

## Evaluation of parameters affecting the isolation and culture of protoplasts of *Hevea brasiliensis* (Muell. Arg.)

Development of protoplast technology has been given considerable attention and has attained significant progress during the past few years. *Hevea brasiliensis*, the major source of natural rubber, is a highly heterozygous and perennial tree with a long breeding cycle. Genetic manipulation through protoplast technologies like somatic hybridization, cybridization or direct gene transfer can be exploited for crop improvement in *Hevea*, provided a reliable and efficient plant regeneration system from isolated protoplasts could be developed. So far there are only a few reports on the isolation and culture of protoplasts of *Hevea*<sup>1-4</sup>. Among these, only Cazaux and d'Auzac<sup>4</sup> could achieve micro callus formation from protoplasts of *H. brasiliensis*. However they observed browning around the micro callus, thereby restricting further development. The present study describes optimization of parameters affecting the isolation and culture of protoplasts of *Hevea brasiliensis*.

Various tissues tried for protoplast isolation were leaf, young stem, friable callus and embryogenic cell suspension derived from immature inflorescence of *H. brasiliensis* (clone RRIM 600). For enzymatic digestion of these different source tissues, several combinations and concentrations of different enzymes were tried as follows: E1 – cellulase RS 1.0% + pectolyase Y 23 0.5%; E2 – cellulase RS 0.5% + pectolyase Y 23 0.4%; E3 – cellulase RS 1.0% + pectolyase Y 23 0.1%; E4 – cellulase RS 1.0% + pectolyase Y 23 0.5% + rhozyme HP 150 0.5%; E5 – cellulase RS 0.8% + pectolyase Y 23 0.1% + macerozyme R-10 0.5%; E6 – cellulase RS 0.8% + pectolyase Y 23 0.4% + macerozyme R-10 0.4% + rhozyme HP 150 0.5%. Various levels (0.1–1.0 M) of different osmotic stabilizers like sucrose, glucose, mannitol and sorbitol were evaluated in order to select the most suitable osmoticum. In addition, 5 mM MES (2 (N-morpholino) ethane sulphonic acid) and 1.48 g/l CaCl<sub>2</sub>·2H<sub>2</sub>O were also present in all these osmotica. All the samples were kept for digestion at 28°C in the dark

under constant agitation (40 rpm) on a gyratory shaker for different time intervals (6–24 h). The digested samples were filtered through a nylon sieve (45 µm) and protoplasts were pelleted by centrifugation at 80 g for 10 min. The pellets were washed twice and finally resuspended in the osmoticum. Yield of the isolated protoplasts was counted using a hemacytometer. Viability was assessed by fluorescein diacetate staining<sup>5</sup>. Further culturing of the isolated protoplasts was tried in KM<sup>6</sup>, MS<sup>7</sup> and B5 (ref. 8) media amended with

various combinations of growth regulators, viz. 2,4-D, NAA, BA and kinetin at different levels. Different culture techniques employed were: (a) culture in liquid medium; (b) culture in semi solid medium as droplets or as thin layer; and (c) culture on membrane filter of 0.2 µm pore size (sartorius membrane) over solid medium. All the cultures were maintained in the dark at 25°C.

