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SOMATIC EMBRYOGENESIS IN LEAF CULTURES OF *HEVEA BRASILIENSIS*: EFFECT OF SOURCE PLANT

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Somatic embryogenesis and plant regeneration have been achieved from leaf explants of the clone RRII 105 of *Hevea brasiliensis*. The embryogenic competence of callus induced from leaf explants of the same developmental stage taken from three different sources such as plantlets regenerated through somatic embryogenesis, budded plants grown in polybags and mature budded trees of the clone RRII 105 were examined. Callus induction was obtained in modified MS medium containing 3.6 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 58 mM sucrose, supplemented with phytohormones 2,4-D (5.4 µM), BA (4.4 µM) and NAA (1.08 µM). Proliferated fresh callus was cultured for embryo induction in modified MS basal medium (2.11 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 2.0 mM KH_2PO_4) containing B5 vitamins, amino acids, 220 mM sucrose and phytohormones. Significant difference in rate and the time taken for embryogenic callus initiation was observed. The embryo induction medium containing 1.5 mM $Ca(NO_3)_2 \cdot 4H_2O$ along with 234 mM sucrose and solidified with (0.5%) phytagel was found to be optimal for embryogenic callus initiation. Embryogenic callus could be obtained only from callus induced from *in vitro* - somatic plants and polybag grown - budded plants. Leaves from mature trees produced proliferating callus with little embryogenic competence. Embryo induction was simultaneous with embryogenic callus formation. After the induction of embryogenic callus, the rate of embryogenesis (60%) was similar in the proliferated embryogenic calli derived from leaves of both *in vitro* - derived somatic plants and glass house - grown budded plants. Embryo induction was obtained from proliferated embryogenic calli when the cultures were incubated in dark in modified MS basal medium (2.11 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 2.0 mM KH_2PO_4) containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/L), casein hydrolysate (300 mg/L), 175 mM sucrose and phytohormones BA (2.2 µM), GA_3 (2.9 µM), Kin (1.25 µM), ABA (0.75 µM) and NAA (0.54 µM). Further embryo maturation and germination, carried out in media standardized earlier, were not affected by the source of explant. The effect of physiological juvenility of source plants and the influence of culture medium on embryogenic tissue initiation are discussed.

Keywords: Carbon source, Embryogenic calli, Gelling agent, *Hevea brasiliensis*, Juvenility of source plants, Somatic embryogenesis.

INTRODUCTION

Somatic embryogenesis is a valuable tool for micropropagation of plant species as well as for genetic manipulation experiments. This regeneration system is generally more difficult with woody plant species. Success of *in vitro* tree regeneration greatly depends on selection of the

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appropriate explant, age of the source plant, culture medium and environmental conditions employed. Generally, *in vitro* propagation of mature trees is more difficult than that of juvenile plants. However, somatic embryogenesis using explants such as leaves and roots has been possible in mature trees of a few species (Bonga and Aderkas, 1992). Bonga and Aderkas (1992) had reviewed the factors influencing rejuvenation in trees using methods to enhance micropropagation involving application of osmotic, temperature or hormonal stresses. Accordingly a degree of clone rejuvenation as indicated by the improved capacity of the cultures to form plantlets can be obtained by *in vitro* procedures.

Hevea brasiliensis being open pollinated, is highly heterozygous and hence development of amenable plant regeneration systems through somatic embryogenesis from physiologically mature clonal explants is a prerequisite for both micropropagation of elite clones and genetic improvement through transgenic approaches. Several reports are available on somatic embryogenesis and plant regeneration from anther wall callus (Wang *et al.*, 1980), inner integument of immature fruits (Carron *et al.*, 1989; Asokan *et al.*, 1992), immature anther (Jayasree *et al.*, 1999) and immature inflorescence (Sushamakumari *et al.*, 2000) as explants of *H. brasiliensis*. Majority of the reports on somatic embryogenesis and plant regeneration in *Hevea* were from flower/fruit derived explants and the seasonal flowering nature of *Hevea* limits their availability. Mendanha *et al.* (1998) tried the use of leaf explants and could report only callus formation. Recent reports on plant regeneration through somatic embryogenesis from explants such as leaves (Kala, *et al.*, 2005; 2006) and roots derived from *in vitro* somatic plants (Sushamakumari *et al.*, 2006) proved the feasibility of using them in plant production circumventing the limitations of explants used earlier. As reported by Lardet *et al.* (2009), in *Hevea* also, the frequency and time taken for embryogenic callus initiation was found to be highly influenced by the source of explant. The pattern of developmental response of cultured tissue and the requirement of auxins and other plant growth regulators for the initiation of somatic embryogenesis is largely determined by the nature and the developmental stage of the explant used (Litz and Gray, 1995).

