Accomplishing ploidy variation through *invitro* techniques for crop improvement in *Hevea brasiliensis*

Thesis submitted to
Mahatma Gandhi University
for the award of the degree of
DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY
Under the Faculty of Science

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February 2016

DECLARATION

I hereby declare that the thesis entitled "Accomplishing ploidy variation through *invitro* techniques for crop improvement in *Hevea brasiliensis*" is an authentic record of original research carried out by me under the supervision and guidance of Dr. Sushama Kumari S, Senior Scientist, Rubber Research Institute of India, Biotechnology Division in partial fulfilment of the requirement for the degree of Doctor of Philosophy of the Mahatma Gandhi University and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

February, 2016
RRII

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CERTIFICATE

This is to certify that the thesis entitled "**Accomplishing ploidy variation through *invitro* techniques for crop improvement in *Hevea brasiliensis***" is an authentic record of original research work carried out by Smt. Divya U K at the Rubber Research Institute of India, Kottayam-9, under my supervision and guidance for the award of the degree of **Doctor of Philosophy in Biotechnology**, under the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in the thesis has not been submitted earlier for any other degree, diploma or any other similar titles of any university.

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DEDICATED TO MY MOTHER,
FATHER & BROTHER

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and sincere thanks to my respected guide **Dr. Sushama Kumari. S,** Senior Scientist, Biotechnology Division, Rubber Research Institute of India for her learned counsel, meticulous and gracious guidance, sustained interest, constant and inspiring encouragement, sympathy and generosity much beyond her formal obligation, affectionate advice, constructive criticisms and forbearance all through the research work, all of which contributed the most to the completion of the study and the critical scrutiny of the manuscript.

I sincerely express my deep sense of gratitude to **Dr. James Jacob,** Director of Research, RRII for his rare brilliance, keen and unstinted interest and critical comments and ever willing help rendered to me at all stages of this endeavour.

I extent my sincere thanks to **Dr. J.G. Ray,** Professor, School of Bioscience, Mahatma Gandhi University for supports and constant encouragement in the completion of the PhD course work, which aided me to complete the thesis.

It gives me great privilege to express my sincere thanks and obligations to **Dr. A. Thulaseedharan**, Joint Director, Biotechnology Division, and RRII for his constructive suggestions and sustained encouragement at all stages of the work.

It is of great pleasure to express my deep sense of gratitude to, **Dr. Rekha K,** for her valuable guidance and timely suggestions throughout the work. The incessant encouragement and whole hearted cooperation of scientist of biotechnology division **Dr. S Sobha, Dr. Kumari Jayasree** and my friends **Suni Annie Mathew, Glindya, Santosh, Soumya and Maria** are thankfully acknowledged.

The expert and timely help received from **Mr.Aneesh,** Asst.Statistician, and RRII for statistical analysis and interpretation of the data is sincerely acknowledged.

I duly acknowledge with full heart the personal sacrifices, timely persuasion, incessant encouragement and moral support of my father **Mr. R. Unni Krishna Pillai**, my mother **Mrs B. Sheela** and my brother **Rajesh Unni Krishna** which I believe enabled me to complete this venture successfully.

I sincerely thank all my family members for their moral support and constant encouragement throughout the work.

Above all, I bow my head before the **God Almighty** whose blessings were always with me enabling to undertake this endeavor successfully.

Divya V K

Abstract

Hevea brasiliensis, the most economically important member of the genus *Hevea*, accounts for about 99 % of global production of natural rubber (NR). The steady increase in global consumption of NR necessitates crop improvement of *Hevea* aimed at increased productivity. *Hevea* breeding using conventional techniques are quite lengthy and tedious because of its high heterozygous, long breeding and cross pollinated behavior. Hence biotechnological approaches to complement conventional breeding in *Hevea brasiliensis* have become highly essential in order to cross the attained yield plateau and to obtain desirable properties. Development of ploidy variants like haploids and polyploids has shown immense benefits towards crop improvement, especially in tree species. In the present study, three different routes for attaining ploidy variation through *in vitro* techniques were attempted, namely a) Embryo sac culture for the development of gynogenic haploids b) Endosperm culture for the induction of triploidy and c) Chromosome doubling using colchicine for the development of tetraploids from diploid callus. Various parameters were standardised for the isolation of female gametes/gametophytes, protoplasts from endosperm tissue, chromosome doubling of diploid callus by colchicine treatment etc. Optimization of culture conditions for callus induction followed by embryogenesis were carried out for all the explants used for the development of ploidy variants. Experiments were conducted at various stages by changing the growth regulator combinations.

Female gametes/gametophytes could be isolated from mature female flowers one day prior to anthesis. Large number of embryo sacs could be isolated through the pretreatment of ovules. 36 % callus induction was observed from cultured embryo sacs in modified K&M medium fortified with 4.6 µM 2, 4-D and 2.7 µM Kinetin. Embryo induction (30 %) was obtained in modified K&M supplemented with GA_3 (1.73 µM) and BA (7.2 µM) in presence of 8 % sucrose and 0.5 % phytagel. Maturation of embryos with bipolar differentiation was highest (8 %) in modified MS medium fortified with IBA (0.74 µM) and Kinetin (2.3 µM) along with ABA (1.89 µM). Some of the matured embryos responded to germination.

Endosperm tissue from immature fruits (8-10 weeks) was found to be the ideal source 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).

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 in MS medium fortified with GA_3 (2.3 μM) and BA (1.8 μM) and a maturation frequency (45 %) in modified MS medium with Kin (4.7 μM) and ABA (1.9 μM) in presence of 0.6 % phytagel were obtained. In Woody Plant Medium, both embryo germination and plant regeneration were obtained with a germination frequency (30 %) in presence of IBA (7.3 μM) and BA (6.6 μM) and a regeneration frequency (20 %) in presence of IAA (2.8 μM), BA (8.8 μM) and GA_3 (1.4 μM).

All the three ploidy levels have been confirmed using cytological and flow cytometric analyses. The ploidy variants developed through these *in vitro* techniques can be further used in *Hevea* breeding. This is the first report on accomplishing ploidy variation through *in vitro* techniques using appropriate explants followed by confirmation of ploidy in *Hevea brasiliensis*.

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ABBREVIATIONS

ABA	Abscisic acid
BA	Benzyl adenine
2,4-D	2,4-Dichlorophenoxy acetic acid
GA_3	Gibberellic acid
NAA	Naphthalene acetic acid
IAA	Indole-3-acetic acid
IBA	Indol-3-butyric acid
K&M	Kao & Michayluk medium
MS	Murashige and Skoog
WPM	Woody plant medium
RRII	Rubber Research Institute of India
NR	Natural rubber
g	Gram
mg	Milligram
µg	Microgram
pg	Picogram
M	Meter
nm	Nanomètre
l	Litre
µl	Microlitre
ml	Milli litre
µM	Micro molar
w/v	Weight/volume
gfw	Gram fresh weight
MES	Morpholinoethane sulphonic acid

GENERAL INTRODUCTION

Chapter 1

General Introduction

Nature's stability depends on everything present in the universe. Human beings live in the Realm of Nature. Forest occupies a major part of our ecosystem consisting of a huge volume of flora and fauna. Forests account for 75 % of the gross primary productivity of the earth's biosphere, and contain 80 % of the earth's plant biomass (Pan *et al.*, 2013). "In a forest of a hundred thousand trees, no two leaves are alike. And no two journeys along the same path are alike." - Paulo Coelho, *Aleph*

Tree species serve as a protection boon as well as help in the economic development of the nation eg. Teak, mulberry, rubber, coconut, oil palm, cocoa etc. In tree species, angiosperms have been studied much more intensively than other types of plants as it is the dominant plant group on earth and also because of their great economic and agricultural importance. Rubber tree, which is the major source of natural rubber (NR), is an important perennial crop that occupies a dominant position among other plantation crops. Natural rubber is a coherent elastic solid obtained from latex synthesised by over 2500 plant species belonging to 300 genera of seven families viz. Apocynaceae, Asclepiadaceae, Asteraceae, Euphorbiaceae, Moraceae, Papaveraceae and Sapotaceae. Rubber tree belongs to the genus *Hevea* in the family Euphorbiaceae. Based on taxonomic description there are ten species under the genus *Hevea* viz. *H. benthamiana, H. brasiliensis, H.camargoana, H. comporum, H. guianensis, H.microphylla, H. milida, H. pauciflora, H. rigidifolia and H. spruceana* (Wycherley, 1992). Among these different species, *Hevea brasiliensis*, commonly known as Rubber tree, is the only commercial source of NR because of its high yield and rubber quality (Aswatreratanakul *et al.*, 2003).

Hevea brasiliensis is a native to rainforests in the amazon region of Brazil and occupies a prime position worldwide for the production of NR, which is considered as nature's most versatile raw material. Chemically natural rubber is a

high molecular weight isoprenoid polymer (cis 1, 4- polyisoprene) valued for its high performance characteristics. Since several centuries, the usage of rubber is tied up with humankind and material mobility. Practically every movement of life requires this fascinating material ranging from the very basic personal articles to today's transportation including defence and civilian purposes. At the beginning of the 20th century, a series of technological developments in processing research revolutionized the uses of rubber and thereby vastly expanded its application. More than 50,000 rubber based products including tyres, engineering components and latex products are now being manufactured all over the world.

Major rubber producing countries are Thailand, Indonesia, Malaysia, India, Vietnam and China. In India, traditional rubber growing regions include Kerala, Kanyakumari district of Tamilnadu, and Dakshin kannada and Coorg districts of Karnataka. Non traditional regions cover North Eastern states, West Bengal, Konkan region, parts of Madhya Pradesh, Andhra Pradesh and Odissha. Around 90% of NR production in India is from the traditional belt comprising whole Kerala and parts of Karnataka and Tamilnadu. The first rubber plantations in India were set up in 1895 on the hill slopes of Kerala. However, rubber cultivation on a commercial scale was introduced in 1902. The rubber tree is tall, sturdy and deciduous. They are monoecious and bear unisexual flowers in the same inflorescence. The male flowers are much more numerous than the female flowers, which are bigger and found terminating the main branches of the panicle. The flowers are typically pentamerous with a bell shaped, five- lobed perianth. The flowers in most cases are yellow in colour. Leaves are trifoliate which droop backwards with the laminae pressed against each other at emergence. Refoliation and flowering follow wintering. The fruit is a trilocular regma, each locule containing one seed which mature and dehisce during July to September. Seed viability is short and has hard brown mottled seed coat containing the endosperm.

