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Effect of nurse culture on inducing division of isolated pollen protoplasts of *Hevea brasiliensis*

S. Sushamakumari*, Sneha Joseph, S. Sobha, K. Rekha, R. Jayashree and Leda Pavithran

Rubber Research Institute of India, Kottayam-9, Kerala, India

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Abstract

Haploids are of great relevance in crop improvement of *Hevea*, a highly heterozygous tree species with a long breeding cycle. The isolation and culture of pollen protoplasts may be a viable proposition for raising haploid plants/ homozygous lines in *Hevea*. The present work envisages the development of a method for the isolation and culture of pollen protoplasts of *Hevea*. Effect of different nurse cultures on the development of cultured protoplasts has been studied. Intact pollen grains were isolated from mature male flowers of *Hevea* prior to opening. Viable protoplasts in high yield could be isolated from these pollen grains when exposed to a mixture of 0.5 per cent cellulase and 0.05 per cent pectolyase in the presence of the osmotic stabilizers 0.6 M mannitol and 0.3 M sorbitol. These protoplasts were partially purified and cultured in the nutrient medium with three different nurse cultures namely embryogenic calli from *Hevea*, tobacco and carrot. Division of the cultured protoplasts leading to the formation of a few micro-colonies was observed in the medium containing 0.8 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} BA and enriched with *Hevea* nurse culture. Cultures with micro-colonies are dark incubated for further development. This is the first report of division of pollen protoplasts and micro-colony formation in *Hevea brasiliensis*.

Keywords: Hevea brasiliensis, micro-colony, nurse culture, pollen protoplast

Introduction

The demand for natural rubber (NR) continues to rise, in spite of the development of synthetic substitutes. Substantial increase in productivity of the original gene pool of *Hevea brasiliensis*, the prime source of NR, has been attained by genetic improvement. The major limitations in crop improvement of *Hevea* are the narrow genetic base, low fruit set, long gestation period, heterozygous nature and the absence of fully reliable early selection parameters. Also, transfer of useful traits from distantly related species which do not sexually cross with the crop plant is not possible through conventional breeding procedures. Application of biotechnological tools in combination with conventional methods is highly desirable in circumventing such limitations.

Presence of chromosomes in single copy in gametes and the concept of totipotency have together led to the development of haploids in many plant species. Haploids are of great significance in plant breeding as each of their genes, even recessive ones, are expressed and pure lines or homozygous plants can be obtained after diploidisation. This also facilitates easy detection of mutations. More recently, the concept of gameto-clonal variation and gameto-somatic hybridization has been developed, aiming to create unique variability for crop improvement. The production of doubled haploids would offer new possibilities for genetic studies and breeding, especially in perennial crop species which are characterized by a long reproductive cycle with several years of juvenile phase, allogamous nature and a high degree of heterozygosity (Hofer *et al*., 1999). Through *in vitro* techniques like anther or microspore culture, haploids can be obtained in a relatively shorter time span. By doubling their chromosome number, homozygous diploids can be

**Corresponding Author: sushama@rubberboard.org.in*

produced in a single generation. This is of great relevance in a perennial tree crop like *Hevea* with a narrow genetic base and a long breeding cycle.

Pollen grains represent the highly reduced haploid male gametophytic generation in angiosperms. Microspore culture is one of the most versatile and efficient procedures to obtain haploid plants (Srivastava *et al*., 2005). Callus initiation and further development from microspores in culture has been observed to be extremely difficult, probably due to the presence of a thick exine. Removal of the mechanically and chemically resistant wall of pollen facilitates nutrient uptake, growth, division and regeneration. Hence, isolation and culture of pollen protoplasts offers a viable alternative for raising haploid plants. Pollen protoplasts became an attractive research system following the success of somatic protoplast culture since it is a useful haploid system for cell fusion, mutation studies as well as for studying pollen biology (Fang *et al.*, 2006).

Attempts on isolation and culture of protoplasts in *H. brasiliensis* started in the 1970s (Cailloux and Lleras, 1979). Microcallus formation from the cultured protoplasts was reported in 1994 (Cazaux and d' Auzac, 1994). Sushamakumari *et al.* (2000) reported, for the first time, successful plant regeneration from *Hevea* protoplasts isolated from embryogenic cell suspension. So far no reports are available on isolation and culture of pollen protoplasts of *H. brasiliensis*.

