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Direct regeneration of HFRI-5 from nodes internodes and apical shoot explants

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ABSTRACT

The genus Eucalyptus contains some of the most important commercial tree species in the production of raw material for the pulp and paper industry and for a future bioenergy and biorefinery industry. Attempts have been made to set up Eucalyptus regeneration system through in vitro technique. Tissue culture protocol of Eucalyptus HFRI-5 has been developed using juvenile meristematic tissues (leaves and nodal cuttings) as explants. Profuse number of multiple shoots were initiated on Murashige and Skoog's (MS) medium supplemented with vitamins, hormones and coconut milk. Physiological and biochemical parameters of hormones on regeneration system of Eucalyptus were studied. Induction of callus from leaf discs and nodal cutting was found to be regenerated in the presence of coconut water after six to twelve weeks of culture. The texture and response of callus obtained from explants, against different combinations of hormones were studied. Different anti-oxidants were also tested on the media for overcoming the phenolic excudation. Light conditions were also found to be important for regeneration. This protocol will facilitate the regeneration of selected Eucalyptus hybrid HFRI-5.

Keywords: Eucalyptus, HFRI-5, Explants, anti-oxidants

INTRODUCTION

2.1 *Regeneration*

Regeneration problems are one of the main limitations preventing the wider application of genetic engineering strategies to the genus *Eucalyptus* [1]. In woody plants, although micropropagation is possible through shoot tip and axillary bud culture, organogenesis and embryogenesis from callus are limited to only a few species.

Micropropagation can be used to offer high multiplication rates of selected tree genotypes of eucalyptus [2]. But the major problem with the micro propagation of eucalyptus is the browning of explants due to the oxidation of phenolic compounds released from the cut ends of the explants [3]. So, it is necessary to design an efficient regeneration protocol for eucalyptus. Although regeneration efficiency has been improved via somatic embryogenesis or organogenesis, genotype dependent regeneration, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, lack of shoot elongation, difficulties of rooting and browning which causes death of tissues or explants are the problems associaiated with tissue culture systems of eucalyptus [4] . Some of these problems are related to the plant material such as explant age or genotype and others to the culture conditions such as hormones, medium composition, or other physical culture conditions [5].

2.2 *Phenolic excudation*

Phenolic acids are intermediates of phenylpropanoid metabolism and precursors of lignin and phenylpropanoid phytoalexins [6] . Their deposition in cell walls is an important defence mechanism after pathogen infection [7]. When they are excreted from plant root system, they exert inhibitory growth function within adjacent rhizosphere

and they affect bacterial flora of the soil [8]. Being an important group of secondary metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid (IAA) catabolism.They are effective in plant growth regulation, cell differention and organogenesis [9] .There are two opinions on interactions between phenolics and plant growth and development. One indicates that phenolics are negatively related with plant in vitro proliferation while others mention the opposite [10]. In tissue culture studies, phenolic substances, especially oxidized phenolics generally effect in vitro proliferation negatively [11]. Tissue browning and blackening are also one of the major problems for in vitro culturing many economically important plants [12]. When cells are damaged, the contents of cytoplasm and vacuoles are mixed and phenolic compounds can readily become oxidized by air [13]. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants. Liquid media can be used to reduce phenolic oxidation [14]. In addition frequent subculturing, some antioxidants such as citric acid and ascorbic acid, PVP (polyvinyl pyrolidone) and activated carbon, which are added into medium, can also reduce phenolic oxidation and contribute to regeneration from explants [15].

Phenolic concentration is frequently affected by several internal and external factors. Light appears to induce flavonol synthesis in the chloroplasts and cytoplasm. Some nutrieents, especially carbohydrate supplies influence the phenolic composition. Some stress factors like drought, water, radiation, and pathogen infection from injured surfaces effect concentrations of the phenolics in plants [16]. Phenol concentrations can also be increased and decreased in different stages of germination. Therefore, determination of the lowest phenol concentration phase during germination and isolation and culturing of explants in this phase will increase regeneration response and success in tissue culture studies [17],[18],[19]. The purpose of the study reported here was to develop and optimize a simple in vitro protocol for the micropropagation of identified *Eucalyptus* species for raising pulp plantations. For this purpose, fast-growing *Eucalyptus* hybrid *HFRI-5* with better physical and phenotypic traits were identified from Eucalyptus plantations[20].

MATERIALS AND METHODS

3.1 *Explant source*

Nodes, internodes and leaf discs obtained from the natural F1 hybrid of *Eucalyptus*, that is FRI-5 (*E.camaldulensis* Dehn x *E. tereticornis* Sm) grown at New Forest experimental field of Forest Research Institute, Dehradun ranging in age between 4 and 8 years,were used as explants. This hybrid produced higher biomass than the parental progenies and also displayed a very high degree of hybrid vigour and proved 3-5 times superior in growth parameters than the parent combinations.

