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Enzymatic Isolation of the Components of Female Germ units of *Hevea brasiliensis* towards the Development of Haploids

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Abstract

Hevea brasiliensis, the sole commercial source of natural rubber, is a perennial tree crop with a long breeding cycle which makes genetic improvement through conventional breeding much time consuming and laborious. Female gametophytic studies leading to gynogenic haploids can be used as an alternative in vitro *approach towards crop improvement of Hevea. Female Germ Units (FGU) can be isolated and the cells can be cultured in vitro for the development of haploids as well as homozygous diploids which may be used in further breeding programmes. Also the components of FGU, which are all haploid in nature except the central cell, can be utilized for breeding through in vitro fertilization and gameto-somatic hybridisation. The present study was undertaken to standardise an efficient method for the isolation of FGU from female flowers of Hevea brasiliensis. Ovules isolated from mature female flowers, one day before anthesis, were exposed to different combinations and concentrations of the enzymes cellulase, pectolyase, macerozyme and rhozyme for different time intervals (2-20h). An enzyme solution containing 0.6% cellulase (Onozuka R-10), 0.4% macerozyme (R-10), 0.6M mannitol, 5mM MES, 1g l^{-1} potassium sulphate and 1g l^{-1} dextrose was found to be the most effective one in the release of FGUs. Egg cell release initiated from the micropylar end of the ovule within 6 h of enzymatic digestion, followed by the release of antipodals from the chalazal end. Maximum release of FGU components was observed by about 16 h of digestion. All the released cells were collected together by centrifuging at 100 rpm for 2 min and cultured in different media for further development.*

Keywords: Hevea brasiliensis, female germ units, egg cell, haploids, cellulase.

Introduction

Hevea brasiliensis, the major commercial source of natural rubber (NR), is an important cash crop occupying a key position among other plantation crops in India. Being a highly heterozygous perennial tree crop with a long breeding cycle of about 6-7 years, conventional breeding of *Hevea* has been much time consuming and laborious. In order to meet the ever increasing global demand for NR, high yielding clones with superior secondary attributes like stress tolerance, disease tolerance *etc* need to be developed. Employing biotechnological approaches is considered as an alternative to the lengthy and tedious conventional breeding techniques. One such *in vitro* approach which finds immense applications in crop improvement is the development of haploids. Production of haploid plants is advantageous for plant breeders because it provides complete homozygosity and increased efficiency of selection[1,2]. Haploid is the general term applied for plants that contain the gametic chromosome number (n). In 1964 Guha and Maheshwari developed haploids from immature anthers of *Datura innoxia*[3] for the first time. Gynogenic haploids have been developed in more than 30 species using tissue culture technology[4,5]. Androgenisis is less preferred in woody species due to the recalcitrant nature with limited success[6]. Culture of intact ovules and ovaries has been the usual practice adopted in many crop species for inducing gynogenic haploids. However, in this technique of culturing intact organs, there is always an ambiguity of the results due to the presence of tissues with different ploidy levels. Hence in the present study we made an attempt to isolate and culture the components of female germ units (FGU) so as to ensure haploid nature of the regenerants. In angiosperms, the female germ unit (FGU) comprises of an egg cell, two synergids, one central cell and three antipodals[7]. After meiosis of the diploid megasporocyte, four haploid megaspores are obtained, out of which only one is functional and the remaining three degenerate. The functional megaspore will develop into the embryo sac. The haploid nucleus of the embryo sac divides mitotically, forming 8 nuclei. They are one egg cell at the micropylar end, two small synergids on both sides of the egg cell, two polar nuclei which form the central cell and 3 antipodals at the chalazal end of the ovule[8]. Egg cell is a highly differentiated haploid cell in higher plants, which gets fertilized with a sperm cell to form embryo.

