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POLYPHENOL INTERFERENCE IN MICROPROPAGATION OF INDIAN ROSEWOOD (*DALBERGIA LATIFOLIA* ROXB.)

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ABSTRACT

Investigations were carried out to control the polyphenol interference in Indian rosewood, *Dalbergia latifolia* Roxb. under tissue culture conditions. Explants from juvenile (3-4 years old trees) and mature (plus trees above 50-60 years of age) sources were used for the study. Differences in response to the treatments for phenolics were observed between juvenile and adult tissues. Polyphenol problem was practically absent when juvenile explants were inoculated. Ninety five per cent of the established cultures showed no signs of browning. On the other hand, browning of the medium was noticed in all the cultures when explants taken from the plus trees were incubated without taking measures to control the polyphenol interference. No individual treatment was found successful in controlling the polyphenol problem. However, a combination of methods including soaking the explants in a solution of ascorbic acid and citric acid each at 150 mgl^{-1} for 3 hours, incorporation of 150 mgl^{-1} each of ascorbic acid and citric acid and 0.7 per cent polyvinylpyrrolidone in the culture medium was found to be very effective.

Key words : Polyphenols, Browning, Tissue Culture, Rosewood, Dalbergia.

Introduction

A serious problem generally faced in *in vitro* culture of adult tissues from woody species is the exudation of phenolic compounds. Phenols leach out from cut surfaces of explants and turns the culture medium dark brown. Browning of the medium is due to the oxidation of phenols leading to the death of the explant. Growth inhibition is generally severe in species that naturally contain high levels of phenolics. Younger tissues are less prone to browning on excision than older ones (George and Sherrington, 1984). Phenolic compounds undergo oxidation to quinines by the enzymic actions of Mono Phenol Oxidase (MPO) and Poly Phenol Oxidase (PPO). The quinines hence formed are inhibitory to plant cellular growth and destroy enzymatic activities (Loomis and Battaile, 1966). The phenolics and enzymes are released or synthesized when tissues are wounded, cut or when senescence occurs. These phenolics, thus, have to be eliminated or

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neutralized for the establishment of the explant.

Indian rosewood (*Dalbergia latifolia* Roxb.), which ranks among the finest woods for furniture and cabinet works faces many problems in plantation programmes. The main constraints include inadequate supply of seedlings, poor quality of the seedlings and low seed germination per cent (Sita and Swamy, 1992). Hence, *in vitro* methods have been adopted to obtain large number of genetically pure elite populations. In the process of standardizing protocols, the problem of release of phenols in the culture was encountered. The present investigation undertaken at the College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur, India deals with the different approaches attempted to overcome the problems associated with exudation of phenolics.

Materials and method

Selection of Plus Trees : A survey was carried out to identify the candidate plus trees of rosewood in Vellanikkara and nearby areas. Three plus trees were selected based on the morphological and biometric observations (**Table 1**) from the identified candidate trees in the field. Nodal and/or internodal explants were collected from the selected plus trees. Explants from young (3-4 years old) rosewood trees were used to study the difference if any, between juvenile and adult tissues with respect to exudation of phenols.

Collection and Preparation of Explants : Stem segments of around 30-40 cm length with 12-15 nodes were excised from the selected plus trees/young trees and brought to the laboratory as quickly as possible. The cuttings were defoliated and washed to free the dust in running tap water using a detergent. After drying the segments in blotting paper, they were swabbed with cotton dipped in 70 per cent (v/v) ethanol. The stem segments were then cut into nodal segments of size 1.5 cm (approx.) using a sharp sterile blade.

Fungicidal Treatment : In order to control the contamination in cultures, the explants were immersed in a fungicidal solution containing 0.3 per cent each of Bavistin (Carbendazim), systemic fungicide and Indolfil-M-45 (Mancozeb) a contact fungicide, for 1 hour duration.

Surface Sterilization : The explants were immersed in mercuric chloride (0.1 per cent w/v) for 12 minutes with occasional stirring, inside a laminar airflow cabinet. The explants after surface sterilization were removed from the chemical and rinsed thrice in sterilized distilled water to remove traces of sterilant sticking to the surface. The explants then were spread on to a sterile petriplate lined with sterile blotting paper for drying.

Treatments : Various treatments attempted to control the polyphenol interference are given in **Table 2**. They included elimination, neutralization and adsorption of phenololics. A combination of various methods was also applied.

Media : Basal media used for the study included Murashige and Skoog (MS) medium

(1962) and Woody Plant Medium (WPM) (Lloyd & McCown, 1980). Benzyl adenine or 6-Benzyl Amino Purine (BA), Kinetin (Kin) and indole-3-acetic acid (IAA) were the different growth regulators used. The different treatments used for shoot proliferation were MS+BA (2.0 mgl^{-1}) for mature tissues and WPM+Kin (1.0 mgl^{-1}) + IAA (0.1 mgl^{-1}) for juvenile tissues (Warrier and Vijayakumar, 1999).

Culture Conditions: Cultures were incubated in a culture room provided with cool white fluorescent lamps with a light intensity of 2500 lux for 16 hours light (unless mentioned otherwise). The room temperature was maintained at 23±2°C. The experiment was laid out in a Completely Randomised Design (CRD). All the treatments were replicated four times with seven cultures each. Culture period was 6 weeks.

Results

The problem of exudation of phenols was practically absent in explants from young trees (with only a maximum of 5 per cent showing light browning) and hence no treatment was given. However, browning of the medium was noticed in all the cultures when explants collected from plus trees were inoculated without taking measures to control phenolic exudation. Browned cultures died 2-3 weeks after inoculation or showed no signs of growth. No individual treatment was found successful in controlling the polyphenol interference in mature tissues (**Table 2**).

