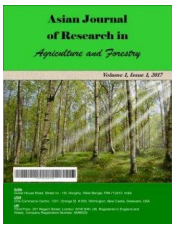

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# Development of Triploid Callus of *Hevea brasiliensis* Using Endosperm

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors KR and SSK designed the study. Author UKD performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KR and SSK managed the analyses of the study. Author UKD managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** To generate a pathway for development of *Hevea* triploids using endosperm tissue as an explant.
**Study Design:** Standardization and optimisation of various parameters for isolation and culture of endosperm tissue and protoplast. Completely randomized design for data from different treatment. Ploidy of the obtained culture was determined.
**Place and Duration of Study:** Department of Biotechnology, Rubber Research Institute of India and duration of the study 12 months.
**Methodology:** Endosperm tissues were collected from *Hevea* seeds of different developmental stages. Somatic embryogenesis from endosperm tissue using callus mediated embryogenesis and direct method of embryogenesis were carried. Endosperm tissue from both immature and mature seeds was cut into thin slices and subjected to enzymatic digestion for the release of protoplasts. Different concentrations and combinations of cell wall digestion enzymes and osmotic agents

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experimented. The callus obtained from endosperm tissue was subjected to cytological analysis and flow cytometric analysis.
**Results:** Endosperm tissue from immature fruits was found to be ideal one week between (8-10 weeks) both for somatic embryogenesis and for the release of large amount of protoplasts. Of the two basal media tried, Nitsch medium favoured callus induction, 6 % callus induction from mature endosperm tissue in presence of 2,4-D (6.3 µM) and Kin (12.1 µM) and 10 % callus induction from immature endosperm tissue in presence of BA (4.4 µM) and NAA (2.2 µM). Direct embryogenesis (2 %) has been obtained from immature endosperm in MS basal medium along with $GA_3$ (2.0 µM) and BA (11.1 µM). A few of the endosperm protoplasts showed division when cultured over K&M medium with NAA (0.1 µM) 2,4-D (0.2 µM) and BA (0.4 µM).
**Conclusion:** Endosperm can be used for the development of triploids of *Hevea brasiliensis*. The ploidy variants i.e. triploids, developed through these *in vitro* techniques can be further used in *Hevea brasiliensis* breeding.

*Keywords: Endosperm; triploid; Hevea seeds.*

## 1. INTRODUCTION

Triploidy, a genomic condition that is favourable for vigor and vegetative productivity have been proved to be beneficial in several crops. Development of triploids with increased biomass is highly desirable in *Hevea* since it may lead to a reduction in the immaturity period as well as an increase in yield. Triploids are generally sterile. Hence triploid development is suitable for plants where seed is not the economic part. The advantage of triploids can be well exploited in *Hevea* since latex, not seed, is the product of commercial value in this crop. Moreover, development of triploids will lead to seedlessness, which can combat Phytophthora disease thereby making the tree resistant to this disease [1].

Traditionally triploids are produced by hybridization between tetraploids and diploids [2]. *In vitro* regeneration of plants from the endosperm, the sole naturally occurring triploid plant tissue, offers a direct single step approach for triploid production. Parenchymatous nature of the endosperm and the absence of vascular tissues make it a unique and excellent experimental system for *in vitro* culture studies [3]. The endosperm in angiosperms is formed via double fertilization and triple fusion (i.e., fusion between 3 different haploid nuclei, 1 from the paternal and 2 from the maternal side), which is a unique process in higher plants and is present in all angiosperm families except Orchidaceae, Podostemaceae, and Trapaceae. Endosperm functions as a nutritive tissue for the growing embryo, as the growth and development of the embryo depends on the presence of the endosperm [4]. Moreover, the endosperm exists as a reserve food in some seeds like cereals. The endosperm represents about 60% of the world's food supply. Failure of the endosperm to develop properly leads to the abortion of the embryo. Endosperm may be fully utilized by the developing embryo (non-endospermous), or it may persist in mature seeds (endospermous). Attempts were made from 1930 by the scientist Lampe and Mills to grow young corn endosperm using *in vitro* techniques [5]. Different developmental stages of endosperm from immature to mature stages were used by different workers for the *in vitro* development of triploids. Culture of endosperm protoplasts is yet another option for the production of triploids. Moreover, triploid protoplasts once isolated are useful for the production of aneuploids through fusion with haploid or diploid protoplasts. Also, plant protoplasts provide a unique single cell system to underpin several aspects of modern biotechnology. Hence experiments such as somatic embryogenesis from endosperm tissue using direct and indirect method and Isolation and culture of protoplast from endosperm tissue.

## 2. MATERIALS AND METHODS

### 2.1 Explant

Endosperm tissue which is the explant used in this study were collected from seeds of different developmental stages. Broadly these source seeds can be divided into two categories mature and immature seeds, depending on their stage of development.

#### 2.1.1 Mature seeds

Seeds (Fig. 1b) were collected from mature fruits (Fig. 1a) from the field grown trees of *Hevea* clone RRII 105, on the day of dispersal. The hard

seed coat was removed mechanically and the seeds were sterilized using 0.1 % mercuric chloride solution with a few drops of Tween 20 for 5 min, followed by several washes in sterile distilled water. Washing was done uniformly throughout the experiment. The inner integuments, as well as the embryos, were removed and the remaining tissue, the endosperm (Fig. 1c), was cut into small pieces and used for the further process.

#### 2.1.2 Immature seeds

Young fruits at different maturity stages of 1-10 weeks (Fig. 1d) were collected from field grown trees of *Hevea* and were surface sterilized using alcohol for 15 min. The developing seeds were separated and cut transversely into two halves (Fig. 1e), of which the half with the micropylar end was cultured in Nitsch medium fortified with growth regulators 2, 4-D (9.0 µM) and Kin (13.9 µM) for inducing endosperm development (Fig. 1f).

