Standard Operating Procedure for Micropropagation of *Melia dubia* Cav. – An Important Fastgrowing Tree

ABSTRACT

The trees having multifarious importance for Timber, Fuel, NTFPs and Medicinal use. Due to increasing demand to meet out industrial requirement as a raw material , soft wood forest species are under tremendous pressure across the globe. The demand of fast growing *Melia dubia* is one of them. The usual approach of regeneration for this plant is through seed is unable to produce large scale plants. Therefore , the present investigation was aimed to develop the Standard Operating Procedure through Tissue culture method for mass multiplication of *M. dubia.* Highest 86.6% shoot initiation response was recorded in Murashige and Skoog medium supplemented with additives, NAA (0.1 mg $l^{-1)}$ and Kinetin (0.5mg $l^{-1)}$. Maximum response of shoot multiplication with highest shoot length of 5.5 cm was obtained in MS medium supplemented with combinations of Ascorbic acid (50 mg $l^{-1)}$ and Kinetin 1 mgl^{-1}. For rhizogenesis, MS + 3.0 mgl^{-1} IBA (93.3 %) demonstrated superior in terms of the percentage of cultures with root induction, the average number of roots, and the average length of roots per explant. In conclusion present study ensures the successful mass multiplication of *M. dubia*, demonstrating the importance of tissue culture in the expansion of this economically significant multipurpose tree.

Key words:- *M. dubia*, Shoot initiation, Multiplication, Response, Explant

1 .Introduction

One of the most important species of industrial trees, *Melia dubia,* has recently spread quickly across the Indian subcontinent. *M. dubia* is a native of tropical and subtropical regions, and it is cultivated largely for its usefulness in industry and medicine. It belongs to the family Meliaceae. The wood of *Melia dubia* is utilised as a primary resource for plywood, paper pulp, and secondary lumber. The wood is also employed in the manufacture of pencils, match boxes, packing cases, cigar boxes, ceiling boards, building materials, agricultural instruments and splints. Due to its multipurpose uses like bioenergy production, paper and pulp manufacturing,

furniture making, building constructions, making musical instruments etc., it is gaining more popularity and is in high demand.(Bhimi Ram *et al.* , 2014)

Melia dubia occurs in the 1,500–1,800 meter-high tropical moist deciduous forests of Sikkim, the Himalayas, North Bengal, and upper Assam, the Khasi Hills of North East India, Orissa, the Deccan, and the Western Ghats. The *Melia dubia* trees are used throughout India; the Nilgiri tribes use the trees as a medicine to treat a variety of diseases. The research suggests that *Melia dubia* fruit is effective in treating colic, dermatitis, and anthelmintic diseases. The tetranotriterpenoids composition and compositolide are said to be present in this tree's leaves and seeds. The development of a new cloned propagation of *Melia dubia* plantation (Malabar Neem tree- a very fast growing, with high calorific value of wood) has opened up new opportunities for small and medium biomass, such as lop & top, for power generation projects. Farmers in Tamil Nadu and other southern Indian regions grow this plant on a large scale. It is a tree with rapid growth and high productivity and it is taking the place of eucalyptus in farmer's fields in the region. Eucalyptus is known to have an allelopathic effect on soil, yet this species doesn't have any detrimental effects.

The primary issue with this species is the very low rate of seed germination and viability, with reports of only 20–30% seed germination from various researchers. In nurseries, this species cannot grow huge numbers of seedlings using typical regeneration techniques. The demand for this species as high-quality planting material can, however, be met through in vitro methods of propagation. The primary benefit of tissue culture is the year-round production of a large number of plants that are true to type in a small amount of time and space. With this technique, we can get disease-free plantlets in a short amount of time. Tissue culture plants (TCPs) could flower sooner, have better branching, be more vigorous, and produce more.

2. Materials and Methods

2.1 Explants source & sterilization

The current study was carried out in the tissue culture lab RRL at the Department of Plant Molecular Biology and Biotechnology in the College of Agriculture, IGKV, Raipur (CG) during 2021 to 2022. Explants were taken from an *M. dubia* tree that had been grown for four years and had been planted in the Research Farm, Department of Forestry, College of Agriculture, IGKV,

