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Contributory Paper 3

Endosperm culture in *Hevea brasiliensis*

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Abstract

The totipotency of triploid endosperm tissue is exploited in many vegetatively propagated crops. *Hevea brasiliensis*, the most important commercial source of natural rubber, is a highly heterozygous perennial tree with a long breeding cycle. Conventional method of triploid production by crossing a diploid with an artificially induced tetraploid is a lengthy and laborious process. Hence *in vitro* regeneration of plants from endosperm tissue provides an easy and direct approach for the production of large number of triploids. *Hevea* fruits at different developmental stages were collected and sterilized using 0.1% $HgCl_2$ for 15 minutes. Seeds were dissected out and the embryos and inner integuments were removed. Endosperm is cut into small pieces and cultured over MS medium supplemented with various levels of sucrose, phytagel and growth regulators viz BA, Kin thidiazuron, 2,4-D, IBA, NAA, zeatin & GA. Organic supplements viz. yeast extract, casein hydrolysate, coconut milk and banana powder were used for callus and embryo induction. Optimum callus induction was obtained in media supplemented with 2,4-D (2.0 mgl $^{-1}$) and Kinetin (3.0 mgl $^{-1}$). On further subculture of the primary calli to medium containing zeatin (1.0 mgl $^{-1}$), embryogenic calli could be obtained. Embryos were induced in MS medium supplemented with ABA (0.3 mgl $^{-1}$) , Kin (0.3 mgl $^{-1}$) and GA (0.5 mgl $^{-1}$). Maturation, germination and bipolar differentiation of the embryos were achieved in MS medium devoid of growth regulators. Root tips from the germinating embryos were subjected to cytological analysis and found to be triploids (3n=54). Regeneration from *Hevea* endosperm is being reported for the first time. The results indicates that like many other crops, *Hevea* endosperm responds well under *in vitro* conditions and endosperm culture could be utilized as a viable technique for triploid plant production.

Key words: *Hevea brasiliensis*, Endosperm , triploids

Introduction

During the past decades, the potential of endosperm tissue for *in vitro* differentiation has been well established. Since endosperm tissue is triploid, the plantlets formed are also expected to be triploids. The significance of triploids in plant improvement is well established. Many of the triploid lines have proved to be superior to the diploid and tetraploid counterparts, especially in yield and nutritive qualities, cold and disease resistance (Bojwani *et al.*, 1977). Triploids can also be utilized to obtain trisomic lines for genetic mapping. The technique of endosperm culture may offer the plant breeders a new method of raising triploids.

Hevea brasiliensis, the most important source of natural rubber, is a highly heterozygous perennial tree with a long breeding cycle. Triploid plants with high yield can be expected in *Hevea,* since the economic product is other than seed. Conventional method of triploid production, by crossing a diploid with artificially induced tetraploids is lengthy and tedious. Hence regeneration of plants from endosperm tissue by somatic embryogenesis is a viable proposition. Somatic embryogenesis and successful plant regeneration has been reported in *Hevea* from different explant sources (Jayasree *et al.*, 1999). However, no reports are available on endosperm culture and successful regeneration of plantlets. Hence the present study was initiated with a view to establish a regeneration pathway from *Hevea* endosperm and to produce triploids.

Materials and methods

Fruits were collected from *Hevea* clone RRII 105 at different maturity (3-6 months) and the endosperm along with inner integument was sterilized with 0.1% $HgCl_2$ for 15 minutes. The inner integument was removed carefully and the excised endosperm was inoculated into the medium. Two pieces of endosperm were placed per culture tube, each containing 20 ml medium.

For callus induction, 3 basal media, MS, WPM, and N_6 and varying levels of growth regulators viz GA, BA, Kinetin, NAA, TDZ, 2,4-D and Zeatin were tried at different levels. (1-5 mgl $^{-1}$) .The pH of the medium was adjusted to 5.8 before autoclaving at 1.5 kg/cm^2 and 120°C.

For embryo induction, reduced levels of cytokinins, GA, and ABA were incorporated in the medium. Effect of phytagel on embryo maturation and germination were studied by incorporating varying levels of phytagel in the medium (2-6%). For maturation and germination, hormone free MS medium was used. The complex organic supplements such as banana powder, yeast extract, casein hydrolysate, and coconut milk were also incorporated in the medium for maturation and germination. The media for embryo induction, maturation and germination were also supplemented with 0.2% activated charcoal. All the cultures were incubated under 16/8 hrs photoperiod at 28°C. Observation on percentage of callus induction, embryo induction, maturation and germination of embryos were recorded.

The roots from the germinating embryos were pretreated in pDB for 2 hrs and fixed in Carnoys fluid for 24 hrs. Squash preparations were made using acetocarmine for confirmation of ploidy.

Result and discussion

It was observed that, the stage of the endosperm, basal medium, and type and concentration of growth regulators influenced callus induction from *Hevea* endosperm. Among the 3 basal media tried, MS medium was found to be ideal for callus induction.

