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IN VITRO CULTURE OF IMMATURE EMBRYOS OF *HEVEA BRASILIENSIS*

In vitro culture of plant embryos is a technique to obtain viable offspring following inter specific and inter generic hybridization where routine fertilization fails to produce a full term embryo. The technique has generally been adopted for rescue of the immature hybrid embryos from incompatible crosses and for overcoming short viability of seeds. Although successful fertilization and early embryo development occur in most cases, a number of irregular events take place subsequently, resulting in the eventual death of the embryo and collapse of the seeds (Raghavan and Torry, 1963). A major cause of early embryo abortion is the failure of the endosperm to develop properly. Aseptic culturing of the embryos in a nutrient medium can often overcome this problem. However, the success of the technique depends upon many factors like isolation of embryos from ovules or immature fruits without injury, use of a suitable nutrient medium and induction of continued embryogenic growth and seedling formation.

A very low fruit set (0-3.5 %) is observed in the natural rubber tree, *Hevea brasiliensis* (Warmke, 1951, 1952; Gandhimathi and Yeang, 1984), which could be due to environmental, physiological or genetic reasons. However, normal plants can be raised from mature embryos of *Hevea* using embryo culture technique (Das *et al.*, 1998) thus permitting the incorporation of useful characters into cultivated rubber (Chen, 1984). In spite of several attempts on *in vitro* techniques for multiplication and improvement of *Hevea*, information on embryo culture techniques for rubber is scanty. The present investigation attempts development of a suitable nutrient medium for *in vitro* culture of immature zygotic embryos of *H. brasiliensis*.

Immature fruits (3-16 week old) were collected from the experimental farm of Rubber Research Institute of India at Taranagar, Agartala in Tripura State. After washing thoroughly with detergent, the fruits were surface-sterilized in 0.05 per cent mercuric chloride solution with a drop of Tween 20 for 15 to 20 min and washed well with distilled water. Subsequently, intact seeds were isolated by dissecting the fruits. Two techniques were adopted for excision of embryos avoiding injury. Embryos from 3 to 8 week old fruits were excised by making an incision at the micropylar end and separating out the tiny embryos by applying force at the opposite end while those from 9 to 16 week old fruits were excised by using the blunt end of the scalpel after dissecting the seed longitudinally.

Widely used basal media like White's 'W' (White, 1963), Gamborg's 'B5' (Gamborg *et al.*, 1968) and Murashige and Skoog's 'MS' (Murashige and Skoog, 1972) were tried for culturing embryos and based on the performance, MS medium was selected as the basal medium for embryo germination.

Further growth of the embryos was induced by modifying the MS medium, by incorporating half the concentration of macro and double the concentration of micronutrients and adding 0.05 to 0.2 g/L of Na_2PO_4 and 2-8 per cent sucrose. Coconut water (CW) was supplemented at different doses (5-20%) as the natural plant extract. Casein hydrolysate (CH), the amino acid complex, was tested at different concentrations (50 – 500 mg/L) to observe the growth response of the embryos.

For regeneration, different concentrations (0.1-5 mg/L) of various auxins such as IAA, IBA, NAA and cytokinins like kinetin, BAP and 2iP were tested. GA_3 was added in the media for healthy growth of the leaves. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C under 1.26 kg/

cm^2 pressure for 20 min. All the cultures were incubated in the dark for the first 7 to 10 days and subsequently transferred to 16h light period at 25°C. The embryo cultures were sub-cultured regularly at 3 week intervals.

To initiate rooting, the isolated shoots were cultured on media with four different doses of IBA and NAA (0.5-2.0 mg/L). The embryos were transplanted at the two to three true leaf stage. Plants with six to nine leaves and well-developed tap root system were removed from agar media, root portions washed thoroughly with sterile distilled water and fungicide (carbendazim 0.1%) solution and then planted in sterilized soil:sand (2:1) mixture pre-soaked with the basal medium.

The screening of the three basal media (MS, B5 and W) for germination of excised zygotic young embryos of *H. brasiliensis* revealed that the MS medium supported growth of the very young embryos after germination. On B5 medium, the survival rate was very low while none of the immature embryos survived on W medium. Similar observations have been reported by Monnier (1978) for *Capsella*. Immature embryos, on MS medium, were first kept in the dark for two weeks and then transferred to light. Within one week, the embryos enlarged, became swollen and turned from white to yellowish green colour. In the modified MS medium, the embryos germinated into multiple shoot buds as reported by Chen (1984) for rubber. The presence of traces of Na_2PO_4 (0.17 g/L) accelerated the germination process, as reported by Chin *et al.* (1988) for embryos of *H. brasiliensis*.