Latex is synthesized by and contained in specialized cells known as laticiferous cells which permeate bark, leaves and other parts of *Hevea* plants. The white or yellow latex occurs in latex vessels in the bark, mostly outside the phloem. These vessels spiral up the tree in a right-handed helix which forms

an angle of about 30 degrees with the horizontal, and the tree can grow as high as 45 ft. Latex is harvested from the rubber tree through controlled wounding of the bark, a process termed tapping, whereby the latex vessels are opened up and the latex exuding from the cut ends is collected for further processing. The main components of productivity are: (a) growth of the trunk that determines the duration of immature phase, (b) evolution of yield per tree along time, and (c) stability of the stand (number of tapped trees per unit area) related with resistance to stress factors like tapping panel dryness (TPD), wind damage, varied diseases, low temperature, higher altitude and moisture deficit (Priyadarshan and Demange, 2004). Latex yield and growth are hardly separable. On the other hand, growth along with tapping is negatively related to yield due to the partitioning of assimilates.

Aimed at crop improvement, breeding and selection is generally carried out to bring together all the beneficial traits and characteristics in a particular crop. In *Hevea* cultivation, both seedlings and clones were used as planting materials, the latter being popular and very widely adopted. The former is the resultant of generative reproduction (pollination) and the latter of vegetative methods (budding/tissue culture). Major objective of rubber tree breeding is the development of potential clones with high rubber yield combined with secondary characters such as high initial vigour, smooth and thick bark with good latex vessel system, good bark renewal, and good growth after initiation of latex harvest, biotic and abiotic stress tolerance etc. Conventional breeding of *Hevea* is too tedious and time consuming, due to the heterozygous nature and a long juvenile phase of 6-7 years before latex harvest. Even though such limitations are there, improved clones have been produced through conventional breeding, the most popular ones being RRII 105 and clones of RRII 400 series. However, adoption of *in vitro* techniques to complement conventional breeding is highly desirable especially in the present scenario where the population pressure and climate change pose additional production constraints and challenges. Plant tissue culture system can be employed to develop sustainable and durable production strategies. As an emerging technology, the plant tissue culture has a great impact on both agriculture and

industry, through providing plants needed to meet the ever increasing world demand. It has made significant contributions to the advancement of agricultural sciences in recent times and today they constitute an indispensable tool in modern agriculture. *In vitro* approaches applied to rubber tree include micropropagation, microcutting, somatic embryogenesis, micrografting, genetic transformation, protoplast culture, mutation breeding and molecular breeding.

Ploidy variation using *in-vitro* techniques has been accepted and adopted as an efficient and reliable tool for bringing about genetic improvement in several crops. Haploids and homozygous diploids are well known for their beneficial effects in plant breeding as well as in genetic research of many crops including woody species. Development of homozygous lines through conventional techniques requires several backcrosses which makes the whole process quite lengthy and practically unachievable in one's lifetime, especially in the case of a perennial tree crop like *Hevea*. In this context, exploitation of *in vitro* techniques as an alternate method for developing haploids is highly desirable. Doubling of the chromosome number of *in vitro* raised haploids, is a viable approach for making homozygous diploids in a single step. Such doubled haploids are of great importance in plant breeding, especially because of their increased efficiency of selection. Moreover, haploids find significance in the study of fundamental and applied aspects of genetics in higher plants.

The concept of exploiting polyploidy technology for crop improvement was prevalent even in the early ninetees. In several crops like sugarcane, potato etc. spontaneous alteration in ploidy, leading to aneuploids and polyploids, has led to substantial increase in productivity. In crops where there is no spontaneous ploidy variation, it can be induced artificially. *In vitro* chromosome doubling using antimitotic agents like colchicine, oryzalin, dinitroaniline that disrupt mitosis has been employed for this purpose. Polyploids show high photosynthetic rates, increased vigour and biomass. Triploids, being sterile, promote vegetative growth by preserving huge amounts of photosynthetic energy normally channelled to seed and fruit production. Hence crop improvement through triploid production is worth

trying in crops where seed is not the economic part and propagation is through vegetative means.

In *Hevea brasiliensis*, attainment of homozygous diploids finds application in breeding programmes and in genetic studies and mutation breeding. Development of triploids and higher polyploids with increased biomass and vigour is highly beneficial towards crop improvement since such polyploids are ideal candidates with increased vegetative growth leading to enhanced latex yield. Also such plants will attain early tappability thereby reducing the immature phase. Both these will ultimately result in boosting up of the per tree productivity. Crop improvement programmes through *in vitro* pathways for the development of ploidy variants in the past have been limited. Even though works have been carried out but further establishment in this area have so far not been achieved. Hence, realising the advantages of haploids and polyploids in plant breeding and in genomic studies, development of *in vitro* pathways for the production of ploidy variants in *Hevea brasiliensis* has been taken up in the present study.

REVIEW OF LITERATURE

Chapter 2

Review of Literature

Of all the great feats of the era of botanical discovery, none was more imposing than that of the domestication of rubber tree (Dean, 1987). Earlier, rubber was considered as an unstable product and it remained for more than three centuries, a mere curiosity.

2.1 History of *Hevea brasiliensis*

From 16th century rubber was popularised in Europe. Christopher Colombus had taken a few rubber balls on his return from West Indies to illustrate one of the wonders of the New World (Schurer, 1957). Before the discovery of the new world, native Indians used the latex of various plants for making balls, bottles, crude foot wear and water proofing fabric. From its "discovery" in the late 1500's until the 1900's, rubber remained largely a curiosity in Europe. Elastics were produced for use in clothing and attempts were made to create tubing for medical uses. Although the production of natural rubber began in the Americas, the introduction of *Hevea brasiliensis* seedlings by the British into other countries has led to predominance of the industry in Southeast Asia.

Year	History
(1703-70) and (1701-74)	Francois C. Fresneau and Charles de La Condamine familiarized rubber to the scientific community in Europe.
1763	The French called rubber as caoutchouc and found that caoutchouc could be dissolved in naphtha and can be used for waterproofing and clothing.
1770	Joseph Priestly reported that the marks in the paper can be rubbed using the material obtained from *Hevea* latex, hence the name India rubber, and now simply "rubber".
1823	Mackintosh manufactured waterproof raincoats by coating fabric with rubber dissolved in naphtha.

1824	Hancock suggested plantation growing of rubber.
1839	Goodyear and/or Hancock discovered vulcanization. When rubber was heated with sulphur, rubber retained physical properties from 0 to 100°C. This led to rubber boom. Interest in rubber with vulcanization process, led to the increased demand and exploitation of wild *Hevea* trees (*Hevea* was the native word). Native tappers hacked trees and spoiled them for later tapping.
1870	Sir Clements Markham of India, suggested that rubber along with cinchona (source of quinine) be obtained from tropical America and grown in Asia.
1872	James Collins reviewed rubber producing plants and published monograph entitled Caoutchouc of Commerce.
1873	Seeds from Brazil were sent to Kew Gardens; 12 plants were raised and sent to Calcutta, but failed to establish.
1875	Second consignment of seed also failed to germinate.
1876	Markham sends Robert Cross to Panama (for Castilla) and to Para, Brazil where he obtained 1000 plants of *Hevea*, but no plants reached the East. At this time H.A. Wickham, an Englishman residing at Manaus (center of the rubber boom in Brazil), sent 70,000 seed from Central Amazon basin (he received 10 £ /100 seed) in an arrangement financed by the government of India. This provided the basis for the world's rubber industry. The seeds were sent to Kew. 2899 plants were raised. 1911 seedlings were sent to Ceylon and 50 plants to Singapore, and a few to Java.
1878	The cultivation of rubber in India from the rooted cuttings imported from Royal Gardens, Heneratgoda, Ceylon
1888	In Singapore there were 9 trees of the original introduction, 21 five-year old trees and 1000 seedlings. Ceylon had 20,000 seedlings. H.N. Ridley, Scientific Director of the Botanical gardens at Singapore developed the rubber industry. He demonstrated that *Hevea* was the superior rubber bearing plant, discovered excision method of extracting latex and devised method for coagulating latex, time of tapping and retapping.
1898	Dunlop rediscovers pneumatic tires (Motor cars invented in 1885). (Today, 70% of rubber involves transportation, 6% footwear, 4% wire and cable).
1898	First planting of *Hevea* in Malaysia by a Chinese grower named Tan Chan Yoy. At this time coffee prices slumped and there was interest in establishing a new industry.

1910	Rubber boom; rubber reaches $3 a pound.
1922	The first rubber factory in India was established in Bengal by Dixie Ave for the proofing of fabrics.
1955	The Rubber Research Institute of India was established to undertake scientific research on aspects relating to production, cultivation and processing.
1956	Ridley dies at the age of 101.
1980	RRII 105 clone evolved by the Rubber Research Institute of India and currently enjoying maximum popularity in the country. Parents are Tjir1 and Gl 1.
2005	RRII 400 series clones released, parents are RRII 105 and RRII 100

2.2 Botanical Characteristics

Rubber is synthesized in over 7,500 plant species, confined to 300 genera of seven families: Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Backhaus, 1985 and Cornish *et al.*, 1993). Rubber tree, the only commercial source of natural rubber is a member of the family Euphorbiaceae and genus *Hevea*. The Euphorbiaceae family is extremely diverse and considered to be polyphyletic (Webster, 1994). It is characterized by typical pentamerous flowers and a tricarpelar ovary (Dornelas *et al.*, 2005). *Hevea* is easily recognizable from its characteristic trifoliated leaves. The genus is basically composed of 10 species: *H. brasiliensis, H. guianensis, H. benthamiana, H. pauciflora, H. spruceana, H. microphylla, H. rigidifolia, H. nitida, H. camporum,* and *H. camargoana* (Wycherley, 1992).