Endosperm tissue obtained from both mature and immature seeds were used as the source for protoplast isolation and culture and also for callus mediated and direct embryogenesis.

### 2.2 Somatic Embryogenesis from Endosperm Tissue

#### 2.2.1 Callus mediated embryogenesis

The endosperm tissue from both the sources were cut into small segments and cultured for callus induction in two basal media, MS and Nitsch, fortified with different combinations of the growth regulators 2, 4-D (4.5 - 9.0 µM), Kinetin (9.3 - 13.9 µM), BA (2.2 - 8.8 µM) and NAA (1.1 - 5.3 µM). Best combination for callus induction was worked out and the calli induced in this medium were transferred to embryo induction medium after proliferation. Two basal media *viz*. MS and WPM fortified with $GA_3$ (0.87- 2.9 µM) and BA (4.4 - 13.3 µM) were used for embryo induction.

#### 2.2.2 Direct embryogenesis

For direct embryogenesis, the endosperm tissue from both the sources were kept in two different embryo induction media, MS and WPM fortified with different levels of $GA_3$ (0.87- 2.9 µM) and BA (4.4 - 13.3 µM). Cultures were maintained in dark.

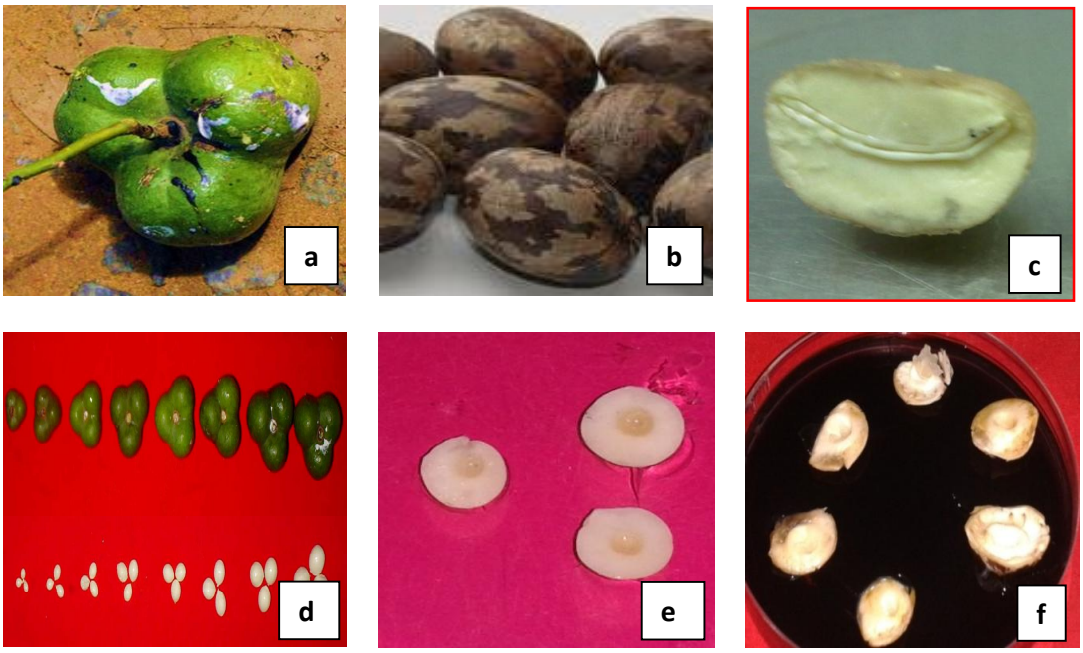


**Fig. 1. Explants used for endosperm culture**
**a) Mature fruit, b) Mature seeds, c) Mature endosperm tissue, d) Immature fruits & corresponding ovules at different developmental stages (1 to 10 weeks), e) Cross section of young seed for half ovulo culture, f) Endosperm developing from cultured immature seeds**

## 2.3 Protoplast Culture

### 2.3.1 Isolation of protoplasts

Endosperm tissue from both immature and mature seeds were cut into thin slices and subjected to enzymatic digestion for the release of protoplasts. Different concentrations and combinations of cell wall digestion enzymes and osmotic agents were experimented with a view to enhance the protoplast yield.

### 2.3.2 Osmoticum

Experiments were carried out for identifying the suitable osmoticum for protoplast release. Cell and protoplast washing medium (CPW medium*) was used as the basal medium. Different concentrations (0.2 - 1.0 M) of the sugars and sugar alcohols viz: sucrose, glucose, mannitol and sorbitol were tried for identifying the right osmoticum. Also different combinations of the two sugar alcohols were tried for optimization of osmoticum. In addition, the osmotic stabilizer MES (5mM) was incorporated in all the above solutions and autoclaved at 120ºC and 15lb/sq pressure for 15 min and stored at 25ºC.

{*CPW medium- $KH_2PO_4$ (27.4 $mgl^{-1}$), $KNO_3$ (101$mgl^{-1}$), $CaCl_2.2H_2O$ (1480 $mgl^{-1}$), MgCl2.7H2O (276 $mgl^{-1}$), KI (0.16 $mgl^{-1}$), $CuSO_4.5H_2O$ (0.025 $mgl^{-1}$), pH- 5.8}

### 2.3.3 Cell wall digestion enzymes

In order to identify the type and concentration of digestion enzymes for protoplast release, different concentrations and combinations of the enzymes cellulase onozuka RS (0.5, 1.0, 2.0 %) and pectinase Y23 (0.05, 0.1, 0.2, 0.5 %) were tried. The enzymes were dissolved in the osmoticum and the pH was adjusted to 5.7. The solution was filter sterilized using a membrane filter (Millipore 0.22 µm), stored at 4ºC and thawed to room temperature before use. Thin slices of endosperm tissue were incubated in different enzyme solutions and incubated for different time intervals (2 - 6 h).

