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Secondary embryogenesis and plant regeneration from leaf derived somatic embryos of *Hevea brasiliensis*

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Abstract

During somatic embryogenesis from leaf derived callus, it was observed that some of the regenerating plantlets produced secondary embryos profusely from the hypocotyl region. Compared to primary embryos, most of these secondary embryos were larger and healthy. These secondary embryos were separated and experiments were carried out for plant regeneration using different media combinations. Embryo maturation and shoot induction was obtained in WPM containing BA (0.5 mg/l), KIN (0.5 mg/l) and GA_3 (0.8 mg/l). Plant regeneration could be achieved in MS basal media and comparable with regenerated plants of primary embryos. Plant regeneration capacity of the secondary embryos exhibited significant difference as compared to primary embryos. It was found that the rate of embryo maturation and shoot induction in secondary embryos was around 40% compared to 75% in normal primary embryos. 25% of the secondary embryos with shoot induction could be converted into healthy plantlets.

Key words: Hevea brasiliensis, hypocotyls, leaf explants, secondary embryogenesis, somatic embryogenesis

Introduction

At present the elite *Hevea* clones are propagated by bud grafting, but *in vitro* propagation is highly desirable since true to type plants could be produced using the explants of elite genotypes. Research to develop somatic embryogenesis as an *in vitro* propagation method for *Hevea* began in the 1970's (Paranjothy and Rohini, 1978). Efficient protocols for plant regeneration through somatic embryogenesis from different explants such as inner integument, immature anther and inflorescence have been reported in *Hevea* by different groups (Wan *et al.*, 1982; Carron *et al.*, 1982; Etienne *et al.*, 1993; Seneviratnae *et al.*, 1996c: Jayasree *et al.*, 1999, 2001; Sushamakumari *et al.*, 2000). However, large scale plant production has not been achieved due to problems in hardening. Most of the micro propagation systems developed earlier were from floral explants of *Hevea*. Since flowering in this crop is seasonal and climate dependant, explant availability is limited to few months. Besides, initiation of culture from floral explants is time consuming. Therefore, experiments were carried out to develop alternate system from other explant source like leaves which are available throughout the year. In many other crops, leaf discs have been reported as a suitable explant for somatic embryogenesis and *Agrobacterium* mediated genetic transformation (De Block, 1988). Though work has been done earlier with the objective to regenerate plants from *Hevea* leaves, only callus formation have been reported (Mendanha *et al.*, 1998). Recently a protocol has been developed for somatic embryogenesis and plant regeneration from leaf derived callus of *Hevea* (Kala *et al.*, 2005, 2006). It was noticed that during plant regeneration from leaf derived primary embryos, few plantlets (10%) even after the induction of shoot and root produced secondary embryos from the hypocotyls. Most of these plantlets failed to show continued growth. These embryos were healthy and normal in appearance. Therefore, the present study is aimed at the assessment of regeneration capacity of the secondary embryos.

Materials and Methods

Primary somatic embryogenesis and plant regeneration from leaf

Somatic embryogenesis from leaf derived callus of clone RRII 105 was induced following the protocol developed earlier (Kala *et al.*, 2005, 2006). Leaf explants from *in vitro* shoot cultures were inoculated in callus induction medium. The leaflets were removed from the petiole and cut into pieces (1x1 cm) transversely across the leaf lamina and cultured on MS medium (Murashige and Skoog, 1962) with the adaxial surface in contact with the medium. Leaf explants were cultured with addition of calcium nitrate (850 mg/l), casein hydrolysate (1.0

