Environment and Ecology 41 (1C) : 633—639, January—March 2023
ISSN 0970-0420

Problem and its Remedy of Micropropagation in Woody Trees: A Review

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Received 15 October 2022, Accepted 23 January 2023, Published on 16 March 2023

ABSTRACT

There are over 600 species of tropical timbers in the world, many of which are commercially valuable in the international trade of plywood, roundwood, sawnwood, and veneer. Many of these tree species are being threatened and are endangered because of logging practices, conversion to agricultural lands, non-optimal management strategies and overall deforestation rates that cannot keep up with natural regeneration of native forests. Several wood species (*Tectona grandis, Cedrus deodara*) have very good natural resilience against destructive wood agents, but global demand has outstripped supply or availability in the marketplace. Furthermore, timber production from government forest areas in India accounts for 3.35% of total demand, or 153 million m^3 in 2020 (projected), while potential timber production accounts for 45% of total demand for raw wood by various wood-based industries (Brocco *et al*. 2017, Vanam 2019). This demand is found to be doubling or tripling in all sectors except for agriculture, where demand is stagnant because of increased mechanization. Plant tissue culture is an important tool to propagate the plants in large scale through the eminent way in the short. Culture of plant and various parts in the aseptic condition with the concept of totipotency. A special media fortified with inorganic nutrients, vitamins, carbohydrates and environmental factors are added *in vitro* condition. Cell totipotentiality and cellular plasticity is the major physiological principle behind the plant tissue culture. Cell plasticity responses for the division and differentiation capacity of the culture cells. The ability of the single cell to transform into a whole plant alike as the mother plant. The tissue culture techniques are successfully used to overcome barriers for plants which are difficult to propagate. Some problem in tissue culture of forest tree species like Contamination of cultures, Browning of the medium (*Tectona grandis*, *Dalbergia latifolia*, cashew), Juvenility-Maturity problem, Vitrification (*Mangifera indica*), Rooting the *in vitro* produced shoots is another problem in many tree species like rosewood, acclimatization. The present review article discusses the limitation or problem of tissue culture and their solution.

Keywords Totipotency, Differentiation, Deforestation, Vitrification, Acclimatization.

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INTRODUCTION

As the gap between the supply and demand of wood and wood products is widening, there is an urgent

Table 1. List of woody plant species and their suitable explant type for tissue culture.

Woody plant species	Explant type	References
Ziziphus jujuba	Leaf explant	Feng *et al.* (2010)
Eucalyptus camaldulensis	Nodal explants	Girijashankar (2012)
Bixa orellana	Nodal segments	Joseph *et al*. (2011)
Gmelina arborea	Shoot tip, node and internode explants	Kumar *et al.* (2010)
Morus alba	Leaf explants	Lee *et al.* (2011)
Pterocarpus santalinus	Shoot tip explants	Balaraju *et al.* (2011)

need to improve the quality and quantity of trees. The ever-increasing demand for forest products and the progressive deterioration of natural forests means that the forest industry cannot continue to rely on the exploitation of natural forests. Recent modern techniques of propagation have been developed which could facilitate large scale production of true-to-type plants and for the improvement of the species using genetic engineering techniques in the next century. According to the "Forestry Statistics India" (2019) survey in India about 24% of total land is under forestation also there are about 140 species of timber in Indian forests. But a very little of these are commonly used most important and valuable tree species is Teak (*Tectona grandis*), Sal (*Shorea robusta*), Rose wood (*Dalbergia latifolia*), Red sander (*Pterocarpus santalinus*), Ailanthus (*Ailanthus excelsa*), Eucalyptus, *Casurina equistifolia*, Indian kino tree this species are highly used in the timber, non-timber forest produce, gum, resin, essential oil, pulp paper industry. Recently rapid industrialization, urbanization and over exploitation are the main factors of depletion of forest resources and permanent loss of forest (Kumari *et al.* 2019). Now a day's it has become the major concern to increase the forest product by 50% to meet the growing demand of the global population. The above problem can be sorted out through proper management and utilization of forest resources. That will also help to prevent the extinction of endangered species and mass destruction of the forest. Global Forest Resources Assessment (FRA) proclaimed that 3.16% declination of global forest area from 1990 to 2015. Therefore, it is now high time to conserve the forests which an essential part is already lost. Tissue culture techniques are one among them for the production of quality planting stock because of its higher multiplication rate with instant silvicultural gains which involves the culture of cells or tissues in the laboratory. Many factors can affect the *in vitro* establishment as well as micropropagation of different woody plants for instance, type of explants, physiological status of explant, genotype and age of donor plant, media, plant growth regulators, photoperiod, antioxidants. Some problem in tissue culture of forest tree species like Contamination of cultures, Browning of the medium (*Tectona grandis*, *Dalbergia latifolia*, cashew), Juvenility-Maturity problem, Vitrification (*Mangifera indica*), Rooting the *in vitro* produced shoots is another problem in many tree species (rosewood), acclimatization.

