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Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell.) Arg.

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High frequency somatic embryogenesis and plant regeneration were achieved from immature anthers of *Hevea brasiliensis* – the natural rubber-producing tree. Optimum callus induction was observed on modified Murashige and Skoog (MS) medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l Kn. Subsequent culturing of callus on medium containing various concentrations and combinations of auxin/cytokinin, resulted in embryo induction. Maximum number of somatic embryos were produced on medium supplemented with 0.7 mg/l Kn and 0.2 mg/l NAA. Further development of the embryos into plantlets was achieved on a hormone-free medium. Cytological analysis revealed that all the plantlets tested were diploid. Plantlets transplanted to polybags were maintained under greenhouse conditions, and subsequently established in the field.

OVER the past two decades, considerable progress has been made on *in vitro* techniques for multiplication and improvement of *Hevea*. However, a protocol for large-scale commercial application has not yet been evolved[1]. Recently, there has been an increasing interest in the induction of somatic embryogenesis especially for use in genetic transformation. Somatic embryogenesis has been reported from anthers as well as inner integumental tissue[2–5]. However, the technique is still not sufficiently developed to be utilized in crop-improvement programmes. Reliable somatic embryo formation is limited to only a few genotypes of *Hevea*. Embryogenic capacity is fugacious, and the rate of conversion of the embryos to plantlets is very low[6]. Pollen-derived plants from 13 different clones have earlier been transplanted and established in the field[7]. However, while only a few clones exhibited a high frequency plantlet induction (22%), many clones exhibited a very low embryoid and plantlet regeneration rate and some clones did not show any embryoid induction at all[1]. To our knowledge no reports are available on plant regeneration from immature anthers for Indian clones of *H. brasiliensis* to date. The present study was therefore carried out for developing a reliable regeneration system from immature anthers via somatic embryogenesis for Indian clones of rubber, *H. brasiliensis*.

Floral buds (0.5–1.0 mm length) were collected from clone RRII 105 of *H. brasiliensis*. After ascertaining their developmental stage under microscope, they were surface-sterilized for 5 min with 0.5% sodium hypochlorite solution containing a few drops of Tween 20 followed by thorough washing with sterile water. Immature anthers at the diploid stage (before microsporogenesis) were dissected out aseptically, and 7–8 anthers were transferred into each tube. Effect of Murashige and Skoog[8] (MS) based medium and modified MS medium on callus induction was studied. MS medium was modified by lowering NH_4NO_3 to 1.0 gm/l and by replacing MS vitamins with B_5 (ref. 9) plus 5% sucrose. For callus induction, various auxins namely 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), indol-3-butyric acid (IBA), indol-3-acetic acid (IAA) at 1.0 or 2.0 mg/l in combination with kinetin (Kn) at 0.5 or 1.0 mg/l were used. Effect of 2,4-D on callusing was further evaluated using modified MS basal medium supplemented with different concentrations of 2,4-D (0.0–3.0 mg/l) along with 0.5 mg/l Kn. Calli were then subcultured on embryo-induction medium (20 ml/tube) containing 7% sucrose and 0.2% activated charcoal fortified with different auxins in combination with Kn to investigate their effect on somatic embryogenesis.

After maturation, embryos were transferred to the same media containing 3% sucrose but without the hormones. All media were solidified with 0.21% gelrite and the pH was adjusted to 5.6 before autoclaving for 15 min at 121°C. For the induction of callus as well as the induction of embryo, cultures were incubated under dark at 25 ± 2°C and maintained in light for plant regeneration under a 16 h photoperiod (40 $\mu E\ m^{-2}\ s^{-1}$) at 25 ± 2°C. Regenerated plants were planted in polybags and grown in greenhouse initially. Each experiment was repeated twice with 3 replications. Data on percentage of explants producing callus and embryos were based on visual observations. To analyse the factorial effect of NAA/Kn on embryo induction, analyses of variance were performed. Mean number of embryos were compared using LSD test. Cytological analyses were carried out to confirm the ploidy level of the regenerants, using the acetocarmine (2%) squash method.