gm/l), Gamborg's B5 vitamins (Gamborg *et al.*, 1968), sucrose (20 g/l) and phytohormones 2,4-D (1.2 mg/l), NAA (0.5 mg/l) and BA (1.0 mg/l) for callus induction. The calli formed were detached from the explants and sub cultured on the same medium containing 2,4-D (0.4 mg/l) and sucrose (40 g/l) for further proliferation. Proliferated calli were then sub cultured for embryo induction in modified MS medium (calcium nitrate 360 mg/l) supplemented with BA (0.5 mg/l), KIN (0.3 mg/l) and GA_3 (0.8 mg/l). The media also contained B5 vitamins and amino acids, organic supplements such as coconut water (5%) and casein hydrolysate (500 mg/l), sucrose (60 g/l), activated charcoal (0.2%). After 60 days of culture, callus along with somatic embryos of different developmental stages were aseptically removed from culture vessels and transferred onto fresh medium for further development (Fig. 1). For maturation of embryos, Woody Plant Medium (Lloyd and Mc Cown, 1980) containing BA (0.5 mg/l), KIN (0.5 mg/l) and GA_3 (1.0 mg/l) was used. Enlarged embryos having shoot and root apex were sub cultured for plant regeneration on full strength MS basal medium.

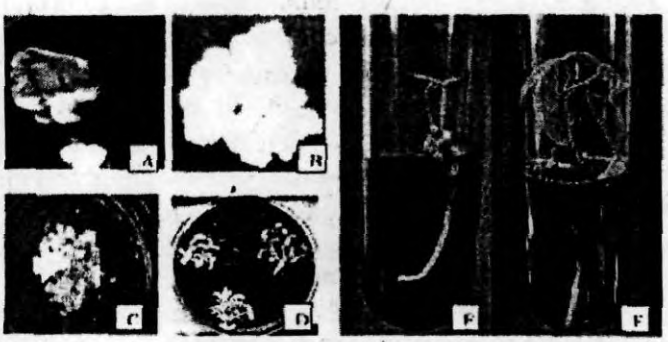

Fig.1. Somatic embryogenesis from primary embryos derived from leaf explants

A. Callus induction in leaf section; B. Embryogenic calli; C. Embryo induction; D. Different stages in embryo maturation; E. Regenerating plantlet; F. Fully developed plantlet

Secondary embryo formation

During the process of plant regeneration from the mature primary somatic embryos, few plantlets after 2-3 weeks of growth developed high frequency secondary embryos from the hypocotyl region. Secondary embryo formation was initiated as a small callus mass which enlarged further and each clump produced nearly 30-50 embryos. The embryos formed enlarged within two weeks and were normal and with good vigor (Fig. 2). Once secondary embryos were formed, the plantlets showed problems in shoot and leaf development and hindrance of root growth which arrested its further development.

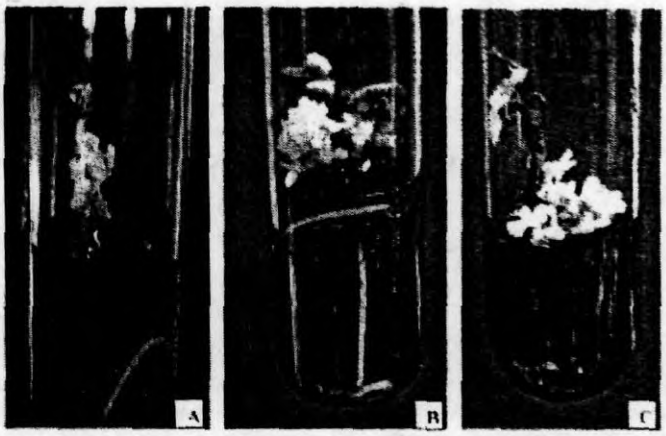

Fig. 2. Secondary embryogenesis from plantlets regenerated from primary embryos

A. Secondary embryo initiation B. Globular embryos C. Cotyledonary stage embryos

Maturation and apex induction of secondary embryos

For maturation and shoot induction, these embryos were separated and sub cultured on the shoot induction medium. The media used for shoot induction in primary embryos was used in the present experiment along with modified media (M1, M2, M3). These media also contained B5 vitamins, sucrose (40 g/l) and organic supplements coconut water (10%), malt extract (100 mg/l) and casein hydrolysate (500 mg/l) in addition to BA (0.3, 0.5 and 0.8 mg/l), KIN (0.3, 0.5 and 0.8 mg/l), and GA_3 (0.5, 1.0 and 1.5 mg/l). Optimum concentration of the hormones were identified and this was used for further experiments on embryo maturation. Basal medium along with phytohormones BA (0.5 mg/l), KIN (0.5 mg/l) and GA_3 (0.8 mg/l) which were found to favour embryo maturation were used. All media contained charcoal (0.2%) and were solidified with phytagel (0.25%). Ten embryos were tried in each experiment with three replications.

