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PLANT REGENERATION *VIA* SOMATIC EMBRYOGENESIS FROM ROOT EXPLANTS IN *HEVEA BRASILIENSIS*

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An efficient plant regeneration pathway through somatic embryo induction has been established in *Hevea brasiliensis* using root explants. In this experiment, actively growing roots of germinating somatic embryos were used as the initial explants. Different basal media *viz.* modified MS, white's low salt base, N6 basal and woody plant medium fortified with growth regulators 2, 4-D, NAA, IBA and BA were tried at various stages of the pathway. Compact calli could be induced at a high frequency (80%) when root explants were cultured over modified MS containing 2.0 µM 2, 4, D and 1.0 µM Kinetin. Modified MS and WPM were found to be effective for induction of friable embryogenic calli. A combination of 4.35 µM GA_3 and 8.8 µM BA was optimum for embryogenesis (50%). A plant regeneration frequency of 60 per cent could be achieved on modified WPM medium fortified with 2.9 µM GA_3 and 8.8 µM BA. Regenerated plants were successfully hardened. This system is reproducible and efficient in terms of frequency of embryogenesis and plant development and can provide a constant supply of target tissues for genetic transformation, which now depend upon the seasonal availability of floral explants. Moreover, after appropriate modifications, this pathway can be utilized for propagation of elite root stocks identified for specific desirable characters.

Keywords: Based media, *Growth regulators,* Root explants, Somatic embryogenesis

INTRODUCTION

Hevea brasiliensis, the natural rubber producing tree, is a cross pollinated, highly heterozygous, perennial tree crop with a very long breeding cycle, thus rendering crop improvement through conventional techniques of breeding and selection much laborious and time consuming. Biotechnological approaches hold much relevance in bringing about crop improvement in *Hevea*. Availability of established cell and tissue culture techniques and efficient protocols for regeneration of whole plants from these cultures is an essential pre-requisite for the application of genetic transformation technologies for plant improvement. In *Hevea*, plant regeneration has been reported from different explants like integumental tissue (Asokan *et al*., 1992, a,b; Carron *et al*., 1995), anther (Wang *et al*., 1980; Chen, 1984; Jayasree *et al*., 1999) and pollen (Chen *et al*., 1979). Sushamakumari *et al*. (2000) reported an efficient and reproducible plant regeneration pathway through somatic embryogenesis using immature

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inflorescence as the initial explant. Plant regeneration through somatic embryogenesis from leaf tissue was reported by Kala *et al.* (2005). Availability of the floral explants is often restricted to a short, yearly time window and the time taken for completion of the entire pathway is 9-10 months. This necessitates the development of a system having a shorter time requirement and higher efficiency so that it can be more effectively utilized in genetic manipulation experiments. Also an explant, the availability of which is not restricted by season, will be highly useful especially in genetic transformation studies. There are several reports on plant regeneration through somatic embryogenesis from root cultures of other species like rice (Asit *et al.*, 2003), papaya (Chen *et al.*, 1987) and *Tylophora indica* (Sulekha and Rana, 2010) *etc*. Somatic plants are juvenile in nature resembling seedlings. Hence roots of somatic plants can serve as ideal explants for the plant regeneration pathway. This paper is an attempt to explore the feasibility of using root explants of *Hevea* to induce embryogenesis and achieve subsequent plant regeneration.

MATERIALS AND METHODS

Plant material and culture conditions

Explants used in this study are actively growing, tender roots from two different sources.

a) Germinating somatic embryos

Somatic embryos were raised from immature inflorescence of *Hevea*, clone RRII 105 according to the protocol already standardized (Sushamakumari *et al*., 2000). These embryos, after maturation were transferred to plant regeneration medium where germination of the embryos characterized by elongation of both roots and shoots take place. At this actively growing stage the elongating roots were separated, cut into thin segments and used as the initial explant for callus initiation.

b) Germinating zygotic embryos

Seeds of *Hevea* were taken from mature fruits and surface sterilized by dipping in 0.2 per cent mercuric chloride for 5 minutes followed by several rinses with sterile distilled water. The zygotic embryos were excised out and cultured over Murashige and Skoog (1962) (MS) medium supplemented with 0.3 mgL^{-1} BA for germination. Tap roots of the emerging seedlings were cut into thin segments and used as the explants.