3.2 *Preparation of explant and surface sterilization:*

The explants were washed thoroughly under tap water for 1–2 h with a few drops of Tween-20 to remove adhering dust and any other foreign material. The cuttings were first defoliated, leaving the petiole attached to the stem, and then cut to smaller sizes (each piece with 4–5 nodes) to facilitate proper cleaning during the decontamination procedure. The surface sterilization procedures and inoculation of plant materials were carried out in a clean air laminar flow cabinet under aseptic conditions. Small shoots containing three to four nodes were surface-sterilized by a 30-s submersion in 70% ethanol, followed by treatment with a 0.1% (w/v) solution of mercuric chloride for 2 min and then a thorough washing with sterile double-distilled water to completely remove the mercuric chloride.

3.3 *Culture media and growth conditions*

Murashige and Skoog medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration from tissues and callus. Due to its content of K and N salts it is a “high salt” medium. We used basal MS media. Sucrose was added to the media as an external carbon source. Sucrose was preferred because it is cheap and easily available. Also 10gm of poly venyl-pyrolidone (PVP) was added as an antioxidant to prevent the browning of media.We also used 50 mg/l of the coconut water as a natural growth regulator. Myo-inositol classified as the member of Vitamin-B complex was added which plays role for the growth of cells in tissue culture. After proper mixing of sucrose in media pH of solution was adjusted to 5.8 which was necessary for the addition and dissolving of agar. At pH below 5.5 agar will not solidify properly while at above 6.0, agar will become too firm. Also the required pH was necessary to support different phases of growth and differentiation. pH was adjusted by adding NaOH or HCl. After that agar was added and heated at 100°C for proper mixing.

RESULTS AND CONCLUSION

Different combinations of phytohormones were used to see the frequency of the formation of callus from leaves and nodal cuttings. When nodal cuttings and leaf explants taken from rooted plantlets growing in vitro were cultured on different media in order to induce callus formation, in most cases the explants turned brown. Large quantities of

dark brown exudates (presumably phenolics) were secreted into the medium. Healthy callus was formed from leaf and nodal explants in the dark within 10 to 12 days (Fig. ia). Explants grown in light generally turned brown and callus formation was poor (Fig. ib).Of the different concentrations tested, 1 mg/l BAP supplemented with 0.1 mg/l NAA yielded the best callus from leaves in terms of growth rate and appearance and 0.5 mg/l BAP plus 5 mg/l 2.4-D yielded the best callus from nodal cuttings (Table i and Table ii).

Table I. The effect of different concentrations of BAP, kinetin, 4-D and NAA on the induction of callus from leaf explants of *E. HFRI-5* on MS medium.

Formulations (Basal MS media)	Cytokinin(mg/L)		Auxin (mg/L)		No. of explants used	Callus forming explants
	BAP	Kinetin	NAA	2,4 D		
F1	0	0	0	0	5	0
F2	**1**	**-**	**0.1**	**-**	**5**	**4**
F3	2	-	0.5	-	5	2
F4	3	-	1	-	5	2
F5	5	-	2	-	5	1
F6	-	1	-	0.5	5	1
F7	-	3	-	0.1	5	0
F8	-	5	-	1	5	0
F9	-	6	-	2	5	3
F10	1	-	-	0.1	5	3
F11	3	-	-	1.5	5	2
F12	5	-	-	1	5	1
F13	10	-	-	5	5	0
F14	-	0.1	5	-	5	4
F15	-	1	10	-	5	1
F16	-	10	1	-	5	0
F17	-	20	0.1	-	5	0

Table II. The effect of different concentrations of BAP, kinetin, 4-D and NAA on the induction of callus from nodal cuttings of *E. HFRI-5* on MS medium.

Formulations (Basal MS media)	Cytokinin(mg/L)		Auxin (mg/L)		Total explants used	Callus forming explants
	BAP	Kinetin	NAA	2,4 D		
F1	0	0	0	0	5	0
F2	1	-	0.1	-	5	0
F3	2	-	1	-	5	1
F4	3	-	0.5	-	5	1
F5	**5**	**-**	**0.5**	**-**	**5**	**3**
F6	-	1	-	0.5	5	1
F7	-	3	-	0.1	5	0
F8	-	5	-	1	5	0
F9	-	6	-	10	5	2
F10	0.1	-	-	5	5	1
F11	**0.5**	**-**	**-**	**5**	**5**	**3**
F12	5	-	-	10	5	1
F13	10	-	-	0.1	5	0
F14	-	0.1	5	-	5	2
F15	-	1	10	-	5	1
F16	-	10	1	-	5	0
F17	-	20	0.1	-	5	0

The callus was compact hard and greenish white in the beginning and turned brown if allowed to grow on the same medium for more than 3-4 weeks (Fig Ib).