For the explants from plus trees, a combination of methods including soaking the explants in a solution of ascorbic acid and citric acid each at 150 mgl^{-1} for 3 hours (elimination) and incorporation of 150 mgl^{-1} each of ascorbic acid and citric acid (reducing agents) and 0.7 per cent polyvinylpyrrolidone (adsorbent) in the culture medium was found very effective in controlling the problem. Application of the above approach resulted in obtaining 80 per cent of the cultures without browning.

Other treatments like placement of cultures in dark after inoculation, frequent subculturing and addition of charcoal did not help in checking the exudation of polyphenolic compounds in mature tissues.

Discussion

Polyphenol interference in culture establishment has been reported by several workers (Anderson, 1975; Llyod and McCown, 1980; Amin and Jaiswal, 1988 and Gill & Gill, 1994). Explants frequently turn brown or black shortly after isolation and when this occurs, growth is inhibited and the tissue dies. Growth inhibition occurs as a result of high levels of tannins or phenols. Various approaches were adopted to overcome this problem in the micropropagation of Indian rosewood. The study revealed that juvenile tissues were practically free from polyphenol interference. On the other hand, mature tissues required reducing agents (ascorbic acid and citric acid) and adsorbent (poly vinyl pyrrolidone) treatments in addition to leaching. Use of antioxidants like ascorbic acid and citric acid for controlling polyphenol exudation has been reported in Rhododendron (Anderson, 1975) and *Musa textiles* (Mante

and Tepper, 1983). Similar concentrations of antioxidants were used in the present study, which proved very effective. These antioxidants act by rapidly removing the quinones formed in the medium (Loomis and Battaile, 1966). Presence of poly vinyl pyrrolidone in the medium also helped in neutralizing the phenols released from the explants. Removal of phenolics by poly vinyl pyrrolidone has been achieved in teak (Gupta *el al.,* 1980), Hamamelis (Christiansen and Hoffenbech, 1975) and Guava (Amin and Jaiswal, 1988). Incorporation of activated charcoal and transfer of explants to fresh medium to reduce phenolics have been proved by Wang and Huan (1975) and Broome and Zimmerman, (1978) respectively. However, these two approaches did not yield good results in the present study. Gill and Gill (1984) observed seasonal variation in quantity of phenols exuded in the medium. They observed that maximum exudation occurred in the months of May-June and September-October, while during the period November-April, it was low.

George and Sherrington, (1984) suggest that no single method could be effective on all species. Often, a combination of treatments must be applied. In eucalyptus, soaking for 3 hours in sterile water followed by keeping the cultures in dark for the initial 8 days helped to reduce the formation of brown exudates at the base (Cresswell and Nitsch, 1975). In the present investigation, there was a variation in response of the juvenile (explants from 3-4 year old trees) and mature tissues (explants from plus trees). When juvenile explants could be inoculated without any treatment to control the phenol problem, mature explants required a combination of methods to overcome polyphenol interference. Further, the study suggests that the phenolic content of shoots could be used as a biochemical marker to differentiate juvenile and mature tissues in *Dalbergia latifolia*.

Table 1 – Biometric observations from the selected plus trees of *Dalbergia latifolia*

Tree No.	Total Height (m)	Clean Bole Height (m)	Girth (m)	Total Volume (m^3)	Clean Bole Volume (m^3)	Age (Approx.)
TR-1	19.42	9.29	1.52	2.50	1.54	Above 60 years
TR-2	19.32	5.76	1.12	1.35	0.52	Above 50 years
TR-3	20.02	8.70	1.22	1.66	0.93	Above 60 years

Table 2 – Treatment attempted to control *in vitro* polyphenol interference in mature tissues of *Dalbergia latifolia*

TREATMENTS	Response
A. ELIMINATION METHODS (LEACHING)	
a. Soaking the explants in distilled, sterile water for 3 hours	++
b. Soaking the explants in solution of citric acid at 150 mg l^{-1} for 3 hours	++
c. Soaking the explants in solution of ascorbic acid at 150 mg l^{-1} for 3 hours	++
d. Soaking the explants in solution of ascorbic acid and citric acid at 150 mg l^{-1} each for 3 hours	
B. NEUTRALISATION METHODS	
(i) Use of antioxidants or reducing agents in the media to reduce the redox potential	
a. Addition of ascorbic acid at the rate of 150 mg l^{-1}	+
b. Addition of citric acid at the rate of 150 mg l^{-1}	+
c. Addition of ascorbic acid and citric acid each at the rate of 150 mg l^{-1}	+
(ii) Removal of phenolics by using adsorbing agents in the media	
a. Addition of polyvinylpyrrolidone (PVP) at the rate of 7 g l^{-1}	+
b. Addition of activated charcoal at the rate of 0.25-1.00 g l^{-1}	+
(iii) Combination of antioxidants and adsorbents in the media	
a. Addition of 150 mg l^{-1} each of ascorbic acid and citric acid and 7 g l^{-1} of PVP	+
(iv) Reduction in enzyme activity and substrate availability	
a. Placing the cultures in the dark for the initial 8-10 days of culture	++
b. Subculturing the explant at short intervals	+
C. A COMBINATION OF METHODS	
a. Soaking the explants in a solution of ascorbic acid and citric acid each at 150 mg l^{-1} for 3 hours and incorporation of 150 mg l^{-1} each of ascorbic acid and citric acid and 0.7% PVP in the media	–

+ Release of polyphenols into the medium occurs but no death of the explants.

++ Release of polyphenols into the medium occurs followed by death of the explants.

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