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OPTIMIZATION OF PARAMETERS AFFECTING SOMATIC EMBRYOGENESIS IN *HEVEA BRASILIENSIS*

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Culture conditions and other parameters for improving the efficiency of somatic embryo induction and germination were investigated. Explants precultured in liquid medium for 10 days followed by 25 days culture in solid medium was most suitable for primary callus induction. Fifteen day old primary callus was found to be ideal for first subculture onto embryo induction medium. Histological analysis confirmed the relationship of callus age and its morphogenetic potential. Polyamines had no significant effect on somatic embryogenesis except for a slight enhancement with 0.5 mg/l spermidine. Embryo induction efficiency was promoted by supplementing 200 mg/l glutamine and 400 mg/l casein hydrolysate in embryo induction medium. Dark incubation favoured induction and proliferation of callus as well as induction of embryogenesis whereas plantlet regeneration was found to be light-dependent. From these results, an efficient method for proliferation of callus, induction of embryos and their maturation is proposed.

Key words : Callus induction, Embryo germination, *Hevea brasiliensis*, Somatic embryogenesis.

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INTRODUCTION

The application of conventional breeding techniques for crop improvement programmes in *Hevea brasiliensis* is limited due to its high heterozygosity and long generation cycle. Recent developments in cellular and molecular biology opened up an alternative and new avenue for crop improvement by the introduction of DNA transfer technology. However, the utilization of these technology is dependent on the availability of an efficient plant regeneration protocol compatible to the gene transfer methods. Plant regeneration through somatic embryogenesis is currently receiving much attention, since this is a very useful technique for the exploitation of the natural

gene transfer system mediated through *Agrobacterium* (Thulaseedharan *et al.*, 2000). In *Hevea*, somatic embryogenesis and subsequent plant regeneration has been reported from different explants (Wang *et al.*, 1980; Wan *et al.*, 1982; Carron and Enjalric, 1985; Asokan *et al.*, 1992). However, in most of these studies only a low frequency somatic embryogenesis and plant regeneration were reported. Recently a new procedure has been developed for somatic embryogenesis using immature anthers as explants (Jayasree *et al.*, 1999). Although this protocol greatly increased the percentage of calli showing somatic embryos and plant regeneration, the system needs further improvement. Embryogenesis from somatic tissue of different genotypes of *Hevea* remains difficult, with a low frequency of occurrence (Thulaseedharan *et al.*, 2000). Media components sometimes become a major obstacle limiting high frequency embryogenesis. It is, therefore, essential to evaluate the effect of certain parameters that significantly influence somatic embryogenesis for any particular clone to utilize this system in crop improvement programmes. The present work was thus aimed to study the effect of explant preculture in liquid medium, age of callus for embryo induction, polyamines, glutamine, casein hydrolysate and light for the enhancement of somatic embryo induction and germination in RRII 105, a high yielding *Hevea* clone from India.

MATERIALS AND METHODS

Floral buds were collected from mature trees of *H. brasiliensis* (clone RRII 105) and surface-sterilized for 5 min in 0.5% sodium hypochlorite solution containing a few drops of Tween-20 followed by repeated rinses with sterile distilled water. Immature anthers at diploid stage (before microsporogenesis) were dissected out and cultured for callus induction, embryo induction and plant regeneration (Jayasree *et al.*, 1999).

Explant preculture in liquid medium

To test the effect of explant preculture in liquid medium, six anthers, each were transferred to 100 ml conical flask containing 15 ml liquid callus induction medium. The callus induction medium consisted of modified MS medium (Murashige and Skoog, 1962), supplemented with 2.0 mg per litre 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg per litre kinetin (KIN). Cultures were kept in rotary shaker at 80 rpm for 0, 5, 10, 15 and 20 days. For each treatment five replicate cultures were raised and the experiment was repeated twice. After the treatment, explants were transferred to semi-solid callus induction medium as reported earlier (Jayasree *et al.*, 1999).

Age of callus for embryo induction

To determine the ideal age of callus for transfer to embryo induction medium, the primary calli obtained from the explants precultured for 10 days in liquid medium followed by 25 days in solid medium, were further maintained in the same medium for 0, 15, 30, 45 or 60 days prior to subculturing to embryo induction medium.