For mature embryo culture, carbohydrates, mostly sucrose, are used not only as an energy source but also as osmotic agents. Although sucrose is generally used at a concentration of 2 to 4%, in the present study, it was observed that younger embryos required higher levels. Use of 6% sucrose in the basal medium resulted in multiple shoots initiation. This is in line with the observations of Monnier (1978) for *Capsella* suggesting that a fluid of high osmolarity surrounds the proembryos *in situ*. Kost *et al.* (1992) working with *Arabidopsis* also concluded that younger embryos require higher levels of sucrose / carbohydrates in order to develop multiple shoots.

The growth promoting factors in CW proved to be very effective. Supplementing BA with CW (15%) supported embryo germination and growth. Overbeek *et al.* (1941) demonstrated that the globular embryos of *Datura stramonium* attained germination and subsequently grew into plantlets only after supplementing the medium with coconut water. However, Muzik (1956) reported that CW had no apparent effect on the germination of mature embryos of *Hevea*.

In the present study, it was observed that 10 mg/L CH in the medium promoted the initiation of multiple shoots (Fig. 1).

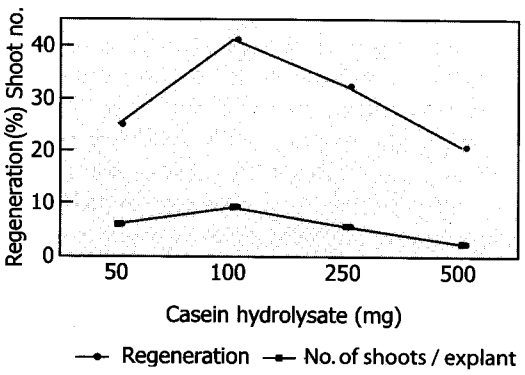

Fig. 1. Effect of casein hydrolysate concentration on multiple shoot formation in immature embryos of *Hevea brasiliensis*

Maheswari and Baldev (1961) made similar observations in *Cuscuta reflexa*, suggesting that CH triggers adventive embryo formation. In the medium with 15% CW and CH (100 mg/L), the germinated embryos developed a number of tiny finger like structures,

which represented the multiple shoot buds.

Exposure to light / dark period is an important criterion which controls shoot bud development. In this study, incubation in dark for two weeks was optimum while three weeks of exposure in the dark resulted in no shoot bud formation as reported by Biondi and Thorpe (1982) and Villalobos *et al.* (1984) for *Pinus radiata.*

Germinated young embryos with very minute shoot buds developed into multiple shoots when they were incubated first on the medium with kinetin (0.5 mg/L) for three weeks and then on medium containing kinetin (0.5 mg/L), 2iP (1.0 mg/L) and NAA (0.5 mg/L). It was found that BAP, kinetin or 2iP, alone or in combination, could induce shoot buds. However, for attaining full growth, combinations of kinetin and BAP/2iP were found to be the best (Table 1). Evans *et al.* (1981) suggested that a mixture of two cytokinins is more effective than when used individually. It has also been reported that kinetin may enhance the division of the cells of suspensor or endosperm tissues or any of the cells in the embryo sac, resulting in polyembryony. These embryos, perhaps triggered by the presence of 2iP, then formed multiple shoots (Ho, 1987). Addition of GA_3 (0.5 mg/L) enhanced the rate of germination as well as promoted leaf development in this experiment, supporting the observations of Nagl (1974) and Cionini *et al.* (1976) for *Phaseolus.* Without GA_3, the shoots elongated abnormally and had stunted leaves.

IBA and NAA are commonly used for rooting in woody plants (Pierik, 1987). In the present study, the isolated shoots were first cultured on a medium devoid of growth regulators for a week and then incubated on the medium with IBA (1.5 mg/L). This promoted the development of healthy taproots (Table 2). It was also observed that increased concentration of IBA promoted hairy root development that inhibited further root growth as has been reported for *Pinus* (Horgan and Aitken, 1981; Bansal and Pandey, 1993).