Rubber is planted at 15°N to 10°S with a temperature of 74 – 95°F, with well distributed rainfall of 75–100 inches per year. The rubber tree may grow to 30 m (100 ft) or more where it occurs in the wild, although plantation trees generally reach heights of around 17 m (60 ft). The thick, leathery leaves, up to

60 cm (24 inch) long, are compound, with 3 elliptic leaflets, each with entire (unserrated) margins and prominent secondary veins. *Hevea* generally has got one flowering seasons that is from February to April, but sometimes off season flowering occurs in the month of September and October. The inflorescence is a panicle of separate staminate and pistillate flowers born in the axils of basal leaves of new shoots that grow out after wintering. Rubber tree is monoecious, with lateral inflorescences (branched panicles) bearing both staminate and pistillate flowers that appear in the last phase of the defoliation-refoliation process during wintering (dry season). Hermaphroditism and the occurrence of bisexual flowers in *Hevea* have been reported by Cuco and Bandel, (1998). Flowers are greenish yellow, with bell shaped perianth having five triangular lobes. Staminate flowers have 10 anthers arranged over a staminal column in two whorls of five each. Each anther contains two pollen sacs that split longitudinally on dehiscence. Pollen grains are smooth, sticky and trifoliate. Pistillate flower consists of 3-celled ovary with 3 short sessile stigmas and they are seen at the tip of the panicle. For each pistillate flower, about 70 staminate flowers are found. When fully developed, they are recognized by their relatively bigger size and the green basal disc, torus. The gynoecium is tricarpellary and syncarpous with an ovule in each locule. The stigma is short styled and trilobed. *Hevea* appears to be obligatory insect-pollinated (Rao, 1961) and predominantly cross-fertilized (Simmonds, 1982). Sticky pollen and stigmatic surfaces indicate the typical entopophilous nature of the flower. After fertilization the ovary develops into a three lobed dehiscent capsules (regma) with three large mottled seeds. Fruits ripen 5-6 months after fertilization (Mydin and Saraswathyamma, 2005). Low fruit set and its variation among clones, notably in the case of self-pollination, may be regarded as a general characteristic of *Hevea brasiliensis* reproductive biology that is not confined to specific incompatible crosses (Hamzah *et al.*, 2002) and this is a major limitation to genetic recombination in rubber breeding. The development of flowers to fruits is estimated to be very low, around 5% (Husin, 1990). Gandimathi and Yeang, (1984) demonstrated and Sedgley and Attanayake, (1988) confirmed that all three ovules of one fruit need to be

fertilized for fruit setting. Rubber fruits mostly have 3 carpels (sometimes 4, and very rarely 5) and fruits with one or two seeds are almost never observed.

In *Hevea brasiliensis* latex is confined in vessels within the inner bark hence modern tapping methods take this advantage and latex is drained from the tree by making a shallow cut in the bark. This technique causes no permanent damage to the tree and allows for a productive life span of up to 15 years. The sap, harvested from the inner bark is then coagulated. Latex is thought to be a defence against insect predators for the tree (Dean, 1987). Latex vessels are modified sieve tubes of the phloem which run in counter clockwise direction and therefore tapped in clockwise direction. *Hevea* has articulated laticifers located in every tissue of the tree, notably in the soft bark (liber) of the trunk from which it can be extracted by tapping. These laticifers develop into ramose tube like structures only through the addition of new primordia to existing ones and not through the growth of individual cells (Hall *et al.*, 2004). These primordia are seen in longitudinal rows, and during initial stages of germination, the end walls of these primordia break down and cell rows are seen converting into vessels. Laticifers are found to differentiate acropetally in newly formed plant parts, such as the stem, leaves, flowers and fruits. Vessels lying nearby are found to produce protuberances fusing together (anastomosis), therefore forming a paracirculatory ramified structure of a laticiferous system (syncytium). The laticifers successively generated by the cambium are organized in cylindrical rings that are not interconnected. There are no plasmodesmata between the latex vessels, or between them and their surroundings (de Faÿ and Jacob, 1989), there are also no associated companion cells in contrast with sieve tubes. Connections exist between the laticifer systems of the stock and the scion, evidenced by the transport of latex (Bonner and Galston, 1947). The latex is a cytoplasm that contains predominantly rubber particles, as well as lysosomal microvacuoles known as lutoids; it also contains double-membrane organelles rich in carotenoids, which look similar to plastids, but their role has not yet been fully elucidated (Paardekooper, 1989). Depending on the species of plant, the latex

may be found in a number of locations. It may be within the cells or intercellular spaces of the roots, stems or leaves of the plant.

Earlier studies on *Hevea* anatomy were limited to bark structure and laticifer characters (Bobilioff, 1923; Gomez, 1982; Premakumari *et al.*, 1985b). Anatomical and histochemical aspects of bark regeneration in *Hevea brasiliensis* have been reported by Vinod *et al.*, 1995. Anatomical studies have also been carried in the leaf of rubber tree clones (Martins and Zieri, 2003). According to Willemse and Van, (1984) much work has been done on megagametophyte development in angiosperms but information is scarce on the function of the ovular tissues that envelop the embryo sac. Work on the ovule itself has mainly been from a purely structural or taxonomic standpoint (Bouman, 1984) and the developmental changes that occur in these tissues during the reproductive process have been largely neglected. The fact that in most angiosperm species the embryo sac is enclosed in a well differentiated ovular structure.

Cytological examinations are generally used to determine the chromosome number. In any crop cytogenetical studies serve as an essential prelude for scientific crop improvement. Cytogenetic studies on *Hevea* spp. accomplished so far had shown that all of them carry 2n = 36 with a base number of x = 18 (Majumder, 1964). The chromosome counts made by various investigators showed variations and were reported as 2n = 16, 34 and 36. However, detailed cytological investigations have confirmed the chromosome complement of rubber tree in the somatic cells as 2n = 2x = 36 (Ramaer, 1935; Saraswathyamma *et al.*, 1984). The chromosomes are small and vary in length and the total chromosome length of the species is 89.7μm. Critical analysis of karyomorphology revealed significant differences between clones with reference to centromeric position and total chromosome length (Sankariammal and Saraswathyamma, 1995). There are no chromosomal or genetic barriers between the 10 *Hevea* species. Triploid plants with 2n = 3x = 54 (Nazeer and Saraswathyamma, 1987) and induced tetraploids with 2n = 4x = 72 (Saraswathyamma *et al.*, 1988) were also reported. Wide range of meiotic abnormalities was noticed in the triploids and tetraploids of *Hevea* (Saraswathyamma, 1997). The cytophotometric determination of DNA

content of various cytotypes revealed 44.2 pg in diploids, 62.40 pg in triploids and 89.37 pg in tetraploids (Saraswathyamma and Panikkar, 1988).

2.3. Economic importance

Rubber is a valuable commodity in today's economy. A vast number of products are made from it, including washers, gloves, gaskets, tubing, waterproof clothing, toys, erasers, belts, elastics, bottle stoppers and insulation for electrical wiring. The main use of natural rubber (NR) is in automobiles mainly tyres and tubes, which consumes 60% to 70% of the total world production each year. In addition to tyres a modern automobile has more than 300 components made out of rubber. Many of these are processed from natural rubber. In addition to this, NR now finds extensive use in soil stabilisation, in vibration absorption and in road making. Demand for rubber has grown remarkably since the beginning of the industrial revolution. Commercially useful rubber-producing species include *Hevea benthamiana* (Para rubber), *Hevea guianensus* (Para rubber), *Manihot glaziovii* (Ceara rubber), *Manihot dichotoma* (Jeque rubber), *Castilla elastica* (Panama rubber) etc.

The world supply of NR is barely keeping up with an expected global demand for 12 million tons by 2020. Rubber content of raw *Hevea* latex accounts for 30-40 per cent of the dry weight of latex. Most of the latex is processed at the site of production by coagulating the colloidal rubber with acetic or formic acid, and converted into sheets, crepe, blocks or granules. The rubber trade became a mainstay of the Brazilian economy, providing at its height almost 40 % of its export revenues. It was not long before the idea was conceived of domesticating rubber. However, Brazil was not the site of the successful commercialization of rubber. Rubber cultivation was, instead, transferred to Southeast Asia and soon abundant and cheap, rubber was put to thousands of uses. Its reduced cost was an important factor in the emergence of a mass market of automobiles; from two-thirds to three-quarters of the demand for rubber soon came from the makers of tires and tubes for motor vehicles.

India is the fifth largest producer and second largest consumer of NR. The country accounted for 7.1% of the global production and 8.5% of the global consumption during 2013. The NR production in India was 844,000 tonnes during 2013-2014, from 518,000 ha of yielding area with 1,629 kg/ha as average yield. Rubber occupied 776,000 ha including area under immature trees at the end of 2013-2014. Rubber growing units having area up to 10.0 ha are referred to as holdings. The geographical region covering the state of Kerala and Kanyakumari district in Tamil Nadu is considered as the country's traditional rubber growing region with reference to agro-climatic suitability. This region accounted for 74.7% of the area and 90.3% of the production of natural rubber during 2012-2013. The north eastern region accounted for 18.6% of the area and 6.1% of the production during the same year. An estimated 73.8% of natural rubber produced in the country during 2013-14 was traded in the form of ribbed smoked sheet (RSS). Solid block rubber (ISNR) and concentrated latex respectively constituted 12.6% and 8.1% of the total output during the year.

2.4 Breeding strategies

Hevea breeding programmes are aimed to develop ideal clones with high production potential combined with desirable secondary attributes like high initial vigor, thick bark having a good latex vessel system, good bark renewal, high growth rate after opening and tolerance to major diseases. In addition to maximum yield, attention has to be paid on producing clones with low incidence of tapping panel dryness. Additionally, the production of location specific clones should also be taken into consideration. Clones with early attainability of tapping girth and high initial yields are also preferred in the later phase of exploitation (George and Jacob, 2000).

The conventional methods adopted for crop improvement in *Hevea* are introduction, ortet selection and hybridization. Introduction is the programme in which different clones were introduced from other rubber growing countries from time to time in India. This serve as a source of valuable genes for breeding programmes. By 1956, thirty five clones originating from Indonesia, Malaysia and Sri lanka were available to the plantation sector. From these introductions,

selected clones have been used as parents in hybridization programmes, which have resulted in the evolution of some very successful cultivars like RRII 105. So far, 127 exotic clones have been introduced from other rubber growing countries (Saraswathyamma *et al.*, 1992). Under the ortet selection programme which is the oldest selection method adopted in *Hevea brasiliensis*. In this method extensive seedling areas are screened for identifying elite plus trees which are further cloned and evaluated for the development of primary clones. Selected trees are cloned and the clones are evaluated in small scale, large scale and block trials, then the promising ones are released for commercial cultivation. So far, over 2000 ha of seedling areas have been screened for this purpose. RRII 5 a promising selection for yield and RRII 33, a clone showing tolerance to abnormal leaf fall disease are some of the primary clones evolved and released in India through ortet selection. Systematic crop improvement programmes through hybridization and clonal selection were initiated in 1954, just before the inception of the RRII. Hybrid clones designated as RRII 100 series, 200 series and 300 series have been developed by the Institute over the years (Premakumari *et al.*, 1984), among which 10 hybrid clones are included in the planting recommendation of the Rubber Board. RRII 105, the most outstanding hybrid clone in terms of yield, enjoys the maximum popularity in the country. The demand for higher yielding clones has increased ever since the commercial release of RRII 105 during 1980, which was developed through hybridization of Tjir and Gl 1. Through *Hevea* breeding programmes promising clones having better yield than RRII 105 like RRII 400 series clones have been released for cultivation since 2005. They are the resultant of hybridization between RRII 105, as female parent and RRIC 100, a vigorous and disease tolerant clone of Sri lankan origin, a male parent. Crop improvement activities are being continued further using RRII 400 series clones with selected Wickham clones as parents aiming at improving the yield and secondary traits of present varieties (Mydin *et al.*, 2011).