### 2.3.4 Protoplast purification

After enzyme incubation, protoplast suspensions were first filtered through nylon sieves of different mesh sizes (30, 64, 71, 85 and 100 µm) to remove the debris and undigested tissue. The filtrate was then transferred to centrifuge tube and the protoplasts were pelleted by centrifugation at 500 rpm for 2 min.

The supernatant was removed using a pasteur pipette and the pellet was re-suspended in the osmoticum, mixed well and again centrifuged. This was repeated three times to remove traces of enzymes. Finally the purified pellet was re-suspended in 1ml of osmoticum and the suspension was used for culturing. The protoplast yield per gram fresh weight was determined with the help of a haemocytometer.

$$\text{Yield/gFW} = \frac{\text{Total number of protoplasts from four 1mm squares of haemocytometer}}{\text{Sample volume in 4 squares}} \times \text{Total volume of protoplast suspension}$$

### 2.3.5 Culturing of protoplasts

Three different basal media viz. MS [6], WPM [7] and KM [8] were used for plating the isolated protoplasts. Sucrose and glucose were provided as carbon source at concentrations of 20 $gl^{-1}$ and 10 $gl^{-1}$ respectively in all these three media. Different growth regulators combinations of 2, 4-D (0.1 - 1.0 µM), NAA (0.1 - 1.0 µM), Kinetin (0.1 - 1.0 µM) and BA (0.1 - 1.0 µM) were also added. Aliquots of purified protoplasts were plated over these different media combinations and all the cultures were incubated in the dark at 28ºC until growth of callus was obtained. They were examined routinely to detect cell division, multiplication, growth and callusing.

## 2.4 Statistical Analysis

All experiments were conducted in completely randomized design (CRD) and analyzed using SPSS 16.0 software. The data was subjected to square root/arc sine transformation and analyzed using ANOVA with a significance of $p \leq 0.05$.

## 2.5 Confirmation of Ploidy

### 2.5.1 Cytological analysis

The proliferating callus obtained from the endosperm tissue was subjected to cytological analysis [9]. Callus with actively dividing cells were pre-treated with 0.2 mM 8-hydroxyquinoline for 5 h at 4˚C. After this pretreatment the solution was drained off, the callus was washed with distilled water and transferred to cold freshly prepared fixative, Carnoys fluid II (3:1 ethanolacetic acid), for 48 h at room temperature. Afterwards the fixative was drained off and the callus was washed thoroughly to remove traces of fixative. Then the samples were stained with 1 % Snows carmine for 4 hrs. The

samples were smeared in 45% acetic acid with a glass rod and mounted on slides as per standard protocol. The slides were observed under a light trinocular microscope (Leica).

#### 2.5.2 Flow cytometric analysis

The ploidy analyzer I (Partec GmbH, Germany) was used to determine the ploidy level of the endosperm derived callus. For sample preparation, the callus was crushed in galbriath's* buffer and kept for 5 min incubation. The suspension containing the nuclei was mixed by pipetting up and down several times and then filtered through a 50 µm nylon mesh. The filtrate containing the nuclear suspension was stained with 50 µg/ml propidium iodide and incubated at room temperature for 5 min. 50 µg/ml RNAse was then added and mixed and this mixture was used for ploidy analysis [10]. The position of peak G1 nuclei of the control (Diploid callus derived from immature anther) was established at channel 400 on a 1024-channel scale, after which the instrument setting was kept constant and the test samples were run under the same parameters.

## 3. RESULTS

### 3.1 Somatic Embryogenesis from Endosperm Tissue

#### 3.1.1 Callus mediated embryogenesis

Of the two basal media tried, Nitsch medium favored callus induction. Among the different growth regulators, a combination of 2, 4-D and Kin responded towards callus induction from mature endosperm. 6 % callus induction was obtained in a combination of Nitsch medium supplemented with 2, 4-D (6.3 µM) and Kin (12.1 µM) (Fig. 2a & b). At higher concentrations of 2, 4-D and Kin there was no callus induction (Table 1). When the mature endosperm tissue was kept in the callus induction medium for longer periods, root organogenesis was observed in a combination of 6.6 µM BA and 4.3 µM NAA (Fig. 2c).

**Table 1. Effect of 2,4-D and Kin on callus induction from mature endosperm tissue**

| 2,4-D (µM) / Kin (µM) | 4.5 | 6.3 | 8.1 | 9.0 |
|---|---|---|---|---|
| 9.3 | 0.0(0.7) | 0.0(0.7) | 0.0(0.7) | 0.0(0.7) * |
| 11.2 | 1.0 (1.2) | 4.5(2.2) | 3.0(1.8) | 2.5(1.7) |
| 12.1 | 2.0(1.5) | 6.0(2.4) | 4.0(2.1) | 3.0(1.8) |
| 13.0 | 1.0(1.2) | 3.5(1.9) | 3.0(1.8) | 2.5(1.7) |
| 13.9 | 1.5(1.4) | 3.0(1.8) | 3.0(1.8) | 1.5(1.4) |

*CD=0.31

** CD-Coefficient of determination*
**callus induction (%) from mature endosperm tissue*
*Data were subjected to square root transformation and transformed means are given in Parenthesis*

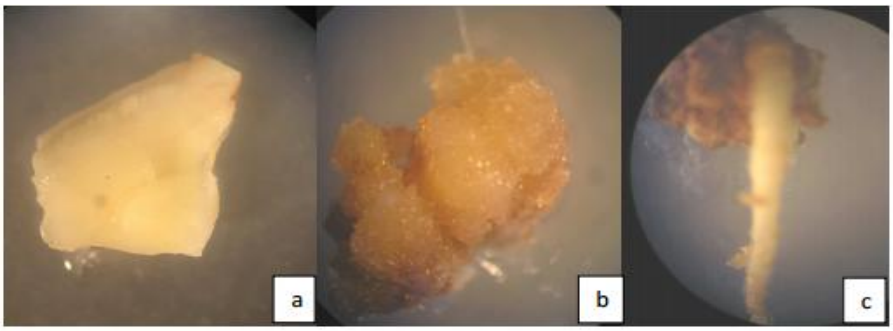


**Fig. 2. Culture of mature endosperm**
**a & b) Cultured mature endosperm isolated from mature seeds and induction of callus from this cultured endosperm {2, 4-D (6.3 µM) Kin (12.1 µM)}**
**c) Root induction from mature endosperm (6.6 µM BA and 4.3 µM NAA)**

