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Influence of 2,4-D and Sucrose on Repetitive Embryogenesis in Rubber (*Hevea brasiliensis* Muell. Arg. cv GT1)

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ABSTRACT

Repetitive embryogenesis system in rubber (*Hevea brasiliensis* cv GT1) was studied. Primary somatic embryogenesis was generated on callus derived from integumental tissue, cultured on B-5 medium supplemented with 0.5 mg/l NAA 2.0 mg/l kinetin, 0.5 mg/l IAA. Repetitive embryogenesis was induced from the primary somatic embryos cultured on B-5 medium supplemented with 0.5 mg/l NAA, 2.0 mg/l kinetin and 0.5 mg/l IAA and 4.0 mg/l 2, 4-D. It was markedly enhanced by the presence of 4.0 mg/l 2, 4-D and 5% sucrose.

Keywords: Repetitive embryogenesis, *Hevea brasiliensis*, somatic embryogenesis.

INTRODUCTION

Large scale *in vitro* plant production is achieved through either shoot organogenesis or somatic embryogenesis. Plant regeneration may occur in both pathways, but the relative merit of the system depends in part on operational convenience leading to cost-effectiveness. Two types of somatic embryogenesis have been distinguished by Sharp et al. (1984); direct and indirect. Direct somatic embryogenesis is considered as the development of an embryo directly from the explant source; in indirect somatic embryogenesis, embryos derived from callus or cell suspension or from cells or group of cells or somatic embryos. The latter process is considered "repetitive embryogenesis" (Tulecke 1987). Embryos derived through this pathway are sometimes described as adventitious or secondary. Although repetitive embryogenesis has been described in a number of species (Baker and Wetzstein 1995, Durham and Parrot 1992, Muraleedharan et al. 1989). There have been no reports of this phenomenon in rubber (*Hevea brasiliensis*).

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The purpose of this study was to develop as well as to enhance the efficiency of a repetitive embryogenesis system in rubber. In this report we describe a successful procedure facilitating embryo production by repetitive embryogenesis influenced by 2, 4-D and sucrose levels in the media.

MATERIALS AND METHODS

Induction of somatic embryogenesis: Immature fruits, 4 to 6 weeks after pollination, were collected from field grown plants of *Hevea brasiliensis* cv GT1, rinsed for 15 min in water, transferred to 40% ethanol for 2 min followed by 10 min wash in 0.5% sodium hypochlorite and then thoroughly rinsed several times with sterile water. The integuments were excised and cultured on MI-1 medium (Table 1) containing B-5 formulation supplemented with 2.0 mg/1 2,4-D, 3.0% (w/v) sucrose and 0.2% Gelrite with a PH of 5.6. Each culture tube contained one explant weighing about 0.2 g and they were kept in the dark for 6 weeks at 25±1°C for callus induction.

The callus was subcultured on two media formulations (MI-2 and MI-3; Table 1). MI-2 contained B-5 medium with the following growth regulator regimes: 0.0 – 4.0 mg/1 NAA in combination with 0.0 – 5.0 mg/1 kinetin (both in increments of 0.5 mg) and 0.5 mg/1 IAA (common to all combinations). The MI-3 contained B-5 medium supplemented with 0.5 mg/1 NAA, 2.0 mg/1 NAA, 2.0 mg/1 kinetin and 0.5 mg/1 IAA (Asokan et al.1992a and b). Both MI-2 and MI-3 contained 3.0% (w/v) sucrose, 0.2% Gelrite at pH 5.8 and cultures kept under 18 hr photoperiod (6.9 Wm) at 25±1°C for 6-7 weeks, and replicated 3 times.

Repetitive embryogenesis: Two procedures were followed for the induction of repetitive embryogenesis, one using primary embryos and other using newly differentiated plantlets.

Primary embryos: About 6-7 week old cotyledonary type somatic embryos, were dissected individually and subcultured on three media formulations: MII-1, MII-2 and MII-3 (Table 2). The medium MII-1 contained B-5 supplemented with 0.5 mg/1 NAA, 2.0 mg/1 kinetin, 0.5 mg/1 IAA and

Table 1: Effect of kinetin and NAA on somatic embryogenesis in *Hevea brasiliensis*. The means (±SD) of number of embryos originated within each kinetin/NAA combination were pooled from 3 tests (figures were rounded off to eliminate decimals.)