Plant regeneration

Well developed mature embryos were obtained after three weeks of culture in maturation medium. These were transferred for plant regeneration on MS and WPM with and without hormones. The medium also contained coconut water (5%) and sucrose (30 g/l) in addition to growth hormones such as BA (0.3 mg/l), GA_3 (0.3 mg/l) and IBA (0.1 mg/l).

Results and Discussion

Somatic embryogenesis from leaf

Nature of the leaf explant played a major role in callus induction. Light green leaves of medium maturity responded well. Callus induction could be obtained

within four weeks of culture under dark (Fig.1 A). After 50 days, somatic embryos in all developmental stages, including mature embryos in the cotyledonary stage were formed in the embryo induction medium (Fig. 1 B,C). Maturation and shoot induction of embryos occurred in WPM containing organic supplements and cytokinins such as BA (0.5 mg/l), KIN (0.5 mg/l) and GA_3 (1.0 mg/l) (Fig.1 D). Cytokinins were reported to favour embryo germination in *Hevea brasiliensis* (Jayasree *et al.*, 2001b). Presence of activated charcoal was essential for embryogenesis and plant regeneration. Embryos with shoot formation were found after three weeks when single embryos were sub cultured and maintained under dark. Plant regeneration could be achieved when mature embryos at the cotyledonary stage were cultured on MS basal medium and kept in light (Fig. 1 E, F).

Secondary embryogenesis

Regenerating plantlets (10 %) exhibited secondary embryo formation from the hypocotyl. It was observed that only a small percentage of plantlets showed this phenomenon during their course of regeneration. Once embryo formation was initiated, the plant started showing hindrance in continued growth, either by multiple shoot formation, cessation of root growth or leaf senescence. Embryogenesis was initiated from the hypocotyl region of the plantlet as a very small clump which proliferated vigorously to produce high frequency somatic embryos. Within two weeks of culture majority of the embryos became healthy (Fig. 2 A, B, C). Cailloux *et al* (1996) have reported high frequency secondary embryogenesis from isolated cotyledonary stage embryos of *Hevea brasiliensis*. Asokan *et al.* (2002) have also reported repetitive embryogenesis from primary somatic embryos derived from integument tissue of *Hevea brasiliensis* cultured on B5 medium supplemented with NAA, KIN, IAA and 2,4-D. In all these cases, secondary embryogenesis means the embryo induction from the primary embryos. Such phenomenon is termed by different groups as either secondary embryogenesis or repetitive embryogenesis. However, in the present context secondary embryogenesis was from regenerating plantlets having both shoot and root system, and not from the primary embryos as reported earlier (Asokan *et al.*, 2002). Some endogenous factors might have triggered secondary embryogenesis rather than influence of any exogenous phytohormones since regenerating plantlets were cultured in hormone free medium. The plantlets which exhibited this phenomenon showed problems in shoot and root development which hindered its further development.

Table 1. Composition of major elements of different media used for maturation and shoot induction of secondary embryos

Media Constituents	MS (mg/l) (M1)	WPM (mg/l) (M2)	Modified MS (mg/l) (M3)
NH_4NO_3	1650 mg	400 mg	500 mg
$CaNO_3$	——	260 mg	——
KNO_3	1900 mg	132 mg	900 mg
$CaCl_2$	333 mg	370 mg	333 mg
$MgSO_4$	181 mg	270 mg	260 mg
KH_2PO_4	170 mg	990 mg	360 mg
K_2SO_4	——		270 mg