Developmental stage of the explant

In order to study the response of different portions of the root towards somatic embryogenesis, intact roots were divided into three categories *viz*. distal, middle and proximal with respect to the root tip. Explants belonging to each group were cultured over callus induction media and observations were taken after one month to evaluate callus induction frequency.

Basal media

Different basal media namely modified MS (Murashige and Skoog, 1962), White's low salt base, and Woody Plant Medium (WPM; Lloyd and McCown, 1980) were tried at various stages of the pathway. Growth regulators included in this study were the auxins (2, 4-D, NAA, IBA), cytokinins (BA and Kin) and giberrellic acid (GA_3).

All media were solidified with 0.2 per cent phytagel after adjusting the pH to 5.6 and autoclaved at 120 ^{o}C for 15 min. Cultures were grown in glass culture tubes containing

20 ml medium and incubated at 25 °C in the dark for callus and embryo induction. For further development of the embryos, the cultures were kept under cool white fluorescent light under a 16 hour photo period. All the experiments were repeated thrice.

Callus induction

Root segments (Fig. 1a) were cultured over callus induction medium fortified with varying concentrations (0.5-5.0 μM) of the auxins 2,4-D, NAA and IBA individually as well as in combination with KIN (0.2-2.0 μM). The cultures were maintained in the dark and subculturing was done at 30 days interval.

Callus proliferation

For proliferation of the induced callus, the optimized callus induction medium was modified by lowering the growth regulator concentrations. The culture conditions were kept the same and the cultures were dark incubated.

Embryo induction and maturation

Proliferated calli were transferred to modified MS and WPM basal media incorporating various levels of GA_3 and BA (1.0- 10.0 μM) along with 1. 0 μM NAA. For embryo maturation, the developing embryos were subcultured on the embryo induction medium fortified with a higher concentration of phytagel as well as on hormone free maturation medium solidified with 0.4 per cent phytagel concentration.

Plant regeneration

The embryos developed were transferred to hormone free maturation medium solidified with a higher phytagel concentration (0.4%). Later on the mature embryos were transferred to plant regeneration media. Different basal media, modified MS and WPM containing different concentrations, (1-10 μM) of GA_3 and BA were tried for plant regeneration**.** MS basal medium was modified by reducing the NH_4NO_3 and KNO_3 concentrations to half strength and incorporating 400 mgL^{-1} $CaNO_3$ $4H_2O$ instead of $CaCl_2$, whereas WPM was modified by eliminating $CaCl_2$ and adding 900.0 mgL^{-1} KNO_3.

Acclimatization

Regenerated plants were subjected to acclimatization as follows. Plantlets with well-developed leaves were carefully removed from the culture tubes and washed gently in running tap water to remove adhering medium and the dead tissues if any near the cotyledons. Plantlets were dipped in 0.2 per cent bavistin for 3 minutes and were blotted to remove the adhering water particles by keeping for 2 minutes in tissue paper. Then they were planted in earthenware pots (5 diameter x 8 cm height) containing sterile sand, soil and soil rite mixture and kept in the controlled conditions in growth chamber with a RH of 90, and temperature 25 °C and left uncovered. Humidity was decreased by 2 units at 2 days interval. Simultaneously, temperature was gradually increased from 25 °C to 30 °C. These plants were supplied with dilute Hoagland mixture weekly once and watering was done at two days interval. After about three weeks, the surviving plants were transferred to poly bags filled with garden mixture (1:1:1 soil-sand-cow dung) and kept in the growth chamber itself. After the emergence and maturation of a new flush, the hardened plants were taken out from the growth chamber and maintained in the shade house. Acclimatized plants were transferred to big polybags in the shade house.