NAA (mg/l)	Kinetin (mg/l)									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
0.5	17(±8)	20(±4)	31(±6)	47(±6)	21(±11)	23(±7)	20(±10)	14(±2)	13(±10)	11(±10)
1.0		8(±4)	19(±8)	21(±9)	17(±3)	14(±7)	8(±5)	12(±11)	15(±13)	-
1.5					8(±4)	19(±7)	7(±3)	10(±8)	9(±6)	
2.0								7(±5)		

Table 2: Media response during primary and repetitive embryogenesis of integumental tissue derived callus of *Hevea brasiliensis*. The mean (±SD) of number of embryos originated in each combination were pooled from 3 tests

Primary embryogenesis	Media	The mean (±SD) number of primary somatic embryos per explant	
MI-1	B5 + 2.0 mg/l 2,4-D	0	
MI-2	B5 + 0.0-4.0 mg/l NAA + 0.0-5.0 mg/l Kinetin + 0.5 mg/l IAA	16(±9)	
MI-3	B5 + 0.5 mg/l NAA + 2.0 mg/l kinetin + 0.5 mg/l IAA	47(±6)	
Repetitive embryogenesis	Media	The mean (±SD) number of repetitive embryos per explant	
		From primary embryos	from plantlets
MII-1	B5 + 0.5 mg/l NAA B5 + 2.0 mg/l kinetin B5 + 0.5 mg/l IAA B5 + 4.0 mg/l 2,4-D	86 (±7)	28(±6)
MII-2	B5 + No hormone	8(±3)	0
MII-3	B5 + 0.5 mg/l NAA +2.0 mg/l kinetin +0.5 mg/l IAA	13(±5)	7(±4)

4.0 mg/l 2, 4-D, where MII-2 was hormone free B-5 medium. MII-3 was similar to MII-1 but lacked 2,4-D. The other media constituents and culture conditions were the same as for the induction of primary somatic embryogenesis.

Plantlets: Morphologically fully differentiated plantlets developed through the process of primary somatic embryogenesis, having distinct root and shoot bipolarity were subcultured on to MII-1, MII-2 and MII-3 media (Table 2). The other culture components and conditions were same as for primary somatic embryogenesis.

Effect of sucrose levels on repetitive embryogenesis: The frequency of repetitive embryogenesis was tested with the following sucrose levels: 0 – 80 g/1, in increments of 10 g (Table 3). The medium used was MII-1 (Table 2) since it was the optimal formulation for repetitive embryogenesis. The Gelrite composition was 0.2%, pH was adjusted to 5.8 and media were autoclaved at 121° C for 20 min. The culture conditions were similar to that of induction of primary somatic embryogenesis.

Histological study: Callus having embryogenic protuberances and somatic

Table 3: The effect of sucrose levels on repetitive embryogenesis of integument derived callus of *Hevea brasiliensis*. The mean (±SD) of number of embryos originating at each sucrose level were pooled from 4 tests

Sucrose g/l	Mean (±SD) number of embryos/explant after 4 weeks	Mean (±SD) number of embryos/explant after 8 weeks
10	0	11(±9)
20	16 (±9)	26(±12)
30	12(±6)	76(±7)
40	18(±3)	80(±11)
50	68(±13)	134 (±9)
60	17(±11)	62(±13)
70	0	19(±12)
80	0	0

embryos were fixed in FAA dehydrated through graded TBA series, infiltrated and embedded in paraffin wax (Johansen 1940). Serial sections of 6-8 µm thick were cut on a rotary microtome (Reichert Histostat) and stained with periodic Acid- Schiff's reagent for general histology (Jensen 1962).

Plant regeneration: Plants regenerated from bipolar embryos were planted in a sand and soil (3:1) mixture in polybags (9x3 cm). Relative humidity (RH) of 70% was maintained for 3-4 weeks, then plants were transferred to larger polybags (30x50) cm and relocated to a shade house and subsequently planted in the field.