When either the leaf or the stem callus was excised and transferred to the fresh medium with different concentrations of BA alone, no shoots were produced in any case.

Excised callus pieces were transferred to medium containing 10% coconut milk and 500 mg/l PVP. BAP was added in different concentrations to the shoot induction media and its effect was studied. It was seen that the best result from leaf callus was obtained at 5 mg/l of BAP (Fig IIa) and in case of callus obtained from nodal cuttings, at 3 mg/l of BAP comparatively a high number of shoot proliferation was observed (Fig IIb). No shoots were produced in BAP concentrations lower than 3 mg/l or higher than 5 mg/1 (Table iii and Table iv).

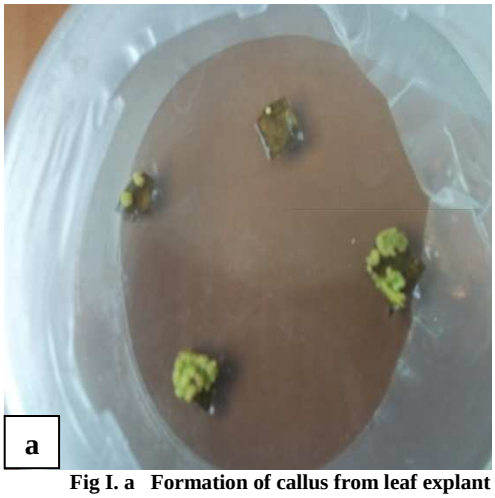

Fig I. a Formation of callus from leaf explant

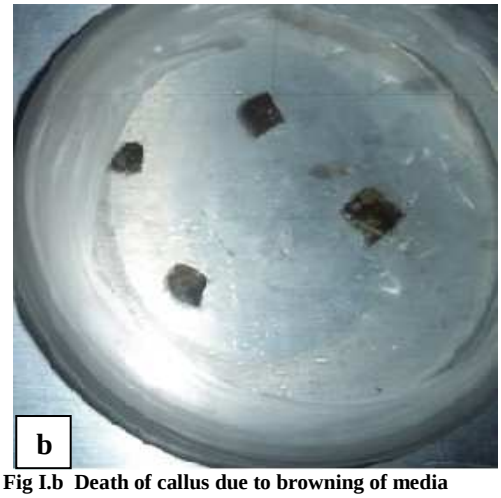

Fig I.b Death of callus due to browning of media

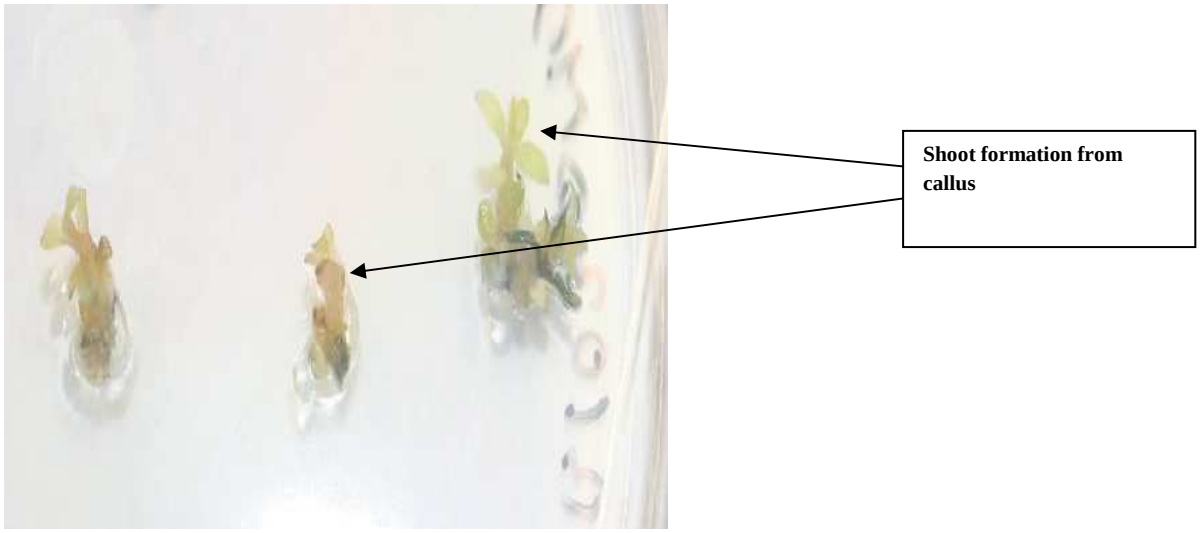

Fig II (a). Regeneration of the shoots from leaf callus

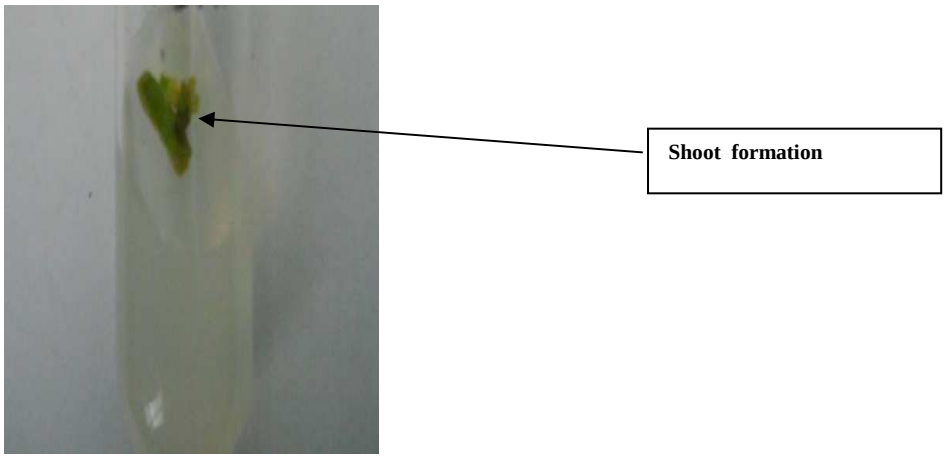

Fig II (b). Regeneration of the shoots from leaf callus

Table III. The effect of different concentrations of BAP on shoot regeneration from leaf callus of *Eucalyptus.HFRI-5*.

BAP (mg/l)	Total explants	Explants forming shoots
0	4	0
0.05	4	0
0.5	4	0
3	4	1
5	4	2
10	4	0

Table IV. The effect of different concentrations of BAP on shoot regeneration from nodal cutting callus of *Eucalyptus.HFRI-5*.

BAP (mg/l)	Total explants	Explants forming shoots
0	4	0
0.05	4	0
0.5	4	1
0	4	2
5	4	1
10	4	0

DISCUSSION

Several authors have reported methods for in vitro regeneration of eucalyptus. However, these regeneration methods were developed for specific varieties. They do not solve the problem of phenolic compounds killing explants. Therefore, in our study we examined the effects of phytohormones and polyphenol adsorbent and antioxidants on regeneration in different types of explants and identified the adsorbent, antioxidant and hormonal combination of explant that would be most effective for in vitro regeneration of this crop.

