Original Research Article

Development of *In vitro* Tetraploid plants of *Hevea brasiliensis*

Abstract

Increase in global consumption of natural rubber necessitates crop improvement of *Hevea* aimed at increased productivity. As conventional breeding of *Hevea* is very elaborate and time consuming. Hence in the present study development of tetraploids through chromosome doubling of diploid callus from *Hevea* using colchicines were attempted. Chromosome doubling of the diploid callus occurred when treated with 1.25 µM colchicine for 3 days. In higher concentrations as well as at longer exposure periods, the callus texture and viability were affected. 48 % embryo induction and a maturation frequency of 45 % were obtained. Embryo germination and plant regeneration with a germination frequency (30 %) and a regeneration frequency (20 %) were obtained. Cytological and flow cytometric analyses confirmed the tetraploid nature of the colchicines treated callus. *Invitro* tetraploid plant developed through these *in vitro* techniques can be further used in *Hevea brasiliensis* breeding.

Keywords: *Hevea brailiensis,* Colchicine, *In vitro*, Tetraploids

Introduction

Tetraploids are polyploids with four sets of chromosomes per cell. In nature, a large number of angiosperm species are available with one or more episodes of polyploids which often results in good quality, high yielding plants with increased resistance to environmental stress, pests and diseases. Differential responses are observed in morphological and physiological characters of species due to tetraploidy. The cells of a tetraploid are much larger than that of diploid, as tetraploids have twice the number of sets of chromosomes per cell. Greater the number of chromosomes per cell, greater is the proportion of cell contents relative to cell wall material. It usually exhibits increased biomass mainly due to their high photosynthetic potential compared to diploids. In *Hevea brasiliensis* the economic life starts after 6-7 years of planting. Any attempt to reduce the immature (juvenile) phase would be quite rewarding. Even a reduction in the immature phase by 6 month or 1 year would enable the farmer to harvest the crops earlier and help to fetch the farmer with a reasonable income. *In-vitro* approaches to increase the vigour and biomass will naturally lead to shortening of the immature phase thereby enabling early tapping. The yield per tree per tap increases with increase in girth of trees due to increase in length of tapping cut. Polyploids either arise spontaneously or produced artificially. Artificial induction plays an important role in polyploid breeding. Manmade synthetic polyploids from wild plants have contributed to improvement of cotton, wheat and peanut Chen ZJ and Ni Z (2006). With the advent of *in vitro* techniques for chromosome doubling using antimitotic agents, polyploids have been produced in a large number of species. *In vitro* induction of tetraploids through colchicine treatment has been achieved in many plants such as *Pyrus communis* (Sun et al. 2009), *Morus alba* (Chaicharoen et al. 1995), *Cinchona* ledgeriana (Nair PKP 2010) etc. Colchicine ($C_{22}H_{25}NO_5$), which is an alkaloid contained in seeds and bulbs of *Colchicum autumnale* L, has affinity for tubulin, a microtubule-sub unit protein, and inhibits spindle function thereby

preventing both cell and nuclear division. *In vitro* induction of polyploids by treating the diploids with colchicine has been successful in many plants like oil palm, sesame and ginger (Nair PKP 2010).

Tetraploid/polyploid plants of *Hevea*, once generated through this technique, will be having greater vigor and increased biomass which can lead to a reduction in the immaturity period. Also, development of such plants with increased biomass may result in high girthing trees which can be employed for cultivation as latex-timber clones. Hence the objective of the study was to develop polyploid callus through colchicine application and regeneration of polyploid plant by confirmation done using flow cytometer and cytological analysis.

Materials and Methods

Plant material

In this study, embryogenic callus derived from immature inflorescence, which is diploid in nature, was used as the target material for colchicine treatment.

Embryogenic callus from immature inflorescence was raised using the earlier developed protocol (Sushamakumari et al. 2000). The immature inflorescence were washed thoroughly in running tap water for 10 min and surface sterilized with 0.1% (w/v) mercuric chloride solution containing two drops of Tween-20 for three minutes followed by rinsing 3 times with sterile distilled water. These explants were cut into small pieces and cultured for callus induction on MS basal medium supplemented with growth regulators 2, 4-D (4.5 µM), NAA (2.7 µM) and Kinetin (2.3 µM). The calli induced were cultured over modified MS medium supplemented with Kinetin (4.6 µM), BA (0.44 µM) and GA_3 (1.4 µM) for embryogenic callus induction. After 3-4 subcultures, cultures were transferred to same medium with high phytagel and charcoal. Embryogenic callus emerged in this medium was used for chromosome doubling through colchicine treatment (Fig. 1).

