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OPTIMISATION OF MEDIA COMPONENTS FOR SOMATIC EMBRYOGENESIS FROM ANTHERS OF *HEVEA BRASILIENSIS*

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Somatic embryogenesis was initiated from immature, anther-derived callus of *Hevea*. Embryogenic callus with prominent proembryos were obtained using modified MS medium along with BAP (1 mg/L), NAA and 2,4-D (1 mg/L) each. Somatic embryos were obtained from these calli when cultured on media containing kinetin (1 mg/L) and IAA (0.1 mg/L). Histological studies of embryogenic callis revealed that the meristematic cells at the periphery of the calli produce somatic embryos. Clonal variation was also observed in callus induction and somatic embryogenesis.

Key words: Anther, Clonal variation, Multiple shoots, Phytohormones, Somatic embryogenesis.

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INTRODUCTION

Tripura is the smallest state of North-East India and the second largest State in area under rubber (*Hevea brasiliensis*) cultivation in the country. The climate of Tripura a non-traditional rubber growing region, is widely different from that prevailing in the traditional region, in terms of low winter temperature, which falls below 10°C (Jacob *et al.*, 1999) with partial soil moisture deficiency (Saseendran *et al.*, 1993) and high summer temperature. In the non-traditional areas of India rubber trees flower during late February to April (Meenattoor *et al.*, 1989; Sudhasowmyalatha *et al.*, 1997) while in the traditional belt, flowering starts in January. Microsporogenesis and anther formation are likely to be affected by variations in climatic pattern. Plant regeneration through somatic embryogenesis and development of synthetic seeds may become advantageous in overcoming such problems in propagation. This method will also facilitate shortening the breeding cycle. Genetic transformation during somatic embryogenesis can also be attempted. Hence induction of haploids from immature anthers of *H. brasiliensis* was attempted at the Regional Research Station of Rubber Research Institute of India at Agartala in Tripura. The optimisation of media components was taken up as this is the most important pre-requisite for successful somatic embryogenesis.

MATERIALS AND METHODS

Young flower buds of RRII 105 and SCATC 93/114 were collected. Their developmental stages were examined under a microscope using aceto-carmine squash method and flower buds of optimum size studied. After thorough washing with Teepol, the buds were surface sterilized with 0.02 per cent mercuric chloride and placed in few drops of Tween 20 for 5 to 7 min, followed by thorough washing with sterilized distilled water. Anthers were dissected out aseptically and cultured on induction medium (20 ml/tube). Half strength of the MS (Murashige and Skoog, 1962) inorganic salts was added to the medium with the normal quantum of organic nutrients. Casein hydrolysate (CH, 100 mg/L), adenine sulfate (AdS, 2 mg/L) and 10 per cent coconut water (CW) were added as organic supplements along with sucrose (3%) and phytagel (2%) for inducing callus proliferation. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. For callus in-

duction, different auxins like indole-acetic-acid (IAA), indole-buteric-acid (IBA), naphthalene-acetic-acid (NAA) and 2, 4-dichlorophenoxy-acetic-acid (2, 4-D) were used at different concentrations (0.05 – 5 mg/L) in combination with kinetin (Kn) or benzyl-amino-purine (BAP). Cultures were incubated in the dark for six weeks at 25°±1°C. After callus induction and proliferation, the calli were subcultured on media containing BAP and a combination of 2,4-D and NAA for induction of proembryos. Different levels of auxins and cytokinins were tested in all possible combinations in the media containing 5 per cent sucrose and devoid of CH and CW.

For histological studies, embryogenic tissues containing embryos were fixed in formaldehyde/glacial acetic acid/ethanol (FAA, 5:5:90 v/v/v) for 24 h at 10°C, dehydrated through a graded ethanol series and embedded in saturated paraffin wax. Embedded materials were sectioned at 5 mm thickness on a rotary microtome. Paraffin wax was removed with xylene prior to rehydration of tissues through a graded ethanol series. Staining was done with 1 per cent (w/v) safranin.

Percentage response for callus induction was estimated after six weeks of culture. Somatic embryo formation and number of embryos per explant were recorded after 18 to 20 weeks of culture in the regeneration medium. Experiments each consisting of 35 cultures were repeated three times.