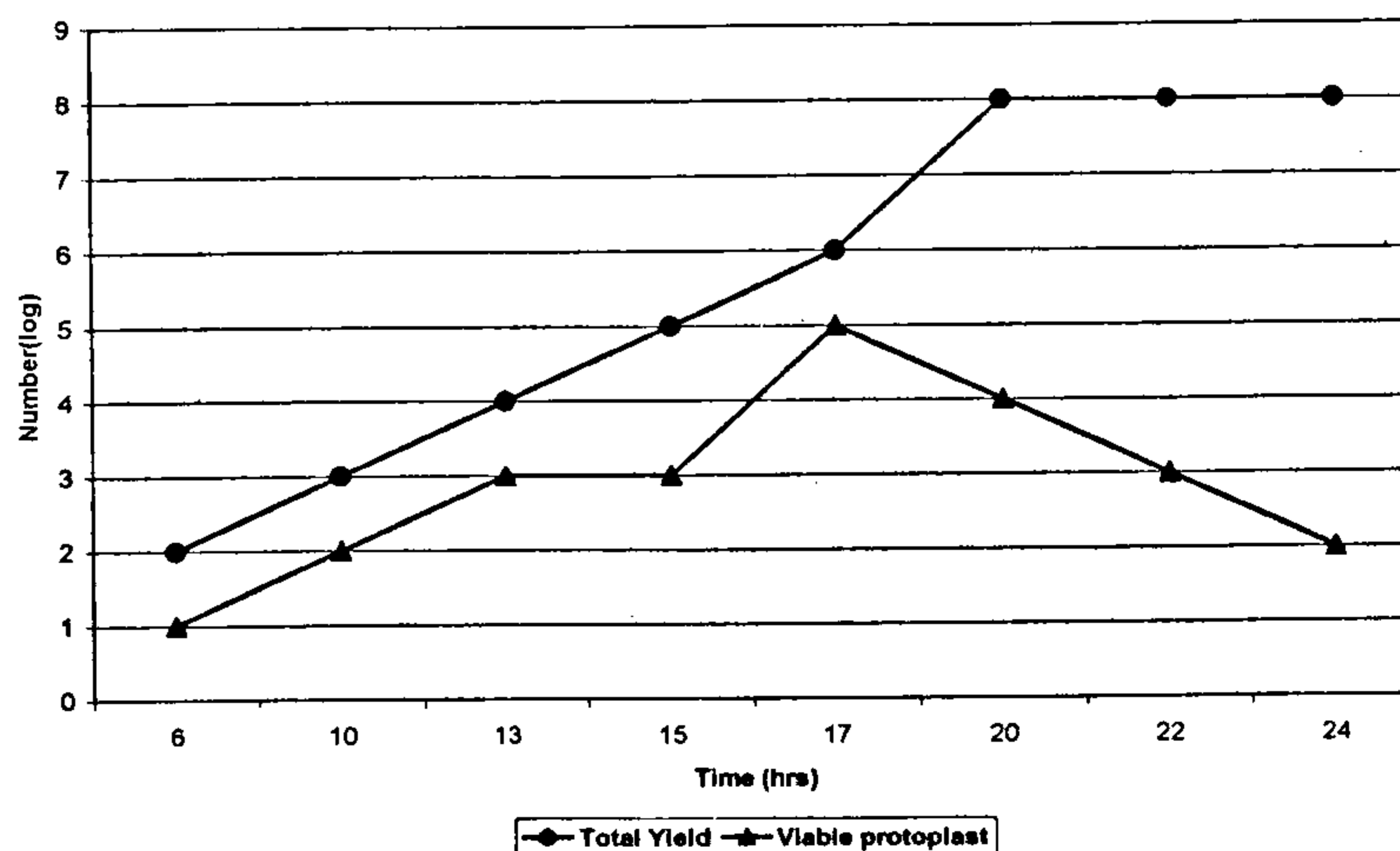
Results of the experiment combining the source tissue and enzyme mixtures are presented in Table 1. Mesophyll tissue and the stem released protoplasts