This paper envisages the isolation and subsequent culture of protoplasts from pollen grains of *H. brasiliensis*. Efficiency of different nurse cultures in promoting sustained division of the cultured protoplasts was evaluated.

Materials and methods

Isolation of pollen

Mature male flowers prior to anthesis (Fig. 1) were collected from field grown trees of *H. brasiliensis*, clone RRII 105. Surface sterilisation was carried out using 0.15 per cent mercuric chloride solution containing a few drops of detergent, Tween 20, for 3 min after which the explants were washed several times with sterile distilled water for removing the sterilizing agent. Anthers were dissected out from these flowers, kept in the osmoticum and crushed

Fig. 1. Mature male flower buds of *Hevea*

gently to release the pollen grains into the osmoticum. This solution was filtered through a 71 µm mesh sieve to remove pieces of anther tissue and larger debris. The pollen suspension was then centrifuged at 800 rpm for one minute to pellet the pollen grains. The supernatant was discarded and the pollen pellet was used for enzymatic maceration (Fig. 2).

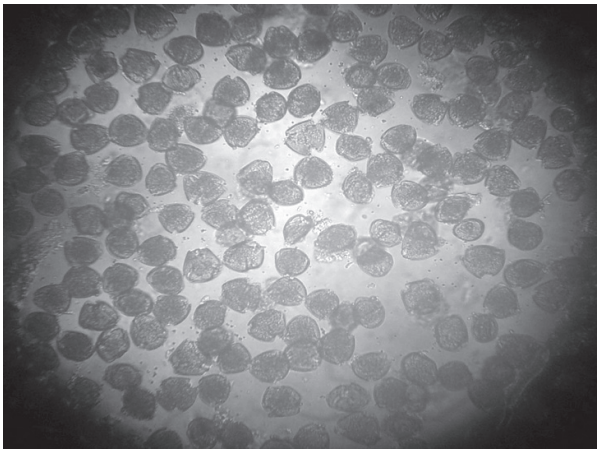

Fig. 2. Isolated pollen grains

Osmoticum

The osmoticum used in this experiment consisted of CPW medium supplemented with 5 mM MES (2- N morpholino ethane sulphonic acid). In addition, 0.6 M mannitol and 0.3 M sorbitol were incorporated as the osmotic stabilizers. This solution was autoclaved at 120 °C, 15 lb sq^{-1} pressure for 15 min and stored at 25 °C.

Enzymatic isolation of pollen protoplasts

The enzymes tested for their effect on protoplast isolation from pollen of *Hevea* were

Cellulase Onozuka RS, Pectolyase Y 23 and Macerozyme R10. Different combinations and concentrations of these enzymes were experimented for optimizing the right enzyme combination effecting successful protoplast release from the pollen grains. The enzymes were dissolved in the osmoticum, pH of the solutions were adjusted to 5.7 and filter sterilized with Millipore (0.22 μM) membrane filter. Combinations of the enzymes tested were

Cellulase RS (0.2-2.0%) and Pectolyase Y23 (0.05-0.2%)

Cellulase RS (0.2-2.0%) and Macerozyme R10 (0.2-1.0%)

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Enzyme solution (5 ml) was added to the pollen pellet and mixed thoroughly. The suspension was dispensed into sterile, disposable Petri dishes and sealed with parafilm. The plates were incubated at room temperature (28 °C) in the dark, one set with gentle intermittent shaking and another set as static cultures. Observations were taken by examining the plates under an inversion microscope at different time intervals up to 16 hrs in order to assess the impact of incubation period on protoplast release.

Percentage release of protoplasts and viability

The release of protoplasts was estimated by counting the number of pollen and protoplasts per microscopic field and was expressed in terms of percentage release. Viability of the isolated protoplasts was determined by FDA staining (Widholm, 1972).

Purification of protoplasts

Two methods were employed for purification of pollen protoplasts

1) Density gradient centrifugation using sucrose, ficoll and PEG

The protoplasts were washed three times, each time resuspending the pelleted protoplasts in the osmoticum, centrifuging at 800 rpm and discarding the supernatant. After three rinses, the pelleted protoplasts were subjected to density gradient centrifugation.