UNDER PEER REVIEW

Raipur, Chhattisgarh. In order to surface sterilise the explants, 2.5 to 3.0 cm long nodal segments were excised after the leaves were removed. After being placed in a bottle with a cap, the explants (nodal segments) were thoroughly cleaned under running water. Explants were treated with 0.1% (v/v) Polyoxyethylene sorbitan monooleate (Tween - 80; Himedia, India) liquid solution for 15 minutes followed by 3 to 4 times thorough washes in double-distilled water and then 0.1% (w/v) solution of Bavistin (Carbendazim 50% WP- a systemic fungicide) for 30 minutes, followed by 5 to 6 thorough washes in double-distilled water, after that explants were treated with 0.1% (w/v) HgCl2 solution for 5 min. Following each treatment, explants were washed five to six times in autoclaved double distilled water to remove any lingering steriliser residues. To reduce leaching, the cut ends of the surface sterilized explants were trimmed aseptically before being vertically inoculated on culture medium.

2.2 Culture initiation

Prior to use, the laminar air flow chamber was sterilized by UV light for roughly 20 to 30 minutes. In the laminar air flow chamber, the floor was cleaned with 70% alcohol, and sterilized forceps and scalpels were employed. Then the sterilized *Melia dubia* nodal explants were placed in culture bottles containing 40 ml of MS basal medium that had been supplemented with additives and various growth hormones. For shoot initiation various treatments comprised of Murashige and Skoog basal media, Kinetin (0.5mg l^{-1}) , NAA(0.1 mg l^{-1}) , IAA(0.1 mg l^{-1}) alongwith additives Ascorbic acid(50 mg l^{-1}) ,Casein hydrolysate(500 mg l^{-1}) and Adenine sulphate (25 mg l^{-1}) were used in various combination. After that, cultures were incubated in a culture room, where the temperature was maintained at 26±2ºC, humidity at 70-80% and under a photoperiod of 16 hours light and 8 hours dark for a period of 15–21 days while incorporating fresh media in the same cultural contexts. Each treatment had three replicates, each of which contained ten explants. The data was recorded at the end of 2nd week. To study the effect of PGRs on shoot initiation various concentration and combinations of cytokinins and additives viz. Ascorbic acid, Adenine sulphate, Casein hydrolysate were used.

2.3 Shoot multiplication

After 21 days, explants with started shoots were moved to glass bottles containing a new multiplication media . Plant growth regulators with varying doses and additives were evaluated in

UNDER PEER REVIEW

MS basal medium for multiplication. MS media ,Kinetin (0.5 mg l^{-1} and 1mg l^{-1}) along with additives Ascorbic acid (50 mg l^{-1}) ,Casein hydrolysate(500 mg l^{-1}) and Adenine sulphate (25 mg l^{-1}) were used in various combination. When the shoots had fully utilized the explants' stored nutrients able to independently absorb nutrients from the nutrient medium, the shoots were separated from the explant. Every 15 to 21 days, sub-culturing with basal media was done. The dried leaves, damaged cells/material, shoots, and sheath were removed during each subculture to provide room for the micro shoots to expand. Each treatment had three replicates, each of which contained ten explants.

2.4 Root induction

Long shoots originating from the node were transferred to ½ MS medium supplemented with different concentrations of IBA and IAA for rooting. The rooting medium consisted of ½ MS with 3% sucrose and solidified with 0.7% of agar. IAA (1 mg l^{-1} , 2 mg l^{-1} and 3 mg l^{-1}) and IBA (1 mg l^{-1}, 2 mg l^{-1} and 3 mg l^{-1}) were used individually or in combination. The percentage of rooted shoots, the total number of roots and the root length were evaluated after 6 weeks of growth on the rooting medium.

3. Result and Discussion

3.1 Shoot induction

Explants showed the presence of high levels of phenolic compounds after inoculation. Exfoliation of phenolic compounds from the shoot tip has resulted in the medium being completely brown and shoot tip explants have not been able to survive in most cultures. Cultures inoculated with nodal explants were observed after two weeks, in which highest shoot initiation percent (86.66%) was obtained on MS media supplemented with Ascorbic acid (50 mg l^{-1}), NAA (0.1 mg l^{-1}) and Kinetin (0.5mg l^{-1}) highest shoot initiation percent (86.66%) was obtained . MS media devoid of PGRs and additives showed very less shoot initiation percent (26.66%) (Table-1). The rate of in vitro shoot induction was accelerated by the addition of cytokinins (Kn) and modest concentrations of auxins (NAA & IAA). When NAA (0.1 mg l^{-1}) and Kinetin (0.5mg l^{-1}) were added to the MS medium, the in vitro shoot initiation increased significantly (from 5.3 to 8.6) (Table 1). However, addition of IAA was less effective than NAA for initiating shoot induction. According to Mascarenhas *et al*. (1982), kinetin is a typical natural cytokinin

UNDER PEER REVIEW

which is frequently utilized in plant tissue culture. The beneficial effect of cytokinin was noticeable in a variety of tree species, including *Butea monosperma* (Kavitha *et al.,* 1996) ,*Azadirachta indica (*Su *et al*., 1997, Mallika Vanangamudi *et al.*, 1997), *Albizia procera* (Majumdar *et al*., 1998), *Eucalyptus tereticornis* (Parthiban *et al.,* 1999).