Effects of polyamines, glutamine and casein hydrolysate

The effect of polyamines, spermine and spermidine, on embryogenesis was tested at four concentrations (0, 0.5, 1.0 and 2.0 mg/l). Spermine and spermidine were filter-sterilized and added to the autoclaved embryo induction medium containing

0.7 mg per litre KIN and 0.2 mg per litre α–naphthaleneacetic acid (NAA). Effects of L-glutamine and casein hydrolysate were evaluated by supplementing different concentrations of glutamine (0, 50, 100, 150 and 200 mg/l) and casein hydrolysate (0, 100, 200, 400 and 600 mg/l) to the culture medium. Calli were transferred to all combinations and the experiment was repeated three times with three replications for each treatment. For callus and embryo induction, cultures were maintained under dark at 25°C. Efficiency of embryogenesis was calculated on the basis of embryos produced after eight weeks of subculture. Data (embryo induction) were analysed by estimating significant difference between each treatment using least significant difference test.

Effect of light

A separate experiment was carried out to study the effect of light on somatic embryogenesis. Inoculated explants were incubated in complete darkness and continuous light (20 $\mu E/m^2/s$) at 25 ± 2°C. Induction and proliferation of callus, embryogenesis and subsequent germination of somatic embryos were observed.

Plant regeneration

Mature cotyledonary stage embryos were transferred to plant regeneration medium as described previously (Jayasree *et al.*, 1999). All media were gelled with 0.25% gelrite and the pH was adjusted to 5.6 before autoclaving for 12 min at 120°C. Plantlets regenerated in light (40 $\mu E/m^2/s$) under 16 hour photoperiod were transplanted into polybags containing autoclaved sand and soil (1:1) and maintained in green-house for hardening.

Histological analyses

Histological analyses were performed to examine the relationship of callus age to morphogenetic potential. Fifteen and 60-day old calli were used in this study. Callus was fixed in formaldehyde – acetic acid – alcohol mixture (Johansen, 1940) and further processed for dehydration through graded series of tertiary butyl alcohol prior to embedding of the material in paraffin. Serial microtome sections of 6-8 µm thickness were cut and stained with Toluidine Blue–0 for general histology and observed under light and polarization microscopes.

RESULTS AND DISCUSSION

Explant preculture in liquid medium

In *Hevea*, when explants were inoculated directly to semi-solid medium, callus initiation from immature anthers started 50 days after explant culture followed by explant swelling. However, explants precultured for five days in liquid medium followed by culturing on solid medium had a stimulatory effect and callus initiation started 45 days after explant inoculation (Table 1). Ten day precultured explants underwent callus induction, after 25 days, on solid medium suggesting that only 35 days were required for callus induction (Fig. 1). When preculturing was prolonged

Table 1. **Effect of explant preculture in liquid medium for callus induction**

No. of days in liquid medium	No. of days on solid medium	Total days for callus induction	Calli formation (%)
0	50	50	80
5	40	45	85
10	25	35	80
15	15	30	60
20	5	25	50

the total time requirement was further reduced. When the explants were precultured for 20 days, only 25 days were required suggesting that the time requirement was reduced by increasing the preculture period. This may probably be due to the fact that the explants were more in contact with the medium during liquid culturing. Although the exact mechanism is not well understood, the absence of a gelling agent may have increased the availability of water and dissolved substances to the explant (Debergh, 1983). However, the percentage of calli formation, showing the embryogenic texture, was affected adversely during preculture (Table 1). Production of calli increased slightly with five day precultured explants when compared to those which were not precultured (control). When explants were precultured for 10 days, the calli formation reduced a little and this was same as the control but required only 35 days for callus initiation as compared to 50 days for control. When explants were cultured for 20 days in liquid, the embryogenic texture of calli was reduced to 50% suggesting that continuous exposure in liquid may cause increased availability of nutrients including the high level 2,4-D. This was in agreement with the report of Kuijpersel *et al.* (1996) for cucumber where increase in embryogenic calli was observed only in the initial culture period on high 2,4-D medium but decreased in prolonged culture.

Ideal age of callus for embryo induction

Age of primary callus or time of first callus transfer to embryo induction medium greatly influenced the induction of embryos. Upon transferring to embryo induction medium, 15-day old callus produced 50% friable embryogenic callus while 30-day old calli produced 40% (Table 2). The percentage of embryogenic callus was drastically reduced to25% for 45 days and 10% for 60-day old callus. Increasing the duration of maintenance of primary callus in callus induction medium, the callus became relatively more hard which might have caused the loss of morphogenetic potential. It was observed that after callus initiation, the calli that proliferated within the first 15 days were ideal for subculturing to embryo induction medium. Ferriere and Carron (1989) reported a drastic reduction in the formation of embryogenic calli derived from integumental tissues of *Hevea* by frequent subculturing in callus induction medium. This indicates that the age and frequency of subculturing are very critical to maintain the embryogenic potential. Though morphogenetic potential is dependent upon various factors, reduced regeneration potential due to callus ageing may be because of the accumulation of inhibitory substances (Halperin, 1986) or due to interactions between growth regulators (Van Staden and Mooney, 1987).