Establishing embryogenic cultures in the shortest possible time is very important for the application of somatic embryogenesis in the clonal propagation of tree species. To develop an efficient somatic embryogenesis pathway, a number of critical physical and chemical treatments should be applied with proper timing (Arnold *et al.*, 2002). Embryogenic efficiency and performance are related to genotype – medium interaction in *Hevea* (Montoro *et al.*, 1993; Carron *et al.*, 1995; 1998). There are reports on the influence of medium desiccation, medium osmolarity, carbohydrate metabolism and phytohormones on embryogenic callus initiation and somatic embryogenesis in *Hevea* (Etiennie *et al.*, 1993; Veisseire *et al.*, 1994; Sushamakumari *et al.*, 2000; Jayasree *et al.*, 2001; Blanc *et al.*, 2002). In several crops, embryogenic potential is found to decrease with explant maturity and is eventually lost (Muralidharan and Kallarackal, 2004). For effective plant regeneration, it is essential to reduce the time taken for embryogenic callus formation and embryo induction. Hence the present study was attempted to assess the embryogenic potential of callus derived from different leaf sources of varying maturity stages of the

source plant to trigger embryogenic callus initiation by modifying the chemical composition of the culture medium.

MATERIALS AND METHODS

Plant material and culture initiation

Leaves of the same developmental stage identified as being responsive for callus induction after extensive optimisation experiments, were used for culture initiation (Kala, *et al.*, 2005). Accordingly, immature leaves with light green colour and shining appearance were collected from different sources such as *in vitro* plantlets regenerated through somatic embryogenesis and kept in culture tubes, one-year-old budded plants grown in polybags in glass house and mature budded trees, and used for culture initiation. Leaves taken from *in vitro* plantlets derived through somatic embryogenesis were cut into small pieces and inoculated directly in callus induction medium. Leaves collected from budded plants and mature trees were surface sterilised with 0.15 per cent mercuric chloride containing a few drops of Tween 20 for 3 min, thoroughly rinsed in sterile distilled water and blotted dry with sterile filter paper. The leaves were cut into pieces transversely across the leaf lamina and cultured with the adaxial surface in contact with the medium.

Callus induction and proliferation

Callus induction was tried from leaf cultures, initiated from the three different sources as described earlier, in MS (Murashige and Skoog, 1962) medium modified with the addition of calcium nitrate (3.6 mM) and containing B5 vitamins (Gamborg *et al.*, 1968), sucrose (58 mM) and growth regulators NAA (1.08 μM), 2,4-D (5.4 μM) and BA (4.4 μM). The calli were sub-cultured in the same basal medium used for callus induction, but with reduced $Ca(NO_3)_2 \cdot 4H_20$ (2.11 mM) and 2,4-D (2.4 μM) and increased sucrose (117mM) for proliferation of the callus tissue. The callus was transferred to fresh medium once in a month. The cultures were grown in the dark at 27°C in culture tubes (25 x 150 mm) containing 10 ml of the sterilised medium. Forty cultures were used for each treatment, and the experiment was repeated thrice.