Table 1: Effect of growth hormone on Shoot initiation in *M. dubia*.

Treatments	Mean±SE	Initiation %
MS+ Ascorbic acid(50 mg l^{-1})+NAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	8.6±0.33	86.66%
MS+ Ascorbic acid(50 mg l^{-1})+IAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	7.6±0.33	76.66%
MS+ Casein hydrolysate(500 mg l^{-1})+NAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	5.3±0.33	53.33%
MS+ Casein hydrolysate (500 mg l^{-1})+IAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	4.6±0.33	46.66%
MS+ Adenine sulphate (25mg l^{-1})+NAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	7.3±0.33	73.33%
MS+ Adenine sulphate (25 mg l^{-1})+IAA(0.1 mg l^{-1})+ Kinetin (0.5mg l^{-1})	6.6±0.33	66.66%
MS (control)	2.6±0.33	26.66%
SE(m)=0.33 CD (0.01)=1.021		

UNDER PEER REVIEW

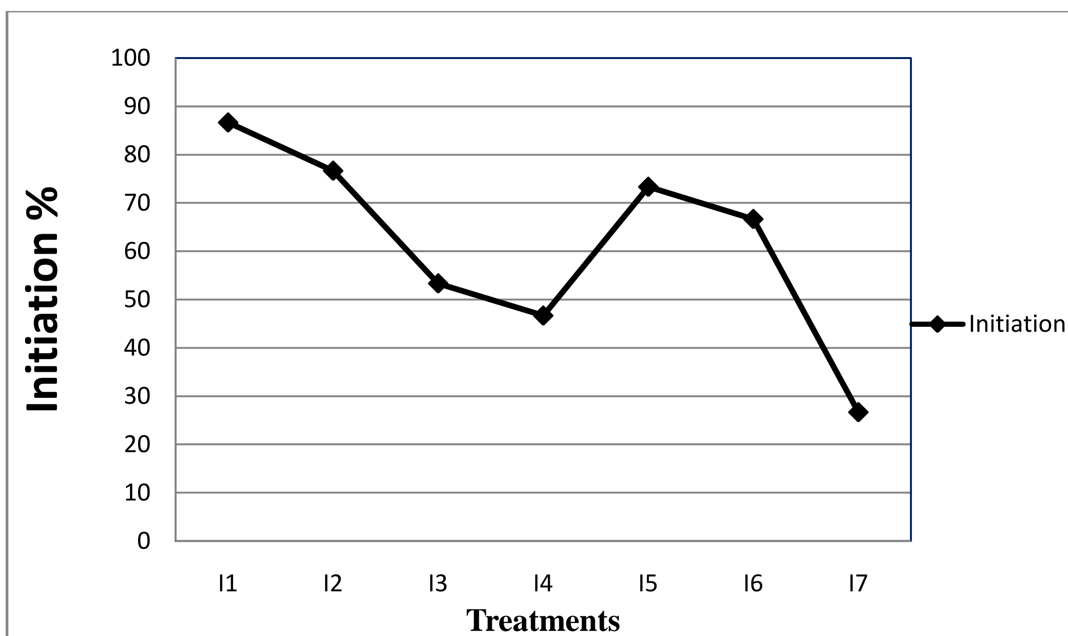

Fig 1:- Graphical representation of effect of growth hormone on shoot initiation in *Melia dubia*