Crop improvement of perennial crop in general and particularly in rubber, by conventional breeding is very complicated and time consuming as in many

other tree crops. The major limitations of conventional breeding in *Hevea* are its very narrow genetic base, high heterozygosity, very long breeding cycles, seasonal nature of flowering, low fruit set, lack of fully reliable early selection methods and pronounced interaction of genotype and environment (George and Jacob, 2000). *Hevea*, dependence on bud grafting has become inevitable for the propagation of selected clones and maintenance of clonal integrity. Since the seedlings are genetically divergent, it is often implicated as the source of large tree to tree variation in growth and yield of bud grafted *Hevea* trees.

Day by day population pressure is increasing towards the demand for rubber productivity. The traditional areas have been already saturated and now people are shifting to non-traditional areas. To overcome the population pressure and also the climatic changes caused due to global warming, there comes the need for the usage of new techniques to suppress the production constraints. Modern tools of biotechnology can be employed to break the attained yield plateau and to neutralize the prevalent intraclonal variation. Plant tissue culture technology is being widely used for large scale plant multiplication. In recent years apart from their use as a tool of research, plant tissue culture techniques have major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. As an emerging technology, the plant tissue culture has a great impact on both agriculture and industry, through providing plants needed to meet the ever increasing world demand. It has made significant contributions to the advancement of agricultural sciences in recent times and today they constitute an indispensable tool in modern agriculture (Garcia *et al.*, 2010).

Tissue culture techniques have been used to overcome problems associated with conventional propagation. From the published information, tissue culture in rubber was first started by Bouychou in 1953. Successful somatic embryogenesis in *H.brasiliensis* has been reported using anther walls, immature anthers and immature inflorescence (Seneviratne and Nayanakantha, 2007). Protocols for somatic embryo induction and plant regeneration for clone RRII 105 have also been developed using various explants like immature anther

(Jayasree *et al.*, 1999), immature inflorescence (Sushamakumari *et al.*, 2000), leaf (Kala *et al.*, 2005) etc. A high frequency of secondary embryogenesis system was induced from isolated early cotyledonary stage somatic embryos of *H. brasiliensis* (Cailloux *et al.*, 1996.). Various *in vitro* approaches applied to rubber tree (tissue culture, haplogenesis, microcutting, somatic embryogenesis, protoplast culture, germination of immature embryos) has been reviewed by Carron *et al.*, (2001, 2005). Microcuttage and somatic embryogenesis were studied in *Hevea* to achieve rapid clonal propagation as alternatives to the drawbacks of the use of cottage and budding techniques with mature trees. Somatic embryogenesis as a means of regeneration opens up new possibilities for transgene technology. *In vitro* culture is made of the application of many laboratory protocols involving hormones, nutrients, culture medium, and of histo-cytological controls; details can be found in the works of Etienne *et al.*, (1997a) ; Carron *et al.*, (1992) ; Housti *et al.*, (1992) ; Montoro *et al.*, (1993) ; Wang and Chen, (1995) ; Seneviratne and Wijesekara, (1996) ; Cailloux *et al.*, (1996) ; Linossier *et al.*, (1997) ; Sushamakumari *et al.*, (2000) and Jayashree *et al.*, (2001).

In *Hevea*, increased growth and vigour have already been reported for plants regenerated through tissue culture (Carron *et al.*, 1995; 2000). Even a very small yield increment per tapping will be a great attribute to a tree crop like *Hevea* with an economic life span of about 30 years. Biotechnology provide powerful tools for plant breeding, and among these ones, tissue culture, particularly haploid and doubled haploid technology, can effectively help to select superior plants. In fact, haploids (Hs), which are plants with gametophytic chromosome number, and doubled haploids (DHs), which are haploids that have undergone chromosome duplication, represent a particularly attractive biotechnological method to accelerate plant breeding. Similarly polyploids have shown their immense benefits of high vigour and biomass in many crop species. The table below shows the various *in vitro* approaches so far been achieved in *Hevea brasiliensis*.

Table 1 Various *in vitro* approaches carried out in *Hevea brasiliensis*

Explants used	Results	References
Micropropagation		
Shoot apices	PD, RD	Paraanjothy and Ghandimathi,1975,1976
Somatic embryos	PD	Carron and Enjalric, 1982
Axillary buds from young trees	PD,RD	Enjalric and Carron, 1982
Rubber rootstocks	PD	Carron and Enjalric, 1983
Shoot tips, internodes	PD	Te-chato and Muangkaewngam, 1992
Apices from mature shoots	PD,RD	Perrin *et al.*, 1994
Axillary buds	PD,RD	Seneviratne *et al.*, 1995
Nodal explants from juvenile plants	PD,RD	Seneviratne and Flegmann, 1996
Shoot tips	PD	Seneviratne, 1991
Axillary buds from mature clones	PD, RD	Perrin *et al.*,1997
Axillary buds	PD	Seneviratne and Wijesekara, 1996
Axillary buds	PD	Lardet *et al.*,1999
Nodal explants	PD	Lardet *et al.*,1999
Axillary buds	PD,RD	Mendanha *et al.*,1998
Shoot tips	PD	Kala *et al.*, 2004
Organogenesis		
Young Stem	CD,RD	Wilson and Street, 1974
Stamens	CD,PD	Wilson and Street, 1974
Cotyledon, hypocotyls, epicotyls	RD	Paranjothy and Ghandimathi, 1975,1976
Mature embryonic axes	PD	Paranjothy and Ghandimathi, 1976
Leaves	CD,ED	Carron and Enjalric, 1982
Leaves	CD,PD	Mendanha *et al.*, 1998
Callus from inner integument	ED	Blanc *et al.*, 2002
Anther/microspore culture		
Anther	CD	Satchuthananthabale and
Anther	CD,ED	Irugalbandra,1972

Anther	CD,CD	Satchuthananthabale, 1973
Anther	CD,ED,PD	Paranjothy, 1974
Anther	CD,ED,PD	Ghandimathy and Paramjothy, 1975
Anther	CD,ED,PD	Paramjothy and Rohani, 1978
Anther	CD,ED,PD	Chen *et al.*, 1978,1979
		Shijie *et al.*, 1990
Somatic embryogenesis		
Anther	CD,ED	Paranjothy, 1974
Anther	CD,ED,PD	Carron and Enjalric, 1982
Integument tissue	CD,ED,PD	Asokan *et al.*, 1992
Stamens	CD,ED,PD	Wang and Chen 1995
Immature anthers	CD,ED,PD	Jayasree *et al* 1999
Immature inflorescence	CD,ED,PD	Sushamakumari *et al.*, 2000
Leaf	CD,ED,PD	Kala *et al.*, 2005
Roots	CD,ED,PD	Sushamakumari *et al.*, 2014
Protoplast culture		
Immature leaves	PI,PF,PD	Cailoux and Lieras, 1979
Stem pith cell suspension	PI,PD	Rohani and paranjothy, 1980
Anther callus and cell suspension	PI,PD	Wilson and Power, 1989
Embryonic callus	PI,PPD,MCF	Cazaux and d'Auzac, 1994
Stem tissues	PI,PD	Cazaux and d'Auzac, 1994
Embryogenic cell suspension	PPD	Sushamkumari *et al.*, 2000
Pollen protoplast	PC	Sushamkumari *et al.*, 2010
Embryo culture		
Embryo rescue	PD	Rekha *et al.*, 2010
Half ovulo culture	PD	Rekha *et al.*, 2012

CD - Callus development, ED- Embryo development, PD-Plant development, PI-Protoplast isolation, PP- Protoplast proliferation, PPD - Protoplast division, MCF- Microcallus formation, PC-Protoplast culture.

Crop improvement programmes through the development of *in vitro* pathways for the production of ploidy variants like haploids, triploids and tetraploids in *Hevea brasiliensis* in the past have been limited. Eventhough works have been carried out but further establishment have so far not been achieved. From 1980 onwards efforts have been taken for the development of gynogenic plants of *Hevea* using egg cell (Guo *et al.*, 1982), unpollinated ovule (Chen *et al.*, 1987). Production of pollen plantlets (frequency of about 3%) through another culture in *Hevea brasiliensis* has also been reported by Chen (1986).

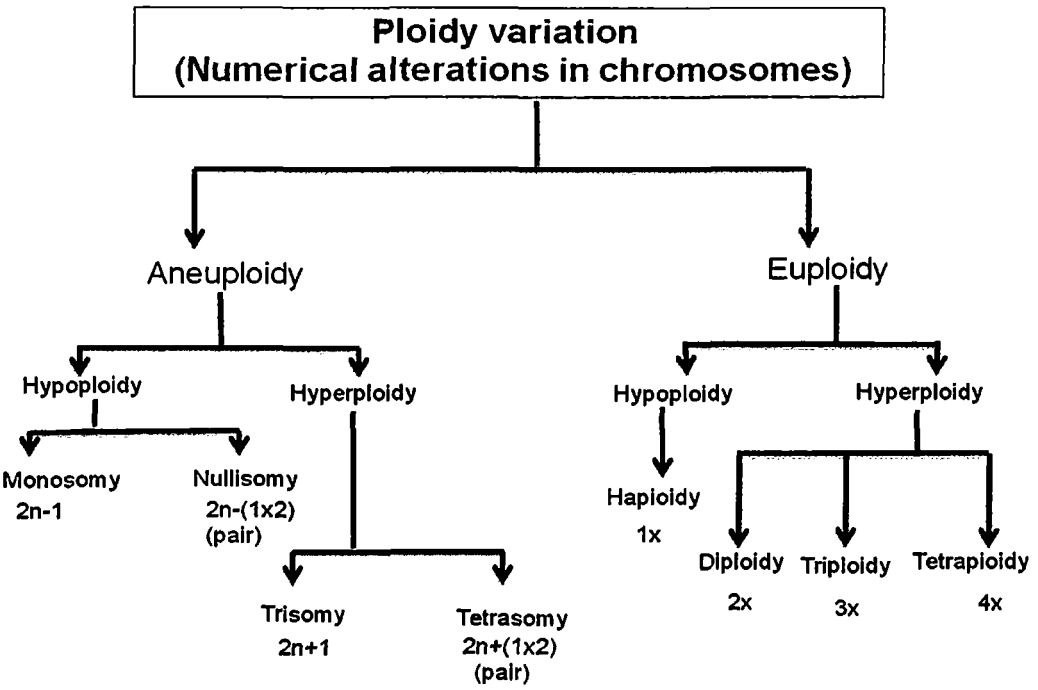
As a process, for widening the genetic base of *Hevea*, polyploidy and induced mutations have been tried on a limited scale. Many researches have been carried out in the field of polyploid induction in *Hevea*. Mendes & Mendes *et al.*, (1963) in Brazil, Shepherd (1969) in Malaysia, Markose (1975) in India have attempted polyploidy induction in *Hevea*. Their initial results created great interest in this field because of the increased vigour of the resultant plants. But subsequent developments in this work have not been further reported. Pinheiro *et al.*, (1980) also reported an increase in yield in several tetraploid clones. Chimeral problem have been observed during the development of polyploids and quality improvement is receiving attention to improve the technique and reduce this problem (Moraes, 1977). Paulo de *et al.*, (1983) induced polyploids in *Hevea* through chemical treatment. Vegetative buds were subjected to coichicine in order to induce polyploids. Saraswathyamma *et al.*, 1984 observed some indication of enhanced yield and production of useful secondary attributes in the induced polyploids. But the polyploid breeding has not increased the enthusiasm of scientists due to the development of chimeras (Zheng *et al.*, 1980). In *Hevea* an artificial triploid has been produced by crossing a diploid and a tetraploid (Saraswathyamma *et al.*, 1988). Naturally occurring triploids have also been reported elsewhere (Nazeer and Saraswathyamma, 1987), but currently it must be acknowledged that all planting materials being utilized throughout the rubber-growing countries are common diploid clones. Recently effect of different concentrations of colchicine upon certain morphological characteristic of rubber

tree seedlings aged 13 month and also the effect of colchicine on the amount of latex released from an artificial wound was also studied (Wiwat, 2013).