Selection of explant

Explant selection is first step in plant tissue culture any part of the plant can be selected as explant. It has long been known that juvenile explants show better response under *in vitro* conditions. Explant collected should be healthy, disease-free mother tree. If the explant collected from greenhouse condition is considered as better in survivability in many forest trees, coppice shoots are being used for macro / micro propagation. Shoot tip and internodes were found as the best source of explants for sterilization and better growth (Dar *et al.* 2012). Type of explants like leaf, nodal, shoot tip, node and internode and root explant significantly effect on tissue culture process of plants (Table 1) (Feng *et al.* 2010, Girijashankar 2012, Lee *et al*. 2011, Kumar *et al.* 2011).

Pre-treatment of explants

Surface sterilization

Surface sterilization of explant is the essential step in the plant tissue culture. If explant taken from the external environment was exposed to microbial contamination will leads to mortality of explant. Fungal and bacterial contamination in the external part of explant can be sterilized with the autoclave water and chemical substances include ethanol, sodium hypochloride bavistine, mercuric chloride.

Table 2. Sterilization process of different explant in different wood species.

Tree species	Explant	Sterilization
Teak (*Tectona grandis*)	Bud, shoot, nodal	10 min with 1% $HgCl_{12}$
Rosewood (*Dalbergia latifolia*)	Shoot	0.2% (w/v) Bavistin
Sandal wood (*Santalum album*)	For tender stem of sandal	0.05% $HgC1_2$ was used for 5 minutes
	Thicker stems and leaves	0.1% of $HgCl_{12}$ was used for 5 minutes

Explants were prepared for culture by removal of dead tissue and cleaning by tap water containing a few drops of detergent followed by 1-2 washes with distilled water to remove the detergent. This was followed by surface sterilization. Mercuric chloride solution prepared in double distilled water was used for surface sterilization of all the plants used in this study. The concentrations and duration of the $HgCl_{l2}$ treatment varied with the type of tissue used. For shoot tips and nodal explants of teak, rosewood and sandal wood, 0.1-1.0% of $HgCl_{12}$ (w/v) was used for 5-10 minutes. For tender stem of sandal 0.05% and for thicker stems and leaves 0.1% of $HgCl_{12}$ was used for 5 minutes (Table 2). Pre-treatment or culture in the presence of antibiotics and actimycotics was also tested to control contamination in explants. Bavistin a systemic fungicide was used as a 0.2% (w/v) solution for 1 hr soaking treatment or as an additive to the media. Streptomycin and Gentamycin were added to the media at a concentration of 100 mg/l after filter sterilization through a 0.22 m membrane (Millipore) to autoclaved media just before gelling.

High rates of fungal and bacterial contamination in the initial cultures of teak the contamination was dependent on the season of collection and was particularly high during the months from June to September. This coincided with the rainy season and the period of high relative humidity in the area from where the specimens were collected. The contaminants proliferated particularly on the cut edges of the explants or when tissues expanded or when abscission of bracts and leaves took place. The contamination is hence suspected to be mostly endogenous in origin since the contaminant was not directly in contact with media. In teak fungal contamination.

Table 3. List of tree species presence phenolic.

Sl. No.	Tree species presence phenolic content
1	Mango (*Mangifera indica*)
2	Pomegranate
3	Rosewood (*Dalbergia latifolia*)
4	Teak (*Tectona grandis*)
5	Cashew

In teak buds collected during May to November gave 100% fungal contamination even with sterilization for 10 min with 1% $HgCl_{12}$. Higher concentration or time resulted in rapid killing of the explants. Contamination rates were minimum in buds taken in March and April (45%). Such high doses of $HgCl_{12}$ were, however found to be damaging to the tissues in the present experiment.

In rose wood high rates of contamination (greater than 80%) was observed in shoot tips and nodes collected in all months from June to February. Survival of buds without contamination could be obtained only in the dry season. Use of 0.2% (w/v) Bavistin in the medium was effective in reducing the contamination to less than 60% and in delaying the appearance of the contamination.