RESULTS AND DISCUSSION

Though it has been reported that MS-S-4 medium could induce callus from anthers of rubber clone PB 5/51 (Das *et al.*, 1994), the experimental materials used in the present investigation (RRII 105 and SCATC 93/114) did not respond well in this medium. The concentration of inorganic salts played a critical role in induction of callus from the anthers (Table 1). Half concentration of inorganic salts in the MS medium induced callogenesis. After the second subculture, no further growth was observed. Addition of 100 mg/L of CH and 2 mg/L AdS increased the proliferation of callus. The concentrations as well as combinations of different phytohormones were also seen to be very critical. Of all the combinations of auxins and cytokinins, 1.5 mg/L of 2,4-D along with 1 mg/L of BAP was found to be optimum for proliferation of friable callus from the anthers. Friability or compactness of callus was seen to be induced by higher concentrations of 2,4-D and BAP respectively. Similar observations were also recorded using anthers of *Hevea*. Induction of embryogenic callus i.e., the callus containing greenish white, minute, shiny, proembryos, as obtained using MS medium with necessary modifications of phytohormones *viz.*, BAP (1 mg/L) and a combination of NAA and 2,4-D (1 mg/L each). It was observed that these proembryos did not grow further to develop into mature somatic embryos even after culturing them two to three times on the same medium. Several

Table 1. **Effect of different doses of MS inorganic constituents on callus induction from immature anthers of *Hevea brasiliensis***

Strength of constituents in MS medium	Clones			
	RRII 105		SCATC 93/114	
Major full + minor full	++	SW	++	SW
Major half + minor full	++	CD, Blw, C	++	SW
Major full + minor half	+++	CD, W, C	++	CD, W, C
Major half + minor half	++++	CD, Yw, F	+++	CD, Yw, F

MS : Murashige and Skoog medium; SW : Swelling only; CD : Callus development; Blw : Blackish white callus; Yw : Yellowish white callus; C : Compact callus; F : Friable callus; W : White callus
Response rating: + less than 25%, ++ less than 50%, +++ more than 50%, ++++ more than 70%.

manipulations were made in the regeneration medium by addition and deletion of growth regulators and other organic components. The concentration of sucrose was enhanced to 5 per cent and CW and AdS were omitted. It was found that a combination of Kn ranging from 0.1 to 5 mg/L and IAA ranging from 0.05 to 2.5 mg/L was best for induction of healthy somatic embryos. Out of the 63 different combinations of Kn and IAA tried, only five combinations responded by generating somatic embryos. Combinations and concentrations of the media in which there was response were (A) Kn 0.5 mg/L + IAA 0.05 mg/L; (B) Kn 1.0 mg/L + IAA 0.5 mg/L; (C) Kn 1.0 mg/L + IAA 0.25 mg/L; (D) Kn 1.0 mg/L + IAA 0.1 mg/L and (E) Kn 0.5 mg/L + IAA 0.1 mg/L.

The anther culture of RRII 105 showed highest percentage of somatic embryogenesis in medium D, followed by medium A (Fig. 1). In medium D, 15 to 22 somatic embryos were observed from each explant. Embryos were well formed and were loosely arranged on the peripheral tissues of the callus mass. In medium A, although a good number of somatic embryos were formed, they were small and compactly associated with the surrounding tissues. In a similar study with SCATC 93/114 under the same combinations of media, a similar trend of regeneration in somatic embryos was observed. However, the percent regeneration was much less compared to RRII 105. Thus medium D was most suitable for somatic embryo induction from anther derived calli of *Hevea*. The somatic embryos obtained showed a range of diversity in their morphology i.e., from globular, torpedo to cotyledon shaped (Fig. 2-4). The better response of RRII 105 to induction of embryogenesis from anther derived callus compared to that of the SCATC 93/114 also suggests that the embryogenic potential of *Hevea* is genotype-dependent. Such an observation was also made by El Hadrami *et al.* (1991) while working on *Hevea* callus culture derived from internal integuments of immature seeds and by Jayasree *et al.* (1999) with immature anther.