Table 2. **Effect of callus age for embryo induction**

Age of calli at the time of subculture (days)	Rate of callus proliferation [a]	Friable embryogenic calli [b] (%)
0	2	30
15	5	50
30	7	40
45	9	25
60	10	10

0 = Callus induced from liquid + solid (10+25 days) culture

[a] = amount of callus proliferated during maintenance in callus induction medium (rating scale (1-10) :1=low, 5 = medium and 10 = abundant calli proliferation)

[b] = Visual observation after 50 days of transfer to embryo induction medium

Effect of polyamines

Polyamines have been shown to influence various biochemical events such as protein biosynthesis, DNA synthesis and conformation and membrane stability (Tiburcio *et al.*, 1988). Polyamines seemed to influence somatic embryogenesis in many species including carrot (Mengoli *et al.*, 1989), celery (Zappia and Pegg, 1989), *Hevea* (El Hadrami *et al.*, 1989a, b). In the present study, polyamines did not show much influence on somatic embryogenesis, but a slight enhancement was noticed with 0.5 mg per l spermidine (mean number of embryos produced at this concentration being 22.6). Other concentrations of spermidine and spermine did not improve somatic embryogenesis, although enhanced calli proliferation was observed (data not shown). In contrast, El Hadrami *et al.* (1989a, b) reported the requirement of polyamines for somatic embryogenesis in *Hevea*. The insufficient availability of endogenous polyamines is one of the limiting factors for somatic embryogenesis in *Hevea*. This may be attributed to the clonal variability in endogenous polyamine content (El Hadrami *et al.*, 1989b), explant source variability or may be controlled by the exogenous or endogenous growth regulators. In grapevine, a high level of polyamine content suppressed somatic embryogenesis as well as caused abnormal development of embryos and a low rate of germination frequency (Faure *et al.*, 1991). In *Gnetumula*, spermine and spermidine helped proliferation of embryogenic callus but had no effect on embryogenesis (Augustine and Souza, 1999).

Effects of glutamine and casein hydrolysate

Beneficial effects of glutamine and casein hydrolysate on somatic embryogenesis of *Hevea* were observed in this study. All concentrations of glutamine tried produced friable, highly embryogenic calli and induced embryos. The rate of both processes was increased with increasing concentrations. Maximum response was observed when the medium was supplemented with 200 mg per litre glutamine (Table 3). At this concentration although, highest percentage of friable embryogenic calli was observed, the increase beyond 150 mg per litre was only marginal. Glutamine was reported to be effective for somatic embryogenesis in soybean

Table 3. **Effect of glutamine and casein hydrolysate on embryo induction**

Additives	Concentration (mg/l)	Friable highly embryogenic calli (%)	Mean No. of embryos
Glutamine	0	46	21.12
	50	48	24.25
	100	51	30.08
	150	59	42.25
	200	61	44.75
CD ($P \leq 0.05$)			2.51
Casein hydrolysate	0	45	20.00
	100	47	23.25
	200	52	25.62
	400	56	33.12
	600	59	27.12
CD ($P \leq 0.05$)			1.88

