2005 747

National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops : Issues and Strategies. 10 - 12 January, 2005; Kerala Agricultural University, Thrissur, Kerala

Contributory Paper 18

In vitro regeneration of *Hevea brasiliensis* from leaf explants

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Abstract

A protocol has been developed for the induction, maturation and germination of somatic embryos from leaf explants of *Hevea brasiliensis* (clone RRII 105). Leaf explants were cultured with their adaxial sides on modified Murashige and Skoog (MS) medium supplemented with different combinations of phytohormones such as 2,4-D & BA, NAA & BA as well as 2,4-D, BA and NAA. Compact calli could be developed from the cut ends of the explants on media containing 2,4-D (1.5 mg/l) and BA (1.0 mg/l) whereas pale yellow friable calli was obtained on media which contained NAA (0.2 mg/l) along with 2,4-D (1.2 mg/l) and BA (1.0 mg/l). The calli formed were detached from the explants and sub cultured for proliferation in medium containing reduced auxin (0.4 mg/l 2,4-D) and slightly increased level of sucrose (40 g/l). Proliferated calli were then sub cultured for embryo induction in modified MS medium supplemented with different auxins and cytokinins. Embryo induction could be achieved in modified MS medium containing BA (2.0 mg/l), GA_3 (1.0 mg/l) and NAA (0.2 mg/l) and maturation occurred in WPM medium containing BA (0.3 mg/l), TDZ (0.5 mg/l) and GA_3 (1.5mg/l). The cotyledonary stage embryos developed into plantlets on transfer to hormone free ½ MS medium. A distinct feature of this study is the induction of somatic embryogenesis from leaf explants of *Hevea* which has not been reported previously. This explant could be used further as an ideal target tissue for *Agrobacterium* mediated genetic transformation in *Hevea*.

Key words: Callus induction, Somatic embryogenesis, Plant regeneration.

Introduction

Hevea brasiliensis belonging to the family Euphorbiaceae is an important commercial crop cultivated as a source of natural rubber. Since it is open pollinated and highly heterozygous, commercial propagation is by budgrafting. Work on *in vitro* culture of *Hevea* was initiated with a view to develop protocols for micropropagation and genetic improvement through transgenic approaches. Efficient plant regeneration from a range of explants such as inner integument immature anthers and immature inflorescence has been reported earlier in *Hevea* (Sushamakumari *et al.*, 2000). However, reports on somatic embryogenesis are relatively few for woody species since most of them are recalcitrant to *in vitro* culture. The system already developed has been widely used successfully as a tool for genetic transformation in the last decade (Arokiaraj *et al.*, 1994; Jayasree *et al*; 2003). In most of the reports on other crops, leaf tissue has been mentioned as a potential target tissue for *Agrobacterium* infection (De Block, 1988) and plant regeneration. Both direct and indirect somatic embryogenesis from leaves has been reported in several crops (Hulme *et al.*, 1992). Though work has been done earlier with a view to regenerate plants from *Hevea* leaves, only callus formation have been reported (Mendanha *et al.*, 1998). Hence attempt has been made to regenerate plants through somatic embryogenesis from callus cultures of leaf explants.

Materials and Methods

Leaf explants were collected from glass house grown plants of *Hevea* clone RRII105, washed in commercial bleach and then thoroughly rinsed in tap water. They were surface sterilized by soaking in an agitated solution of 0.15% $HgCl_2$ containing a few drops of Tween 20, thoroughly washed in sterile distilled water and the explants were blotted dry on sterile filter paper. The leaflets were separated from the petiole and cut into pieces (1x1 cm) transversely across the leaf lamina. Leaf segments were cultured with the adaxial surface in contact with the medium. The cultures were maintained in the dark.

Callus induction from leaf explants was tried on both MS and WPM (Lloyd and Mc Cown, 1980) media supplemented with different phytohormones. The effect of growth regulators was tested using phytohormones 2,4-D and NAA in combination with BA and KIN. The pH of the medium was adjusted to 5.7 with 1N KOH before autoclaving at 121° C for 10 min. Two way factorial of auxins 2,4-D and NAA along with cytokinins BA and KIN was done so as to identify the ideal concentration for callus induction. Concentration of auxins tried for callusing varied from (0 – 2.0 mg l^{-1}) and cytokinins (0 – 2.5 mg l^{-1}). Three leaf sections were placed in each culture tube. Total 20 cultures were kept for each treatment. The cultures were incubated at 27 ± 2°C in the dark. The frequency of callus formation was determined one month after culture initiation.