2.5 *In-vitro* induction of ploidy variation

Ploidy refers to the number of complete sets of chromosomes in a cell. The symbol 'x' is used to indicate the basic number of chromosomes in each set or genome. Monoploids have one set (1x) and diploids have two sets (2x) of chromosomes. The symbol 'n' indicates the haploid or gametic chromosome number of the species and the 2n denotes the diploid or somatic number of the species. Ploidy variation involves changes in the number of whole sets of chromosomes. Ploidy variation are generally of two types i.e aneuploidy and euploidy. Aneuploidy caused due to the variation in the number of chromosomes in a set, and euploidy caused due to the variation in the number of sets of a chromosome. Aneuploidy gives rise to monosomy, nullisomy, trisomy, tetrasomy etc. Euploidy give rise to haploidy, diploidy, triploidy and tetraploidy. The number of chromosomes possessed by a crop influences its genetics and the strategies applied for its improvement. Plant breeders can alter chromosome number to modify and exploit genetic variability.

Haploidy indicates the presence of single set of chromosomes and polyploidy is the generic term used for the presence of more than two sets of chromosomes. Haploid and polyploid plants have shown there immense benefit especially in tree crops. From the reviews of many plants, the importance of haploid and polyploid can be known. The chart below shows the types of ploidy variation developed by alterations in the number of chromosomes.



(x = basic chromosome number, 2n = somatic chromosome number, n = chromosome number of a gamete).

Fig. 1 Types of ploidy variation

It is not compulsory that all plant species should be diploids. Haploids and polyploids exist both naturally or as a result of various induction techniques. Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium*. Similar observations were reported in other plant species e.g. *Nicotiana tabacum* (Clausen and Mann, 1924), *Triticum compactum* (Gaines and Aase, 1926) and in several other species (Forster *et al.*, 2007). Earlier itself the role of haploids and doubled haploids in breeding and genetics studies have been recognized and researches have been carried out for the development of haploids, taking into account the different methods and factors for its improvement (Kimber and Riley, 1963). *In vitro* anther culture of *Datura innoxia,* lead to a great achievement in the field of development of haploids in higher plants (Guha and Maheswhari, 1964). This technique was used as well as established in many plants but less idea was there regarding the mechanism behind it. Since 1970s, haploids have been produced

through gametic embryogenesis for breeding purpose. In several economically important crop species, including major cereals and cabbages doubled haploid techniques have been well reported (Wedzony *et al.*, 2009). Some examples of doubled haploids produced in fruit crops: *Actinidia deliciosa, Annona squamosa, Eriobotrya japonica, Carica papaya, Feijoa sellowiana, Malus domestica, Morus alba, [Musa balbisiana (BB)], Prunus armeniaca, Prunus avium, Prunus domestica, Prunus persica, Pyrus communis, Pyrus pyrifolia, Olea europaea, Opuntia ficus-indica, Vitis vinifera* (Germana, 2011).

Polyploidy is an exciting phenomenon with intriguingly, a lot of practical potential. As mentioned earlier it has been an area of interest right from the eighteenth century and still continues to be an area of interest, as seen from the increasing number of publications being generated continually on various aspects of polyploidy over the last decade (Mable, 2003). 75% of all angiosperms are polyploids, which are characterized by having more than two sets of chromosomes in their somatic cells. About 70% of undomesticated grasses and 255 of legumes are polyploids. Among domesticated crop species in general, 75% have been found to be polyploidy, with roughly similar percentages found for both annual and perennial species (Schneeweiss, 2013). Major crops such as wheat, alfalfa, potato, cotton and sugarcane are polyploids. There are also plants that do not possess complete sets of chromosomes. Most polyploids display novel variation or morphologies relative to their parental species leading to the processes of speciation and eco-niche exploitation (Rieseberg and Willis, 2007). Polyploid plants can arise spontaneously in nature or can be induced through chemical treatment. Both autopolyploids (e.g. potato) and allopolyploids (e.g. canola, wheat, and cotton) can be found among both wild and domesticated plant species. Many of our crop plants are polyploids and a number of them have and are being synthetically produced. Yet, it has been impossible to predict with certainity, which diploid genomes when merged will coexist stably. Therefore, transforming the present 'hit and miss' process into a more exact science, will form an area of focus in future.

2.6 Induction of haploids

Basically *in-vivo* and *in-vitro* techniques are used for the development of haploids. The basic techniques involved under *in-vivo* method are hybridization, chromosome elimination, parthenogenesis and haploids developed spontaneously. Haploids have also been developed by a cross between either random or selected parents within the same or different species (Dunwell, 2010) i.e. intraspecific hybridization and interspecific hybridization example, development of haploid from diploid material in *Haplopappus* (Jackson and Jordan, 1975). Similar process identified in Citrus plant, where haploids were generated from an interploidy cross between diploids and triploids (Germane and Chiancone, 2001). Haploid obtained through selective chromosome elimination depends on several factors such as a range of genetic (Bitsch *et al.*, 2000) and experimental variables, as well as the intensity of light at early stages of embryo development (Campbell *et al.*, 2001). Wide crosses using interspecific pollinations for haploid induction has been successful in many species (Wedzony, 2009).

The haploid plants have tremendous application in genetics, plant breeding, physiology and embryology. Many attempts have been made since then for the development of *in vitro* haploid, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (reviewed in Maluszynski *et al.*, 2003). In fact, under optimal conditions, doubled haploids (DH) have been routinely used in breeding for several decades, although their common use is still limited to selected species. There are several reasons for this. These might be categorized as biological, based on plant status (annual, biannual, perennial, authogamous, allogamous, vegetativelly propagated) and flower morphology, which are the result of the feasibility and efficiency of DH induction protocol. Induction protocols substantially vary, in fact, not only among species but also among genotypes of the same species (Murovec and Bohanec, 2012).

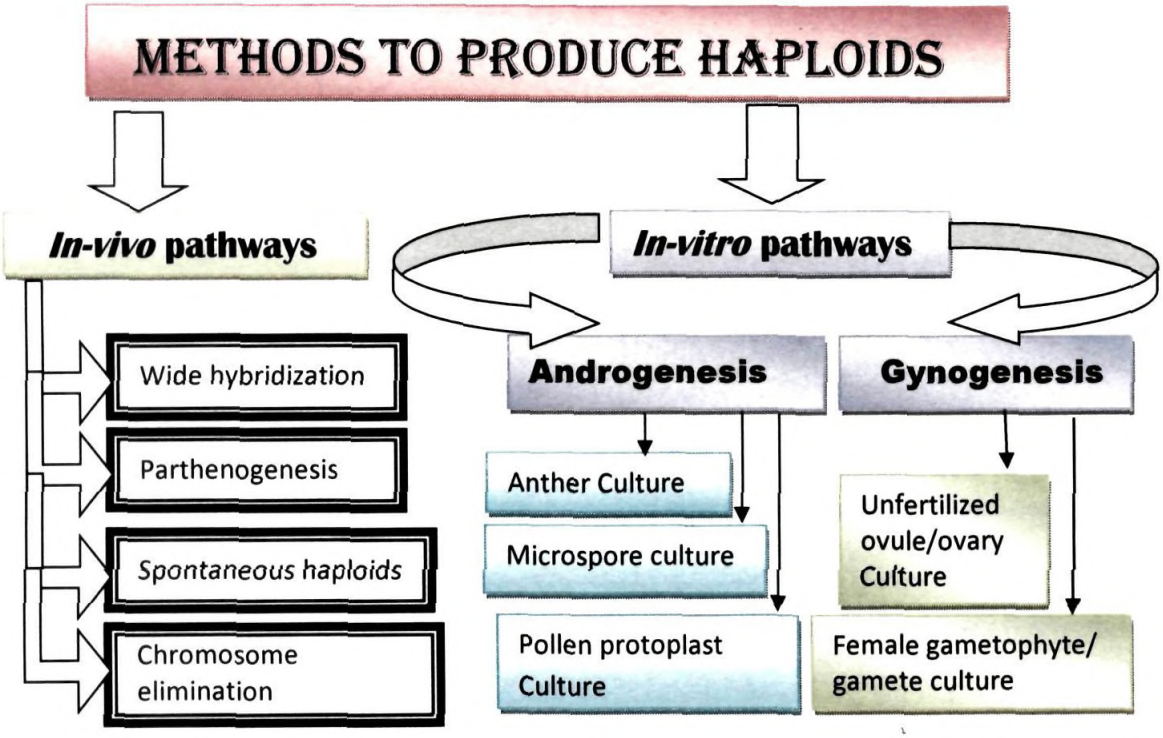


Fig. 2 Different methods employed for the development of haploids

Generally androgenesis and gynogenesis are the two basic *in vitro* pathways used for the development of haploids. In Androgenesis, cultured anthers or isolated microspores undergo embryogenesis/organogenesis directly or through intermediate callus and in gynogenesis, cultured unfertilized isolated ovules or ovaries of flower buds develop embryos from cells of the embryo sac (Palmer and Keller, 2005). Since 1970 extensive research has been carried out for haploid production in tree species using gametic embryogenesis breeding method (Chen, 1986; Germana, 2006). Generally, haploids can be produced by two ways i.e. by regeneration from the male gamete or from the female gamete. Haploid production has been reported majorly through androgenesis in more than 250 plant species, belonging to 100 genera and 40 families (Mishra and Goswami, 2014). Haploid technology made possible through gametic embryogenesis serve as the single-step development of complete homozygous lines from heterozygous parents. Already it had a huge impact on agricultural systems of many agronomically important crops, representing an integral part in their crop improvement programmes. There are several methods available for the

development of haploids and doubled haploids of which *in-vitro culture* technique is the most effective and widely used.