Effect of explant : Explants are small pieces of plant parts or tissues that are aseptically cut and used to initiate a culture in a nutrient medium. Plant parts like shoots, leaves, stems, flowers, roots can be used as explant in plant tissue culture. The source of explants has been considered a critical variable for *in vitro* culture in pomegranate. All explants are not equal in terms of regenerability. It is likely that different selective pressures would be exerted against different explants. This could result in different frequencies and spectrums of regeneration among plants from different explants.

Type of explant is one of the important factors in optimizing the tissue culture protocol. Type of explants like leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root explant

Table 4. List of chemicals to reduce phenolic content.

Chemical	Concentration
Citric acid	150 mg/l
PVP	0.5%
Charcoal	0.25% (w/v)

significantly effect on tissue culture process of plants. This may be due to the different level of endogenous plant hormones present in the plant's parts.

Endophytic organism cause contamination

Epiphytic microbes in the explant were removed by surface sterilization but the occurrence of endophytic microbes in the explant leads to contamination in the culture.

Role of antibiotics

Usage of antibiotics play essential role in the elimination and inhibition of microbial contamination. by adding appropriate amount of antibiotics like streptomycine, tetracycline, Gentamicine, Cifotaxime in the medium may eradicate the contamination in culture. Benomyl and streptomycine were added in the medium provides better culture of nodal explant.

Dedifferentiation

The main problems during callus induction of Indian sandalwood include the low frequency of callus formation and poor repeatability by different researchers. When Murashige and Skoog (MS) medium was used as the basal medium (Murashige T, Skoog F 1962) some researchers used 2,4-dichlorophenoxyacetic acid (2,4-D) alone to induce callus from hypocotyls or stem segments (Zhang X 2016) while other researchers found that callus induced from hypocotyls on callus induction medium (CIM) containing 2,4-D alone could not regenerate any callus (Crovadore J, 2012) or that the callus induction efficiency of stem segments was extremely low.

In rosewood leaf explants cultured on media containing 2,4-D or NAA or 2, 4-D and NAA gave rise to callus on all the concentrations. Coconut milk was not essential to obtain callus growth. The callus which developed at the cut edges of the leaf explants was brownish white in color and soft and friable in texture. The media below the callus often turned brown in many of the cultures. The use of PVP was ineffective in reducing browning in callus cultures. Since the browning did not appear to be inhibitory to callus growth, other antioxidants were not tested. When subculture to fresh medium the callus could be maintained in a healthy growing state. The maximum callus formation was obtained on 2 mM 2, 4-D.

Effect of antioxidants : Browning and necrosis is a common problem in cultures of woody species and have been generally attributed to the oxidation of phenolic compounds in explant tissues (Dobránszki and Teixeira da Silva 2010). After oxidation these components become toxic to the explants and results in retardation of growth and eventually leads to complete failure to survivability of the explants. Some tree species listed in (Table 3) presence of phenolic compounds.

Medium browning is a major problem in pomegranate due to the exudation of high amount of phenols, especially in mature explants (nodal segment and shoot apex). In perennial fruit crops like pomegranate, establishment of explants requires special procedures to escape the problem that associated with exudation of phenolic compounds from cut surface.

Different attempts has been made to eliminate browning problem in woody plant species like pre-socking of explants in antioxidants solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants (Ahmad *et al.* 2013). An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation reactions can produce free radicals. Free radicals can cause damage or cell death. Antioxidants remove free radical intermediates and inhibit other oxidation reaction.

Phenolic substance leaches from the cut surface of the explant and oxidizes later results in turning of media brown. Browning of tissue process is caused by the oxidation of tannin and polyphenols and the formation of guinones which are highly reactive and toxic to the tissue. Phenolic compounds contain at

least one hydroxyl group on the benzene ring. Several oxidases such as monophenolase (Tryosinase), polyphenol (Catecholoxidase) oxidized the hydroxyy group resulting in the formation of guinone and water (Onuoha *et al.* 2011). After oxidation these components become toxic to the explants and results in retardation of growth and eventually lead to complete failure to survivability of the explants. Medium browning is a major problem in pomegranate due to the exudation of high number of phenols, essentially in nodal segment and shoot apex explants (Naik *et al.* 1999).

In explants of *Tectona grandis* leaching of phenolic compounds into the medium commenced within a few hours of inoculation. After 24 hrs browning of the media was severe in mature teak explants and to a lesser degree in teak seedling and rosewood explants.

