 2, 4-D incorporation singly or in combination with cytokinin resulted in callus formation. 2, 4-D (2.0 mg l^{-1}) alongwith 1.0 - 5.0 mg l^{-1} BAP showed extensive callus formation and shoot development.

In vitro raised shoots were made into segments having one node and were again cultured on medium containing 5.0 mg l^{-1} Kin for the next shoot harvest. Shoot production from original explant continued over ten times without any adverse affect on multiplication and shoot quality.

5.2.3. *In vitro* rooting and plant establishment : Half of the *in vitro* developed shoots those having over 3.5 cm length were used for rooting. Half MS medium with 0.5 mg l^{-1} NAA found to be best for root induction. IBA (2.0 mg l^{-1}) gave 60% rooting. Developed roots were further elongated into 2-2.5 cm within 6 weeks.

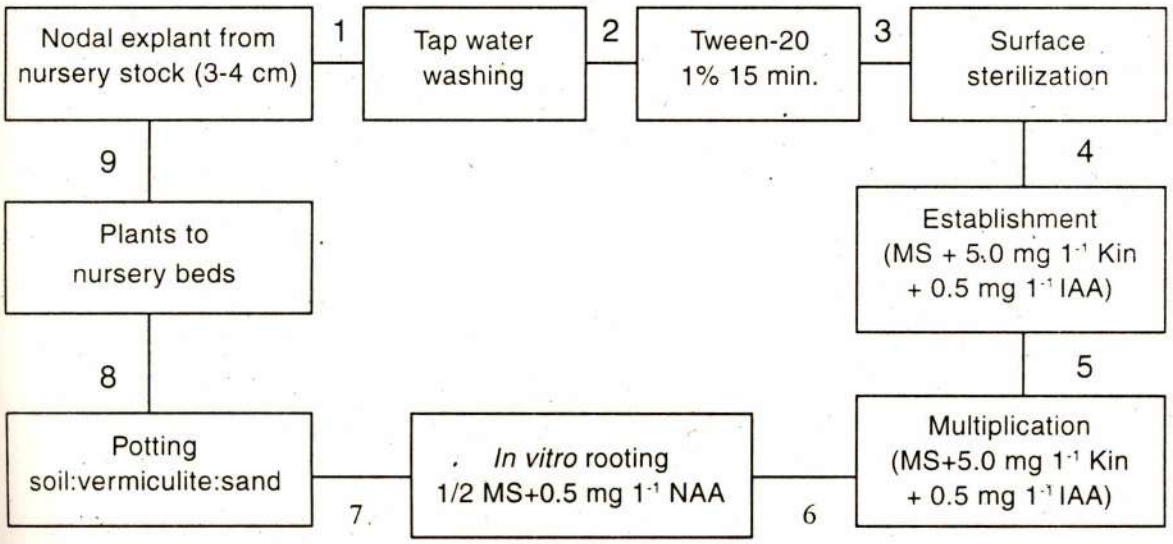
Well rooted shoots were transferred to potting mixture prior to planting. Shoots were washed in tap water and were potted in soil:sand:vermiculite (1:1:1) mixture and placed in mist chamber showed 40% recovery. They were later successfully established in the field. The

efficiency of developed protocol has been evaluated and indicated following advantages ; i. very short time period for plantlet production (3-4 months) ii. high multiplication rate, iii. year round production.

5.3. Protocol summary (Re-cap)

1. Establishment and multiplication: MS + 5.0 mg 1^{-1} Kin +0.5 mg 1^{-1} IAA
2. Root induction:1/2 MS+0.5mg 1^{-1} NAA
3. Potting mixture : soil : sand : vermiculite (1:1:1)

5.4. Protocol: Schematic representation



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