Embryogenic callus initiation

Proliferated calli obtained from the three sources were transferred to disposable petri plates (90 x15 mm) containing sterile embryo induction medium standardised earlier for embryogenic callus initiation (Kala *et al.*, 2006). Embryo induction medium used was modified MS medium [$Ca(NO_3)_2 \cdot 4H_20$ - 2.11mM and KH_2PO_4-2.0mM] supplemented with phytohormones such as BA (2.2 μM), GA_3 (2.9 μM), Kin (1.25 μM) and NAA (1.08 μM). Basal medium also contained B5 vitamins, amino acids, organic supplements, and 220 mM sucrose. Amino acids present in the medium were glutamine (2.05 mM), proline (0.87 mM), cysteine HCl (0.13 mM), serine (0.19 mM), and arginine (0.18 mM). The organic supplements used were casein hydrolysate and coconut water.

Embryogenic callus was obtained in the above medium. However, to reduce the time taken and improve the rate of embryogenic callus initiation, an attempt was made to modify the existing medium, for which proliferated callus obtained from leaves of *in vitro* - derived somatic plants were used. To trigger embryogenic callus formation, osmotic stress was provided by increasing sucrose concentrations and water stress was

imposed by varying the level of phytagel. A factorial experiment was done to find the combined effect of calcium and sucrose on embryogenic callus initiation. A range of calcium concentrations ($Ca(NO_3)_2 \cdot 4H_20$- 0.7-3.6 mM) was tried along with different levels of sucrose (109-292 mM) for varying osmolarity, while the concentration of phytagel was kept at 0.3 per cent. Data were recorded from 30 callus clumps and the experiment was repeated thrice. The medium combination from which embryogenic callus emerged in the shortest time was used to test the effect of different levels of water stress by using different concentrations (2 - 8 g/L) of phytagel. The experiment was repeated three times, with data being recorded on 30 clumps in three petri dishes. All cultures were maintained in dark and observed once a week for culture contamination and callus initiation.

After identifying the optimal medium for embryogenic callus initiation, proliferated calli from leaves collected from the three different source plants were cultured and compared.

Embryo maturation and plant regeneration

The embryos formed were separated and subcultured for maturation and apical meristem induction. Developing embryos were separated from the callus and cultured singly in maturation medium in petri plates and kept in the dark. The maturation medium used was WPM (Lloyd and McCown, 1980) with reduced K_2SO_4 (2.58 mM) containing organic supplements, sucrose (183 mM) and phytohormones. The medium was solidified with 0.2 per cent phytagel. WPM containing organic supplements such as 0.5 per cent CW, 100 mg/L malt extract, 400 mg/L casein hydrolysate and phytohormones BA (2.2 µM), Kin (1.38 µM), IBA (0.49 µM) and GA_3 (5.3 µM), that was found optimum in earlier reports (Kala, *et al.*, 2006), was used. Sucrose level was reduced to 117 mM with 0.2 per cent charcoal and the medium was solidified with 2.0 g/L phytagel. Bipolar embryos at the cotyledonary stage, with the apex induced, formed after three weeks in this medium, were transferred to MS medium devoid of phytohormones but containing 89 mM sucrose, 0.3 per cent charcoal and 2 g/L phytagel for plant regeneration in culture tubes.

RESULTS AND DISCUSSION

Callus induction and proliferation

Callus was induced in leaf explants from the three sources cultured in modified MS medium (Fig.1A). The rate and the time taken for callus induction varied with the source of the explant (Table 1). In leaves derived from somatic plants, callus was induced within 20 days of culture. In leaves collected from budded plants and mature trees, callus induction was observed after 30-40 days. The reasons for variation in time of callus induction in *in vitro* and *ex vitro* - derived leaves may be the effect of

Table 1. **Rate and time of callus induction in leaves collected from different sources**

Source of leaf explants	Time taken (days) for callus induction	Callus induction (%) after 40 days culture	Mean (%)
In vitro somatic plants	20	80-90	84.16 ± 2.89
Budded plants	40	40-50	46.66 ± 3.82
Mature trees	40	40-50	41.45 ± 1.44