Addition of excess phytohormones or growth regulators in the initial culture me-

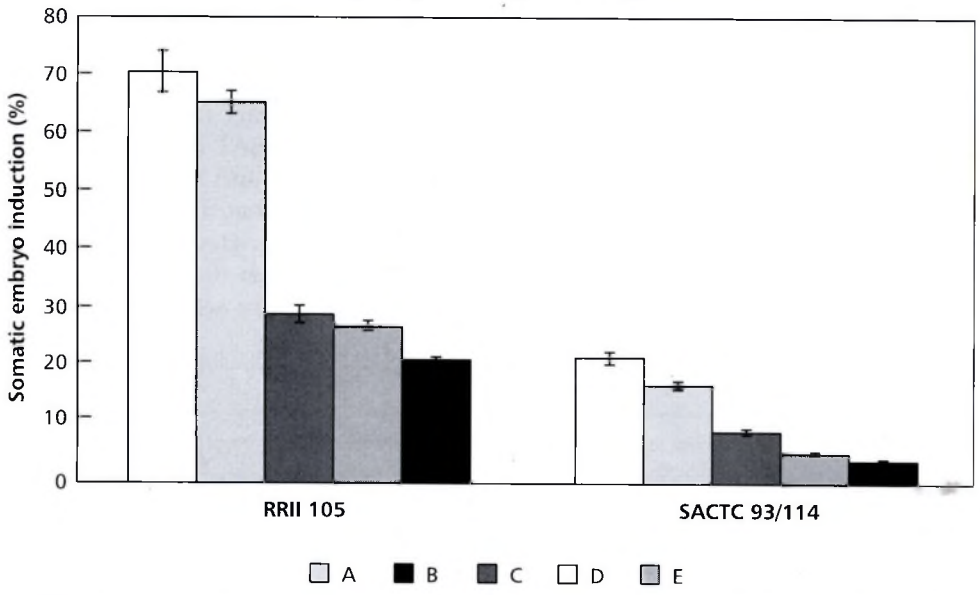
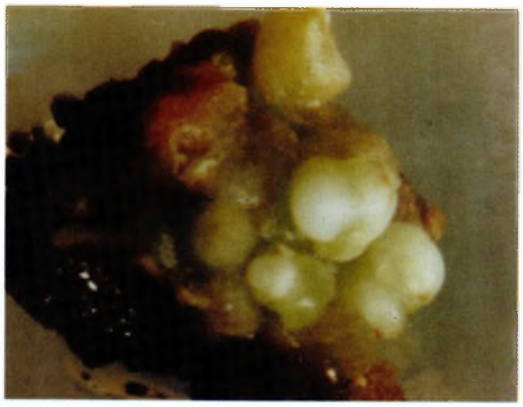
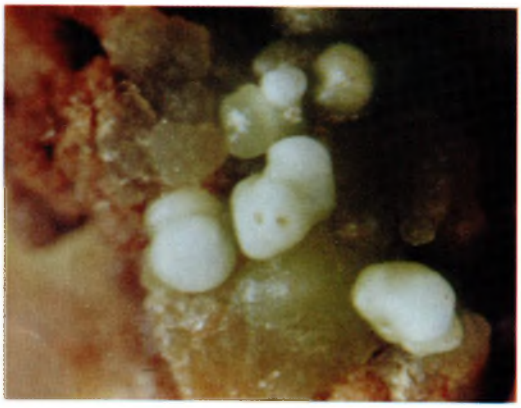


Fig. 1. Effect of different phytohormones on induction of somatic embryos from anther-derived callus of *H. brasiliensis*; A: Kn 0.5 mg/L + IAA 0.05 mg/L; B: Kn 1.0 mg/L + IAA 0.5 mg/L; C: Kn 1.0 mg/L + IAA 0.25 mg/L; D: Kn 1.0 mg/L + IAA 0.1 mg/L and E: Kn 0.5 mg/L + IAA 0.1 mg/L.





Figs. 2 and 3. Formation of globular or torpedo shaped somatic embryos from the anther derived callus of *Hevea brasiliensis*.

dium has negative effect on embryogenic potential in rubber. This may be due to arrested meristematic activity (Michaux – Ferriere and Carron, 1989). Thus, emphasis was always given to the use of optimum dosage of external growth regulators. Other investigators have recorded similar observation while working with rubber (El Hadrami *et al.*, 1991) and *Medicago sativa* (Meijer and Brown, 1987). On the contrary, in crops like *Vicia narbonensis* (Pickardt *et al.*, 1989) and alfa alfa (Stuart *et al.*, 1985) the excess concentration of phytohormones like 2,4-D induced greater number of somatic embryos.