RESULTS AND DISCUSSION

The integumental tissue, cultured on a B-5 medium supplemented with 2.0 mg/1, 2,4-D, generated friable embryogenic callus after 6 weeks. This callus, when subcultured on the same medium supplemented with NAA (0.0 – 4.0 mg/1), kinetin (0.0 – 5.0 mg/1) and IAA (0.0 – 5.0 mg/1) MI-2 medium) regime, produced somatic embryos (Fig. 1A), predominantly cotyledonary types, followed by globular and fasciculated abnormal types, after 4 to 5 weeks of culture. Embryogenesis was confined to 0.5 –5.0 mg/1 kinetin in combination with 0.5 –2.0 mg/1 NAA combinations and 0.5 mg/1 IAA (Table 1). The mean (±SD) number of embryos obtained within this wider growth regulator regime was 16 (±9) which could be enhanced over almost three times in a growth regulator combination of 0.5 mg/1 NAA with 2.0 mg/1 kinetin and 0.5 mg/1 IAA. Some of our earlier work (Asokan et al. 1992a and b) also indicated that kinetin was preferable to other cytokinins as reported in anther culture of *Hevea brasiliensis* (Chen 1984). However, Carron et al. (1989) reported that 0.5 mg/1 BA was essential for embryo generation in rubber. Two types of explants were used for repetitive embryo induction, primary cotyledonary somatic embryos and well differentiated bipolar plantlets, both derived from callus subcultured on MI-3 media. The primary somatic embryos when subcultured, underwent repetitive embryogenesis (Fig. 1b). Among the 3 media formulations used for adventive embryo induction, 2 media displayed several cycles of asynchronous embryo development. The first emergence of these embryos was noticed as early as 10