The plant life cycle (Fig. 3) proceeds via alternation of generations of sporophytes and gametophytes where the sporophyte is the dominant life form of higher plants with 2n genomic constitution. It is formed as a result of fertilization of male and female gametes, each of which contributes a set of chromosomes. Haploid plants are sporophytes with gametophytic number of chromosome (n) (Kasha and Maluszynsky, 2003 ; Germana 2011a), produced under *invitro* conditions from naturally occurring haploid cells in the gametophytic phase of higher plants in their ovules and pollen.

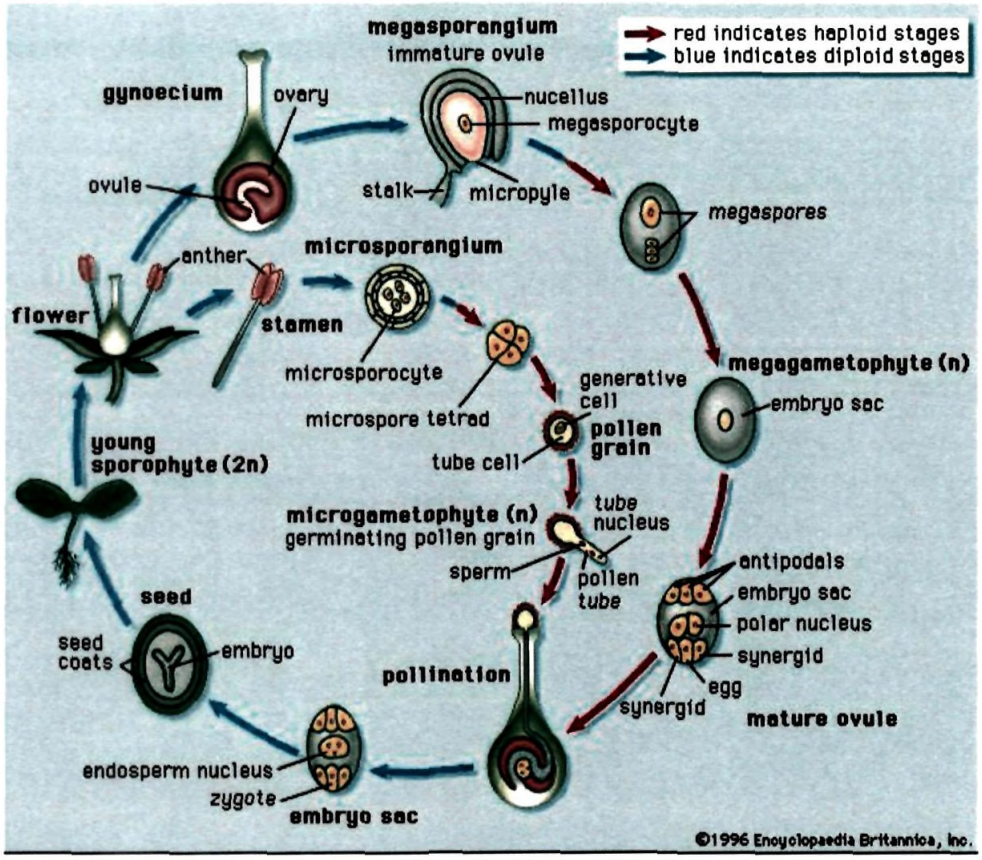


Fig. 3 The plant life cycle

In vitro induction of maternal haploids, so-called gynogenesis, is another pathway to the production of haploid embryos exclusively from a female gametophyte. It can be achieved with the *in vitro* culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower

buds. Although gynogenetic regenerants show higher genetic stability and a lower rate of albino plants compared to androgenetic ones, gynogenesis is used mainly in plants in which other induction techniques, such as androgenesis and the pollination methods above described, have failed (Forster *et al.*, 2007). Gynogenic induction using un-pollinated flower parts has been successful in several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley etc. (Bohanec, 2009 and Chen *et al.*, 2011) but its application in breeding is mainly restricted to onion and sugar beet. Female haploids in seeds of angiosperms are frequent among seeds germinating with two or more seedlings (Clayton and Yawney, 1972). Induction of female-derived haploid embryos following pollination with irradiated pollen has been used successfully in many species such as Apple (Zhang and Lespinasse, 1991), Pear (Inoue *et al.*, 2004) and Citrus (Froelicher *et al.*, 2007). Uchimiya *et al.*, (1971) first reported induction of haploids from megaspores by culturing unpollinated ovaries of *Zee mays* and *Solanum melongene*. Later, different researchers reported haploid production through megaspores (Keller and Korzun, 1996).

Female gametophytic cells have successfully been isolated in several monocot species such as *Zea mays* (Kranz *et al.* 1998), *Hordeum vulgare* (Holm *et al.* 1994), *Triticum aestivum* (Kovács *et al.* 1994), *Oryza sativa* (Khalequzzaman and Haq 2005; Uchiumi *et al.*, 2006) and *Alstroemeria aurea* (Hoshino *et al.*, 2006), as well as in dicot species such as *Vicia faba* (Zhou and Yang, 1982), *Helianthus annuus* (Zhou, 1987), *Torenia* (Hoshino and Mii, 1999), *Plumbago zeylanica* (Huang and Russell, 1989) and *Dianthus* (Hoshino *et al.*, 2000). In angiosperm plants, the FGU refers to the minimum complement of cells required to accomplish double fertilization *in vivo* (Huang and Russell, 1992). Typical FGU consists of an egg, two synergids and a central cell, which fulfills the role of pollen tube attraction, sperm discharge to the receptive cells, transportation of sperm nuclei and double fertilization (Higashiyama *et al.*, 2001). However, it is still difficult to manipulate FGUs because they develop as parts of embryo sac, which is embedded within the sporophytic tissues of the ovule. The techniques developed so far for isolating female gametes such as embryo sac and

free FGUs mostly have several steps comprising the combination of enzymatic maceration and manual procedures (agitation and microdissection) with or without some additional steps such as plasmolysis for facilitating the liberation of embryo sac from ovule or of FGU from embryo sac wall (Kranz *et al.*, 1998; Imre and Kristof, 1999). Beside the use of enzymes, Kovács *et al.*, (1994) reported the isolation of viable egg cells of wheat that has been achieved by mechanical disintegration of softened ovule tissues 3 to 7 days after applying 2, 4-D to the stigmas. All parts of female germ unit are important in proper pollen tube guidance and bursting, to release the sperm cells (Chen *et al.*, 2007). Synergids are essential for the fertilization process of angiosperms (Punwani and Drews, 2008. In some plants, e.g., in Plumbago and Plumbagella where synergids are absent, the egg cell takes over the functions of the synergid (Huang *et al.*, 1990) and also possesses a synergid characteristic-a filiform apparatus (Russell and Cass, 1988). The embryo sacs are present in a diversity of developmental pathways, however, the most common is the monosporic polygonum type, in which the functional megaspore passes through three mitotic divisions producing a seven celled embryo sac (Chasan and Walbot, 1993; Li and Ma, 2002) consisting of three antipodal cells, one central cell formed by two polar nuclei, two synergid cells and the egg cell (Kagi and Groß-Hardt, 2007; Yang *et al.*, 2010).

Enzymatic procedures for the isolation of female gametes have been described for several plant species, including *Torenia fournier* (Katoh *et al.*, 1997), *Brassica napus* (Hoshino *et al.*, 2000), *Dianthus* species (Popielarska and Przywara, 2003) and *Helianthus annuus* (Yoichiro *et al.*, 2006). The use of the female gametophyte is an alternative way for production of haploids (Bohanec *et al.*, 1994; Picard *et al.*, 1994). Gynogenic haploids have been developed in more than 30 species using tissue culture technology (Keller and Korzun, 1996). Study of progressive developmental stages of gynogenic embryos have been done in dicotyledonous plants such as sugar beet (Ferrant and Bouharmont, 1994) and in sunflower (Yan *et al.*, 1987). Unpollinated ovule culture has been used successfully for haploid production in sugar beets and onions. In case of *Nicotiana rustica* ovules with placenta were isolated from flower buds and were

cultured on N6 medium supplemented with growth regulators (Katoh and Iwai, 1993). Embryo sacs have been isolated from ovules using either digestive enzymes or through microdissection (Wagner *et al.*, 1988). Digestive enzymes are used to weaken cell walls so that nucellar cells break away and expose embryo sacs, resulting in completely isolated embryo sacs. Access to embryo sacs using this approach, unfortunately, results in disruption of cell walls within embryo sacs as well, which may adversely affect embryo sac viability (Leduc *et al.*, 1995). In addition, the nucellar cells removed during isolation may be important for initiating and controlling the early stages of embryogenesis (Campenot *et al.*, 1992). Microdissection on the other hand, involves physically cutting nucellus away from embryo sacs. This can be labour intensive, but enzymes are avoided thus leaving embryo sacs intact. As well, it is virtually impossible to remove all of the nucellus from the embryo sac, and therefore visual access to individual embryo sac cells is often hampered.

Isolation of viable (living) embryo sacs is of considerable interest. *In vivo* as well as *in vitro* fertilization can be studied in living embryo sacs (Kranz and Lorz, 1993). This approach creates the potential for selection of desired embryo sacs and sperm cells and provides the ability to manipulate the *in vitro* environment during fertilization. Crosses that are not occurring naturally due to incompatibility, could result in the creation of new crops. As well, transgenic sperm or embryo sacs can be used for *in vitro* fertilization procedures to produce transgenic plants. Similarly transgenes could be introduced into fertilized embryos sacs which could develop into transgenic crops (Leduc *et al.*, 1996). Embryogenesis and endosperm development can be studied from fertilization, providing information on the early stages of development. These stages can be studied with both the nucellus intact or with it removed, which may provide a means to study the role of nucellus in stimulating embryogenesis. Cellular as well as genetic and biochemical information can be obtained. As well, manipulation of embryo sacs during these early stages may reveal genes that are important and exclusive to these stages. Manipulation of these genes may further provide understanding of seed development and lead to crop improvement.