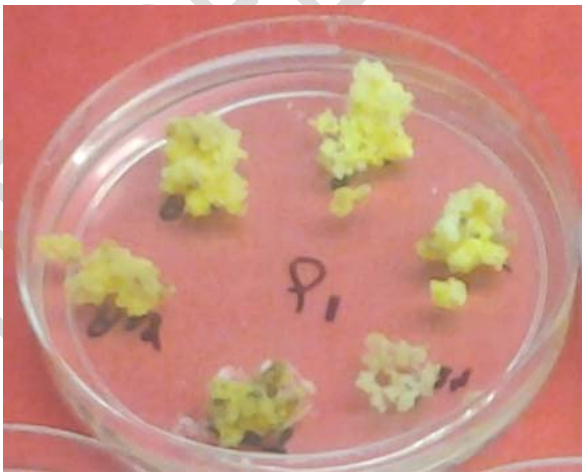

Fig. 1 Embryogenic calli derived from immature Inflorescence

Colchicine treatment

Colchicine treatment of the embryogenic callus was carried out using two different methods.

Direct exposure to colchicine

The embryogenic callus was suspended in different concentrations (0.25 - 2.5 µM) of filter sterilized colchicine solution and incubated for different time intervals (2 - 24 h) with

continuous shaking at 1000 rpm, after which they were transferred to callus proliferation medium.

Different levels of colchicine (0.75 - 7.5 µM) were incorporated in the callus proliferation medium and the embryogenic callus was cultured in these media for different time intervals (2-10 days) followed by transfer to proliferation medium without colchicine.

Filter sterilized solution of colchicine was added to the autoclaved medium just before solidification, mixed well and poured into petri dishes and allowed to solidify. A stock solution of 1000 ppm colchicine (Plant Cell Culture Grade-Sigma) was prepared in distilled water and kept in amber coloured bottle, since the solution is light sensitive.

Callus proliferation

Modified MS medium supplemented with 2, 4-D (4.5 µM), Kin (0.9 µM), BA (0.8 µM) and GA_3 (0.14 µM) was used as the callus proliferation medium. After colchicine treatment, the calli were transferred to the proliferation medium. Sub culturing to the same medium was carried out at one month interval for callus proliferation. Cytological analysis was carried out using this callus to confirm the ploidy.

Embryo induction

The proliferated calli were transferred to different embryo induction media consisting of three basal media namely Nitsch, MS and WPM fortified with different levels of BA (0.9 - 4.6 µM) and GA_3 (0.57 - 2.9 µM). Observations on embryo induction were recorded after 2-3 months in this medium and percentage of embryo induction was calculated.

Embryo maturation

The developing embryos were transferred to embryo maturation media consisting of modified MS and WPM supplemented with different levels of kinetin (2.3 – 9.3 µM) and ABA (1.1 – 3.8 µM). Basal medium without any growth regulators was also tried. Effect of phytagel on embryo maturation was evaluated by solidifying with different levels of phytagel (0.2 - 1.0 %).

Promotive effect of three amino acids viz. L- glutamine, L- asparagine and L- alanine on embryo maturation was evaluated by adding different concentrations (5, 10, 15, 20 mM) and combinations of these amino acids into the maturation medium. Four different combinations were tried as follows-

(1) L glutamine (5mM) + L asparagine (5mM) + L alanine (5mM)

(2) L glutamine (10mM) + L asparagine (15mM) + L alanine (15mM)

(3) L glutamine (15mM) + L asparagine (10mM) + L alanine (10mM)

(4) L glutamine (20mM) + L asparagine (20mM) + L alanine (20mM)

Embryo germination and plant regeneration

Two different basal media, modified MS and WPM were used for germination and plant regeneration experiments. The growth regulators experimented for germination was BA (2.2 - 8.8 µM) and IBA (2.5 - 9.9 µM). For plant regeneration, the germinated embryos were transferred to media fortified with various levels of IAA (1.7 -5.7 µM), BA (6.6- 13.3 µM) and GA_3 (1.4 µM) along with organic supplements like coconut milk (10 % v/v) and banana powder (500 mg/l).

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 and arc sine transformation and analyzed using ANOVA with a significance of $p \leq 0.05$.