There are a few reports on the isolation of single gametes, embryo sacs, fertilised egg cells and zygotes [9,10]. In 1960 Cocking employed, for the first time, enzymatic digestion for the isolation of higher plant protoplasts[11]. Later on, enzymatic procedures were employed for the isolation of female gametes or embryo sacs in various plant species, including *Torenia fournieri*[12], *Brassica napus*[13], *Dianthus species*[14] and *Helianthus*

annuus[15]. Cellulase, hemicellulase and pectinases are enzymes which are generally used for cell wall degradation and protoplast isolation.

The present study aims at standardisation of a protocol for the enzymatic isolation and culture of intact FGU components of *Hevea brasiliensis* towards the development of haploids.

Material and Methods

Plant material: Mature female flowers, one day prior to anthesis, were collected from *Hevea* clone RRII 105 (figure-1a) and surface sterilized using 0.1% of mercuric chloride with 2 drops of Tween 20. Afterwards these flowers were washed 4-5 times with sterile distilled water. Ovules were isolated from the sterilized flowers using surgical blade and forceps.

Enzymatic digestion: Isolated ovules were subjected to enzymatic digestion for the release of female germ unit components. Cellulytic and proteolytic enzymes tried in this experiment were cellulase, pectolyase, macerozyme and rhozyme. Different combinations of these enzymes *viz*. cellulase R-10 and macerozyme R-10, cellulase R-10 and pectolyase y-23 and cellulase R-10 and rhozyme HP-150 with concentrations ranging from 0.2% to 1.0% were tried. The enzyme solutions were prepared in an osmoticum[17]consisting of Mannitol (6M), MES (0.5mM), Potassium sulphate (0.1%) and Dextran 40 (0.1%). The enzyme solutions were filter sterilised after adjusting the pH to 5.7 and the ovules were incubated in the enzymes for different time intervals (2-20h). Ten ovules were kept per treatment and each treatment was replicated five times. Observations were recorded on the number of FGU components released.

Purification of the released FGU components: To collect the released FGU components without debris, ovules were removed from the enzymatic solution through sieving and the enzymatic solution was centrifuged at 100 rpm for 2 min to pellet the FGU components. The supernatant was removed and the pelleted FGU components were suspended in liquid MS medium.

Culture conditions: The purified FGU components were then cultured over callus induction medium with various combinations of growth regulators. Two basal media namely MS and KandM were tried, with a growth regulator combination of 2,4-D (0.1-1mg/l) and BA (0.1mg l^{-1} - 1mg l^{-1}).

Results and Discussion

Intact ovules could be isolated from mature female flowers one day prior to anthesis. The isolated ovules were ivory in colour with reddish markings on the outer integument (figure-2b). In the case of *Hevea,* 3 ovules could be isolated from a single trilocular ovary.

Among the different enzyme combinations tried, a mixture of cellulase (0.6%) and macerozyme (0.4%) was found to be effective for the release of FGU components from the ovules (table-1). Similar results were shown by Yoichiro *et al*.,[16] where a combination of cellulase and macerozyme was effective in the isolation of viable egg cells from *Alstroemeria.* Increasing concentrations of both the enzymes resulted in the damage of the released FGU components.

Table-1
Effect of enzyme combinations on the release of FGUs from *Hevea* ovules

Cellulase Macerozyme	0.2	0.4	0.6	0.8	1
0.2	5	8	4	3	2
0.4	2	5	24	5	3
0.6	-	4	6	20	-
0.8	-	-	1	2	-
1	-	-	-	-	2

It is already established that enzyme combinations and concentrations for effective release varies with species. Mehri[17] reported that different combinations and concentrations of enzyme were needed for the release of protoplasts from different tissues. According to their report, the digestion of leaf mesophyll tissue needed cellulase R-10 (onozuka) and pectolyase y-23 while callus protoplasts of the same material could be isolated with cellulase R-10 (onozuka) and macerozyme R-10. Similarly, rhozyme was used along with cellulase and pectolyase for the effective isolation of banana protoplasts[18]. FGUs including central cell could be successfully isolated in *Torenia fournieri*[12] and *Nicotiana tabacum*[19] by using enzyme solution containing macerozyme R-10. Earlier researchers have reported that high enzyme concentrations adversely affected the survival [18,20, 21] and functions of isolated female gametophytic cells[22, 23].