Maturation and apex induction in secondary embryos

These embryos were detached from the plantlets and sub cultured for maturation and shoot induction along with the primary embryos as control. Of the three different media (M1, M2, M3) tested for shoot induction, WPM containing BA (0.5 mg/l), KIN (0.5 mg/l) and GA_3 (0.8mg/l) (M2) was found superior (Table 2). GA_3 influenced germination of the embryos. Jayasree and Thulaseedharan (2001) have reported that incorporation of 2 mg/l GA_3 increases germination percentage in anther derived embryos of *Hevea*. Significant difference in shoot induction could be observed between primary and secondary embryos. It was found that the rate of embryo maturation and shoot induction in secondary embryos was 40% compared to 75% in normal primary embryos. Most of the embryos did not form shoot and root apices and showed abnormal enlargement of the cotyledons with callusing (Fig. 3 B). Secondary embryos with shoot development were larger as compared to primary embryos.

Table 2. Frequency of shoot induction in primary and secondary embryos

Treatments	Number of embryos	Shoot induction frequency		Mean
		Primary embryo	Secondary embryo	
M1	30*	7.000	4.333	5.667
M2	30	7.667	3.667	5.667
M3	30	6.333	3.333	4.833
Mean		7.000	3.778	

CD(A)=1.5907

* In each treatment 30 embryos were cultured with 10 embryos in each replication

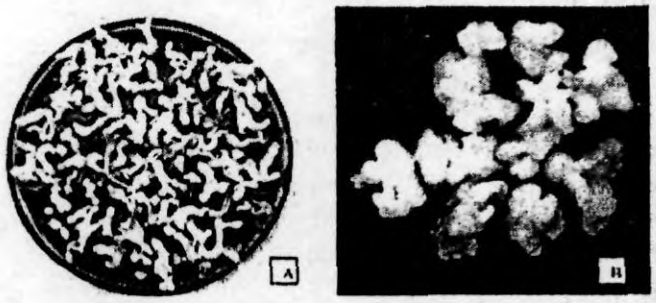

Fig. 3. Maturation and Apex induction of primary and secondary embryos A. Primary embryos; B. Secondary embryos

Plant regeneration from secondary embryos

Experiment on plant regeneration was carried out in MS and WPM with and without hormones. No significant difference between treatments was observed. Significant difference in plant regeneration could be observed between the shoot induced primary (75%) and secondary embryos (40%). When shoot induced secondary embryos were sub cultured for plant regeneration, it was found that only 25% showed continued growth. Since the regenerating plantlets from the secondary embryos were healthy they showed good growth in MS basal media itself and were comparable to normal regenerating embryos (Fig. 4 A,B,C). The plantlets obtained were healthier with increased shoot and root growth than the ones obtained from primary embryos. Complete plant development from these embryos could be achieved within three weeks and successfully transferred to polybags.

Fig. 4. Plant regeneration from secondary embryos
A. Shoot and root elongation; B. Leaf development; C. Fully developed plantlet

Table 3. Frequency of plant regeneration from apex induced embryos

Treatments	Number of apex induced embryos	Plant regeneration frequency Primary embryo	Secondary embryo	Mean
M1 (MS)	10	7.667	3.000	5.333
M2 (MS-H)	10	7.333	3.000	5.167
M3 (WPM)	10	7.000	2.000	4.500
M4 (WPM-H)	10	6.333	2.333	4.500
Mean		7.083	2.667	
CD(A)=0.90				

Conclusion

In the present study, efficiency of secondary embryos to regenerate into complete plants was assessed. Significant difference in shoot induction was found between primary and secondary embryos. The rate of embryo maturation and shoot induction in secondary embryos was 40% compared to 75% in primary embryos. Only 25% of the shoot induced secondary embryos could regenerate into complete plants. It was observed that though numerous secondary embryos were formed and appeared to be healthy, they had low regeneration capacity since the embryos had more tendency for callus induction. However, refinement of the existing protocol is expected to be useful for regeneration and conversion of plantlets on a large scale.

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