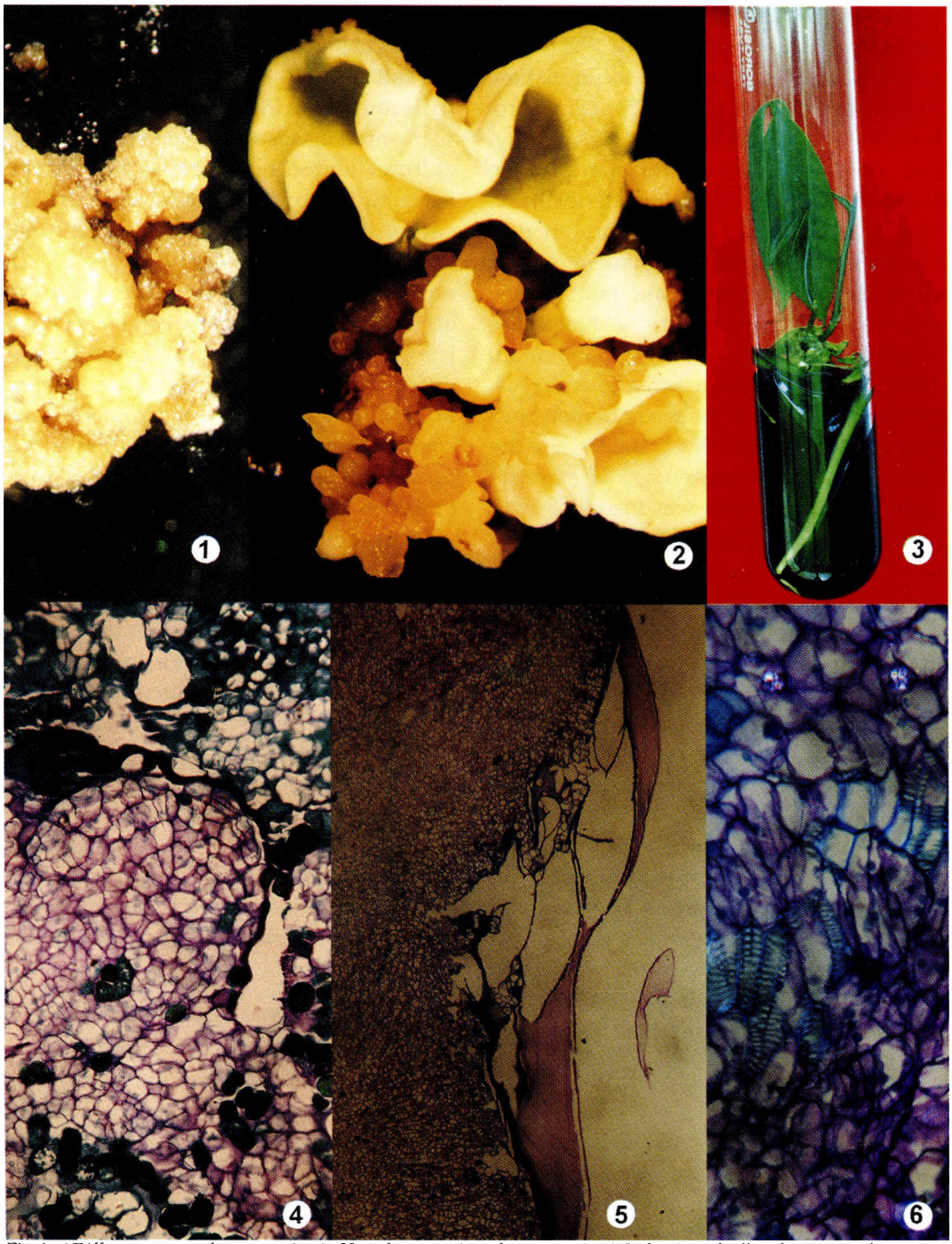

Fig. 1 - 6 Different stages of regeneration in *Hevea* by somatic embryogenesis : 1. Induction of callus showing embryogenic texture; 2. Embryo development from friable embryogenic callus; 3. Development of full plant; 4. Cross section of 15 day old callus showing embryo emergence enseathed with embryogenic callus; 5. Detachment of dead cells with agglomerated phenolic content; and 6. Differentiation of tracheary elements and crystal deposition.

(Tetu *et al.*, 1987) and in some species of gymnosperms (Khlifi and Tremblay, 1995). This amino acid enhances protein accumulation. According to Merkle *et al.* (1995) storage proteins are the markers for embryo maturation and consequently the quality marker of the regenerated plants. Therefore, the inclusion of glutamine caused proper maturation of the embryos thus leading to higher vigour of plantlets. In carrot somatic embryos, the total protein content increased three times when glutamine was added (Dodeman, 1995) and in oil palm somatic embryos, glutamine enhanced the accumulation of 7S globulins (Morcillo *et al.*, 1999). In the present study, the percentage of embryogenic callus formation was increased with increasing concentration of casein hydrolysate (Table 3). However, the embryo induction was decreased when casein hydrolysate was added beyond 400 mg per litre suggesting that higher levels of casein adversely affected somatic embryogenesis in *Hevea*. Proliferation of friable embryogenic calli were higher at 600 mg per litre, however, maximum embryo production was obtained at 400 mg per litre casein hydrolysate (Fig. 2). Similar effect of casein hydrosylate for somatic embryogenesis of French bean has been reported (Martins and Sondahl, 1984). Casein hydrolysate was found to stimulate adventitious buds as well as somatic embryogenesis in *Panax ginseng* and the effect was maximum at 500 mg per litre (Tang, 2000).