Confirmation of ploidy

Cytological analysis

Proliferated calli from various colchicine treatments was subjected to cytological analysis (Rekha K 1993). After plant regeneration, developing root tips of the regenerated plants were also subjected to cytology in order to reassure ploidy of the regenerants. 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 ethanol acetic acid), for 48 h at room temperature. Afterwards the fixative was drained off and the callus was washed thoroughly to remove traces of fixative, if any. 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).

Flow cytometric analysis

The callus which showed good embryogenic response was subjected to flow cytometry analysis. 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 (Rashmi RH and Rakhi Chaturvedi 2013). 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.

*($MgCl_2$ 45mM, MOPS 20mM, Sodium citrate 30mM, Triton X 100- 0.1% (vol/vol),

pH-7 and stored at -20℃ as 10 ml aliquots)

Results

Among the two different methods of colchicine application, addition of colchicine in the culture medium was found to be ideal. In the first method of direct exposure to colchicine, both colour and texture of the callus changed after colchicine treatment. Texture of the callus changed from friable to spongy and simultaneously yellowish colour of the callus first turned white and gradually became brown in colour. Rate of such changes was proportional to the colchicine concentration to which the callus was exposed. At higher concentrations of colchicine (1.0 – 2.5 µM) the calli turned brown and spongy within 12 h whereas in the calli exposed to lower concentrations of colchicine (0.25-1.0 µM), these changes took place only slowly. Also it was observed that duration of colchicine treatment did not have any significant effect. The spongy, brown callus obtained after direct exposure to colchicine, did not undergo any further growth or development, instead it gradually dried up. All the treatments responded almost in the same way.

In the second method embryogenic calli were cultured, for different duration over media supplemented with colchicine. The colchicine treated calli kept in the proliferation medium first turned white in colour and watery, irrespective of the concentration of colchicine and duration of exposure (Fig. 2a). However, emergence of new yellow

embryogenic callus occurred, within 4-6 weeks, from the calli exposed to lower concentrations of colchicine (Fig. 2b). In this experiment this concentration of colchicine is crucial for the emergence of new callus.

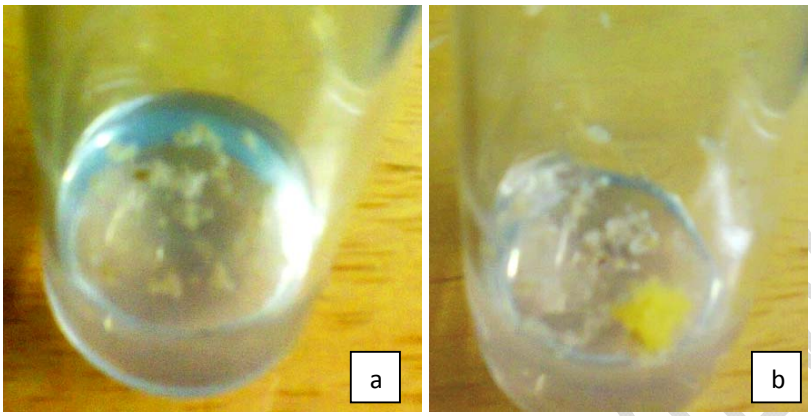

Fig. 2 Colchicine treatment of diploid callus
a Callus after colchicine treatment
b Emergence of friable yellow callus after colchicine treatment

As evident in Fig.3, lower levels of colchicine (0.75-2.5 µM) did not affect the viability of the treated callus and led to the emergence of new callus whereas those cultures exposed to higher levels (5 and 7.5 µM) of colchicine did not give rise to any new callus, instead they just got dried up on prolonged culture. Also it was observed that emergence and proliferation rate of the callus varied with the period of exposure. Highest callus proliferation frequency of 73% was observed in the cultures exposed to 1.25 µM colchicine for 3 days. With the increase in concentration of colchicine, along with increase in days of exposure, callus proliferation rate was reduced. On the high side of colchicine (5 and 7.5 µM) and longer duration of exposure (8 and 10 days), no callus emergence could be observed (Table 1).