The histological study showed actively dividing meristematic cells at the periphery of the callus that gradually formed compact masses consisting of parenchymatous cells (Fig. 5). These developing peripheral layers burst and cut off after intense metabolic and mitotic activity and evolved the embryonic units (Fig. 6). These units were identified as the globular proembryos. A similar observation was also recorded in *Hevea* by Michaux-Ferriere *et al.* (1992). Longitudinal sections through torpedo or cotyledon shaped embryos suggested the presence of an apical tip with a bilateral symmetry and cotyledon formation (Fig. 7). A posterior end

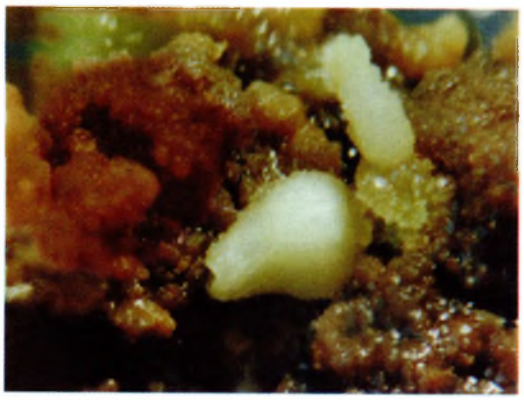


Fig. 4. Single cotyledon shaped somatic embryo from the anther derived callus of *Hevea brasiliensis*.

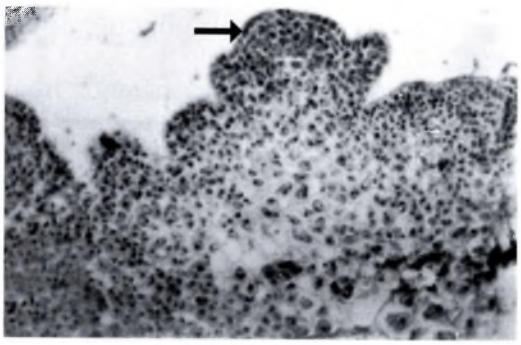


Fig. 5. Logitudinal section through embryogenic anther callus of *Hevea brasiliensis* showing somatic embryos. Arrow represents the formation of compact globular masses from the peripheral layer of the callus.

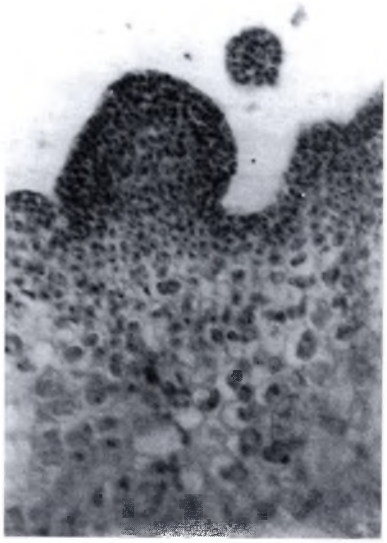


Fig. 6. Longitudinal section through an individual embryonic unit.

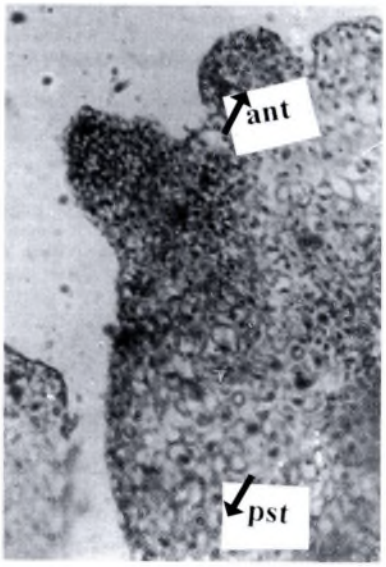


Fig. 7. Longitudinal section of cotyledon shaped somatic embryo. Arrows show the formation of bilateral symmetry (ant : anterior end, pst : posterior end).

was also observed indicating the initiation of root. Similar development has been reported for *Hardwickia binata* (Chand and Singh, 2001). The formation of an individual epidermal layer around the embryo unit separated it from the surrounding callus mass.

The data on callus regeneration from anther derived calli through somatic embryogenesis and the supportive histological evidence showed that RRII 105, grown in the non-traditional belt like Tripura, is a better explant source than the Chinese clone SCATC 93/114. It may be concluded that somatic embryogenesis can be induced from the anther derived callus of clones RRII 105 and SCATC 93/114 in *Hevea brasiliensis* using modified MS medium containing half the concentration of inorganic components and 5 per cent sucrose in combination with Kn (1.0 mg/L) and IAA (0.1 mg/L).

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