**Table 1.** Effect of enzyme combinations on yield and viability of protoplasts from different explant sources

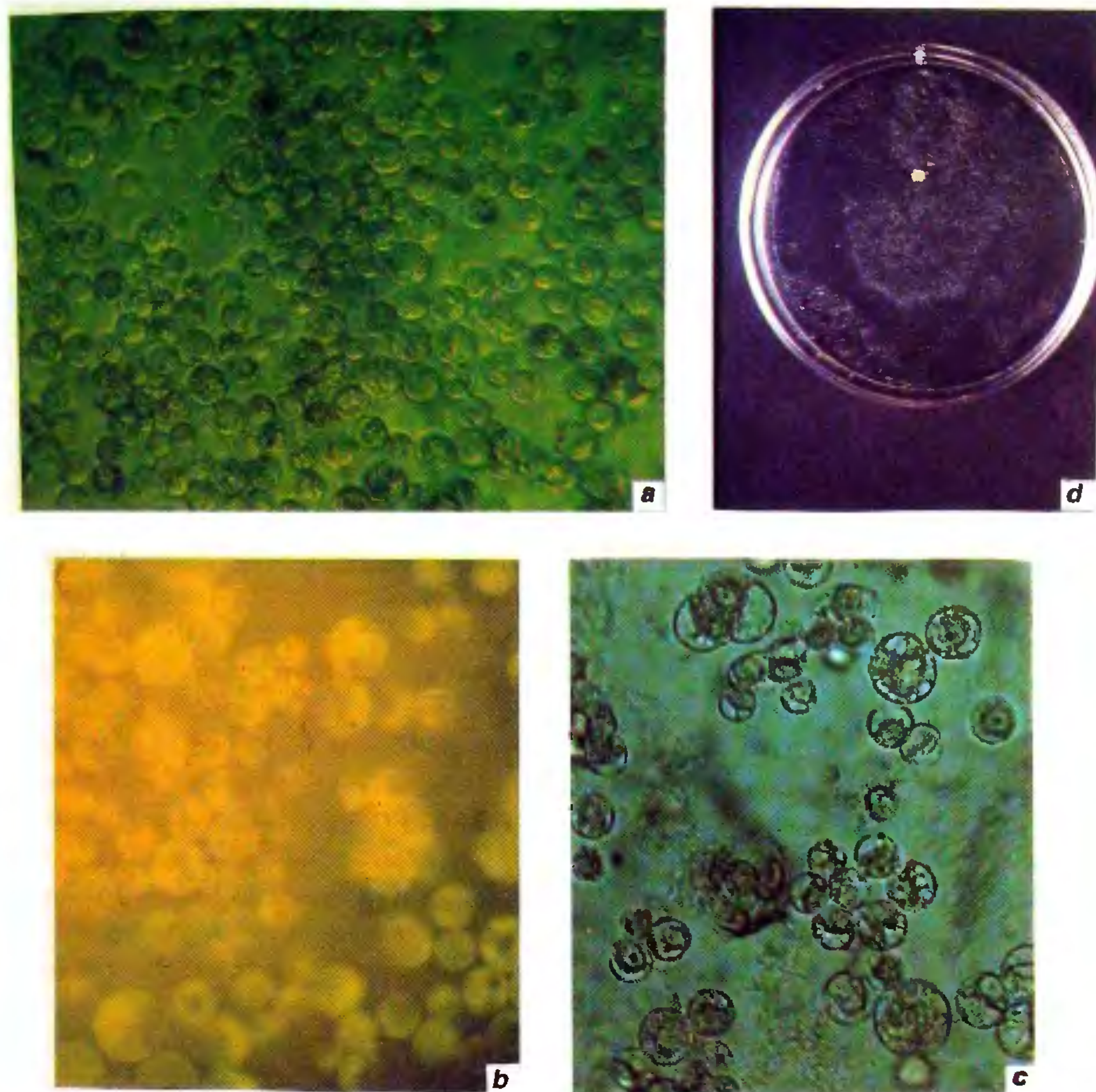
Source	Enzyme combinations					
	E1	E2	E3	E4	E5	E6
	*	–	–	*	–	–
Leaf	+	–	–	+	–	–
Young stem	***	–	–	–	**	**
	+	–	–	–	+	+
Friable callus	*	**	**	**	*	**
	+	+	+++	++	+	+
Embryogenic cell suspension	***	**	****	***	*	**
	++	++	++++	+	+	+

\*Yield (per gram fresh wt); \*, < 10<sup>3</sup>; \*\*, 10<sup>4</sup>; \*\*\*, 10<sup>5</sup>; \*\*\*\*, > 10<sup>6</sup>.