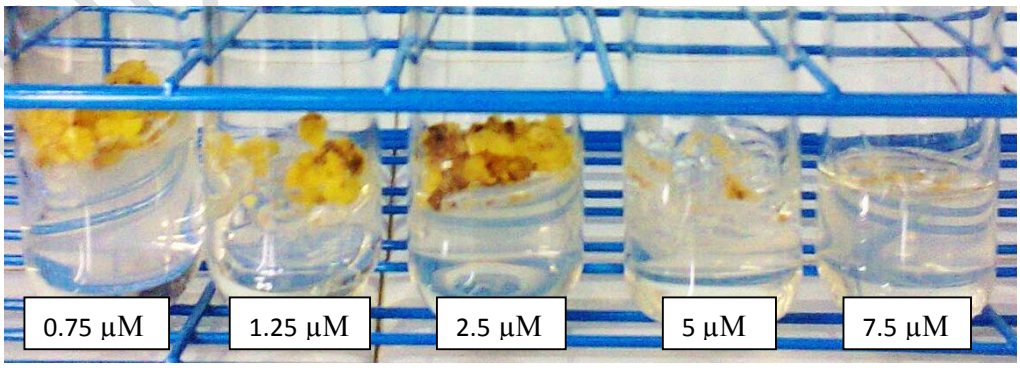

Fig. 3 Callus proliferation rate of calli exposed to different concentrations of colchicine

Table 1 Effect of colchicine concentration and exposure time on callus proliferation rate

Colchicine concentration (µM) → / Days of exposure ↓	0.75	**1.25**	2.5	5.0	7.5
2	46.5(42.9)	53.0(46.7)	33.8(31.0)	6.5 (14.7)	0.00 (0.33)*
3	57.5(49.3)	**73.2(59.0)**	47.5 (43.5)	3.5 (10.7)	0.00 (0.33)
6	25.5(30.3)	33.5(35.3)	6.5(14.7)	3.5(10.7)	0.00 (0.33)
8	7.0(15.3)	14.0(21.9)	0.00(0.33)	0.00 (0.33)	0.00 (0.33)
10	4.0(11.5)	4.0(11.5)	0.00 (0.33)	0.00 (0.33)	0.00 (0.33)

***Callus proliferation rate (%)** *****CD= 1.61

*** CD- Coefficient of determination**

The data were subjected to arc sine transformation and transformed means are given in parenthesis

In the embryo induction process MS medium was found to be most effective for induction of embryogenic callus and subsequent embryo formation (Fig.4a&b). Highest embryo induction frequency of 48% could be obtained in MS medium in the presence of 2.3 µM GA_3 and 1.8 µM BA. At lower concentrations of growth regulators, no embryo induction could be obtained. At higher concentrations also embryo induction was found to be less (Table 2).

Table 2 Effect of different concentrations of GA_3 and BA in embryo induction

GA_3 (µM) → / BA (µM) ↓	0.57	1.2	1.73	**2.3**	2.9
0.9	0.00(0.33)	0.00(0.33)	27.0 (31.3)	31.5(34.1)	23.0 (28.6)*
1.8	0.00(0.33)	0.00 (0.33)	35.5(36.57)	**48(43.5)**	37.5(37.5)
2.7	0.50(3.03)	5.5(13.5)	22.5(28.3)	27.5(31.6)	12.5(20.6)
3.6	1.0(5.7)	4.5(12.2)	16.0(23.5)	19.0(25.8)	5.5(12.0)
4.6	1.0(5.7)	3.5(10.7)	5.0(12.9)	7.5(15.8)	2.0(8.1)

CD – 5.0

*** Percentage of embryo induction**

The data were subjected to arcsine transformation and transformed means are given in parenthesis

Embryos at different developmental stages like globular, heart shaped and cotyledonary stages were obtained (Fig. 4c& d). It was also observed that the percentage of embryo induction as well as the quality of the embryos depends on the colchicine concentration in the treatment phase. Normal and healthy embryos were obtained from the calli exposed to 1.25µM colchicine. Calli exposed to higher colchicine levels gave rise to abnormal embryos, that too at a low frequency.