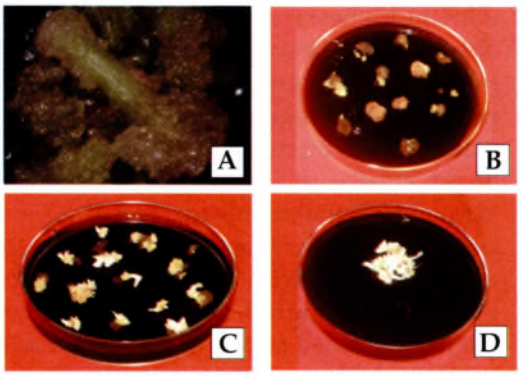

Fig. 1.Emergence of embryogenic callus and embryogenesis
A - Callus induction in leaf explants
B - Embryogenic callus emergence
C - Embryogenic calli with simultaneous embryo induction
D - Embryogenesis from proliferated embryogenic calli

sterilization and/or the juvenility of the explant source. Rate of callus induction in the *in vitro* - derived leaves was above 80 per cent whereas in the *ex vitro* - derived leaves it was above 40 per cent. Proliferation of fresh leaf calli could be achieved after two weeks of culture and the calli had a friable texture with brownish yellow appearance.

Embryogenic callus initiation

When proliferated calli obtained from different explant sources were cultured in the embryo induction medium standardised earlier (Kala *et al.*, 2006), variation was observed in the rate and time taken for embryogenic callus initiation. Embryogenic callus initiation was obtained from proliferated embryogenic callus in modified MS basal medium ($Ca(NO_3)_2 \cdot 4H_20$ – 2.11 mM and KH_2PO_4 - 2mM) containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/L), casein hydrolysate (300 mg/L), sucrose 220 mM and phytohormones BA (2.2 µM), GA_3 (2.9 µM), Kin (1.25 µM), and NAA (1.08 µM). The medium was solidified with 0.3 per cent phytagel. The callus developed from *in vitro* - derived somatic plants initiated embryogenic callus in the least time of three months with a higher frequency (55 %). In callus obtained from leaves of budded plants, the rate was 20 per cent and time taken was 6 months. The callus derived from mature tree leaves did not show any embryogenic potential. Embryogenic callus emergence was highly dependent on the source and maturity of the plant from where the leaves were collected. Lardet *et al.* (2009) also reported that the

Table 2. **Effect of calcium and sucrose concentration on initiation of embryogenic callus in presence of 0.3% phytagel**

Sucrose	Ca $(NO_3)_2 \cdot 4H_20$ (mM)							
(mM)	0.7	1	1.5	1.9	2.3	2.7	3.2	3.6
109	1.67	3.33	3.33	0.00	0.00	0.00	0.00	0.00
205	5.67	2.67	1.67	0.00	0.00	0.00	0.00	0.00
219	10.00	13.67	15.33	11.33	14.67	7.33	2.67	0.00
234	13.00	14.00	18.67	14.00	7.33	6.00	5.00	0.00
248	13.00	13.67	16.33	11.67	10.00	4.67	1.67	0.00
277	9.00	9.33	12.00	10.00	11.67	9.67	9.33	3.00
292	6.50	9.33	9.00	8.00	0.00	0.00	0.00	0.00

CD (P = 0.05) Ca x Suc = 3.44

embryogenic capacity of integument explants was dependent on the physiological aging of the plants from where explants were taken, and initiation of embryogenic callus from primary somatic embryos derived from the different explant sources varied. Liu and Pijut (2008) have also reported higher regeneration efficiency from juvenile source leaf explants of mature black cherry.

Modification of the medium to increase callus frequency and reduce the time taken for embryogenic callus initiation showed that $Ca(NO_3)_2 \cdot 4H_20$ at a concentration of 1.5 mM and sucrose 234 mM could efficiently induce embryogenic callus from the proliferated callus clumps at a phytagel concentration of 0.3 per cent (Table 2). Further, when this combination ($Ca(NO_3)_2 \cdot 4H_20$ at a concentration of 1.5 mM and sucrose 234 mM) was tried with different levels of phytagel, 5g/L (0.5%) phytagel was found to be optimum for embryogenic tissue initiation (Table 3).