2.6.1 Factors influencing gynogenic haploid production

The genotypes of the donor plants are an important factor, which influence the culture response of ovary/ovule (Mukhambetzhanov, 1997). The process of gynogenesis using *in vitro* techniques are much affected from the physiological condition of the donor plant *i.e* the environmental condition and age of the donor plant. The stage of embryo sac is an important determining factor for *in vitro* gynogenesis in various plant species. Unfortunately, there is not sufficient information available on this aspect. The degree of development of the embryo sac is indirectly defined during the stage of development of pollen grain or with the help of histological preparations of ovules (Mukhambetzhanov, 1997). In niger no culture response in ovule culture were observed when collected two or three days before anthesis but when collected before one day of anthesis about 5.0 % and 13.3 % embryogenesis were recorded (Bhat and Murthy, 2007). Although a wide range of stage of embryo sac are responsive to *in vitro* gynogenic development, but, in most cases the later stages give better gynogenic response. e.g., barley (San Noeum, 1979).

The constituents of the basal medium and combinations of growth regulators (with specific concentration) are also an important factor in eliciting successful gynogenesis. Initially in the 1950s, Nitsch medium was used for ovule and ovary culture and from 1970s; Miller used MS or N6 media. In *Gerbera*, MS basal medium was found better than the Knop and Heller medium (Cagnet-Sitbon, 1980) for the induction of gynogenesis. The requirement of growth regulators and culture medium in terms of type and concentration may differ with each and every plant system. Auxins are extensively used for induction of gynogenesis and their optimum concentrations have been reported to vary considerably from species to species (San Noeum and Gelebart, 1986). Gynogenesis in sunflower occurred only in presence of 2, 4-D or NAA in the medium (Gelebart and San Noeum, 1987). Combination of auxin and cytokinin was also reported to be useful for gynogenesis in allium species (Alan *et al.*, 2003), mulberry (Thomas *et al.*, 1999). Maximum gynogenic response was observed in mulberry when excised ovaries from inflorescence segments were

cultured on MS+BAP (8.5 μM) +2, 4-D (4.5 μM) and then transferred to MS +2, 4-D (4.5 μM) + glycine (6660 μM) + proline (1738 μM) (Thomas *et al.*, 1999). On the other hand, in some reports gynogenic haploids of mulberry could be produced only in presence of cytokinin (BA or kinetin) supplemented medium (Lakshmi Sita and Ravindran, 1991). The addition of other substances in the medium such as glutamine, casein, proline, biotin, inositol, coconut water, silver nitrate and polyvinyl pyrrolidone has been reported to influence haploid development (Powell, 1990; Achar, 2002). Similarly, the use of additives in medium has also been reported in ovary culture.

The kind, type and concentration of carbohydrate used in the medium for inducing *in vitro* gynogenesis vary from species to species. High sucrose concentration (8 – 10 %) in the culture medium has been shown to be helpful on some species like sweet potato (Kobayashi *et al.*, 1993) and onion (Campion *et al.*, 1992) whereas in summer squash 9 % sucrose was detrimental for production of any embryos (Shalaby, 2007). In *Cucumis melo* pollination of pistils with irradiated pollen was essential to obtain ovules capable of forming gynogenic haploids (Katoh *et al.*, 1993). A beneficial role of cold treatment on gynogenesis has been reported in some plant species while in others no significant effect on gynogenesis has been observed. Pretreating the capitula of sunflower at 4°C for 24 – 48 h before culture could significantly increase the induction frequency (Yan *et al.*, 1987). Cai *et al.*, 1988 observed a promontory effect of cold treatment of the young panicles of rice at 7°C for 1 day before ovary culture. *In vitro* gynogenesis is also affected by the position of female flower on plant stem. This factor affected induction of embryos from ovule cultures of *Cucurbita pepo* (Shalaby, 2007). One of the possible explanations for enhancing responses of tissue culture could be attributed to indigenous hormonal level (Johansson, 1986).

2.6.2 Advantages of haploids in crop improvement

Homozygous diploid plants are highly important for screening of high yielding lines (Mishara and Goswami, 2014). Especially in perennial tree crops where development of homozygous diploid plants by conventional methods is difficult and time consuming. It takes several years to obtain a pure line by means

of successive inbreeding throughout many generations. The seed set by inbreeding in many trees is so low, usually only a few of ten thousandth or sometimes no seed can be obtained at all therefore, it is impractical to obtain pure lines by inbreeding (Chen, 1986). Homozygous diploid plants have been achieved in a single generation by diploidization of *in vitro* raised haploids by colchicine treatment eg. Oilseed rape (Payam *et al.*, 2011).

Haploids can be used for various genetic studies like linkage mapping, genetic transformation, obtaining new genotypes with alien chromosomes, cytogenetic research etc. Haploids are applicable in detecting linkages associated with quantitative inherited characters and could be used in calculating recombination values between linked genes (Islam *et al.*, 1992; Lashermes *et al.*,1994). Direct gene transfer by microinjection technique offers the possibility of transgenic plant formation by using isolated pollen culture having high regeneration efficiency (Kasha and Maluszynsky, 2003), similar technique can be applied by using embryo sac culture, but still no such work have been carried out. The procedure of interspecific and intergeneric hybridization can be combined with anther or ovule culture techniques (Thomas *et al.*, 2003). Thus, new genotypes with various reconstructed chromosome complements can be obtained after their chromosome doubling. Haploids are useful in several areas of cytogenetic research. These include production of aneuploids, determination of the nature of ploidy, determination of basic chromosome number and evaluation of the origin of chromosomes.

In general, majority of induced mutations are recessive and therefore are not expressed in diploid cells due to presence of dominant allele. Since, haploid plants have only one set of chromosomes, their dominant and recessive characters can be seen simultaneously on separate plants. Haploids may be considered as being mutants in their own right at a genomic level (Blakeslee, 1922). It is extremely advantageous to provide a convenient system for the induction of mutations and selection of mutants with desirable traits in the absence of their dominant counterparts (Bhojwani and Razdan, 1996). In Haploids it is easy to

locate mutations, which may be masked in a diploid (Maluszynski and Kasha, 2002; Jambhulkar, 2007; Szarejko and Forster, 2007).

Protoplast of haploid can be used to produce diploid fertile plant with unique combination of valuable character such as cytoplasmic male sterility. Use of this will save labour and space compared with those required in classical technology (Atanassov *et al.*, 1995).

2.7 Polyploidy

Polyploidy (genome doubling), discovered in 1907, is an important driver of eukaryotic evolution, evident in many animals, fungi, and plants (Hovav *et al.*, 2008; Wood *et al.*, 2009). Most angiosperms are of polyploid origin (Soltis and Soltis, 1999). Polyploid plants have been generated from evolutionary processes, crop domestication, and/or artificial synthesis via chemical or physical mutations. Polyploids can occur among regenerated plants through *in vitro* culture or during the transformation process. The mode of regeneration and regeneration capacity of polyploid tissues cultured *in vitro* is especially interesting. So far, efficient regeneration systems among polyploids have been developed mainly for potato and rapeseed (Ono *et al.*, 1994; Garcia and Martinez, 1995). It is often assumed that polyploids spread because they can tolerate and invade harsher environments than their diploid counterparts owing to increased hardiness or increased genetic buffering provided by extra genome copies (Otto and Whitton, 2000).

Polyploid plants can be classified on the basis of genomic origin, formation approach and time after polyploid formation. Polyploid origin can be divided into autopolyploids, which are derived from a whole-genome duplication event of the same ancestral chromosome set, and allopolyploids, which are derived from a hybridization event of alternate parental genomes followed by genome duplication (Pignatta *et al.*, 2010).

Polyploids can also be divided into natural and synthetic polyploids. Natural polyploids result from spontaneous genome-doubling, whereas synthetic polyploids are the result of induced genome doubling with or without prior genome-hybridization. Paleopolyploid refers to an ancient polyploid that later

again become a diploid due to sequence divergence between duplicated chromosomes. They generally have large basic chromosome numbers (Chand and Sahrawat, 2007). Neoploy ploids is a newly formed auto and allopolyploids (Guha and Maheshwari, 1967). A polyploid could have individuals with a series of ploidy levels within the species thereby giving rise to a 'ploidy series'. The ploidy series may consist of individuals with even or odd multiples of the basic chromosome number (eg: Chrysanhemum (x=9); series 2x, 4x, 6x, 8x,10x) or odd multiples of the basic chromosome number (eg: *Crepis occidentalis* (x=11); series 2x, 3x, 4x, 5x, 7x and 8x forms) (Grant, 1981). Dibasic polyploids are the sum of two different diploid numbers (eg: *Brassica oleracea* (2n=18) and *Brassica campestris* (2n=20) and their tetraploid derivative *B. napus* (2n =4x=38) (Grant, 1981). Polyploids often tolerate the loss of one or more chromosome pairs which at times may give rise to modified polyploid series, what Darlington called a 'polyploid drop'(eg: a modified series found in Hesperis where different species have gametic numbers of n =7,14,13 and 12) (Grant, 1981).

Polyploidy occurs naturally, best example is in the forest tree species (Libby *et al.*, 1969). The highest prevalence of natural polyploids in tree species is found in the angiosperms (Wright, 1962), where it has been observed in a number of genera, for example Betula, Acacia and Populus (Wright, 1962). The finding of the triploid aspen (*Populus tremula*) in 1936 conjured much interest in polyploidy in forestry trees, as this tree exhibited an increased growth rate and favourable wood properties (Libby *et al.,* 1969). Since the identification of these desirable polyploid trees, programmes to research the cultivation of polyploidy trees have been established in a number of countries (Eriksson *et al.*, 2006). There are few naturally occurring polyploid conifers. One example is the giant tree *Sequoia sempervirens* or Coast Redwood which is a hexaploid (6x) with 66 chromosomes (2n = 6x = 66) (Ahuja and Neale, 2002). Natural autoploids include tetraploid crops such as alfalfa, peanut, potato and coffee and triploid bananas. Economically important natural alloploid crops include strawberry, wheat, oat, upland cotton, oilseed rape, blueberry and mustard (Acquaah, 2007; Chen, 2010).

Experimental conversion of diploids to polyploids has been undertaken around the world in tree species from the genus Pinus (Mergen, 1959), Eucalyptus (Janaki *et al.*, 1969; Kampoor and Sharma, 1985) and Acacia (Moffet and Nixon, 1960; Blakesley *et al.*, 2002). Mergen (1959) induced polyploidy in slash pine, loblolly pine, Austrian pine and Mugo pine. In Acacia species, polyploidy has been successfully induced in an attempt to confer infertility to curb excessive seed production (Moffett and Nixon, 1960; Beck *et al.*, 2005). Triploid plants are usually seed sterile and is undesirable where seeds are of commercial value. But in cases where the seedlessness is employed to improve the quality of fruits as in banana, apple, citrus, grapes, papaya etc. induction of triploid plants would be of immense use. Some examples of induced polyploids (Wikipedia contributors) are triploid crops (apple, banana, citrus, ginger, watermelon, poplar, mulberry, tomato, rice). Tetraploid crops (apple, macroni, wheat, cotton, potato, canola/rapeseeed, tobacco, peanut, kinnow and pelargonium). Chrysanthemum, bread wheat, triticale, oat, kiwifruit are some of the examples for hexaploid crops. Octaploid crops include strawberry, dahila, pansies, sugar cane, oca (*Oxalis tuberosa*) and dodecaploid crops like sugarcane hybrids.