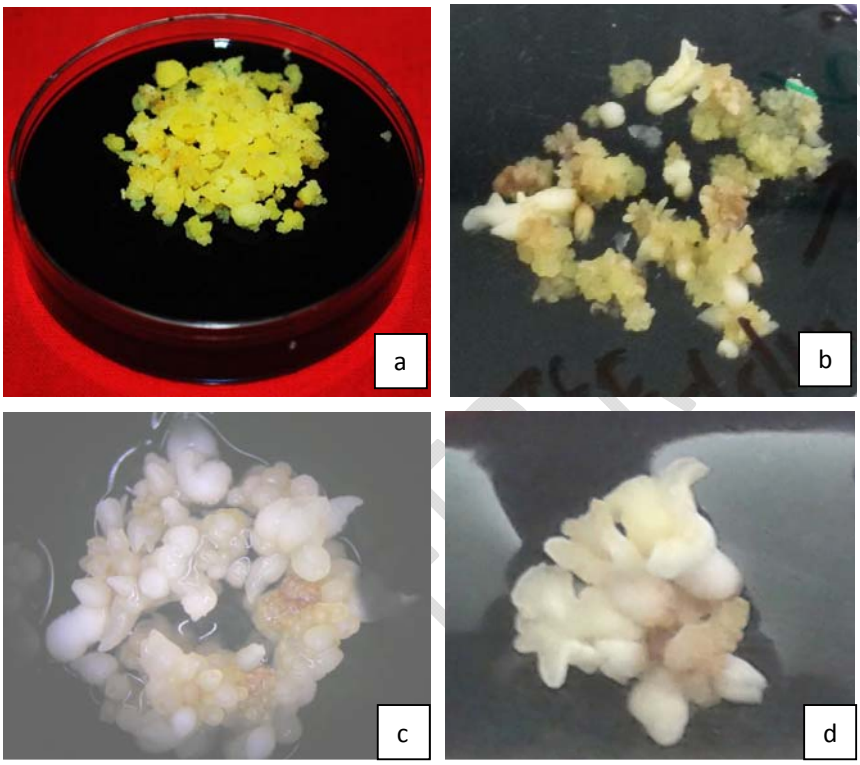

Fig. 4 Embryo induction from colchicine treated calli
a Embryogenic callus
b Induced embryos
c& d Embryos at different stages of development and cotyledonary

In case of embryo maturation a maturation frequency of 30 % could be obtained in modified MS medium fortified with 4.7 µM Kin and 1.9 µM ABA (Table 3). In the hormone free medium, maturation percentage was quite low. No increase in the embryo maturation frequency was obtained by the addition of amino acids. However, quality of the embryos could be improved in medium supplemented with the amino acid combination containing L glutamine (15 mM) + L asparagine (10 mM) + L alanine (10 mM). Well developed, normal and healthy matured embryos could be obtained from this combination.

Table 3 Combined effects of Kin and ABA in embryo maturation

Kin (µM) → / ABA (µM) ↓	2.3	**4.7**	6.9	9.3
1.1	5.0(2.2)	21.5(4.6)	16.5(4.0)	12.0(3.4)
1.9	23.5(4.8)	**30.2(5.5)**	26.5(5.1)	22.5(4.7)
2.7	19.0(4.3)	26.5(5.1)	22.0(4.6)	18.0(4.2)
3.8	16.0(3.9)	14.0(3.7)	10.0(3.1)	6.5(2.5)

*** Percentage of embryo maturation**

CD= 0.25

***Plant regeneration percentage**

The data were subjected to square root transformation and transformed means are given in parenthesis

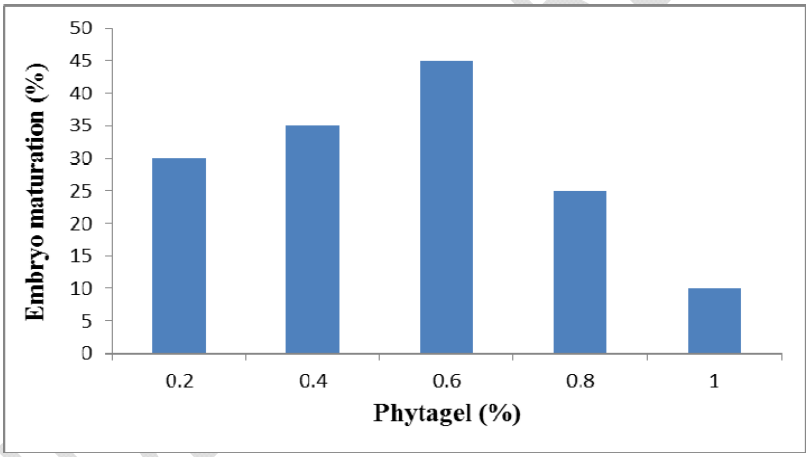

Fig. 5 Effect of phytagel concentration on embryo maturation

Maturation frequency could be further enhanced by increasing the phytagel concentration. It was noticed that in a medium containing all the standardized parameters including basal medium, growth regulators and amino acids, an increase in the maturation frequency from 30 to 45 % could be achieved when the phytagel concentration was increased from 0.2 to 0.6 % (Fig. 5). At higher concentrations of phytagel the maturation frequency decreased, reaching 10% in the presence of 1.0% phytagel. Bipolar differentiation could be observed in some of the matured embryos (Fig. 6a).