Van *et al* [24] reported that egg cells could be isolated only with a combination of enzymatic maceration and mechanical manipulation of the dissected ovules. They also reported that the osmolality of the isolation medium, length and temperature of enzymatic maceration appeared to be crucial for obtaining viable egg cells.

Figure-1 shows the effect of incubation period in the enzyme solution, on the release of female germ unit components. Release of the FGU components started after 4 h. The number of FGU components increased with incubation period and reached maximum by about 16h. Afterwards, it started declining, leaving only very few numbers of intact FGU components after 22 h of incubation. This may be due to the cell wall decomposition of the released cells upon overexposure to the digestion enzymes.

Figure-2d shows the release of egg cells from the micropylar end of the ovule, followed by the release of central cell within 8 h (figure-2e). Figure-2f shows the simultaneous release of various FGU components.

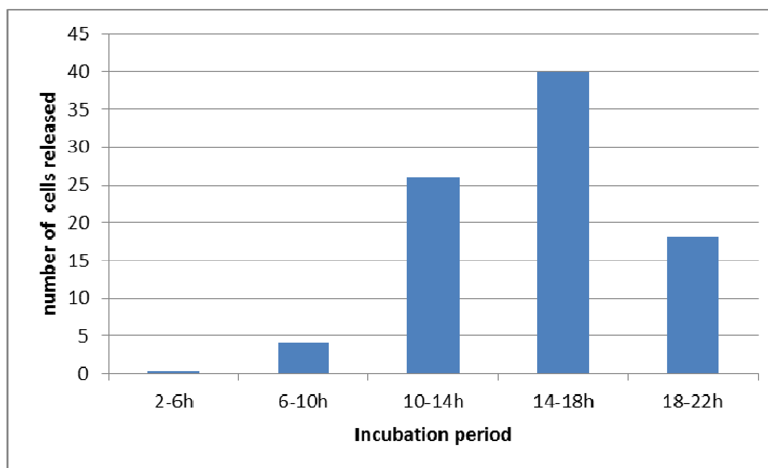

Figure-1
Effect of incubation period in the release of various components of FGU

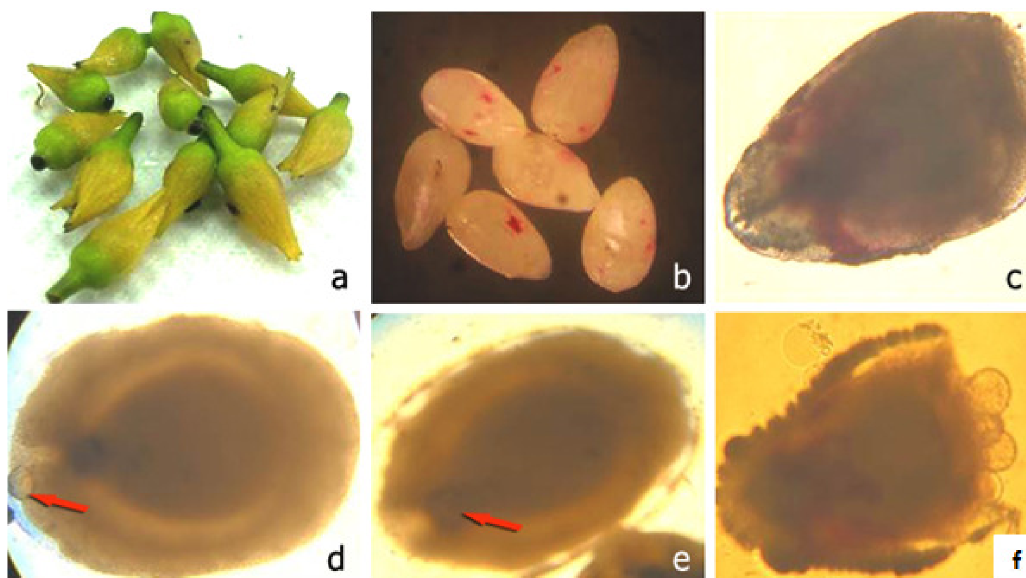

Figure-2
Different stages of FGU release
(a) Mature female flowers (b) Isolated ovules (c) Enzymatic digestion (2h after incubation) (d) Release of egg cell from the micropylar region (e) Release of central cell (f) Release of FGU components (egg cell, synergids, central cell and antipodals)