The protoplasts were layered carefully onto a sucrose gradient in a centrifuge tube. Concentrations of the sucrose solutions used for preparing the gradient were 15, 20 and 30 per cent from top to bottom of the tube. Centrifugation was carried out at 300 rpm for 5 min in Marathon 21K swinging bucket desktop centrifuge. Similarly, the protoplasts were layered onto a ficoll gradient (6% and 9% Ficoll) and centrifuged at 400 rpm for 3 min. Two concentrations of Polyethylene glycol (PEG) (20 and 30%) solution were used to prepare the gradient over which the protoplast suspension was layered.

2) Sieving through nylon mesh

After enzyme incubation, the protoplast suspension was filtered through nylon sieves of different mesh sizes (20, 30, 64, 71, 85 and 100 μm). The filtrate was sedimented to a pellet by centrifugation at 300 rpm for 2 min.

Protoplast culture

The pelleted protoplasts, which could only be partially purified and containing some empty pollen walls and undigested pollen, were washed 3 times in the osmoticum to remove traces of enzymes from the protoplasts and finally pelleted by centrifuging at 300 rpm for 2 min. The pellet was mixed gently with a few drops of osmoticum for further culturing. Two different basal media KPR (Abdullah *et al*., 1986) and Chu N6 (Chu *et al*., 1975) media, fortified with different levels of 2,4-D (0.1-1.0 mg l^{-1}) and BA (0.1-1.0 mg l^{-1}) were used in this experiment. Sucrose and glucose were provided as carbon sources at concentrations of 20 g l^{-1} and 10 g l^{-1} respectively and the media were semi solidified with 0.8 per cent (w/v) Type VII agarose (FMC Bioproducts, Rock land, USA). Partially purified pollen protoplasts were cultured over these media as small droplets and incubated in the dark at 28 °C. Observations were taken routinely through an inversion microscope to detect cell division and growth.

Effect of nurse culture

The promotive effect of three different nurse cultures *viz*. *H. brasiliensis*, tobacco and carrot

cultures on inducing protoplast division was evaluated. For this, actively growing cell cultures were raised from all these three sources. Friable, embryogenic calli from these different tissues were suspended in liquid medium which contained modified MS (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} 2, 4-D as the growth regulator and kept in a shaker with constant agitation. Sub culturing to fresh liquid medium was done at weekly interval. These cell suspensions, 2 days after sub culturing to new medium, were used as the nurse cultures. Liquid KPR medium was prepared at double concentration, filter sterilized, and the nurse cultures in cell suspension were added to the medium (1 ml nurse per 100 ml medium). Equal volume of 1.6 per cent agarose solution in water was prepared, autoclaved, cooled to room temperature and then added to the medium enriched with the nurse calli. After uniform mixing, this medium was poured into petri plates and allowed to solidify. This semi solidified media were used for culturing the protoplasts which were layered on top of a sterilized membrane disc placed over the medium. Cultures were kept in the dark, at 28°C and observations were taken at one week interval by recovering a fraction of the cultured protoplasts through a pasteur pipette into an empty petri plate and viewing through an inversion microscope.

Results and discussion

Effect of enzymes on protoplast isolation

It has been observed that the type and concentration of the cell wall degrading enzymes play a crucial role on the release of protoplasts from *Hevea* pollen. Among the three combinations of enzymes used in this study, cellulase - pectolyase mixture was found to be the most suitable one. Highest yields were obtained with combinations of 0.5% cellulase + 0.05% pectolyase and 0.5% cellulase + 0.1% pectolyase (Table 1). Comparatively lesser but satisfactory yields were obtained with the combination of 1.0% cellulase + 0.1% pectolyase. Enzyme concentrations at the lower and higher ranges yielded few or no protoplasts. Even though some combinations of cellulase and macerozyme could bring about digestion of pollen, the yield was comparatively low. The mixture of macerozyme and pectolyase failed to bring about protoplast release. This could probably be due to the similar action of these enzymes, both of which are pectinolytic in nature and lack the β-1,4 glucanase activity possessed by cellulase.