Better embryo germination and plant regeneration were obtained in the presence of WPM, rather than MS medium. Mature embryos in the cotyledonary/late torpedo stage

germinated (Fig. 6b) in WPM supplemented with 7.3 µM IBA and 6.6 µM BA. A germination frequency of 30 % was obtained with this growth regulator combination. Number of germinating embryos was quite low in media containing lower levels of growth regulators (Table 4). Some of the embryos showed abnormalities in development. Some had multiple cotyledons, some became dormant and some showed only root development.

Table 4 Effect of BA and IBA on embryo germination

BA(µM)→ / IBA(µM)↓	2.2	4.4	**6.6**	8.8
2.5	0.0(1.0)	1.5(1.6)	3.0(2.0)	2.0(1.73)*
4.9	4.5(2.3)	10.5(3.4)	14.5(3.9)	9.5(3.2)
7.3	11.5(3.5)	19.0(4.4)	**30.0(5.4)**	14.5(3.9)
9.9	5.0(2.4)	6.5(2.7)	9.5(3.2)	6.5(2.7)

CD=0.24

*** Percentage of embryo germination**

The data were subjected to square root transformation and transformed means are given in parenthesis

Among the various growth regulator combinations experimented for plant regeneration, 20% plant regeneration could be achieved in the combination of IAA (2.8 µM), BA (8.8 µM) and GA_3 (1.4 µM) (Fig.6c). In other combinations, the regeneration frequencies were low (Table 5).

Table 5 Effect of IAA and BA in presence of GA_3 1.4(µM) on plant regeneration

IAA (µM)→ / BA(µM) ↓	1.7	**2.8**	4.0	5.7
6.6	6.5(2.7)	10.5(3.4)	8.0(3.0)	7.0(2.8)
8.8	7.5(2.9)	**20(4.5)**	14.5(3.9)	4.5(2.3)
11.1	6.5(2.7)	15.5(4.0)	10.5(3.4)	3.5(2.1)
13.3	4.0(2.2	2.0(1.7)	1.0(1.4)	0.0(1.0)

CD=0.27

***Plant regeneration percentage**

The data were subjected to square root transformation and transformed means are given in parenthesis

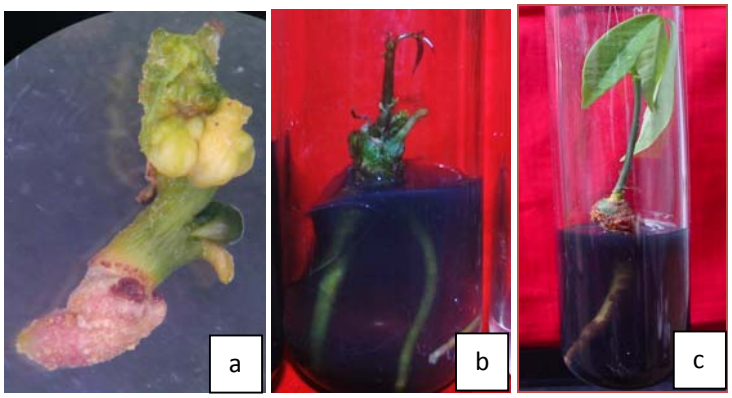

Fig. 6 Plant regeneration from matured embryo obtained from colchicine treated calli

a) Embryo maturation

b) Embryo germination

c) Regenerating plant

Cytological studies have revealed a chromosome count of 4n=72 in the colchicine treated callus, observed at a magnification of X 400 (Fig. 7). Also from the root tip of one regenerated plant similar chromosome count was obtained. This confirms the tetraploid nature of the regenerated plant from the colchicine treated diploid callus.