In the case of immature endosperm tissue, a combination of BA and NAA responded towards callus induction whereas with the combination of 2,4-D and Kin no callus initiation occurred, only enlargement of endosperm tissue was observed. 10 % callus induction was obtained in a combination of BA (4.4 µM) and NAA (2.2 µM) growth regulators (Fig. 3a & b). At higher concentrations of these growth regulators, especially BA, the callus induction was nil (Table 2).

#### 3.1.2 Direct embryogenesis

Endosperm tissue isolated from 8 week old fruits was found to be suitable for direct embryogenesis. Embryo induction at a low frequency (2 %) was obtained directly from immature endosperm, when cultured over MS medium fortified with $GA_3$ (2.0 µM) and BA (11.1 µM) (Fig. 4a & b). These embryos are kept in the same medium for further development. From the mature endosperm tissue, no embryo development could be obtained and later the tissue got dried up.

### 3.2 Protoplast Isolation and Culture

#### 3.2.1 Protoplast isolation

Among the different stages of immature fruits cultured, endosperm development was observed in the 8 - 10 week old fruits (Fig. 1d). Protoplasts could be isolated from both mature and immature endosperm tissue, but the yield from immature endosperm was more compared to the mature endosperm tissue.

**Table 2. Effect of BA and NAA on callus induction from immature endosperm tissue**

| BA (µM) / NAA (µM) | 2.2 | 4.4 | 6.6 | 8.8 |
|---|---|---|---|---|
| 1.1 | 1.0 (1.2) | 6.5 (2.6) | 4.5 (2.2) | 0.0 (0.7)* |
| 2.2 | 7.0 (2.7) | 10 (3.1) | 7.0 (2.7) | 0.0 (0.7) |
| 3.2 | 5.5 (2.4) | 9.0 (3.08) | 3.0 (1.8) | 0.0 (0.7) |
| 4.3 | 2.5(1.7) | 4.0 (2.1) | 1.5 (1.4) | 0.0 (0.7) |
| 5.3 | 2.0 (1.5) | 0.0 (0.7) | 0.0 (0.7) | 0.0 (0.7) |

CD= 0.23

** percentage of callus induction from immature endosperm tissue*
*Data were subjected to square root transformation and transformed means are given in parenthesis*

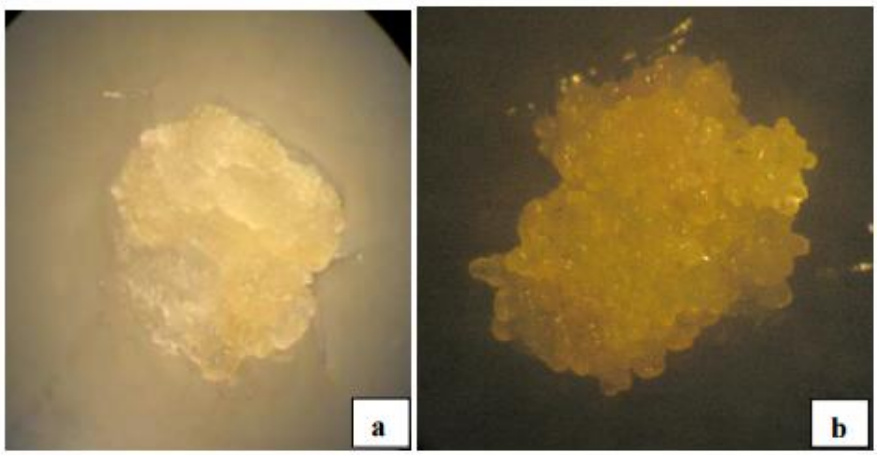


**Fig. 3. Culture of immature endosperm**
**a & b) Cultured immature endosperm and induction of callus from this cultured endosperm {BA (4.4 µM) and NAA (2.2 µM)}**

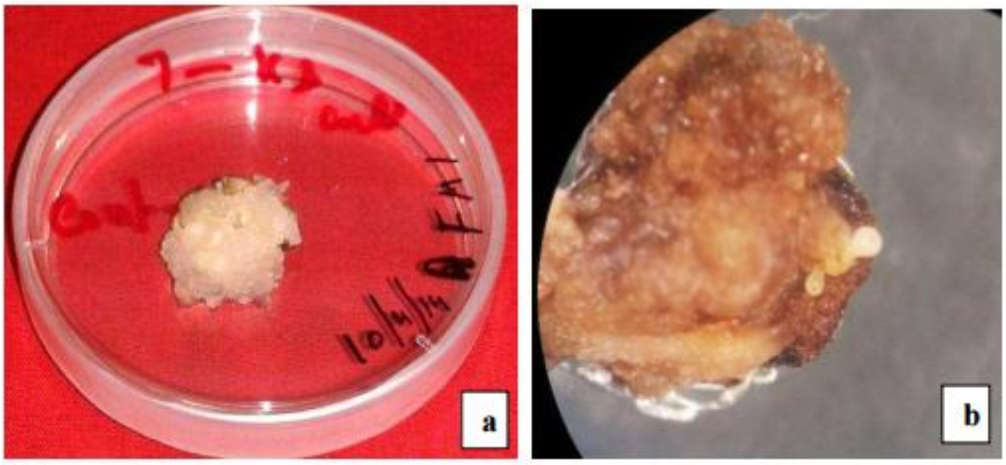


**Fig. 4. Direct embryogenesis**
**a) Cultured immature endosperm tissue (8 week old)**
**b) Direct embryo induction {$GA_3$ (2.0 µM) and BA (11.1 µM)}**