In the optimised medium, the proliferated callus developed from *in vitro*-derived somatic plants took the least time (two months) to initiate embryogenic callus

Table 3. **Effect of phytagel on embryogenic callus initiation and embryogenesis in presence of 1.5 mM $Ca(NO_3)_2 \cdot 4H_20$ and 234 mM sucrose.**

Phytagel (g/L)	Embryogenic callus (mean %)	Globular embryos (mean %)
3.5	50.67	30.00
4.0	54.00	46.67
4.5	60.00	48.67
5.0	68.33	55.00
5.5	43.33	50.00
6.5	28.33	33.33
6.5	19.33	26.67
CD (P = 0.05)	15.41	15.99

with the highest frequency (68 %). In callus obtained from leaves of budded plants, the rate was 35 per cent and the time taken for initiating embryogenic callus was 5 months (Table 4). An increase of 10 per cent could be achieved with a considerable reduction in time. Here the combined effect of calcium and sucrose by varying the osmolarity of the medium, along with medium desiccation provided by higher levels of phytagel, might have aided faster embryogenic tissue initiation. The proliferated calli turned brown in this medium and embryogenic calli emerged as a small yellow clump which then proliferated and induced embryos (Fig.1, B&C). Further embryo induction was obtained from the proliferated embryogenic calli when the cultures were dark incubated in modified MS basal medium ($Ca(NO_3)_2 \cdot 4H_20$ - 2.11 mM and KH_2PO_4 - 2 mM) (Kala *et al.*, 2006) containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/L), casein hydrolysate (300 mg/L), 175 mM sucrose and phytohormones BA (2.2 µM), GA_3 (2.9 µM), Kin (1.25 µM), ABA (0.75 µM) and NAA (0.54 µM). Rate of embryo induction from the proliferated embryogenic calli was about 60-70 per cent. Increase of phytagel from 3.0 - 5.0 g/L along with change in $Ca(NO_3)_2 \cdot 4H_20$ and sucrose concentrations might have favored embryogenic calli formation. The influence of calcium in inducing callus friability in *Hevea* had been reported earlier by Montoro *et al.* (1993). Similar observations had also been made by Li *et al.*, (1998) in loblolly pine where 4.0 g/L phytagel induced embryogenic extrusions, whereas Becwar *et al.* (1995) reported that reducing the gelling agent concentration significantly enhanced extrusion and proliferation. In red spruce, a phytagel concentration of 2.0-3.0 g/L could initiate

Table 4. **Embryogenic callus initiation in callus induced from leaves from different sources in the optimised media containing 0.5 % phytagel**

Source of leaf explants	Time taken (months)	Mean embryogenic callus initiation (%)
In vitro somatic plants	2	68.33 ± 4.64
Budded plants	5	33.33 ± 5.77
Mature trees	—	0

embryogenic callus (Harry and Thorpe, 1991). Sucrose has major influence on somatic embryogenesis of various species (Ammirato, 1983). The findings of the present study are also in accordance with that of Sushamakumari *et al.* (2000) who reported a higher level of sucrose favouring the enhancement of embryo induction in presence of GA_3 in callus derived from immature inflorescence of *Hevea*. Similarly mineral and carbohydrate nutrition also influenced embryogenic calli formation, somatic embryogenesis and embryo maturation in *Hevea* (Etienne *et al.*, 1991; Cailloux *et al.*, 1996). Differential carbohydrate metabolism helps morphogenesis in embryogenic callus of *Hevea* (Blanc *et al.*, 1999 & 2002). GA_3 also regulated embryo induction and germination in *H. brasiliensis* (Jayasree *et al.*, 2001). The manner in which embryogenic potential of the cultured *Hevea* callus is affected leading to embryogenic calli formation followed by embryogenesis could be stabilised by modifying the culture conditions such as hormone balance and time of subculture (Michaux-Ferriere and Carron, 1989).