In the present study, MS medium induced no callus formation even after 2 months of culturing of immature anthers in the presence of different auxins tested, except poor callusing was observed in presence of 2,4-D. However, Chen *et al.*[10] had previously reported callus induction in MS basal medium for *Hevea* anther culture. In this study, swelling of the explant was noticed on modified-MS medium, after 2 weeks of culture, followed by callus initiation between 40–50 days. Callus was obtained only when 2,4-D was added along with 0.5 mg/l

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Kn. Frequency of callus induction decreased with the increase in Kn level (1.0 mg/l). Other auxins, like IBA and IAA, resulted only in explant swelling and NAA showed a poor response. Both friable callus and compact callus could be obtained from the explants. However, the nature of friability and compactness differed depending upon 2,4-D concentration resulting in 3 types of callus: Type I, friable; Type II, less friable; and Type III, compact were obtained (Table 1). Similarly, friable callus and compact callus was observed on MH medium with 3,4-D/Kn by Etienne *et al.*[11]. Friable but loose or watery callus (Type I) was obtained when media contained low concentrations of 2,4-D. However, friability was reduced with an increase in 2,4-D concentration. The optimal concentrations of 2,4-D and Kn for embryogenic callusing were 2.0 mg/l and 0.5 mg/l, respectively. Pale yellow, compact aggregates of cells with a shiny surface and mucilaginous coat (Type II) were found on this combination. The percentage of callusing increased when the concentration of 2,4-D increased from 2.0–3.0 mg/l, but callus was nonembryogenic in nature (Type III).

Subculturing of Type II callus, initially changed to brownish colour, and subsequently yellow friable embryogenic callus was produced. Embryoids started to emerge from these callus cultures after 4–5 months of culturing in embryo-induction medium. The friable embryogenic callus, induced from Type II, displayed active morphogenic growth and asynchronous development of globular structures (Figure 1 *a*). Different stages of embryogenic organizations from globular, heart to cotyledonary stages were observed in the same medium (Figure 1 *b* and *c*). Compact callus was observed to show maximum embryogenic competence. While the histological studies (Montoro *et al.*[12]) revealed that compact callus showed high embryogenic competence *Hevea*, in the present study although compact callus was most embryogenic, Type III callus sometimes remained as it was or produced callus with no embryogenic competence, thus suggesting that high concentrations of 2,4-D beyond 2.0 mg/l in the medium were inhibitory to embryogenesis. Type I callus also turned brownish and produced friable callus, but with no embryos. Montoro *et al.*[12] noticed that modifying the auxin/cytokinin balance resulted in the loss of embryogenic potential of the friable callus.

Table 1. Effect of 2,4-D and kinetin (Kn) on callus induction from immature anthers of *Hevea brasiliensis*

Growth regulators 2,4-D + Kn (mg/l)	Callus morphology	Callus type	Callus scoring
0.0 + 0.5	–	–	–
0.5 + 0.5	Friable, watery or wet, soft, white-creamy	Type I	+
1.0 + 0.5	Friable, little watery, soft, white-creamy	Type I	+
1.5 + 0.5	Less friable or compact, pale yellow	Type II	++
2.0 + 0.5	Compact, shiny surface, pale yellow	Type II	+++
2.5 + 0.5	Compact, hard or dry, pale yellow	Type III	+++
3.0 + 0.5	Compact, hard or dry, pale yellow	Type III	++++

Data were taken after two months of culturing on callus-induction media with modified MS basal media. Callus-induction frequency is expressed as follows:
+ = 1–20%; ++ = 21–50%; +++ = 51–80%; ++++ = 81–100%.

Table 2. Effect of different auxins in combination with Kn on the induction of somatic embryogenesis of *H. brasiliensis*

Growth regulator conc. (mg/l)		Nature of response: Callus browning	New calli proliferation	Embryo formation
2,4-D/Kn	0–0.4/0.5–1.0	less	70% FEC with fast growth	Infrequent development
IBA/Kn	0–0.4/0.5–1.0	abundant	20% FEC with slow growth	Sporadic formation
IAA/Kn	0–0.4/0.5–1.0	abundant	20% FEC with slow growth	Sporadic formation
NAA/Kn	0–0.4/0.5–1.0	moderate	40% FEC with slow growth	Frequent development

Observations were made after 4–5 months of culture in embryo induction medium and the results were noted: FEC = friable embryogenic callus.