### 3.2.2 Osmoticum

Among the different osmotic agents tried, mannitol has been found to be the most efficient, followed by sorbitol for maintaining osmotic stability. The optimum concentration of mannitol and sorbitol was in the range 0.6 - 0.8 M and 0.4 - 0.6 M respectively (Table 3). Protoplast yield was more in immature endosperm compared to mature endosperm in both mannitol and sorbitol. In presence of mannitol a protoplast yield of 17 x $10^4$ /gfw from mature endosperm and 28 x $10^4$ /gfw from immature endosperm were obtained. With sorbitol 12 x $10^4$ /gfw from mature endosperm and 16 x $10^4$ /gfw from immature endosperm was obtained. No protoplast release occurred with sucrose and glucose.

It was observed that a combination of the two sugar alcohols, mannitol and sorbitol, yielded more number of protoplasts when compared to the number of protoplast released when this osmotic agents were added separately. 0.7M mannitol with 0.5M sorbitol was found optimum in both the case of immature and mature endosperm tissue (Fig. 5). The highest yield of protoplast of about 20 ×$10^4$ /gfw from mature endosperm tissue and in case of immature endosperm tissue, 34×$10^4$ /gfw was obtained.

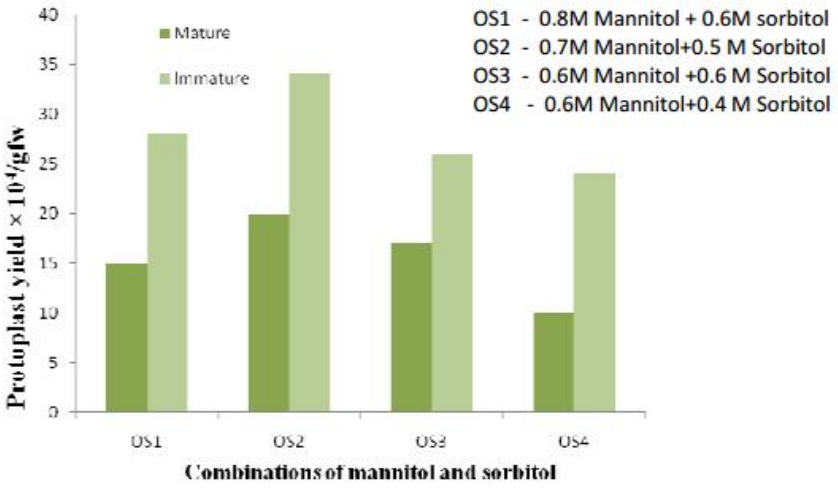


**Fig. 5. Effect of osmoticum on protoplast release**

**Table 3. Effect of sugar alcohols on protoplast release from mature and immature endosperm tissue**

| Source | Mannitol (M) | | | | | Sorbitol (M) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Mature fruit endosperm | 4 | 7 | 15 | 17 | 7 | 6 | 10 | 12 | 6 | 3 |
| Immature fruit endosperm | 10 | 12 | 26 | 28 | 20 | 10 | 15 | 16 | 12 | 9 |

** Protoplast yield × $10^4$ /gfw*

### 3.2.3 Cell wall digestion enzymes

Out of the five enzyme combinations tested, 2.0 % cellulase mixed with 0.2 % pectinase was found to be optimum for the release of protoplasts from mature endosperm tissue. A combination of 1.0 % cellulase and 0.2 % pectinase gave highest protoplast yield from immature endosperm. Protoplast yield from other combinations of these enzymes was quite low in both the explants (Fig. 6). A protoplast yield of 24 x $10^4$ /gfw was obtained from mature endosperm when subjected to enzymatic digestion in the enzyme combination E4 whereas protoplast release from immature endosperm tissue was 36 x $10^4$ /gfw in the combination E3.

With the optimized parameters of osmotica and enzymes, endosperm tissue isolated from immature seeds was found to be ideal for isolation of protoplasts (Fig. 7b) compared to mature seeds. In the case of mature endosperm, even though a good number of protoplasts were released initially but as the enzymatic digestion proceeded for about 2hrs, the osmoticum turned turbid, thereby rendering estimation of protoplast yield much difficult (Fig. 7a). Turbidity may be due to the release of oil granules from the mature endosperm tissue.

### 3.2.4 Purification and culture of protoplasts

Endosperm protoplasts isolated from young developing seeds could be purified easily when compared with the mature tissue due to the lack of oil granules. Protoplasts released from mature endosperm tissue are bigger compared to that from immature tissue (Fig. 8a & b). However, due to the presence of oil granules in case of mature tissue, purification process was difficult and the yield was also less. Purification was carried out using 71µ mesh size sieve for protoplasts from immature endosperm and 100µ mesh size sieve for protoplasts from mature endosperm. Protoplasts from mature endosperm did not respond in culture. A few of the protoplasts from immature endosperm started division in K&M medium supplemented with 0.1 µM NAA, 0.2 µM 2,4-D and 0.4µM BA (Fig. 8c & d). Further division and microcolony formation from these protoplasts is awaited.