Among the different stages of endosperm tried for callus induction, only mature endosperm responded to the cultures. The endosperm from fruits about to dehisce was found to be precise for endosperm culture. Many workers studied the influence of endosperm stage on culture response. It varies with species. Mature endosperm is not amenable to culture in the case of cereals whereas in many plants belonging to *Euphorbiaceae, Loranthaceae* and *Santalaceae*, the mature endosperm responded well . It was also observed that the initial association of the embryo is not essential for culture response in *Hevea*, even though it is reported in several members of *Euphorbiaceae* family.

Among the growth regulators, the cytokinins viz. Kin, BA and TDZ had no response on callusing, when supplied individually (1-5 mg^{-1}). Explants dried and turned brown in most of the cases. Among the auxins 2,4-D induced callusing with a very low percentage and failed to proliferate further. Addition of NAA (2.0 mg^{-1}) induced root formation from endosperm. Among the different combinations of auxins and cytokinins, the combination of 2,4-D with Kin performed well. Results are presented in Table-1. The highest percentage of callus induction (14%) was obtained with 2.0mg^{-1}2,4-D and 3.0mg^{-1} Kinetin. (Table 1). On further increase of Kinetin, reduction in the callusing percentage was observed. Similar response was observed with increase in 2,4-D also. Callus induction and differentiation of embryo like structures from endosperm using 2,4-D and Kinetin is reported in *Ricinus communis, Croton bonplandianum* and *Jatropa* (Reinert and Bajaj, 1977). For further experiments 2.0mg^{-1}2,4-D and 3.0 mg^{-1} Kinetin was used for inducing primary callus.

The primary calli, were then sub cultured into medium containing different cytokinins for obtaining friable embryogenic calli and yellowish embryogenic callus was obtained in the medium supplemented with 1.0 mg/l Zeatin 2.0mg^{-1} 2,4-D and 3.0 mg^{-1} Kin.

Table 1. Effect of 2,4-D and KIN on callus induction from *Hevea* endosperm (%)

2,4-D	KIN (mg^{-1})				
(mg^{-1})	1.0	2.0	3.0	4.0	5.0
1.0	3	4	4	2	0
2.0	5	6.67	14.0	8.66	7.0
3.0	2.0	2.0	8.33	6.0	9.0
4.0	1.0	2.0	4.0	6.0	7.0
5.0	0	7.0	2.66	5.0	5.67

CD (0.05) -1.55

Table 2. Effect of ABA and GA on embryo induction (%)

ABA	GA (mg^{-1})				
(mg^{-1})	0.5	1.0	1.5	2.0	2.5
0.1	11.0	23.0	6.33	3.0	2.0
0.2	13.0	20.3	6.0	1.66	0.67
0.3	52.3	39.6	18.0	5.66	1.3
0.4	45.3	36.3	16.67	3.0	0.6
0.5	30.0	26.33	20.0	14.67	14.0

CD (.05) 1.98

For embryo induction, the auxin was removed from the medium and embryo induction was attempted with other growth regulators. Maximum embryo induction frequency (52%) was obtained in MS medium supplemented with 0.3 mgl^{1} ABA, 0.5 mg/l GA3 and 0.3 mg/l Kinetin (Table 2). On further increase in concentration of GA, embryo induction frequency reduced considerably. However increase in ABA concentration, a slight reduction in embryo induction was observed. Jayasree *et al.* (2001) reported that GA_3 stimulated embryo induction in *Hevea brasiliensis* at lower levels and a progressive reduction in the embryo induction was noticed with increase in concentration, for anther derived calli (Jayasree *et al.*, 2001) Many workers reported the beneficial effect of exogenious ABA in embryo induction as well as maturation in *Hevea* earlier. The highest frequency of transgenic embryos was obtained in *Hevea* with ABA, GA and Kin combinations at lower concentrations (Jayashree *et al.*, 2000). According to Cailloux *et al.* (1996) higher concentrations of ABA promotes long-term embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*.

Table 3. Effect of phytagel on embryo maturation and germination

Phytagel Concentration (%)	Embryo Maturation (%)	Embryo Germination (%)
0.2	5.67	8.67
0.3	11.67	3.3
0.4	18.33	1.6
0.5	24.00	0.67
0.6	16.00	0.33
CD (.05)	2.57	1.33

For embryo maturation and germination hormone free MS medium was found to be ideal. It is also observed that the phytagel plays a significant role in maturation and germination of embryos (Table 3). A drastic increase in the embryo maturation frequency was noticed, when phytagel was increased from 0.2- 0.5% .The highest percentage of embryo maturation (24%) was obtained with 0.5% phytagel. Further increase in phytagel, decreased maturation frequency. The water stress induced by the phytagel may be promoting embryo maturation. Residual effect of ABA from the previous medium also might be contributing towards maturation. Jayashree *et al.* (2003) have earlier reported the promotive effect of high phytagel along with ABA (0.1mg^{-1}) on embryogenesis.