Changes in culture atmosphere (Auboiron *et al.*, 1990) and water status of the medium and explant (Etienne. *et al.*, 1991; Van Winkle *et al.*, 2003) also influenced embryogenic potential of the callus. In this medium, embryogenic calli were originated from more than one region of each callus clump as a small yellow lump within a month of culture which then proliferated to form a mass of friable golden yellow callus. Simultaneous with the formation of embryogenic calli, embryo induction could also be observed in all regenerating clumps from which embryos became visible within two weeks (Fig.1, C & D). Once embryogenic callus was formed, rate of embryo induction from the proliferated embryogenic calli was similar irrespective of the explant source. The physiological age of the source plant, from where the explants were collected, determines the embryogenic potential of the induced callus in leaf explants of *H. brasiliensis*. Interaction and balance of media constituents also determine the efficiency of a system along with the cultured tissue.

Embryo maturation and plant regeneration

After three weeks of culture in WPM, embryos were found enlarged and apex induction also occurred (Fig.2, A & B). Sucrose (183 mM) along with organic supplements and phytohormones, BA (2.2 μM), Kin (1.38 μM), IBA (0.49 μM) and GA_3 (5.3 μM) favoured maturation of embryos (Kala *et al.*, 2006). Cailloux *et al.*,(1996) and Sushamakumari *et al.* (2000) also reported that higher levels of sucrose (234 mM) favoured embryo maturation. Embryo germination was obtained when mature apex induced embryos were transferred to culture tubes containing hormone - free MS medium with 0.3 per cent charcoal and solidified with 0.2 per cent phytagel (Fig.2, C). Full plantlet development occurred within one month in this medium where the sucrose level was

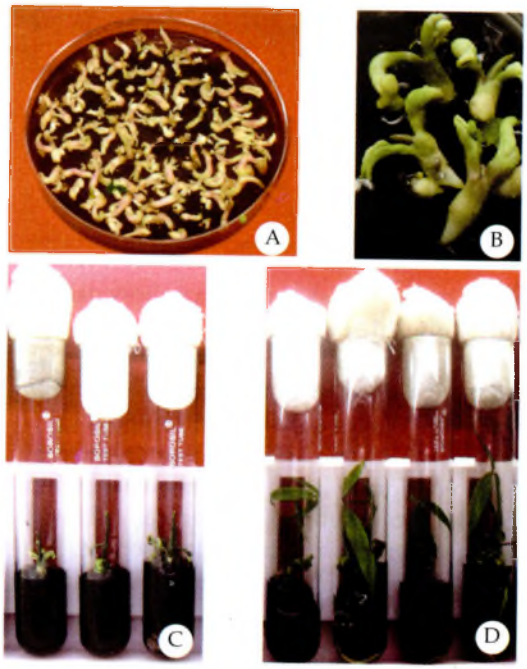

Fig. 2. Embryo maturation and plant regeneration
A - Development of cotyledonary stage embryos
B - Apex induced mature embryos
C - Regenerating embryos showing shoot elongation
D - Fully developed plantlets

further reduced to 89 mM (Fig.2, D). Earlier reports show that reducing sucrose concentration, thereby reducing the osmotic potential, encourages embryo germination (Carron *et al.*, 1995).

CONCLUSION

Physiological age of the source plant from which explants were collected was found to determine its embryogenic capacity. The leaves collected from *in vitro* plantlets derived through somatic embryogenesis, the most juvenile tissue, gave the maximum positive response within the shortest time. The embryogenic potential was found to reduce with maturity of the source plant, with leaves from mature trees having no embryogenic capacity. Between the remaining two sources, rate of embryogenesis from the proliferated embryogenic calli was similar irrespective of the explant source. Embryogenic callus initiation could be increased by the combined effect of calcium, sucrose and phytagel concentration of the culture medium. It was observed that a combination of water stress provided by phytagel and the osmotic stress caused by sucrose in conjunction with the presence of calcium imparts callus friability. This provided a favourable environment triggering the emergence of embryogenic calli and simultaneous embryogenesis. The present study reports obvious differences in embryogenic competence of leaf explants collected from source plants of different physiological maturity as shown by varied response in embryogenic tissue initiation in *H. brasiliensis*.

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