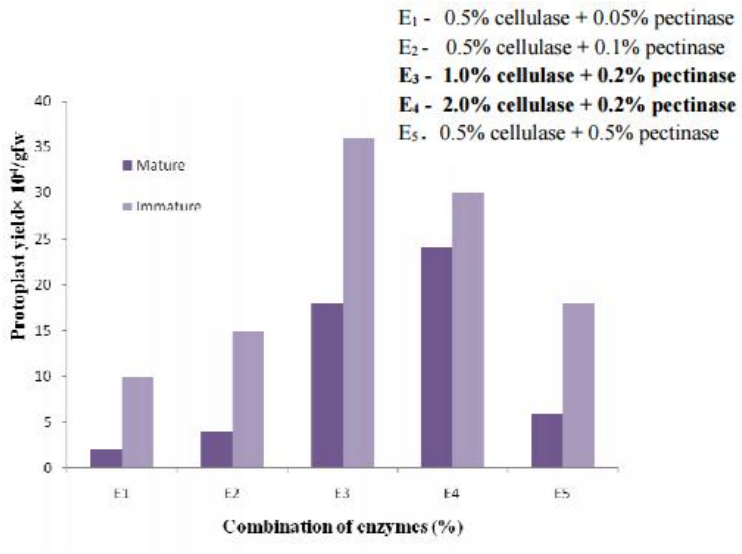


**Fig. 6. Effect of digestion enzymes on protoplast release**

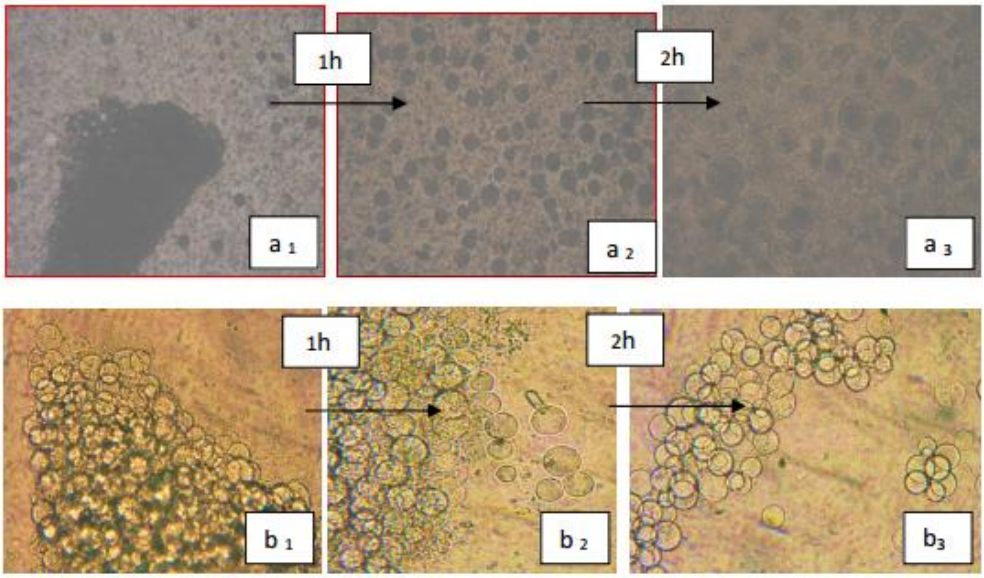


**Fig. 7. Protoplast release from mature and immature endosperm**
**a) Mature endosperm tissue**
**$a_1$ Initial stage of digestion, $a_2$ After 1 hr of digestion, $a_3$ After 2hr of digestion**
**b) Immature endosperm tissue, $b_1$ Initial stage of digestion, $b_2$ After 1 hr of digestion, $b_3$ After 2hr of digestion**

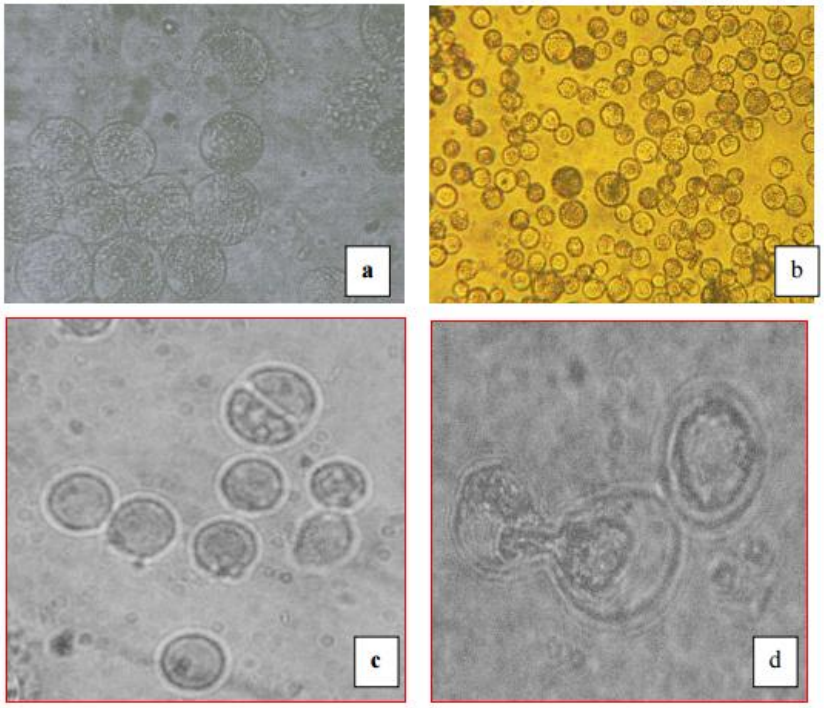


**Fig. 8. Purification and culture of released protoplast**
**a) Purified protoplast from mature endosperm tissue, b) Purified protoplast from immature endosperm tissue, c & d) Protoplast division**

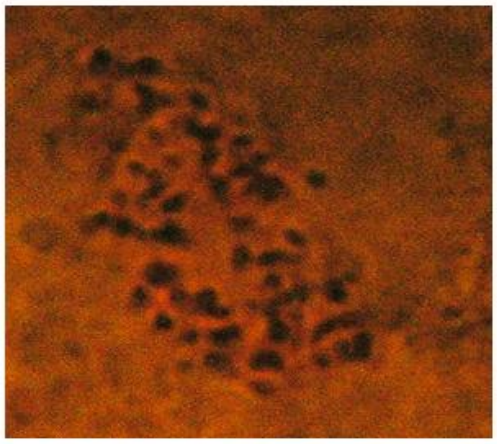

**Fig. 9. Chromosome count from endosperm derived callus (3n=54)**

## 3.3 Ploidy Determination

### 3.3.1 Cytological analysis

Cytological studies showed a chromosome count of 3n=54 (Fig. 9) in the callus obtained from endosperm tissue, observed at a magnification of X 400. Hence it can be confirmed that the endosperm derived callus is triploid in nature.