Among the various auxins tested for embryogenesis, IAA and IBA influenced sporadic formation of embryos either singly or in clusters (Table 2). Incorporation of 2,4-D often led to embryogenic callus proliferation showing a tendency for embryogenic competence or rarely-formed globular embryos. However, these failed to develop further. NAA had a profound influence on embryogenesis in rubber. Table 3 shows the influence 30 combinations of various concentrations of NAA a... Kn on the number of somatic embryos formed. Among the 30 combinations used, embryogenesis was observed in most of the cultures irrespective of the concentration of growth regulator in the media, except in the absence of NAA. Maximum number of embryos were noticed in media containing 0.7 mg/l Kn in association with 0.2 mg/l NAA. Beyond 1.0 mg/l Kn, poor or no embryogenesis was observed. Also embryogenesis decreased when NAA level exceeded beyond 0.3 mg/l.

After 2–3 weeks of growth, mature embryos showed bipolar differentiation (Figure 1 *d*), subsequently developing into plantlets upon transferring to hormone-free medium. Eventhough root and shoot meristems were observed in somatic embryos, simultaneous development of root and shoot formation was infrequent. More often root formation occurred first which either resulted in induction of shoot apex or its noninduction. About 27% of the somatic embryos grew into normal plantlets on

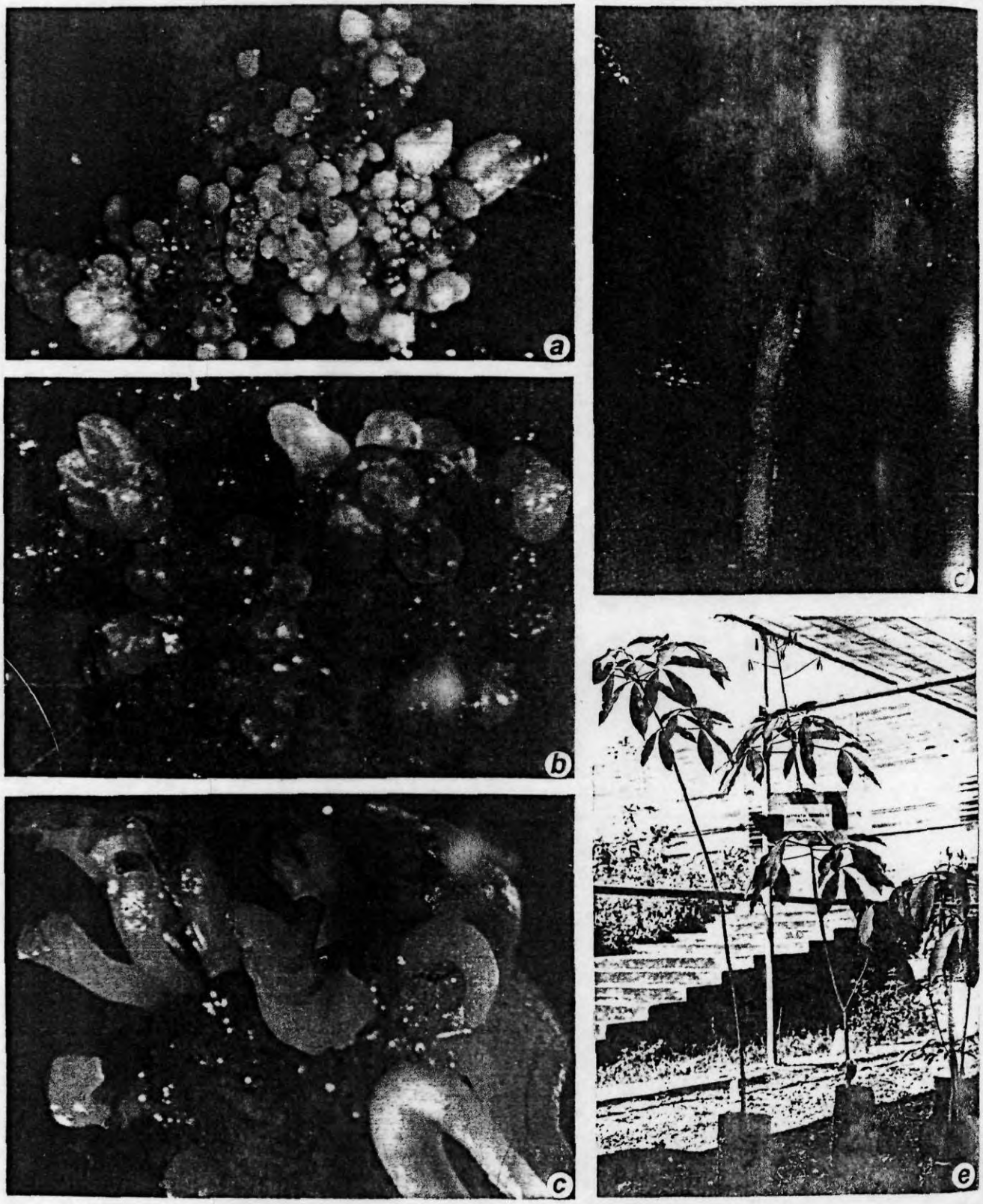

Figure 1. Somatic embryogenesis from immature anthers of *Hevea brasiliensis*: *a*, Globular embryos emerged from yellow callus; *b*, White globular and heart-shaped embryos; *c*, Green cotyledonary embryos; *d*, Bipolar differentiation of embryos with root and shoot poles; *e*, Embryo-derived plants growing in a greenhouse.

hormone-free medium. Cytological analysis of root tip cells from regenerated plantlets revealed only diploid cells. Plantlets that attained 2–3 cm length were planted in small polybags containing soil mix and were maintained in a greenhouse (Figure 1 *e*) prior to being transplanted in the field.

Table 3. Influence of NAA and Kn on the number of somatic embryos formed in *H. brasiliensis*

NAA (mg/l)	Kn (mg/l)					
	0.5	0.6	0.7	0.8	0.9	1.0
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1	4.67 ± 1.75	10.83 ± 1.33	15.33 ± 1.63	11.83 ± 1.72	5.67 ± 0.82	0.7 ± 0.82
0.2	10.0 ± 1.67	18.0 ± 2.37	23.83 ± 1.72	20.67 ± 2.58	10.3 ± 1.75	3.5 ± 2.74
0.3	2.83 ± 1.72	9.0 ± 1.79	14.17 ± 2.93	11.83 ± 1.72	4.67 ± 1.51	1.0 ± 0.89
0.4	0.50 ± 0.84	3.17 ± 1.47	6.0 ± 1.26	3.83 ± 1.47	1.33 ± 1.75	0.0 ± 0.0