5.1 Culture conditions

We studied the effect of different hormone concentrations on the regeneration of *Eucalyptus* and found that1 mg/l BAP supplemented with 0.1 mg/l NAA yielded the best callus from leaves in terms of growth rate and appearance and 0.5 mg/l BAP plus 5 mg/l 2.4-D yielded the best callus from nodal cuttings. Shoot regeneration from the leaf callus was observed with 5 mg/l of BAP and at 3 mg/l of BAP in case of callus obtained from nodal cuttings. Concentrations of BAP higher or lower than this showed negligible results

5.2 Techniques applied for reducing phenolic compound secretion of explants.

One of the most common problems associated with the *in vitro* establishment of many monocotyledonous and woody species is the deleterious effects of oxidized phenols. Oxidized phenolic compounds inhibit enzyme activity and darken the culture medium. The explants brown or blacken, and die. In our experiment we tried to reduce injury associated with phenolic oxidation. An antioxidant is an electron donor (reducing agent) which inhibits the oxidation of labile substrates. The antioxidant compounds utilized in the experimental work were selected because they have been used successfully in the past to delay browning in other monocotyledonous species.

The successful prevention of browning in explants of *Eucalyptus* was seen by using 10% PVP.PVP behaves as a chelating agent bonding to ions responsible for activating polyphenol oxidative enzymes. Activated charcoal was also used in our experiment.Charcoal is able to scavenge oxygen radicals produced when tissue is damaged and therefore cells are protected from oxidative injury. Oxygen radicals are attributed to exacerbating oxidative injury. But activated charcoal when added in the medium as an polyphenol adsorbant increased the chances of contamination. So we concluded that addition of PVP folowed with the frequent subculturing of the media was a better way to decrease the browning of the media and to prevent the tissue from death.

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