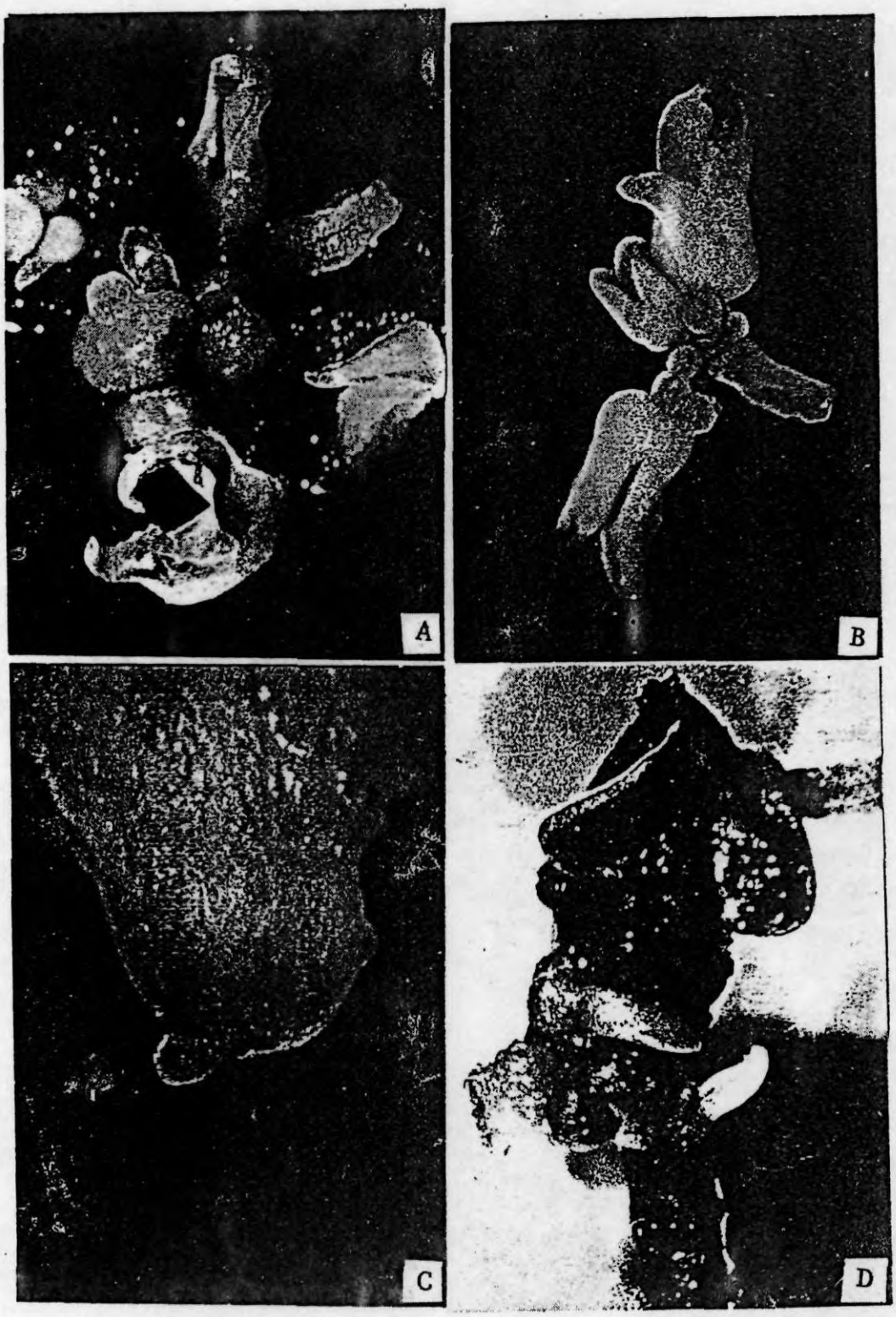

Fig.1: **A.** Somatic embryogenesis emerging from integumental derived callus in *Hevea brasiliensis*; **B.** Repetitive embryogenesis from primary somatic embryo culture; **C.** Point of origin of repetitive embryogenesis of a primary somatic embryo in culture; **D.** Repetitive embryogenesis localized at the hypocotyle region of a fully developed plantlet.

days in culture and point of origin was generally at the base of the primary embryos (Fig.1c). They first appeared as globular protuberances in singles or groups and developed into cotyledonary shapes 3-4 weeks after emergence. Primary embryos gave rise to secondary embryos on all three media (Table 2). Plantlets produced secondary embryos on both MII-1 and MII-3, but no secondary embryos were observed on plantlets cultured on the hormone-free MII-2 medium. The difference between MII-1 and MII-3 media was the presence of 4.0 mg/1 2, 4-D in the former. In MII-1 the mean (±SD) number of visible embryos was 86 (±7); for MII-3 the mean (±SD) number was 13(±5) (Table 2). This six fold increase in embryo production was frequently influenced by the presence of 4.0 mg/1 2,4-D. Enhancement of repetitive embryogenesis by 2,4-D was reported in peanuts (Finer and Nagasawa 1988, Durham and Parrot 1992, Baker and Wetzstein 1994), peach and nectarines (Bhansali et al. 1990). The 2,4-D may progressively insuce embryogenic competence for continued production of primary somatic embryos, leading to a longer duration of generation cycles. Over 4 cycles of repetitive embryogenesis were observed from primary embryos cultured on MII-1 and MII-3 media.

In plantlets derived from primary somatic embryos, most repetitive embryos originated from the hypocotyl region (Fig.1d), emerging as single embryos or asynchronous multiplication giving rise to several clusters. Though the majority of embryos occurred at the hypocotyl region (Fig.1d) embryos were occasionally observed at the root pole. The mean (±SD) number of embryos on MII-1 media formulation (Table 2) was 28 (±6).In the hormone free medium no embryos were observed. MII-3 which is lacking only for 2,4-D produced a mean (±SD) number of 7 (±4) embryos, presumably due to the presence of other auxins. In summary, for plantlets cultured on these media, the presence of 2,4-D markedly influenced the frequency of repetitive embryogenesis.

The influence of sugar on the frequency of repetitive embryogenesis was another factor investigated to enhance the efficiency of this process. Sucrose alone or in combination with other media components has been reported to enhance embryo generation (Konan et al.1994). In rubber adventive embryo formation was enhanced by sucrose concentrations up to 50 g/1 resulting in a maximum of 134 (±9) embryos. However, repetitive embryogenesis in crops such as peach (Bhansali et al. 1990), the optimal sucrose level was 30 g/1 (w/v), substantially lower than the optimal sucrose level in rubber. However in suspension cultures of eastern white pine, 60 g/1 sucrose were required for embryogenesis (Finer et al. 1989). In rubber, sucrose concentrations above 50 g/1 inhibited embryogenesis (Table 3).