+, viability; +, < 20%; ++, 20–40%; +++, 40–70%; +++, 70–90%.



**Figure 1.** Yield and viability of protoplasts against duration of digestion.



**Figure 2.** Micro colony formation in cultured protoplasts of *H. brasiliensis*. *a*, Freshly isolated protoplasts from embryogenic cell suspension; *b*, Viable protoplasts stained with FDA; *c*, Protoplasts undergoing division (after two weeks in culture); *d*, Micro colonies appeared over membrane filter two months after plating.

in a few combinations namely E1, E4, E5 and E6, but the yield and viability were too low. Even though protoplast release was noticed in all combinations in the case of callus as well as cell suspensions, the yield and viability varied considerably. Highest yield ( $>10^6$  protoplasts/g fresh weight) with 70–90% viability could be obtained from the embryogenic cell suspension digested with cellulase RS (1.0%) and pectolyase Y 23 (0.1%). At this enzyme concentration the friable callus also yielded protoplasts with 40–70% viability, but the yield was lesser compared to the cell suspension. It was observed that among the osmotic stabilizers employed, only mannitol (0.7 and 0.8 M) could yield a large number of viable protoplasts, the highest yield being at 0.7 M mannitol.

Regarding the duration of digestion, maximum number of viable protoplasts could be released during 17 h digestion after which the yield was still increasing but the viability was declining sharply (Figure 1).

Protoplasts cultured in liquid medium did not show any mitotic division in any of the media experimented. Instead, the protoplasts remained intact for 2–3 days and were then gradually broken down. When the protoplasts were plated over semi-solidified culture media, it was observed that within one day of culture almost all of them were broken down. Small micro colonies were observed within 2–3 months over KM medium fortified with 2,4-D (1.0 mg/l) and BA (0.5 mg/l) when a membrane filter was introduced in between the culture

medium and the protoplasts. No colony formation was observed on cultures over MS or B5 media. Plating density also influenced protoplast division. Micro colony formation was observed only in cultures with higher plating densities ( $1.0\text{--}2.0 \times 10^6 \text{ ml}^{-1}$ ) (Figure 2). At lower plating densities no micro colonies were developed. Cazaux and d'Auzac<sup>4</sup> obtained microcolonies when protoplasts embedded in alginate beads, at a lower plating density of  $2.0 \times 10^5 \text{ ml}^{-1}$ , were cultured on solid medium containing tobacco nurse cells. The reason for micro colony formation at lower plating density may be the stimulating effect of tobacco nurse cells on protoplast division.

In conclusion, the present results indicate that in *H. brasiliensis*, embryogenic cell suspension is the best source for high yields of viable protoplasts which can lead to the formation of micro colonies. Different nurse cells need to be tried in order to optimize the most suitable feeder cells which can enhance protoplast division and sustained growth of the protoplast-derived colonies of *H. brasiliensis*.

1. Cailloux, M. and Lleras, E., *Acta Amazonica*, 1979, 9, 9–13.
2. Rohani, O. B. and Paranjothy, K., *J. Rubber Res. Inst. Malaysia*, 1980, 28, 61–66.
3. Wilson, Z. A. and Power, J. B., *Plant Cell Rep.*, 1989, 7, 622–625.
4. Cazaux, E. and d'Auzac, J., *Plant Cell Rep.*, 1994, 13, 272–276.
5. Widholm, J. M., *Stain Technol.*, 1972, 47, 189–194.
6. Kao, K. N. and Michayluk, M. R., *Planta*, 1975, 126, 105–110.
7. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, 15, 473–497.
8. Gamborg, O. L., Miller, R. A. and Ojima, K., *Exp. Cell Res.*, 1968, 50, 151–158.

S. SUSHAMAKUMARI  
S. SOBHA  
R. JAYASREE  
M. P. ASOKAN

Biotechnology Division,  
Rubber Research Institute of India,  
Kottayam 686 009, India.