3.2 Shoot multiplication

Sub culturing was found essential within 2-3weeks period on fresh shoot multiplication medium to regenerate more number of shoots and for shoot elongation . Shoot clump of 2-3 shoots proved better than single shoot or shoot segment for further shoot multiplication. MS supplemented with Ascorbic acid(50 mg l^{-1}) , Kinetin (1mg l^{-1}) recorded (13.33) higher number of shoots per explants with highest shoot length 5.5 cm whereas, lowest number of shoots per explants (6.33) and lowest shoot length 2.4 cm was obtained in MS basal media devoid of PGRs (Table-2). The analysis of variance for shoot multiplication of *M. dubia* showed significant results because F-calculated value is greater than F-tabulated value at 1% level of significance. Due to their anti-oxidative qualities, Ascorbic acid, Adenine sulphate, and Casein hydrolysate in the medium had an effect on the frequency of shoot multiplication rate and length of shoot, which indirectly had an axillary effect on shoot multiplication and shoot growth. Ascorbic acid serves as a best precursor to cytokinins or derivatives as compared to other additives. The strategy of using Ascorbic acid as an adjuvant has been adopted effectively for many other plant

UNDER PEER REVIEW

species including *Tectona grandis* (Devi *et al*., 1994), *Acacia catechu* (Kaur *et al.,* 1998), *Pterocarpus marsupium* (Husain *et al*., 2008) , *Melia azedarach* (Husain and Anis 2009; Sen *et al.* 2010).

Table 2 : Effect of additives & different concentration of Kinetin on shoot multiplication in *M. dubia*

Treatments	Mean±SE	Shoot length(cm.)
MS+ Ascorbic acid(50 mg l^{-1}) + Kinetin (0.5mg l^{-1})	11.33±0.66	5.0
MS+ Ascorbic acid(50 mg l^{-1}) + Kinetin (1mg l^{-1})	13.33±0.66	5.5
MS+ Casein hydrolysate(500 mg l^{-1})+ Kinetin (0.5mg l^{-1})	7.66±0.33	3.9
MS+ Casein hydrolysate(500 mg l^{-1})+ Kinetin (1mg l^{-1})	8.00±0.57	4.0
MS+ Adenine sulphate (25mg l^{-1}) + Kinetin (0.5mg l^{-1})	8.66±0.66	4.1
MS+ Adenine sulphate (25mg l^{-1}) + Kinetin (1mg l^{-1})	9.33±0.33	4.4
MS (control)	6.33±0.33	2.4
SE(m)=0.509 CD (0.01)=1.586		

3.3 Root induction

In the present study, rhizogenesis was observed due to addition of auxins *viz,* IAA and IBA either singly or in conjoint addition between two auxins evaluated, all the treatments recorded significantly higher rooting per cent due to increasing concentrations of either IBA or IAA auxins. Highest root induction was obtained on ½ MS supplemented with 3.0 mgl^{-1} IBA (93.3 %) with root length (5.46 cm) followed by ½ MS supplemented with 2 mg l^{-1} IBA (90%) with root length(4.63cm) and lowest rooting was observed in ½ MS (control) ,where no rooting response was obtained (Table 3). The positive effect of IBA on rooting of *in vitro* propagated plants had been established in many tree species, *viz*., *Simarouba glauca* (Sekar,2003), *Bambusa bamboos* (Kamala,2009) and *Simarouba glauca* (Nesamani, 2005) which are consistent with the result of present findings.

UNDER PEER REVIEW

Table 3: Effect of different concentration of auxins on root induction in *M. dubia*			
MS media with different concentrations of IAA and IBA (mg l^{-1})	Mean±SE	Rooting (%)	Root length (in cm.)
½ MS +control	0.000±0.00	0%	0
½ MS +1 mg l^{-1} IAA	2.100±0.30	50%	1.46
½ MS +2 mg l^{-1} IAA	3.233±0.14	63.3%	2.76
½ MS +3 mg l^{-1} IAA	4.133±0.08	73.3%	3.46
½ MS +1 mg l^{-1} IBA	4.200±0.05	83.3%	4.03
½ MS +2 mg l^{-1} IBA	4.633±0.12	90%	4.30
½ MS +3 mg l^{-1} IBA	5.467±0.21	93.3%	4.60
½ MS +1 mg l^{-1} IAA+1 mg l^{-1} IBA	4.267±0.08	86.6%	4.10
½ MS +2 mg l^{-1} IAA +2 mg l^{-1} IBA	4.200±0.11	80%	3.90
½ MS +3mg l^{-1} IAA +3 mg l^{-1} IBA	4.000±0.05	76.6%	3.66
SE(m)=0.146 CD(0.01)=0.433			

UNDER PEER REVIEW

4. Conclusion

This study provides an efficient in vitro propagation method which could be commercially feasible for *M.dubia*. Present investigation showed that MS supplemented with Kinetin and low concentration of auxin i.e. NAA are essential to produce higher shoot initiation percent, and the highest number of micro propagated shoots obtained by the use of Ascorbic acid as additive, Kinetin (1mg l^{-1}) alongwith MS basal media for this fast growing tree species. Whereas, for high rooting percent , IBA was found most appropriate auxin. The standard acclimatization procedure was developed successfully to get high survival percent of micro-propagated plantlet

of *M. dubia* .Thus it is concluded that the current investigation ensures the successful mass multiplication of *M. dubia*, demonstrating the importance of tissue culture in the expansion of this economically significant multipurpose tree.