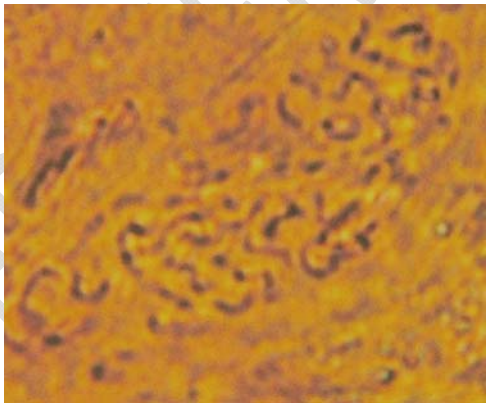

Fig. 7 Chromosome count of colchicine treated callus 4n=72 (X 400)

Using flow cytometry the ploidy of the colchicine treated callus was determined and from the histogram it can be observed that the fluorescence intensity of nuclei from the test sample got the highest peak at channel 780 which is double the value of control sample (Fig. 10). This confirms that after treating the diploid embryogenic callus with colchicine, the nuclei content increased twice as that of the diploid callus, thereby resulting in the development of tetraploids.

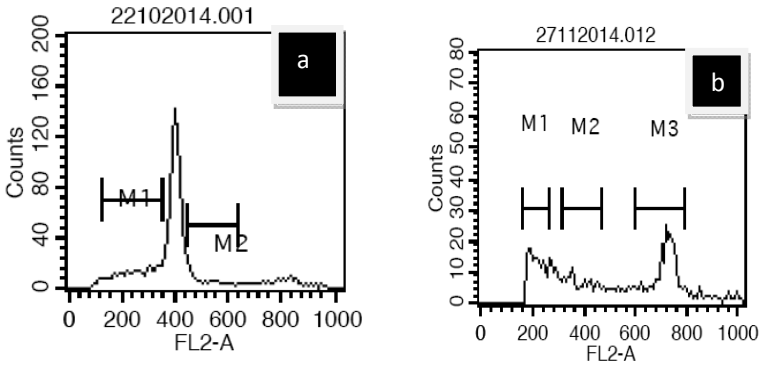

Fig. 8 Histogram showing peaks for

a Diploid (Immature anther derived callus)

b Tetraploid (Colchicine treated embryogenic callus)

Discussion

Development of synthetic polyploids of *Hevea brasiliensis* by applying colchicine on the axillary buds started by 1984 (Saraswathyamma et al. 1984). Success obtained through this method was very low due to the chances of occurrence of chimeras and the difficulty in stabilizing the polyploids. In *Hevea*, when seeds are used for colchicine treatment, it leads to loss of clonal integrity. Usage of explants like shoot apex and nodal segments for treatment, are not preferred because of the chances of obtaining chimeras. In the present study we have used callus as the explant for colchicine treatment. As the somatic embryo arises from a single cell, this method ensures complete tetraploidy of the regenerated plants and the chance for obtaining chimeras is remote. Also the clonal integrity can be maintained except for somaclonal variations. There are also reports in mulberry on the induction of tetraploidy through colchicine treatment of germinating seeds, seedlings and vegetative buds. Percentage of success was maximum (47 %) with callus explant compared to other explants (Chakraborti et al. 1989).

Direct exposure may lead to abnormalities in cell division which can cause chromosome imbalance leading to low survival. Even lower concentrations of colchicine were lethal for direct exposure of callus. Since callus is a mass of loosely arranged single cells, penetration of colchicine into these cells will be more compared to the cells of seeds/embryos which are more compactly arranged. Callus induction from leaf explants of *Dionaea muscipula* soaked in different concentration of colchicines was attempted and they observed that callus induction rate decreased when colchicine concentration was high (Jala A 2014). In the second treatment where colchicine was incorporated in the culture medium, there was no browning of the colchicine treated callus, instead the callus first turned white and watery and later, upon transfer to callus proliferation medium, new friable embryogenic calli emerged. Whitening and browning of the callus after colchicine treatment was also observed in case of winter rose where effect of different concentration of colchicine was experimented (Pickens and Cheng 2006). Colchicine concentration and its duration of application is another factor influencing the success. The exact concentration and time of exposure needs to be standardized in each crop and with different explants. A lot of changes

happen in the cellular level with the application of colchicine. During cell division, colchicine is reported to arrest the spindle fiber formation leading to doubling of chromosome number. Higher concentrations above the optimum will result in abnormalities and lead to low success. Similarly exposure for longer durations is also detrimental. Varying stress symptoms are shown by the explants exposed to colchicine treatment. Callus growth was repressed at higher concentrations and/or longer treatment duration with colchicines (Zeng S, Chen C and Hong L 2006). Most of the cells after colchicine treatment died, owing to persistent lethality of colchicine.