RESULTS AND DISCUSSION

Explant

Swelling of the explants was observed 6-7 days after the explant inoculation and callus induction occurred within 3-4 weeks (Fig. 1b). Root explants from both sources *viz*. somatic and zygotic seedlings could induce calli which later on gave rise to

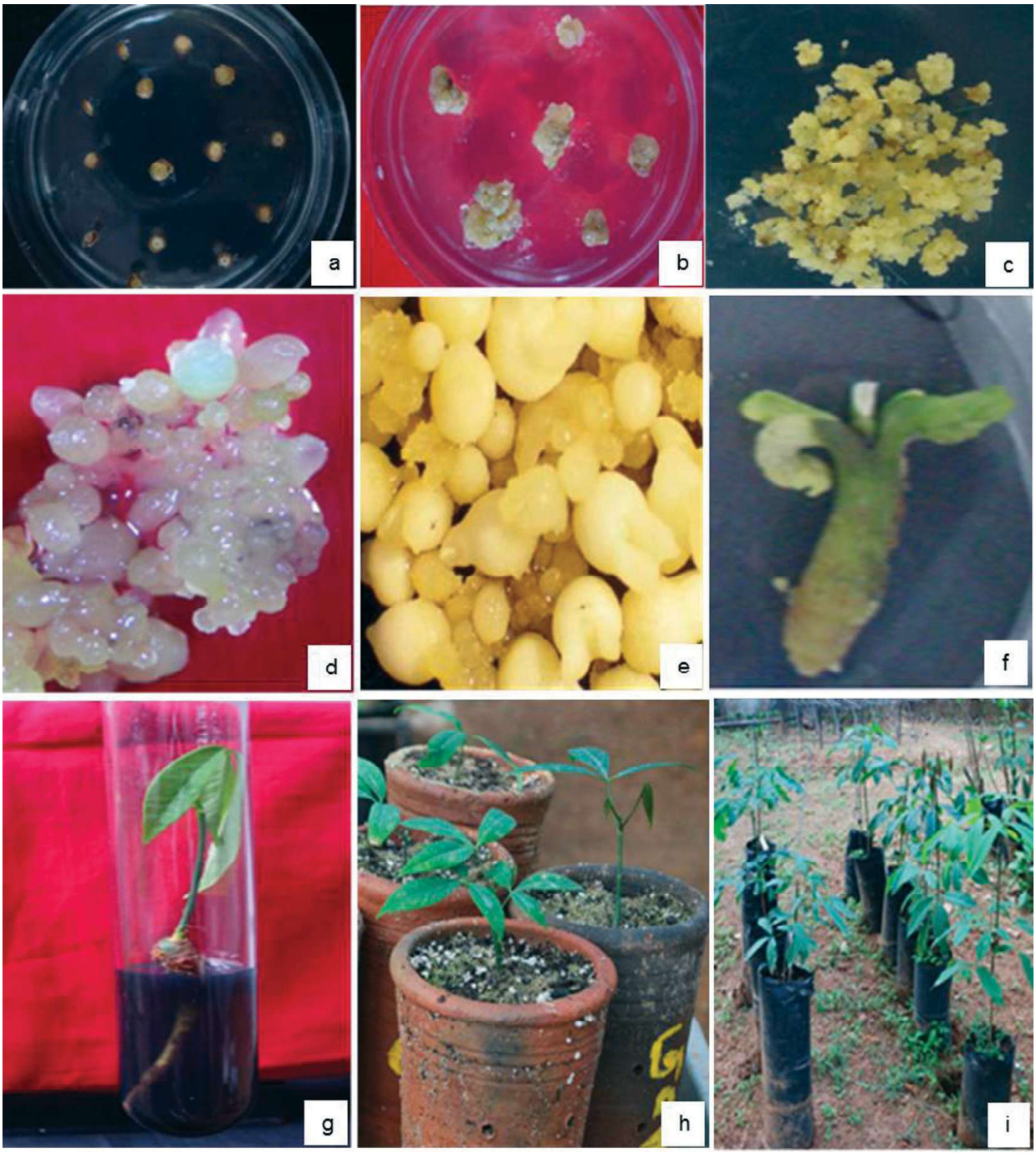

Fig. 1. (a-i) Different stages of somatic embryogenesis pathway from root explants in *Hevea*
a. Cultured root segments, b. Callus induction, c. Embryogenic callus,
d. Embryo induction, e & f. Embryo germination, g. Plant regeneration,
h. Initial stage of hardening, i. Hardened plants in poly bags