References

Bhimi Ram et al. , 2014. “An efficient protocol for micropropagation and genetic stability analysis of *Melia dubia* Cav. - an important multipurpose tree”. International Journal of Current Microbiology and Applied Sciences, 3(7), 533-544.

Devi, Y. S., Mukherjee, B. B. and Gupta, S. 1994. Rapid cloning of elite teak (*Tectona grandis* L.) by *in vitro* multiple shoots proliferation. Indian J. Exp. Bio. 32: PP, 668-671.

Husain, M. K., Anis, M. and Shahzad, A. 2008. *In vitro* propagation of a multipurpose leguminous tree (*Pterocarpous marsupium* Roxb.) using nodal explants. Acta Physiol. Plant 30: PP, 353-359.

Husain, M. K. and Anis, M. 2009. Rapid *in vitro* multiplication of *Melia azedarach* L. (a multipurpose woody tree). Acta Physiol. Plant 31:765-772.

Kaur, K., Verma, B. and Kant, U. 1998. Plants obtained from the Khair tree (*Acacia catechu* Willd.) using mature nodal segments. Plant Cell Rep. 17: 427-429.

Kamala, K. 2009. Screening of thornless bamboos for pulpwood quality and development of *in vitro* propagation techniques. M.Sc. Thesis, Tamil Nadu Agricultural University, Coimbatore.

Kavitha. R.K., H. Smitha and L.D. Souza. 1996. Micropropagation of *Butea monosperma* (Lam) Taub. Biotechnology, 19: 291-293

Majumdar, K., S. Sinha and R.K. Sinha. 1998. *In vitro* regeneration and multiplication of shoots in *Albizia procera* Benth. Adv. Pl. Sci., 11(2): 7-12.

UNDER PEER REVIEW

Mallika Vanangamudi, K.T. Parthiban, C. Surendran and R. Annamalai. 1997. Micropropagation of *Azadirachta indica* A. Juss. from nodal segments of three year old trees. Journal of Non Timber Forest Products, 4(1/2): 34-37.

Mascarenhas, A.F., S. Hazara, V. Potdar, D.K. Kulkarani and P.K. Gupta. 1982. Rapid clonal multiplication of mature forest trees through tissue culture: In: Proceedings of the 5th International Congress on plant tissue and cell culture (Ed) A. Fujiwara Tokyo, Japan. pp. 719-720.

Nesamani, K. 2005. Studies on seed sources, micro propagation and molecular characterization of sex specificity in *simarouba gluaca* DC. M.Sc. Thesis, Tamil Nadu Agricultural University, Coimbatore.

Parthiban, K.T., Malliga Vanangamudi, C. Surendran and S. Balaji. 1999. Callogenesis and organogenesis in *Eucalyptus tereticornis*. Indian J. For., 22(2): 101-105.

Saravana Kumar V.G. et al. , 2017. Development of Proliferation and Acclimatization of *Melia dubia*-Australian Teak Variety through Micropropagation. International Journal of Innovative Research in Science, Engineering and Technology. Vol. 6(1): 1226-1236.

Sen, A. et al., 2010. Pivotal role of plant growth regulators in clonal propogation of *Melia azedarach.* International Journal of Pharmaceutical Sciences Review and Research 5(2) 43-49.

Sekar, I. 2003. Seed storage, seed source variations, molecular characterization and in vitro propagation in *Simarouba glauca* DC. Ph.D. Thesis, Tamil Nadu Agricultural University, Coimbatore.

Shah Syed Naseer et al., 2016.An efficient protocol for in vitro organogenesis and antioxidant studies in *Melia dubia* Cav. African Journal of Biotechnology 15(19), pp. 768-775.

UNDER PEER REVIEW

Su, W.W., H. Hwang, S.Y. Kim and Y. Sagawa. 1997. Induction of somatic embryogenesis in *Azadirachta indica.* Plant cell, Tissue and organ culture, 50: 91-95.

UNDER PEER REVIEW