### 3.3.2 Flow cytometer

Using flow cytometer, ploidy of the endosperm derived callus was determined (Fig. 10a & b). The histogram showed fluorescence intensity of nuclei from the endosperm callus at the highest peak at channel 560 which is almost 1.5 times the value of the control diploid callus (400), thus proving this callus to be triploid. This confirms the presence of an extra set of chromosomes in the endosperm derived callus, thereby rendering it to be triploid.

## 4. DISCUSSION

Endosperm tissue shows a unique characteristic when compared with other explants in terms of origin, development and ploidy level. In our study both mature and immature endosperm tissue were taken as explants for protoplast culture and for direct and callus mediated embryogenesis.

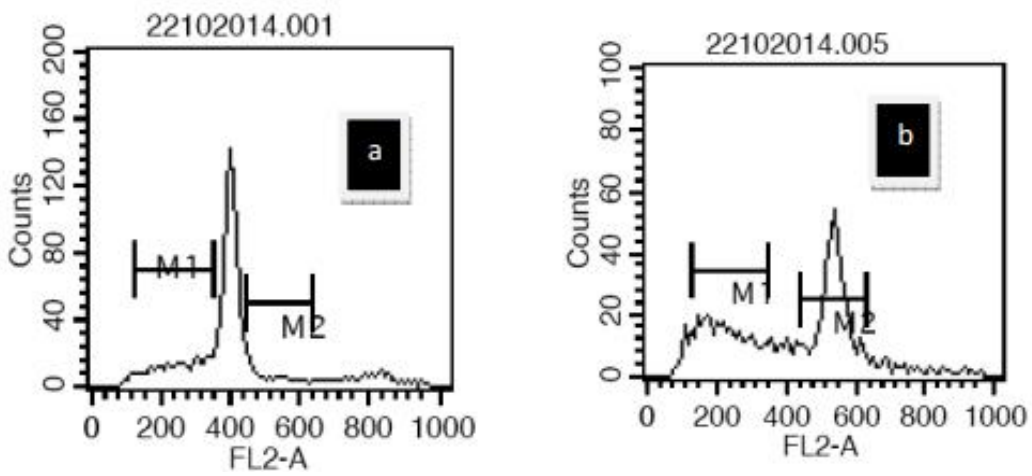


**Fig. 10. Histogram showing peaks for**
**a Diploid (Immature anther derived callus)**
**b Triploid (Endosperm derived callus)**

## 4.1 Somatic Embryogenesis from Endosperm Tissue

### 4.1.1 Callus mediated embryogenesis

In triploids chromosome pairing during meiosis is disrupted and the unequal segregation of the chromosomes produces aneuploid gametes, thereby significantly decreasing fertility. The most direct route to generate triploid plants is to regenerate shoots or somatic embryos from mature or immature endosperm tissue. This has been achieved in a range of plants [11 and 12].

In the present study callus induction, both from immature and mature endosperm tissue could be obtained at frequencies 10% and 6% respectively. Basal medium was the same, namely Nitsch medium. The growth regulator combinations favoring callus induction were quite different, 2, 4-D (6.3 µM) + Kin (12.1 µM) for mature endosperm and BA (4.4 µM) + NAA (2.2 µM) for immature endosperm. This may be due to the difference in requirement of the endosperm tissue during the early stage of development and the late/mature stage. Callus induction from endosperm of mature fruits in MS medium with IAA ($1mgl^{-1}$) + BAP ($1mgl^{-1}$) and also in 2,4-D ($1\ mgl^{-1}$) + Kin ($1\ mgl^{-1}$) had been reported [13]. Callus induction from mature endosperm of *Actinidia deliciosa* was obtained in MS basal medium with 2, 4-D (2 mgl-1) + Kin ($5mgl^{-1}$) [14]. Also different growth regulator requirements for callus induction and subsequent callus proliferation from immature endosperm of neem (*Azadirachta indica*) have been reported [15]. According to them, best callusing (53 %) was obtained when cultured in MS + NAA (5 µM) + BA (2 µM) + CH ($500\ mgl^{-1}$) whereas the percentage of callus proliferation was maximum (45 %) in MS + 2, 4-D (5 µM). In a few cultures, root induction was observed from the mature endosperm tissue upon prolonged culture in the callus induction medium fortified with 6.6 µM BA and 7.5 µM NAA. However, shoot induction hasn't so far been obtained in those cultures. Endosperm exhibits the property of organogenesis in the case of *Exocarpus cupressiformis* [16].

Earlier attempts were made for the development of triploids in *Hevea brasiliensis* using mature endosperm tissue, but due to low plant regeneration frequency, no further work was attempted [17]. In 1947 La Rue, for the first time, reported the possibility of obtaining continuously growing tissues from the cultured immature maize endosperm [18]. In general it has been found that mature endosperm requires the initial association of embryo to form callus but immature endosperm proliferates independent of the embryo. Similar observation for mulberry was reported [19]. The stages of immature endosperm at the time of culture were normally expressed as days after pollination (Thomas et al. [19]). However, some researchers estimated the endosperm stage in relation to the stage of developing embryo [20]. When the callus was transferred to a medium containing BA or Kin, shoot buds differentiated from all over the callus. Maximum regeneration in terms of number of cultures showing shoot buds and number of buds per callus occurred in the presence of 5 M BA [21].