embryogenic calli with regenerative potential. It was also noted that the position of the explant in the original root strongly influenced callus formation and further development. Root segments from the proximal position responded better, giving rise to compact callus at a high frequency (90%) within a short period of around 3 weeks whereas for the other two groups, callus formation was slower and with a lower frequency (data not given). Also calli from the proximal segments were found to have the highest embryogenic competence. This difference in response among the three groups experimented might be due to the variation in hormone levels in various parts of the root segments (Mukhopadhyay *et al*., 1997). Moreover large number of meristematic cells present in the extreme root tip might be more juvenile and hence capable of faster cell division and subsequent differentiation into embryos (Asit *et al*., 2003). Explant selection is a critical parameter to consider while going for somatic embryogenesis and plant regeneration experiments. Different explant types often have varying potential for transformation and certainly for organogenesis or development of somatic embryos (Piqueras *et al*., 2010).

Culture media

Callus initiation was observed in all the basal media, WPM, MS, modified MS and White's low salt base, supplemented with 2,4-D. However, modified MS medium containing B5 vitamins and 2.0 µM 2,4-D in combination with 1.0 µM Kinetin gave maximum compact callus having embryogenic potential (Table 1). Among the growth regulators tried for callus induction, 2,4-D was observed to be a critical component for callus induction and proliferation. Role of 2, 4-D alone or in combination with another auxin and cytokinin, on callus induction and further embryogenesis in *Hevea* from various explants is well documented (Wang *et al*., 1980; Chen, 1984; Jayasree *et al*., 1999; Asokan *et al*., 2002; Kala *et al*., 2005). The synergistic effect of NAA with 2, 4-D in presence of Kinetin on callus induction and proliferation from immature inflorescence of *Hevea* was reported by Sushamakumari *et al.* (2000). However a reduction in callus friability with higher concentrations of 2, 4-D was observed by Jayasree *et al*. (1999).

For callus proliferation, lower levels of 2, 4-D (0.5-1.0 µM) alone was sufficient. After two subcultures in the proliferation medium at one month interval, the calli could be transferred to the embryo induction medium.

Embryo induction and maturation
a. Basal medium

Both modified MS and WPM were found to be effective for induction of

Table 1. **Effect of growth regulators on callus induction (%) from root segments**

Kin (µM)	2,4-D (µM)				
	1	2	3	4	5
0.5	58	62	57.33	46.33	39.66
1	64	77.66	73.33	50.33	42
1.5	60.66	82.66	73.66	53.66	46
2	57.33	74.33	66	52.66	45
2.5	49	67.66	62.33	47.66	41
CD (kin x 2,4-D) = 1.99					

embryogenic callus from the initial compact calli. Within 40-50 days in the embryo induction media, friable yellow calli with embryogenic potential started emerging subsequently giving rise to numerous globular embryos (Fig. 1c &d).