Histological observations of the primary

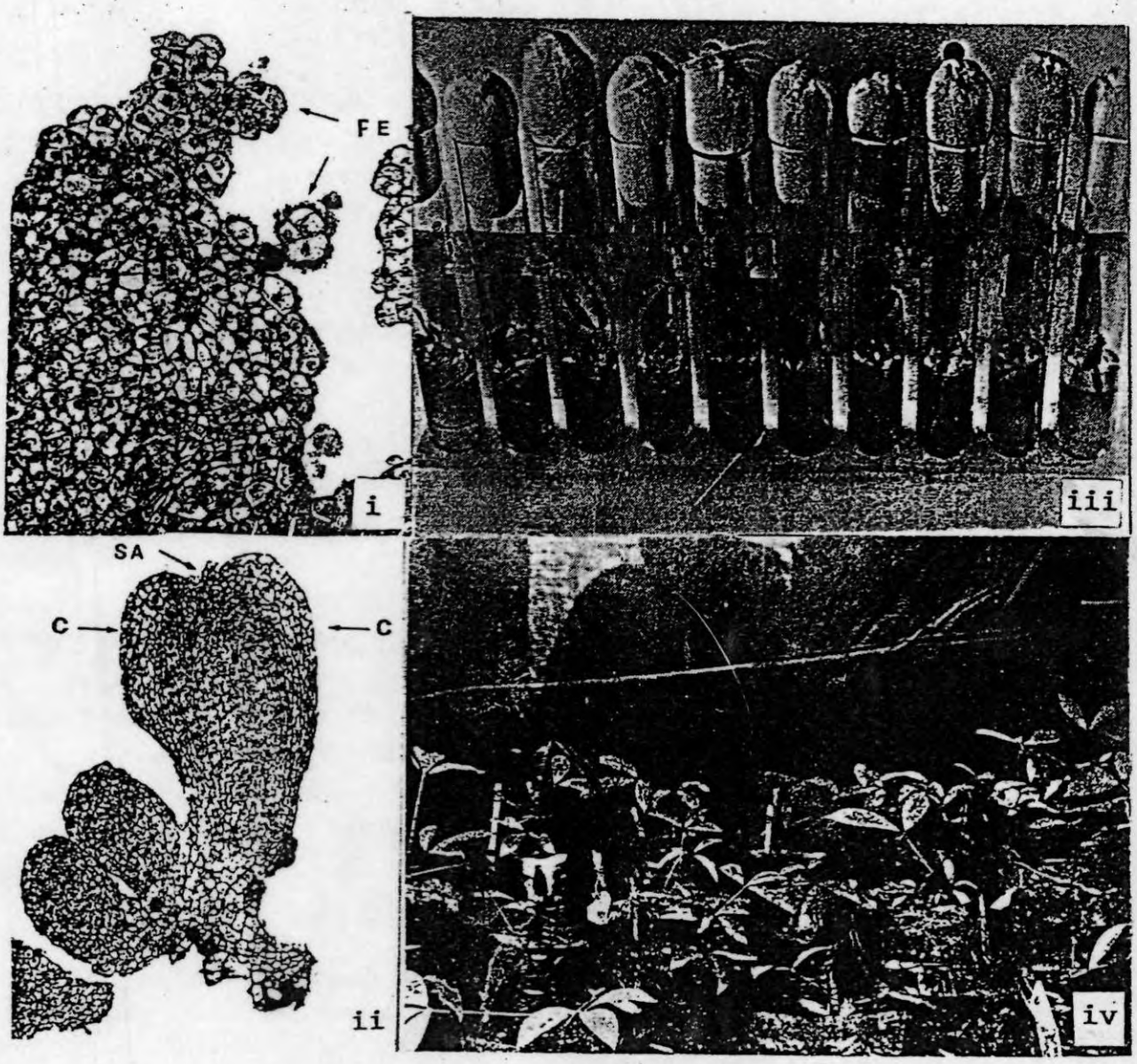

Fig. 2: i) Histology of embryogenic callus with proembryos (PE); **ii)** Somatic embryos cluster showing cotyledones (C) and shoot apex (SA) in the oldest embryo; **iii)** Regenerated plants derived from somatic embryogenesis; **iv)** Somatic embryos-derived plants growing in polybags

somatic embryos revealed that the surface of the embryogenic callus appeared uneven due to nodular proliferations. Microscopic examinations revealed that the nodules at the surface were developed from meristematic centres in the epidermal layer. Large and deeply stained cells with prominent nuclei were observed on the surface of the callus mass (Fig.2i). These enlarged cells divided transversely to form a large terminal cell and a basal cell of which the larger one as suspensor initial. The terminal cell underwent divisions and developed in to globular proembryos. They were frequently visible on the surface of embryogenic callus (Fig.2i). Proembryos developed into embryos. Histological study also showed asynchronous embryo development (Fig. 2 ii). Relatively more mature embryos displayed wing shaped cotyledonary development on the sides with apex origin in the middle (Fig. 2,ii).