In explants of rosewood leaching of phenolic compounds into the medium commenced within a few hours of inoculation. After 24 hrs browning of the media was severe in mature teak explants and to a lesser degree in teak seedling and rosewood explants.

Cryopreservation using vitrification was described for *Mangifera indica* embryogenic cultures variety Zihua' derived from nucellar and cotyledon explants.

Reduce antioxidant effect

Since exudation of phenolic compounds and browning of explants were observed in teak and rosewood, explants were pretreated with running water, activated charcoal (AC), polyvinyl pyrolidone (PVP) or citric acid. Explants were kept after cutting to proper size in running water for 1 hr or for 30 min. in solutions of 0.25% (w/v) AC or 0.5%PVP or 150 mg/l citric acid after which surface sterilization was carried out (Table 4). Potassium citrate is variably better antioxidant in the culture of *Musa pardisiaca* (Onuoha *et al.* 2011). The best way of removal of secondary products from the explants is to collect explants at earlier morning before sunrise (the plant do not start the photosynthesis before sunrise) and immerse the explants in the water immediately after collection as it is a best solvent to elucidate the secondary compound (Singh *et al*. 2012). To overcome phenolic exudation, CA, AC and PVP were investigated and activated charcoal found to be the best (Chinnappan, *et al.* 2012).

Multiplication issue

In rose wood calli transferred to the regeneration media containing BAP and NAA and shifted to light developed green and hard nodules. No evidence of organogenesis was however observed. All the different media reported to induce organogenesis failed to give results in rosewood.

The induction of multiple shoot formation through culture of nodes or shoot tips has not been described in sandal wood by any of the groups working on sandal wood even though the method would have been the most preferred means of clonal propagation. It is to be concluded that no success in regenerating plants was obtained as in the present study. Perhaps a complete re-evaluation of the composition of the basal medium is required to obtain a better response.

Rooting issue

Rooting the *in vitro* produced shoots is another problem in many tree species like rosewood.

Ex vitro rooting has been found highly successful in species like teak. Difficulty with rooting is a major problem in the final stage of shoot organogenesis of Indian sandalwood (Crovadore *et al.* 2012, Bele *et al.* 2012) and is often encountered in the process of somatic embryogenesis as well (Mo *et al.* 2008).

Acclimatization

A major limitation in large scale application of micropropagation technology is high mortality experienced by *in vitro* raised plants during laboratory to land transfer. Micropropagated plants on being transferred to *ex vitro* conditions are exposed to (altered temperature, light intensity and water stress) conditions so need acclimatization for successful establishment and survival of plantlets (Chandra *et al.* 2010). Following

the survey, the candidate realized that understanding the factors which control successful acclimatization of tissue cultured plants needs to be taken up and it is important to recognize and understand the differences between an *in vitro* and greenhouse or field environment. Realizing the importance of acclimatization for micro propagated plants, the candidate has made an attempt to gather available information on morphology and anatomy of *in vitro* and *ex vitro* or field grown plants. A thorough study of the available literature on *in vitro* hardening and *ex vitro* acclimatization of tissue culturally raised plants has been undertaken to know the factors affecting acclimatization and the morphological and anatomical changes that occur in the tissue cultured raised plants during acclimatization. When shoots or plantlets are transplanted from culture room to greenhouse conditions they may desiccate or wilt rapidly and can die as a result of the change in environment, unless substantial precautions are taken to accommodate them.

RESULTS AND DISCUSSION

Economically important plants and tree have been recorded in widespread of India as well as in the World, though some plants and tree species were still in endangered condition. Conservation of plants is the main objective of the plant tissue culture. Many commercial laboratories and national institutes worldwide use *in vitro* culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations, and for secondary metabolite production.

In tissue culture process in plant propagation first step to choose the suitable explants (Table 1) for propagation and these explants are surface sterilized in different chemicals (Table 2) due to prevent the contamination of culture. *In vitro* culture condition there is problem of browning of media in many plant species (woody plants) due to leaching of some phenolic substances (Table 3). To prevent the browning problem in woody plant species use of antioxidants (activated charcoal PVP, ascorbic acid, citric acid) solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants. Shoot regeneration and emergence of root various concentration of auxin and cytokinin used, successful establishment *in vitro* plants are acclimatization in field condition. This review paper is widely focused on the problems that occur during plant tissue culture and suitable solution.

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