b. Growth regulators

Experiments carried out for identifying the ideal combination and concentration of growth regulators required for embryo induction revealed that GA_3 was a critical component for somatic embryo induction. A combination of GA_3 (4.35 µM) and BA (8.8 µM) was found to be optimum for embryogenesis and further development. An embryo induction frequency of 50 per cent could be achieved with this hormonal combination (Table 2). The beneficial effect of GA_3 on somatic embryo induction and further development is well documented (Joshi *et al.*, 2010). A combination of ABA along with Kin and GA_3 positively influenced embryo induction and development from transgenic calli of *Hevea* integrated with MnSOD gene (Jayashree *et al.*, 2003). Moreover, at this growth regulator level, it was observed that embryos were emerging and developing faster in the modified WPM medium. Maturation also started in the same medium itself (Fig. 1e, f), as a result of which these embryos need not be sub cultured on to the maturation medium. Instead, they could be directly transferred to the plant regeneration medium where they germinated into plantlets. In the pathway developed earlier from immature inflorescence, the somatic embryos emerging from the embryogenic calli required a maturation phase of about 30-40 days on a specific maturation medium. Here, the cultures developed from root derived callus could bypass the maturation step and directly germinated into plantlets in the regeneration medium thereby resulting in a reduction in the total time span of the pathway. This in turn has improved the efficiency of the pathway so that it will be highly useful in genetic transformation experiments.

Plant regeneration

Somatic embryos need precise nutrients and cultural conditions for initiation and development of shoots and roots leading to complete plantlet formation. In the present study, plant regeneration was observed in both modified MS and WPM medium. However, modified WPM medium was found to be most suitable for plant regeneration (65%) in the presence of 2.9 µM GA_3 and 8.8 µM BA (Fig. 1g). WPM medium

Table 2. **Effect of growth regulators on embryo induction (%) from root derived callus**

GA_3 (µM)	BA (µM)				
	2	4	6	8	10
1	16.33	27	31.66	38.66	31.33
2	18.66	26.33	36.66	41.66	35
4	21.33	31	41.33	51	41.66
6	17.66	28	34.33	32.33	31
8	16	24	31.33	30.33	28
10	15	28.33	34	25.33	31
CD (GA_3 x BA) = 1.56					

has been used for the germination of transgenic embryos integrated with SOD gene as well as for the germination of rescued immature zygotic embryos (Jayashree 2003; Rekha *et al.,* 2010). Beneficial effect of GA_3 on germination is already reported in *Hevea* (Carron *et al.*, 1995; Jayasree and Thulaseedharan, 2002). A combination of BA (0.3 mgL^{-1}) and GA_3 (0.3 mgL^{-1}) was used for the germination of rescued zygotic embryos in the immature stage in *Hevea* (Rekha *et al.,* 2006). In the case of transgenic *Hevea* embryos integrated with osmotin gene, maximum germination occurred in half strength MS medium supplemented with 1.5 mgL^{-1} BA and 1.5 mgL^{-1} GA_3 (Rekha *et al*., 2013). Incorporation of GA_3 in combination with BA and IBA in the germination medium favoured bipolar differentiation and improved germination followed by plant regeneration in transgenic embryos of *Hevea* integrated with MnSOD (Sobha *et al.,* 2003b). Even though the exact mechanism of the beneficial role of GA_3 on embryo germination is not clear, ultrastructural studies carried out by Choi *et al.* (1999) showed that somatic embryos developed *in vitro* could be dormant after maturation and a breakage of the dormancy is required. The beneficial effect of GA_3 on germination is reported in grapes (Mullins and Sreenivasan, 1976) and in *Panax gingseng*. In *Citrus sinensis* the embryos derived from ovules were germinated by supplementing GA_3 in the medium (Button and Bornman, 1971). The germinated embryos on further transfer to the same medium developed into full plantlets within 3-4 weeks.

Acclimatisation

Plants could be hardened within one month (Fig. 1h). An initial survival rate of about 30 per cent could be achieved. After acclimatization and transfer to big poly bags (Fig. 1i), final survival rate was found to be around 20 per cent.

CONCLUSION

A plant regeneration pathway from root explants *via* somatic embryogenesis has been developed in *H. brasiliensis*. Regenerated plants were successfully hardened. In this system, the time span for the development of plantlets could be substantially reduced compared to the earlier reported pathways from other explants in *Hevea*. This pathway can be utilized in genetic transformation experiments for developing transgenic plants through multiple gene integration. Also, after appropriate refinements, it can be employed for the multiplication of elite rootstocks of *Hevea*.

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