### 4.1.2 Direct embryogenesis

Endosperm tissue, being the storage tissue for the developing zygotic embryo, is expected to be amenable to direct embryogenesis under in vitro condition. In our experiment, direct embryo induction from cultured immature endosperm could be obtained in MS medium supplemented with $GA_3$ (2.0 µM) and BA (11.1 µM). However the frequency was quite low. Hence further standardization needs to be carried out in this direction to perfect a system for direct embryogenesis from the triploid endosperm tissue. Parameters like exact age of the young fruit for endosperm isolation, various media components and other additives need to be standardized. It is technically demanding but the rate of success is generally very low.

### 4.1.3 Protoplast isolation and culture

Successful isolation of protoplasts from both immature and mature endosperm tissues could be achieved. Protoplasts obtained from immature endosperm showed division when cultured for callus induction. Isolation procedures that yield highly purified and functional protoplasts have been described for many species. The isolation of plant protoplasts was first reported more than 50 yr ago [22]. Isolated protoplasts allow the study of various metabolic processes. Freshly isolated protoplasts have been proved to be versatile cell systems for studying a broad spectrum of plant physiology, plant cell biology, plant gene engineering, biomechanics, stress responses and cell death controls [23 and 24].

Enzymatic isolation of protoplasts using cellulase was first reported in tomato from root tips [22].

The easy availability of commercial, purified enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme and hemicellulase has now resulted in an increase in the yield and viability of protoplasts and their subsequent response in the culture medium. Commonly a combination of pectinase and cellulase is used to digest the cell walls and to liberate protoplasts [25]. In our experiment using a combination of 1 % cellulase and 0.2 % pectinase, large number of protoplasts could be released from immature endosperm tissue. Higher concentration of the enzyme cellulase (2.0 %) along with 0.2 % pectinase was found effective in the release of protoplasts from mature endosperm tissue. The concentration and combination of enzymes for the isolation depend upon age, genotype and stage of differentiation of the tissue from which the protoplasts are to be isolated [26]. Protoplasts can be isolated from a variety of tissues, young in vitro-grown plants [27], tissues like callus, cell suspension [28] and explants such as root tips [29], hypocotyl, cotyledons [30] and shoots [31] and leaves from old or mature plants [32]. In our experiment protoplast isolation was tried from both mature and immature endosperm tissue. Similar combinations of enzymes were used by [33] to isolate endosperm protoplast from dwarf rice variety. In legumes the most frequently used cellulase is Onozuka R-10. This enzyme proved to be suitable for efficient protoplast release from primary explants of alfalfa [34 and 35]. Pectinases interact with cells being in different phases of the cell cycle and act like biochemical sorters [36]. The stability, viability and further growth of the isolated protoplasts are closely related to the maintenance of a proper osmoticum during isolation and subsequent culture. In general, osmotic potential is adjusted by adding D-Mannitol, sorbitol, glucose or sucrose to the enzyme mixture [37].

Generally protoplast burst in hypotonic solution and collapse in hypertonic solution [38]. The use of metabolically active osmotic stabilizers like glucose, sucrose along with metabolically inert mannitol is advantageous for protoplast culture. Such substances will be utilized by the protoplasts for growth and cell wall regeneration [39]. At optimum sugar alcohol and enzyme combination the protoplast yield from mature endosperm tissue was less compared to the immature endosperm tissue. A decrease in yield and viability of protoplasts obtained from later stage endosperm due to starch increase has been reported in maize [40] and wheat [41].

Purification of protoplasts from mature endosperm tissue also became difficult due to the presence of oil granules. Thus endosperm tissue from young developing seeds was identified as the suitable explant for protoplast isolation and culture. The characterization of oil and starch accumulation in tubers of *Cyperus esculentus Var. Sativus (Cyperaceae) was studied* where they reported that at the initial stage the starch starts accumulating and along with the development, sugar and protein levels decrease and oil starts accumulating and lipid and fatty acid composition begins to reflect a storage character [42]. Similar observations were obtained in our experiment showing that in *Hevea,* as maturation proceeds there is accumulation of oil granules. Protoplast yield of 36 x 104 gfw was obtained from immature endosperm tissue of *Hevea* and showed division when cultured in the K&M medium.

In *Hevea,* successful protoplast release could be achieved using embryogenic cell suspension derived from immature inflorescence and inner integument of immature fruits [28]. They also reported that the protoplasts, when cultured in KPR liquid medium, underwent division leading to callus formation and embryogenesis. Similar results were obtained which showed first cell division of protoplast when cultured in KM8P medium [43]. The isolated protoplasts from endosperm are generally metabolically active as discussed by Isabel who reported stable transformation of protoplast from maize endosperm [44]. Many useful genes expressing in barley are endosperm specific.

#### 4.1.4 Ploidy determination

The callus obtained from endosperm tissue was triploid in nature as confirmed through both cytology and flow cytometric analyses. Cytology and flow cytometer are two techniques generally used to count the chromosome and the DNA content in many species like coconut [45], kiwifruit [14]. In our result a chromosome count of 54 was obtained from endosperm tissue of *Hevea. Hevea* is a diploid species with a chromosome count of 36. Hence it can be assumed that the calli obtained from endosperm tissue are triploid in nature and can be used for the in vitro development of triploid plants *of Hevea* through endosperm culture. The result was also supported by the flow cytometer data, showing peak at 560 which is 1.5 times more than the peak value of control.

## 5. CONCLUSION

Callus induction could be achieved from both mature and immature endosperm tissue. Direct embryogenesis has been obtained from the immature endosperm.

Different parameters for isolation and purification of endosperm protoplasts have been standardized. Protoplast release could be obtained from both immature and mature endosperm tissues. Protoplast division could be induced in a few cultures derived from the immature endosperm. Isolation, culture and division of protoplasts from endosperm tissue of *Hevea brasiliensis* is reported for the first time. Triploid nature of endosperm callus (3n =54) was confirmed through cytology as well as flow cytometry.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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