Table 1. Effect of digestion enzymes on protoplast release from *Hevea* pollen Percentage release of protoplasts

Pectolyase (%)	Cellulase (%)				
	0.2	0.5	1.0	1.5	2.0
0.02	-	-	-	-	-
0.05	-	80	40	25	-
0.10	-	85	55	25	15
0.15	-	35	45	30	10
0.20	-	20	30	40	10

The effectiveness of cellulase-pectolyase mixture for protoplast isolation in *Hevea* tissue has been reported earlier (Sushamakumari *et al.*, 2000) where, high yields of viable protoplasts could be isolated from embryogenic cell suspensions of *Hevea* using an enzyme mixture of 1 per cent cellulase and 0.1 per cent pectolyase.

In the present study, it was observed that the protoplasts were extruded through the germ pore characterized by the presence of thin walled cells. It is quite evident from the experiment with digestion enzymes that both cellulytic and pectolytic enzymes are essential for protoplast release from the pollen. This indicates the pecto-cellulosic nature of the germ pore.

Release of protoplasts

The thick, mechanically and chemically resistant pollen wall was not at all affected by any of the enzymes tried. It was observed that the protoplasts emerged through a very narrow aperture namely the germ pore. During the natural course of pollen germination, it is through these thin walled regions that the pollen tube emerges. During the enzymatic digestion of the pollen grains, the protoplasts could initially be seen as small bulbous projections (Fig. 3) through any one of the three colpi. This may be due to the plasmolysing action of the osmoticum on the pollen. Simultaneously, the germ pore was enzymatically degraded due to its pecto-cellulosic nature. Gradually the protoplasts were completely expelled, leaving behind empty pollen walls (Figs. 4 & 5). Although the exine is rigid, the presence of thin walled regions (germ pore) facilitates the use of enzymes to release protoplasts

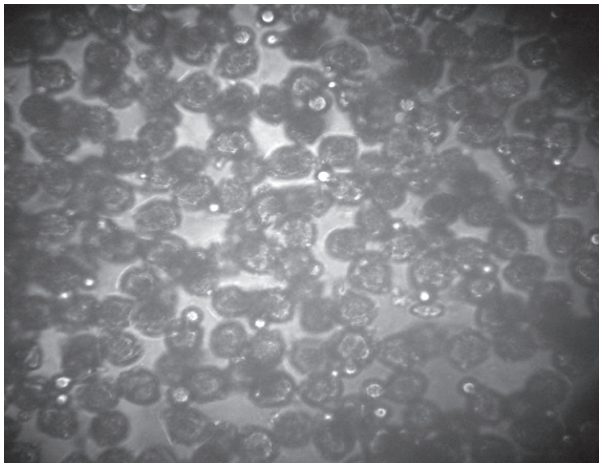

Fig. 3. Initial stage of protoplast release from pollen grains

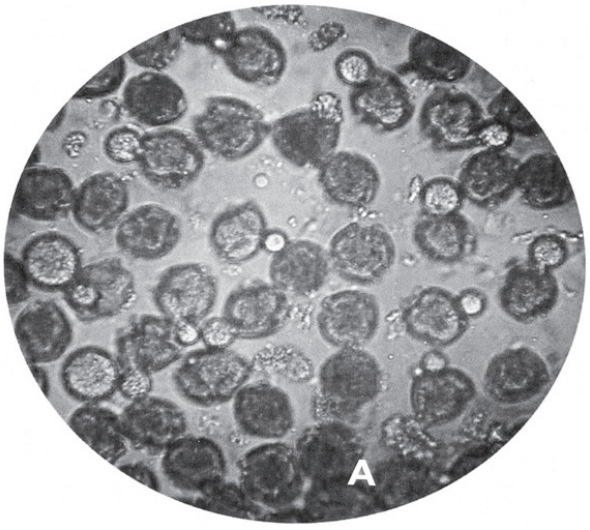

Fig. 4. Pollen protoplasts at different stages of release from pollen grains

from *Hevea* pollen. In previous reports of isolation of viable pollen protoplasts, release occurs in a similar manner (Fellner and Havranek, 1992; Kunitake *et al*., 1993). In some pollen, protoplasts emerge from all the three colpi, leading to the formation of sub-protoplasts.

Average release of protoplasts estimated from different samples came around 70 per cent. When the partially purified protoplasts were subjected to FDA staining, 80 per cent viability was obtained.