The liberated FGUs (egg, central cell, synergids, antipodal and embryo sac) were distinguished from each other by their size and position. Observation through inverted microscope shows that the central cell has larger size compared to the other components of FGU (figure-3b), followed by the egg cell which consists of a large vacuole (figure-3d). Antipodals are smaller in size than the egg cells (figure-3c). Synergids are initially released as attached with the egg cell (figure-3a) and later they get separated. Similarly Ratchada[22] distinguished the isolated FGU from ovules of *Petunia hybrid* on the basis of their specific size and characteristics.

The isolated FGUs were purified by centrifuging at 100 rpm for 2 min and are being cultured for callus induction (figure-3e) in different media combinations.

Conclusion

A protocol for the isolation of FGU components from the ovules of *Hevea* was developed. FGU components including the egg cells, antipodals, central cell and synergids could be isolated successfully and the different components could be identified based on size. The isolated FGUs were purified and cultured for callus induction. Callus once induced will be either haploid (from the egg cell) or homozygous diploid (from the central cell). This is the first attempt of isolation and culture of FGU components towards the development of haploids in *Hevea brasiliensis*.

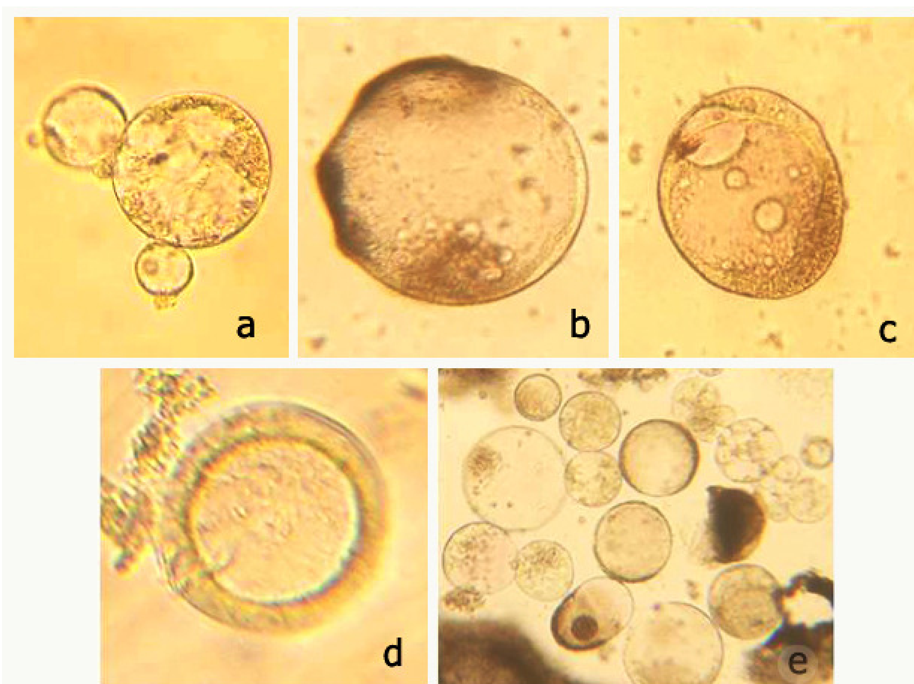

Figure-3
Released FGU components (x 100)
(a) Egg cell with synergids (b) Central cell (c) Antipodal cell (d) Egg cell with large vacuole (e) Pelleted FGU components used for culturing

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