Generally, full plant development from embryoids required 5-6 months. About 1600 embryoids from primary and repetitive embryogenesis were subcultured for the maturation process where the recovery of plantlets was 26%. There was no marked difference in the frequency of plant regeneration from primary and repetitive embryoid groups. The regenerated plants (Fig. 2,iii) were planted in polybags containing sterilised soil mixture. These plants were kept for 5-6 weeks in a hardening facility at 28±2°C with 70 – 80% RH. Subsequently they were kept in the shade house (Fig.2,iv) till beginning of monsoon season (June) which is the field planting season for natural rubber. Field mortality was 8% after 12 months.

Eventhough the embryo production rate by repetitive embryogenesis is higher than primary somatic embryogenesis, the over all rate of plant recovery from embryoids was low. This is not uncommon in many angiosperms including rubber (Carron 1989) and is considered one of the impediments of progress in this direction. The procedure for the induction of repetitive embryogenesis described in this report is prefereable to primary somatic embryogenesis in rubber for obtaining a higher number of embryos per unit time. Moreover, this multiplication technique by secondary embryogenesis may reduce the risk of somaclonal variation associated with prolonged callus culture (Guijaroo et al. 1995). Hence in clonally propagated recalcitrant woody tree crops, such as rubber, utilizing repetitive embryogenesis may be a useful method.

REFERENCES

Asokan MP, Jayasree P and Sushama Kumari 1992a Plant regeneration by somatic embryogenesis of rubber tree (*Hevea brasiliensis*). *International Rubber Conference*, Bangalore:49

Asokan MP, Jayasree P, Sushama Kumari S and Sobhana P 1992b Plant regeneration from anther culture of rubber tree (*Hevea brasiliensis*). *International Rubber Conference*, Bangalore:51.

Baker CM and Wetzstein HY 1994 Influence of auxin type and concentration on peanut somatic embryogenesis. *Plant Cell Tiss Org Cult* **36**: 249-254.

Baker CM and Wetzstein HY 1995 Repetitive somatic embryogenesis in peanut cotyledon cultures by continual exposure to 2,4-D. *Plant Cell Tiss Org Cult* **40**: 249-254.

Bhansali RR, Driver JA and Durzon DJ 1990 Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis. *Plant Cell Rep* **9**: 280-284.

Carron MP, Enjarlic F, Lardet L and Deschamps A 1989 Rubber (*Hevea brasiliensis* Muell. Arg). In: YPS Bajaj (Ed) *Biotechnology in Agriculture and Forestry Vol 5 Trees II* Springer Verlag, Berlin: 222-245 pp.

Chen Z 1984 Rubber *(Hevea)*. In: WR Sharp, DA Evans, PU Ammirato and Y Yamada (Eds) *Handbook of Plant Cell Culture, Vol 2 (Crop Species)* Macmillian, New York: 546-571 pp.

Durham RE and Parrot WA 1992 Repetitive somatic embryogenesis from peanut cultures in liquid medium. *Plant Cell Rep* **11**:122-125

Finer JJ, Kriebel HB and Becwar MR 1989 Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L). *Plant Cell Rep* **8**: 203-206

Finer JJ and Nagasawa 1988 Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill). *Plant Cell Tiss Org Cult* **15**:125-136.

Gamborg OL, Miller RA and Ojima K 1968 Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* **50**:151-158.

Guijaroo BF, Celestrino C and Toribio M 1995 Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus ruber*. *Plant Cell Tiss Org Cult* **41**: 99-106

Jensen WA 1962 *Principles and Practice, Botanical Histochemistry*. WH Freeman and Co., San Francisco.

Johansen DA 1940 *Plant Microtechnique*. Mc Graw Hill Book Co., New York

Konan NK, Sangwan RS and Sangwan BS 1994 Somatic embryogenesis from cultured mature cotyledons of Cassava (*Manihot esculenta* Crantz). *Plant Cell Tiss Org Cul* **37**: 91-102.

Muraleedharan EM, Gupta PK and Mascarenhas AF 1989 Plantlet production through high frequency somatic embryogenesiss in long term cultures of *Eucalyptus citriodora*. *Plant Cell Rep* **8**: 41-43.

Sharp WR, Evans DA Ammirato PV and Yamada Y (Eds) 1984 *Handbook of Plant Cell Culture Vol* 2. Macmillan, New York

Tulecke W 1987 Somatic embryogenesis in woody perennials. In: JM Bonga and DJ Durzan (Eds) *Cell and Tissue Culture in Forestry Vol 2* Martinus Nijhoff Publishers, Dordrecht: 61-91 pp.