After 45 days of culture, the induced calli were first subcultured for proliferation. Further the proliferated calli were experimented for embryo induction.. Modified MS and WPM medium with B_5 vitamins, amino acids, organic supplements, CW, casein hydrolysate and 6% sucrose supplemented with different combinations and concentrations of phytohormones BA, GA_3, KIN, 2,4-D and NAA were tried for embryo induction. The phytohormones were supplemented in the range 0.2 – 2.5 mg l^{-1} BA, 0 – 1.5 mg l^{-1} GA_3, 0.2 mg l^{-1} NAA and 0.1mg l^{-1} 2,4-D. Maturation and apex induction of the embryos were also tried on both MS and WPM medium supplemented with phytohormones BA, GA_3 and TDZ. Enlarged embryos having shoot apex were transferred for plant regeneration to ½ MS medium. All experiments were repeated thrice with twenty replications.

Results and discussion

Leaf explants cultured on basal medium with different combinations of auxin and cytokinin induced callus within 4 weeks of incubation. The optimal stage of the leaf for callus induction was in between immature and mature stage i.e. when the leaf blade attains a light green colour with a shiny appearance. Callus induction could be obtained in MS medium with addition of calcium nitrate (850 mg l^{-1}) and supplemented with casein hydrolysate (1.0 gm l^{-1}), B5 vitamins, sucrose 20 g l^{-1} and phytohormones 2,4-D, BA and NAA (Table 1). Table 1 shows the rate of callus induction and type of callus formed after 40 days in different media combinations. Our results show that medium containing BA (1.0 mg l^{-1}) and 2,4-D (1.5 mg l^{-1}) in presence of low concentration of NAA (0.2 mg l^{-1}) was most effective for inducing good quality callus in leaf explants.

The proliferated calli when sub cultured in MS medium containing B5 vitamins and amino acids with organic supplements and phytohormones NAA, Kin, BA and GA_3 turned black within 50 days after culture. These calli when again subcultured to modified MS medium supplemented with phytohormone combinations produced yellow embryogenic calli which further differentiated into pro embryos after two weeks in the same medium. Embryo induction could be achieved in modified MS medium, with ($CaNO_3$ 360 mg l^{-1}, B_5 vitamins and phytohormones BA (2.0 mg l^{-1}), GA_3 (1.0 mg l^{-1}), NAA (0.2 mg l^{-1}) and 2,4-D (0.1 mg l^{-1}). The embryo induction medium also contained amino acids proline (100 mg l^{-1}), cysteine (20 mg l^{-1}), serine (20 mg l^{-1}) and arginine (37 mg l^{-1}) along with organic supplements coconut water 10% and casein hydrolysate (400 mg l^{-1}). The best result was observed with the combination of BA and GA_3. After 50 days globular stage embryos were removed from culture and transferred onto fresh medium for further development of embryos. Sub culturing single embryos in darkness was found to be more effective for their enlargement and conversion to cotyledonary stage. Cotyledonary stage embryos were formed after three weeks in this medium.

Maturation and apex induction of embryos occurred in WPM medium containing organic supplements CW 10%, malt extract 100 mg l^{-1}, casein hydrolysate 500 mg/l and phytohormones BA (0.3 mg l^{-1}), TDZ (0.5 mg l^{-1}) and GA_3 (1.5 mg l^{-1}). Addition of TDZ in the maturation medium and reducing the level of BA was found favourable for further development of embryos. Apex induced embryos when transferred to hormone free ½ MS medium could produce plantlets within 3-4 weeks of culture.

The earlier reports on somatic embryogenesis of *Hevea* have been from explants like immature anther, inner integument, immature inflorescence etc. In conclusion, we have developed a system for induction of somatic embryos from *Hevea* leaves. This paper is the first report on the induction of somatic embryogenesis from calli derived from leaf explants of *Hevea brasiliensis*.

Table 1. Effect of 2,4-D and BA in presence of 0.2 mg/l NAA on callus induction

Growth regulators BA (mg l^{-1})	2,4-D (mg l^{-1})	Morphology of callus	Rate of callus induction
0.8	0.0	—	—
	0.4	Watery, soft, white creamy	1-20 %
	0.8	Friable, little watery, soft	21–50 %
	1.2	Friable, pale yellow	51-80 %
	1.6	Compact, pale yellow, hard	51-80 %
1.0	0.4	Watery, soft, white creamy	20-50 %
	0.8	Friable little watery	20-50 %
	1.2	Compact, pale yellow	50-80 %
	1.6	Pale yellow, hard	50-80 %
1.2	0.4	Watery, soft, turning green	20-50 %
	0.8	Friable, soft, pale yellow	20-50 %
	1.2	Compact, pale yellow	50-80 %
	1.6	Compact, hard, pale yellow	50-80 %

Data were taken after 50 days of culture

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