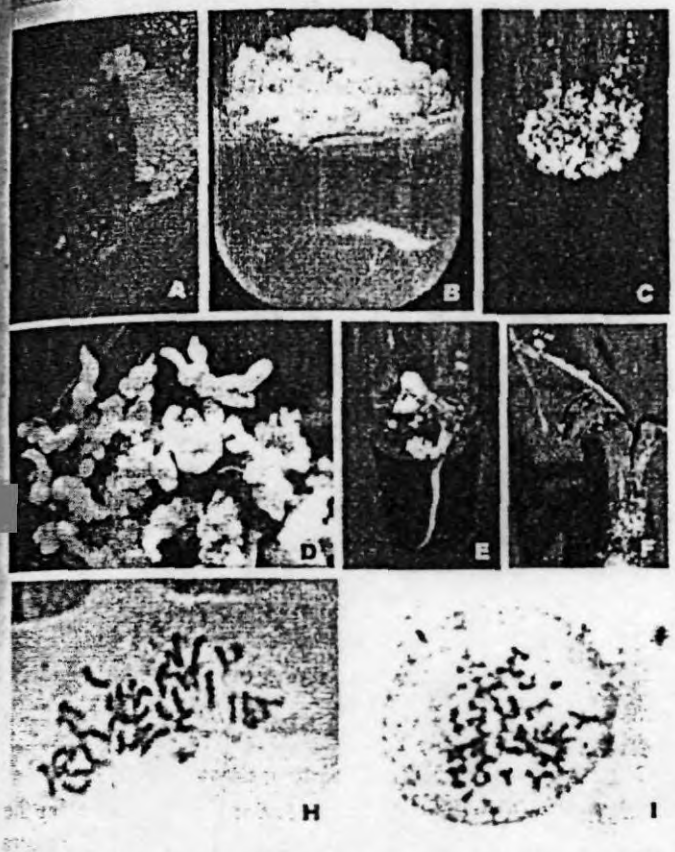

Figure1.A. Callus formation from *Hevea* endosperm. B. Embryogenic calli C. Embryo induction D. Embryos in the maturation medium E. Germinating embryo showing root growth F. Germinating embryo showing shoot emergence H Cell showing diploid chromosome number 2n=36 (1500X) I. Cell showing triploid chromosome number 3n=54 (1300X)

In contrast to maturation, lower levels of phytagel favoured germination. A maximum germination frequency of 8.6 % was achieved with 0.2% phytagel .The germination frequency decreased gradually with increase in phytagel concentration. Germination was also improved with the addition of 400 mg^{-1} casein hydrolysate, 500 mg^{-1}malt extract and 200mg^{-1} banana powder. The germinated embryos are at different stages of development.

The mitotic studies of the few embryos germinated revealed that they are triploids with 3n=54.

In spite of high frequency embryo induction and maturation, the germination frequency was found to be very low. Extensive optimization experiments are to be carried out for refining the technique and for further acclimatization of the plants. However, the present results indicates that *Hevea* endosperm responds well under *in vitro* conditions and this is the first step towards the recovery of triploid plants in *Hevea brasiliensis*.

References

Bhojwani, S.S., Johri, B.M.1971. Morphogenetic studies on cultured mature endosperm of *croton bonplandium*. *New Phytol*. 70:761-766.

Caillioux, F., Julien-Guerrier, J., Linossier, L. and Goudret, A. 1996.Long term somatic embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*. *Pl.Sci*. 120:185-96

Jayasree, P.K., Asokan, M.P., Sobha, S., Sankariammal, L., Rekha, K., Kala, R.G., Jayasree, R. and Thulaseedharan, A. 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell. Arg.). *Curr.sci*. 76: 1242-1245.

Jayashree, R., Rekha, K., Sushamakumari, S., Kala, R.G., Jayasree, P.K., Thanseem, I., Ashokan, M.P. and Thulaseedharan, A. 2000. Agrobacterium mediated Genetic Transformation in *Hevea brasiliensis*. *Placrosym XIV*, Hyderabad, Dec.10-13.

Jayasree, P.K. and Thulaseedharan, A.2001.Gibberelic acid regulated embryo induction and germination in *Hevea brasiliensis* (Muell.arg.) *Ind.l Nat.Rubb.Res* 142: 106-111.

Jayashree, R., Rekha, K., Venkatachalam, P., Uratsu, Jayasree, P.K., Kala, R.G., Sushamakumari, S., Sobha, S., Priya, P., Asokan, M.P., Sethuraj, M.R., Thulaseedharan, A. and Daendekar, A.M.2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis*) with a constitutive version of antioxidative stress SOD gene. *Plant. Cell. Rep.* 22: 201-209.