Age of the embryos played a critical role in regeneration. It was observed that the regeneration percentage and the number of shoots from each embryo decreased with the increase in age (Fig. 2). Maximum rate of regeneration was obtained using 7 to 8

Table 2. **Effect of auxins on rooting of immature zygotic embryo derived shoots of *H. brasiliensis***

Auxin used (mg/L)	Observation
IAA (0.5)	No rooting
IAA (1.0)	Hairy roots
IAA (1.5)	Abnormal root formation
IAA (2.0)	Abnormal tap root
IBA (0.1)	Hairy roots
IBA (1.0)	Tap root formation, no secondary roots
IBA (1.5)	Normal tap root with secondary roots
IBA (2.0)	No rooting

Table 1. **Effect of cytokinin combinations and concentrations on multiple shoot formation in immature zygotic embryos of *H. brasiliensis***

Growth regulator (mg/L)	Regeneration (%)	No. of shoots/explant	Shoot length (mm)*
Kinetin (0.5) + BAP (0.1)	36.8	3-6	6-8
Kinetin (0.5) + BAP (0.5)	40.8	4-8	4-8
Kinetin (0.5) + BAP (1.0)	48.0	5-8	4-10
Kinetin (0.5) + 2iP (0.1)	46.4	6-8	8-10
Kinetin (0.5) + 2iP (0.5)	52.8	4-8	6-9
Kinetin (0.5) + 2iP (1.0)	58.4	7-12	8-15
BAP (0.5) + 2iP (0.1)	32.4	3-5	3-6
BAP (0.5) + 2iP (0.5)	36.2	4-7	5-8
BAP (0.5) + 2iP (1.0)	40.4	4-7	6-8

* After 12 weeks of culture

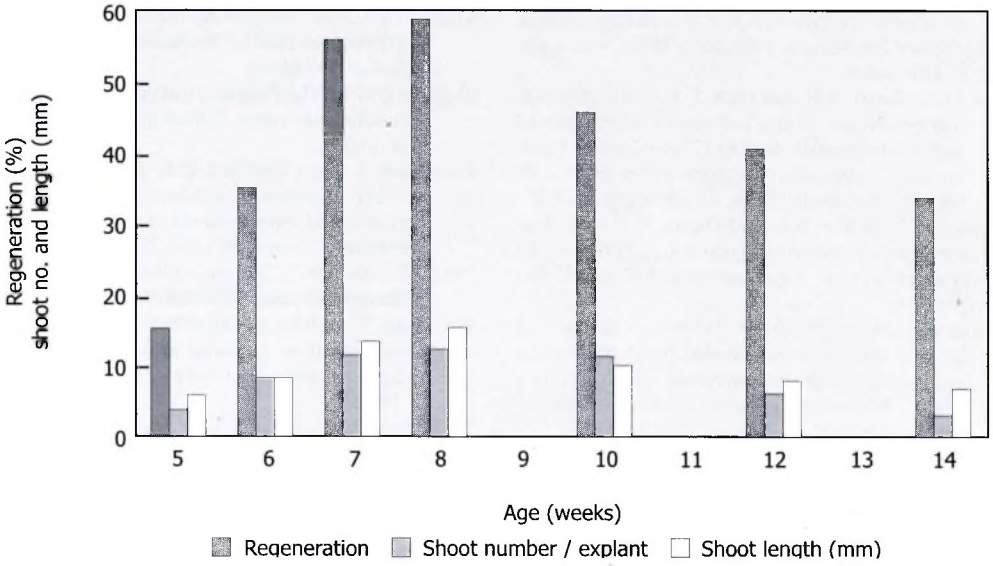

Fig. 2. Effect of age of immature embryos on multiple shoot formation

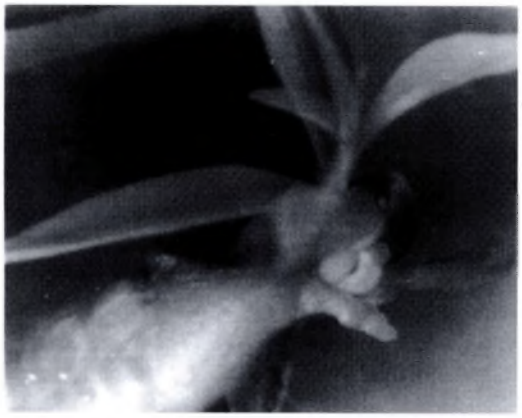

Fig. 3. Multiple shoot development from immature zygotic embryos of *Hevea brasiliensis* in culture

week old embryos. No regeneration was observed when the embryos were less than four weeks or more than 14 weeks old.

It can be concluded from the present investigation that 7 to 8 week old immature embryos of *H. brasiliensis* can be regenerated into shoots using embryo culture technique. Moreover, multiple shoot formation (Fig. 3) can be achieved by inducing polyembryony in the presence of optimum doses of sucrose, CW and CH in combination with kinetin, 2iP, NAA and GA_3 in the modified MS medium. The results open up alternate techniques for regeneration of young embryos of *H. brasiliensis*.