Incubation period

It has been observed that, under optimum conditions, protoplast release was initiated within 10 minutes of incubation. During the initial period of incubation, protoplast release varied linearly with time for about one hour. Prolonged incubation periods did not enhance protoplast yield. On the contrary, viability of the protoplasts was adversely affected upon prolonged exposure to the digestion enzymes as a result of which the yield was found to be decreasing with longer incubation periods.

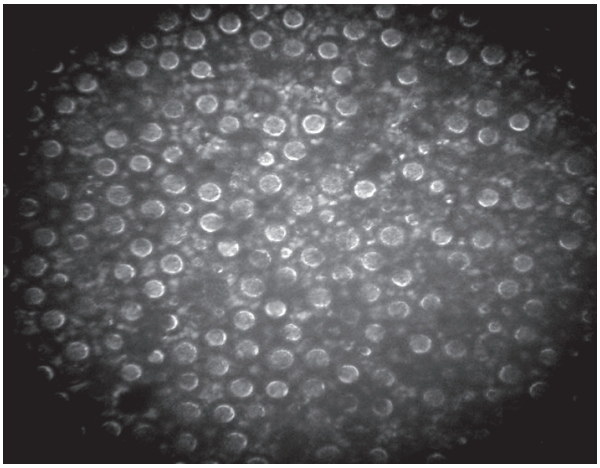

Fig. 5. Isolated pollen protoplasts before purification

Purification of protoplasts

In this study, both density gradient centrifugation and filtration through sieves were attempted so as to purify the isolated pollen protoplasts. With density gradients of sucrose (15, 20 and 30%) the intact pollen formed a pellet but no protoplasts were observed in the supernatant. This may be due to the bursting of protoplasts as a result of the osmotic shock exerted by the sucrose solution. However, sucrose gradients have enabled pollen protoplast purification in many other plant species like *Brassica oleracea* (Liu *et al*., 2007).

Both ficoll and PEG gradients could not successfully separate the protoplasts from the debris. Certain concentrations of ficoll and PEG pelleted down undigested pollen grains, pollen cases and protoplasts during centrifugation. With some other concentrations they were collected at the interfaces. However, it has been noticed that in all such cases protoplasts as well as the pollen settled together, indicating that they have the same density. Hence, this technique could not be employed for the separation of pollen protoplasts of *Hevea.* Generally, protoplasts and empty pollen cases are expected to have different densities thus enabling their separation at suitable density gradients. A possible

explanation for the contradictory observation, in this study, is that the empty but intact pollen cases had taken up the surrounding solution of the osmoticum through the germ pore and acquired the same density as the pollen protoplasts and the undigested pollen. Percoll, a polymer with low viscosity and osmolarity was used to purify pollen protoplasts of *Lilium longiflorum* (Tanaka *et al.*, 1987).

Among the different mesh sizes used, neither the pollen nor the protoplasts passed through the sieves of pore size 20 µm and 30 µm whereas the sieve having a pore size 71 µm facilitated the passage of both protoplasts and pollen. The suspension was partially purified using a 64 µm mesh. This sieve allowed the protoplasts as well as pollen of smaller diameter to pass through, but retained most of the pollen. As a result, only partial purification of the protoplasts could be attained (Fig. 6).