It has been observed that the concentration as well as the duration of colchicine treatment influenced callus proliferation. The proliferation rate of the callus was different for each treatment. Out of the different treatments tried, highest callus proliferation of 73 % was obtained in cultures treated with 1.25 µM colchicine for 3 days. At higher concentrations of colchicine the proliferation rate was low. Similar observations were made in mulberry, where the callus treated with 0.025 % colchicine for 3 days showed the highest percentage of survival (76 %) (Chaicharoen et al. 1995). Mortality rate is very important while applying colchicine to explants for i*n vitro* induction of polyploids (Sajjad et al. 2013). In the present study with *Hevea* embryogenic callus, survival rate decreased with increased concentrations of colchicine. 1.25 µM colchicine was identified as an optimum concentration for the induction of tetraploids without compromising the survival and further proliferation.

The culture conditions and basal media for somatic embryogenesis have already been standardized by many workers in *Hevea* (Sushamakumari et al. 2000). 0.2 mg/l NAA was effective for embryo induction from immature anther (Jayasree et al 1999). In the present study 48 % embryo induction was obtained in presence of 2.3 µM GA_3 and 1.8 µM BA in MS medium. The role of GA_3 (4.35 µM) and BA (8.84 µM) in differentiation of friable embryogenic calli derived from root explants into somatic embryos were demonstrated in *Hevea* (Sushamakumari et al. 2000). Embryo maturation in *Hevea* is generally induced by a hormonal stress (Jayasree et al. 1999). Hence most maturation media do not contain auxins or cytokinins. But in our study 30 % embryo maturation in modified MS medium supplemented with 4.7 µM Kin and 1.9 µM ABA was obtained. Normal maturation of somatic embryos needs an ABA treatment in walnut (Vahdati et al. 2006). Similarly maturation of somatic embryos of Fraser fir was not observed on medium lacking ABA for both precotyledonary and cotyledonary embryos (Kim et al. 2007). 80 µM ABA was most effective in producing cotyledonary stage embryos in *A. fraseri*. The role of phytagel in embryo maturation is well established in *Hevea* a drastic increase in the embryo maturation frequency was noticed when phytagel was increased from 0.2 to 0.5 % [19]. Raising the concentration of phytagel in the medium from 0.4 % to 0.8% improved the maturation of somatic embryos of *Larix eurolepsis* (Teyssier et al. 2011). The use of high concentrations of phytagel as gelling agent reduces water availability. Similarly 0.5 % phytagel was effective in the maturation of anther derived embryos of coconut (Perera et al . 2011).

Earlier reports are there indicating the beneficial effect of GA_3 on germination in *Hevea* (Jayashree PK and Thulaseedharan A 2001). Also a combination of BA (0.3 mgl^{-1}) and GA_3 (0.3 mg l^{-1}) has been reported to favour germination of rescued zygotic embryos in *Hevea* (Rekha et al. 2006). However in our result embryo germination (30 %) from colchicine treated callus was obtained in MS basal medium fortified with IBA (7.3 µM) and BA (6.6 µM). Similar to our results, a combination of BA and IBA was used in mulberry (Chaicharoen et al. 1995)**,** for the induction of shoot and root from the colchicine treated callus. Embryo culture to enhance efficiency of colchicine induced polyploidization in grape fruit reported that even a low level ($0.03mgl^{-1}$) of colchicine was lethal for embryos towards germination (Usman et al. 2012). But in our study such lethality was not observed in the

embryos obtained from callus treated with 1.25 µM colchicine for 3 days. Lower concentrations of colchicine (0.1%, 0.2% and 0.3%) did not affect the rooting behavior (Ramesh et al. 2011). However, the number of roots developed was considerably decreased in the progeny treated with higher concentration of colchicine (0.4% and 0.5%). In our study, root growth was not affected in the embryos raised from cultures exposed to 1.25 µM colchicine. Embryos germinated with well-developed root system. Plant regeneration frequency of 20 % was obtained in WPM medium fortified with IAA (2.8 µM), BA (4.4 µM) and GA_3 (5.7 µM). Similar basal medium (WPM) fortified with GA_3 (2.9 µM) and BA (8.8 µM), brought about 60 % plant regeneration frequency from root explants of *Hevea* (Sushamakumari, 2014).

Ploidy determination of the callus and regenerated plants through cytological and flow cytometry analyses revealed tetraploidy (4n= 72). Now a day's flow cytometer is used for ploidy determination since it is time saving and because of its easiness to predict result. The DNA content in the colchicine treated callus was doubled when compared with the control having the highest peak at 400 channel.

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