REFERENCES

Banasl, Y.K. and Pandey, P. (1993). Micropropagation of *Sesbania aculeata* (Pers.) by adventitious organogenesis. *Plant Cell Tissue and Organ Culture*, **32** : 351-355.

Biondi, S. and Thorpe, T.A. (1982). Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. *Botanical Gazette*, **143** : 22-25.

Chen, Z. (1984). Rubber. In: *Handbook of Plant Cell Culture*. Vol.2 (Eds. W.A. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada), Macmillan Publishing Co., New York, pp. 546-571.

Chin, H.F., Krishnapillay, B. and Alang, Z.C. (1988). Media for embryo culture of some tropical recalcitrant species. *Pertanika*, **11**(3) : 357-363.

Cionini, P.G., Bennici, A., Alpi, A. and Amato, F. (1976). Suspensor, gibberellins and *in vitro* development of *Phaseolus coccinens*. *Planta*, **113** : 115-117.

Das, K., Sinha, R.R., Dey, S.K. and Varghese, Y.A. (1998). Effect of phytohormones on regeneration of mature and immature embryos of *Hevea*

brasiliensis. In: *Proceedings of International Symposium on Tree Science*, 1998, New Delhi, India, pp. 27 (Abstract).

Evans, D.A., Sharp, W.R. and Flick, C.E. (1981). Growth and behaviour of cell cultures: Embryogenesis and organogenesis. In: *Plant Tissue Culture: Methods and Application in Agriculture* (Ed. T.A. Thorpe), Academic Press, London, pp. 45-113.

Gamborg, O.L., Miller, R.L. and Ojima, K. (1968). Nutrient requirement of suspension cultures of soya bean root cells. *Experimental Cell Research*, **50** : 151-158.

Gandhimathi, H. and Yeang, H.Y. (1984). The low fruit set that follows conventional hand pollination in *H. brasiliensis:* Insufficiency of pollen as a cause. *Journal of the Rubber Research Institute of Malaysia*, **32** : 20-29.

Ho, R.H. (1987). Embryo culture. In: *Cell and Tissue Culture in Forestry*. Vol. 2 (Eds. J.M. Bonga and D.J. Durzan), pp. 137-162.

Horgan, K. and Aitken, J. (1981). Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiologia Plantarum*, **53** : 170-175.

Kost, B., Potrykus, I. and Neuhums, K. (1992). Regeneration of fertile plants from excised zygotic embryos of *Arabidopsis thaliana*. *Plant Cell Reports*, **12** : 50-54.

Maheshwari, P. and Baldev, B. (1961). Artificial production of buds from the embryos of *Cuscuta reflexa*. *Nature*, **191** : 197-198.

Monnier, M. (1978). Culture of zygotic embryos. In: *Frontiers of Plant Tissue Culture* (Ed. T. Thorpe), University of Calgary Press, Canada, pp. 271-286.

Murashige, T. and Skoog, F. (1972). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15** : 473-497.

Muzik, T.J. (1956). Studies on the development of the embryo and seed of *Hevea brasiliensis* in culture. *Lloydia*, **19** : 86-91.

Nagl, W. (1974). The *Phaseolus* suspension and its polytene chromosomes. *Zeitschrift fur Pflanzenphysiol*, **73** : 1-44.

Overbreek, J. Van., Conklin, M.E. and Blakeslee, A.F. (1941). Factors in coconut milk essential for growth and development of very young *Datura* embryos. *Science*, **94** : 350-355.

Pierik, R.L.M. (1987). In vitro culture of higher plants. Maritimus Niijhoff Publishers, Dordrecht, 348 p.

Raghavan, V. and Torry, T.G. (1963). Growth and morphogenesis of globular and older embryos of *Capsella* in culture. *American Journal of Botany*, **50** : 540-541.

Villalobos, V.M., Leung, D.W.M. and Thorpe, T.A. (1984). Cytokinin induced switch in development in excised cotyledon of radiata pine cultured *in vitro*. *Physiologia Plantarum*, **61** : 497-504.

Warmke, H.E. (1951). Studies on pollination of *H. brasiliensis* in Puerto Rica. *Science*, **113** : 646-648.

Warmke, H.E. (1952). Studies on natural pollination of *H. brasiliensis* in Brazil. *Science*, **116** : 474-478.

White, P.R. (1963). A handbook of plant and animal tissue culture. Jacques Cattell Press, Lancaster, Pennsylvania.

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