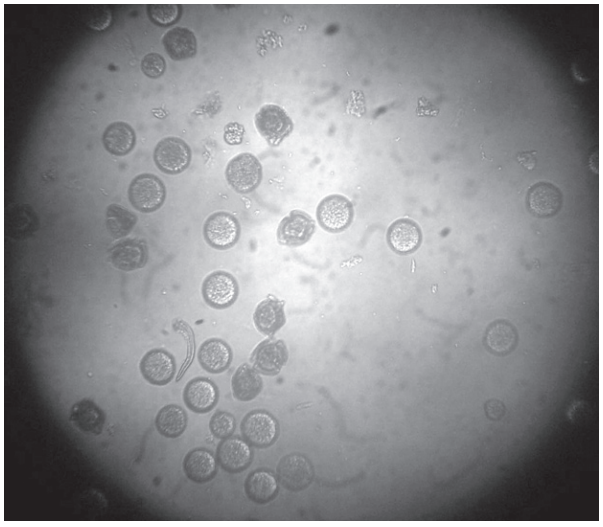

Fig. 6. Partially purified pollen protoplasts

Sieving has been widely used as the method of separating protoplasts from undigested debris. This has been more successful in the case of enzymatic maceration of plant parts like leaf, young roots, stem, cell suspension and calli where there is a clear size difference between protoplasts and debris. This method has been employed in the purification of Asparagus microspore protoplasts (Kunitake *et al.*, 1993). In *Hevea brasiliensis,* a nylon sieve (30 µm pore size) could successfully remove the undigested cells from embryogenic cell suspension derived protoplasts (Sushamakumari *et al.*, 2000) while a 100 µm mesh was required to purify protoplasts from embryogenic callus (Cazaux and d' Auzac, 1994).

Protoplast culture

It has been observed that in those cultures with partially purified protoplasts plated directly over the medium as drops, the protoplasts have got a tendency to fuse together to form giant protoplasts. Such giant protoplasts appeared in all the cultures irrespective of the media and they soon disintegrated. Only the undigested pollen as well as the pollen walls mixed with very few intact protoplasts could be found in those plates after 2-3 days of culture.

Effect of nurse culture

Among the three nurse cultures tried, the one developed from *Hevea* embryogenic calli was found to be effective in promoting the development of the cultured protoplasts. The other two nurse cultures were not beneficial for initiating cell division from the protoplasts.

Division of protoplasts leading to the formation of a few micro colonies (Fig. 7) was observed in N6 medium containing 0.8 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} BA and enriched with *Hevea* nurse culture. In the case of the cultures with KPR basal medium containing *Hevea* nurse culture, even though no micro colony formation could be detected, the protoplasts appeared intact and healthy. It has been proven from earlier studies that modified KM media are highly conducive to the growth and proliferation of plant protoplasts (Kao and Michayulk, 1975). Liquid KPR medium (Abdullah *et al.*, 1986) has been used for culture of suspension derived protoplasts of rice. Effectiveness of KPR

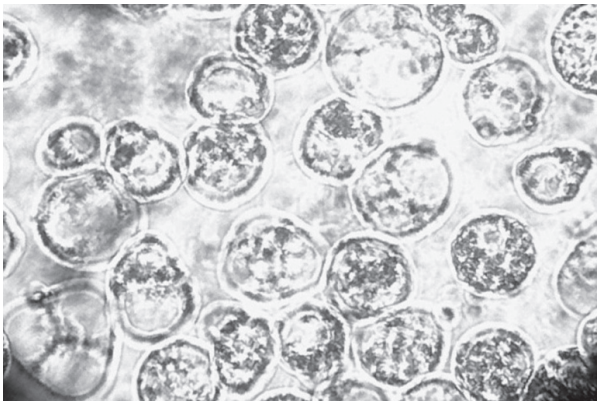

Fig. 7. Cell division and micro colony formation in pollen protoplasts

medium for protoplast division and micro-calli formation from embryogenic cell suspension derived protoplasts of *Hevea* has been reported earlier (Sushamakumari *et al.*, 2000).

Those cultures with micro colonies have been dark incubated for further development. Addition of fresh medium, as drops, to the cultured protoplasts was done at fortnightly intervals. Micro callus formation from these micro colonies is still awaited.

This is the first report of division of pollen protoplasts and micro colony formation in *H. brasiliensis*. Once these micro colonies could be proliferated, it will open the path for the development of haploids in *H. brasiliensis* which has got immense potential in the genetic improvement of this crop.

Conclusion

The lengthy and tedious process of creating a pure line in a highly heterozygous tree species like *H. brasiliensis* can be overcome by raising haploids *in vitro*. The present study demonstrated the isolation, purification and culture of *Hevea* pollen protoplasts. Protoplasts were successfully released in very high yield from pollen grains following enzymatic digestion with a combination of cellulase and pectolyase. The effect of different digestion enzymes on protoplast isolation was studied. The isolated protoplasts could be partially purified by filtering through nylon sieve. The isolated protoplasts were cultured in different nutrient media. Effect of different nurse cultures on further development and growth of the cultured protoplasts was studied by incorporating three different nurse cultures in the culture media. Division of protoplasts leading to the formation of a few micro colonies was observed in modified Chu N6 medium enriched with *Hevea* nurse culture.

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