Mean number of embryos (± SD) were recorded from pooled data after 4–5 months of culturing in embryo induction medium.
F = 10.92**; CD (0.05) = 1.95.
**indicates significant at $P < 0.01$.

A plant regeneration protocol via somatic embryogenesis was developed using immature anthers of *H. brasiliensis* (clone RRII 105). Modified MS medium supplemented with 2,4-D, NAA and Kn as plant growth regulators was found to be ideal for embryogenesis. This protocol could be applied to several Indian genotypes of *Hevea* for production of large-scale plants. The present regeneration system can also be used for developing transgenic plants either by *Agrobacterium*-mediated gene transfer or by particle bombardment with desirable genes.

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In vitro regeneration of a medicinal plant – *Houttuynia cordata* Thunb. from nodal explants

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***In vitro* regeneration of multiple shoots was achieved in nodal explant cultures of a medicinal plant *Houttuynia cordata* Thunb. (family Saururaceae) on Murashige and Skoog (MS) medium supplemented with 1 mg/l of N-6 Benzyladenine (BA). *In vitro* grown shoots were cultured for rooting on MS medium with 1 mg/l of indole acetic acid (IAA). The rooted plantlets were successfully established in soil.**

HOUTTUYNIA cordata Thunb. (family Saururaceae) is a wild perennial herb with creeping rootstocks and swollen nodes. The species exists in two chemotypes – the Japanese chemotype, which smells like orange and the Chinese chemotype which smells like raw fish and fresh coriander leaves[1]. The Chinese chemotype was used in this study. Mainly leaves and occasionally the whole plant of *H. cordata* are used as medicine. Leaves are used to treat cold, measles, dysentery, indigestion, and eye, urine and skin ailments[2,3]. The plant is used as a detoxicant, anti-inflammatory, antipyretic and diuretic agent in traditional medicinal practices of Assam and China[4]. Two pyridine alkaloids, three aporphine-related alkaloids (viz. cepharanone, cepharadione B and 7-chloro-6-demethyl cepharadione B) and aristolactans have been isolated from *H. cordata*[5]. It also yields essential oils[5].

Organized cultivation of *H. cordata* for extraction of medicinal compounds was reported from Vietnam[6]. In Assam, these plants are collected from natural population which is not abundant. *In vitro* propagation of this species could help in raising disease-free healthy clones

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