Effect of light

Callus induction occurred both under the dark and light incubation conditions. However, further callus proliferation was found to be adversely affected by incubation in light. Cultures incubated in dark promoted callus proliferation to 80% while it drastically slowed down to 10% under light incubation (data not shown). Moreover, the calli proliferated under light never got differentiated into embryos. A green pigmentation was also observed with callus cultures under light incubation. During subculturing to embryo induction medium, callus discolouration and subsequent induction of friable embryogenic calli occurred under dark. Once the process of acquisition of embryogenic competence and induction was accomplished, further differentiation occurred either in darkness or in light. The exact role of darkness is not completely understood but it is likely that incubation of plant tissue in darkness preserves light-sensitive endogenous plant growth regulators and other compounds (Hartmann *et al.*, 1977). Similarly light completely suppressed somatic embryogenesis in *Manihot glaziovi* (Joseph *et al.*, 2000), while in *Solanum melongena* embryo induction was light dependent (Gleddie *et al.*, 1983). In our studies *Hevea* needed continuous dark till the acquisition of embryo induction. Further maturation occurred both in darkness or in light, however, light seemed to favour embryo maturation. Similarly light seemed to be a critical factor for plant regeneration eventhough few plantlets regenerated under dark.

Plant regeneration

During the transfer of mature embryos to plant regeneration medium, the size of embryos increased slightly followed by expansion of cotyledons. Subsequently all embryos produced a vigorous long root, however, the shoot development and further elongation were limited. About 35% embryos germinated into full plants (Fig. 3). When they reached a length of 2-3 cm with

more than two mature leaves, they were transplanted to polybags containing sand and soil (1:1 v/v) and grown in a green house after hardening process.

Histological studies

Histological examinations of 15-day old callus revealed the presence of actively dividing cells with deeply stained cytoplasm and prominent nuclei. At this stage, mature cells were larger compared to the dividing cells and most of them were filled with phenolic contents. Similar close association between the developing embryos and phenolic rich cells were reported in myrtle somatic embryogenesis (Canhoto *et al.*, 1999). Eventhough the mechanism was not studied in detail, phenols constitute some kind of barrier that isolates embryos from the surrounding tissue. Moreover, phenolic compounds can interfere with endogenous auxin levels by affecting the activity of IAA oxidase and thus condition somatic embryo development (Delalande *et al.*, 1996). A high cell division activity and emerging of globular embryos (Fig. 4) with the embryonic cuticle were also observed at this age. Intercellular spaces in certain areas of the callus were large and were found to be filled with mucilaginous material. In 60-day old *Hevea* callus, a reduced cell division activity was observed. The presence of phenolic contents was observed and many of the cells discharged the contents into the intercellular spaces to form agglomerated phenolic content within the tissue mass. At a later stage, these agglomerated mass together with cells in groups detached from the callus mass due to the growth activity of the dividing cell (Fig. 5). Developing embryos were not observed in 60-day old callus. Many of the cells showed helical wall thickening due to tracheary element differentiation (Fig. 6). After primary calli induction, the calli were maintained for 60 days in high 2,4-D medium and this might have led to the differentiation of tracheary element instead of cytodifferentiation. The appearance of crystals was another interesting feature noticed in aged calli. This was indirectly in agreement with the observation of Pedroso and Paris (1999) that the embryogenic induced cell did not accumulate calcium oxalate crystals in leaves of *Camellia japonica.*

CONCLUSION

A procedure for enhancing the frequency of embryogenesis from immature anthers of *H. brasiliensis* by optimizing various factors could be evolved. The process includes an initial preculture of explants in liquid medium. The age of callus which is very critical for increased callus proliferation as well as somatic embryogenesis was also identified. Glutamine at 200 mg per litre and casein hydrolysate at 400 mg per litre concentrations considerably enhanced proliferation of embryogenic callus and production of mature embryos. *Hevea* somatic embryogenesis from immature anthers need incubation in dark for embryogenic competence and in light for plant regeneration. This system offers much scope as a micropropagation system and for genetic manipulation studies by *Agrobacterium*-mediated transformation.

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