2.7.1 Induction of polyploids

Natural polyploids are mostly formed through either somatic chromosome doubling or by the fusion of unreduced gametes during sexual reproduction (Otto and Whitton, 2000). Various modes are there for the origin of polyploids. These include somatic doubling during mitosis, production of unreduced gametes, polyspermy (fertilization of the egg by two male nuclei) and endoreplication (replication of the DNA but no cytokinesis). The major pathways involved in polyploidy formation are represented in Fig 2.

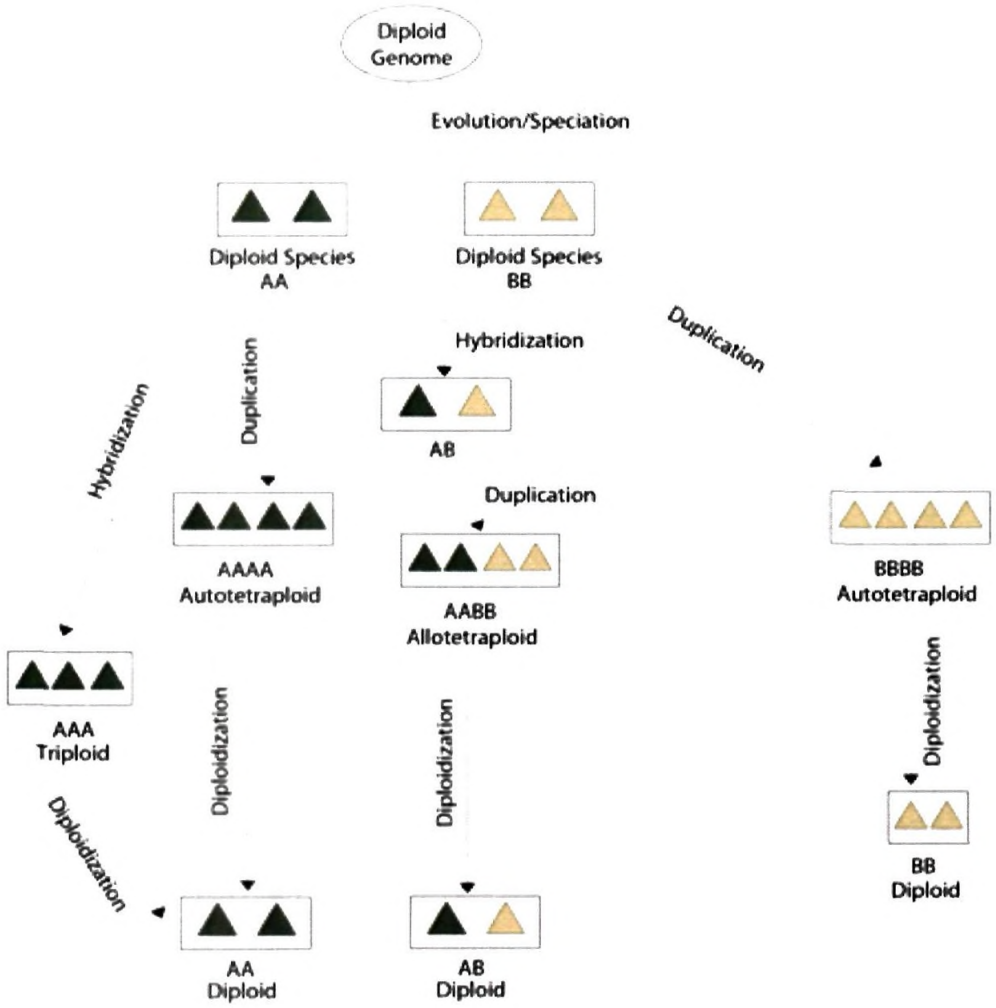


Fig. 4 Major pathways involved in polyploid formation

Doubling of chromosomes can occur either in the zygote to produce a completely polyploid individual or locally in some apical meristems to give polyploid chimeras. Somatic polyploidy is also seen in some non-meristematic plant tissues eg: tetraploid and octoploid cells in the cortex and pith of *Vicia faba* (Ramsey and Schemske, 1998). In somatic doubling the main cause is mitotic non-disjunction (Grant, 1981). Spontaneous somatic chromosome doubling is a rare event (Lewis, 1980) and the only well documented instance of the same was in case of tetraploid *Primula kewensis* which arose by somatic doubling in certain flowering branches of a diploid hybrid. The phenomenon of chromosome doubling in the zygotes was best described from heat shock experiments in which

young embryos were briefly exposed to high temperatures (Lewis, 1980). Zygotic chromosome doubling was first proposed by Winge (1917) and the spontaneous appearance of tetraploids in *Oenothera lamarckiana* and amphidiploid hybrids in *Nicotiana* were as a result of zygotic chromosome doubling (Grant, 1981).

Another route may involve non–reduction occurring in one of the germ lines (pollens or the eggs). A tetraploid individual can then result from a two-step process (sometimes referred to as a triploid bridge mechanism) from the fusion of an unreduced 2n gamete with a reduced 1n gamete to give a 3n zygote followed by the subsequent fusion of a 3n gamete with a normal 1n gamete in the next generation to give rise to a tetraploid individual (as in artificial *Galeopsis tetrahit*) (Grant, 1981). This is a more common route of polyploid formation from unreduced gametes (though the frequent sterility of most triploid hybrids has lead to a questioning of this method by some authors rather than the former one (Ramsey and Schemske, 1998). Polyspermy is observed in many plants but its contribution as a mechanism for polyploid formation is rather rare except in some orchids (Ramsey and Schemske, 1998). Endoreplication is a form of nuclear polyploidization resulting in multiple uniform copies of chromosomes. It has been known to occur in the endosperm and the cotyledons of developing seeds, leaves and stems of bolting plants (Larkins *et al.*, 2001).

Triploids are formed within a diploid population, and backcrossing to diploids, or self-fertilization of the triploid, produces tetraploids. For example, 1% tetraploid progeny were obtained by backcrossing a spontaneous triploid clone of *Populus tremulat*. However, triploids generate small numbers of euploid (x, 2x) gametes (Dermen, 1931) and can also produce 3x gametes via nonreduction (Belling, 1925). Autotriploids can produce tetraploids by self-fertilization or backcrossing to diploids (Zohary and Nur, 1959). Tetraploids are formed directly in a diploid population by the union of two unreduced (*2n*) gametes or by somatic doubling. Traditionally, triploids are produced by hybridization between induced superior tetraploids and diploids. The first step in this process is to produce tetraploids by colchicine treatment of germinating seeds, seedlings or vegetative buds (Sikdar and Jolly, 1994; Chakraborty *et al.*,

1998). Once tetraploids have been produced, their cross with the diploid parent may not be successful in majority of the cases. In successful crosses the seedset, seed germination and survival rate of the seedlings is low (Sikdar and Jolly, 1995). Further, all sexually produced triploids do not behave uniformly, which may be due to segregation both at tetraploid level (Dandin, 1990).

Protoplast fusion has become an important tool for the generation of novel genotypes and ploidy manipulation in plant improvement schemes. The development of this technology became possible following the first successful isolation of plant protoplasts by Cocking, (1960). This was followed by the first successful report on somatic hybridization in tobacco by Carlson *et al.*, (1972). Since this time, hundreds of reports have been published during the past four decades which extend the procedures to additional plant genera and that evaluate the potential of somatic hybrids in many crops including citrus, rice, rapeseed, tomato and potato. Excellent general reviews of the subject have appeared over the years including those by Bravo and Evans, (1985); Johnson and Veilleux (2001) and for specific commodities including potato (Orczyk *et al.*, 2003) and citrus (Grosser and Gmitter, 2005; Grosser *et al.*, 2000).

Artificial polyploid induction began early in the 20th century (Elliot, 1958). Induction methods included subjecting germline material of seed and meristematic tissue to chemicals and temperature extremes (Blakeslee and Avery, 1937), which permitted breeders to create an array of varieties not available in their diploid relatives (Kehr, 1996). Tetraploidy can be induced using the chemicals like colchicine, oryzalin, trifuralin, N_2O, dinitroanilines etc. In polyploidy induction, chemicals are generally applied to actively dividing cells (Elliot, 1958). These include seeds, apical meristematic structures and actively dividing cells in culture (Dermen, 1940). Seed is frequently used in chemical polyploid induction because of its high success rate (Dermen, 1940). The chemical inducers are usually applied to seed in two different ways: either by first soaking the seed in a solution of the chemical and then allowing the seed to germinate (Avery *et al.*, 1947); or by germinating the seed directly in a solution of the chemical, usually on chemical soaked filter paper, allowing the seed to

imbibe the inducer (Ahokas, 1998; Rubuluza *et al.*, 2007). For successful induction of polyploidy, different concentrations of a particular chemical inducer needs to be tested so as to employ the most effective concentration (Dermen, 1940). Colchicine an alkaloid originating from *Colchicum autumnale* (Lehre *et al.*, 2008), was first used to induce polyploidy in Datura (Blakeslee and Avery, 1937), and later in other plants such as *Acacia mearnsii* (Moffett and Nixon, 1960), *Colophospermum mopane* (Rubuluza *et al.*, 2007). The success of this alkaloid lies in its ability to prevent the formation of microtubules during cell division, mitosis (Dhooghe *et al.*, 2009). Similarly, oryzalin has been used to induce polyploidy in *Acacia dealbata* and *Acacia mangium* by Blakesley *et al.*, (2002). Apical meristems (AMs) and shoot apical meristems (SAMs) are also used as a source of plant material in chemical polyploid induction (Dermen, 1940). Chemical containing lanolin or agar paste has also been used to induce polyploidy in *Carica papaya* (Hofmeyr and Elden, 1942) and Solanum (Chauvin *et al.*, 2003). Cells in tissue culture are another source of plant material used in polyploid induction (Shao *et al.*, 2003; Yang *et al.*, 2006). The chemical inducer is usually added to the tissue culture medium (Shao *et al.*, 2003). This *in vitro* method of polyploidy induction has reported to yield more tetraploids and fewer chimeras than treating seed and axillary buds ex vitro (Shao *et al.*, 2003 and Yang *et al.*, 2006). Polyploidy has been successfully induced using colchicines supplemented media for the culturing of shoot tips of Alocasia (Thao *et al.*, 2003) and root tips of Musa - 20 - acuminata (Van *et al.*, 1996). Similarly oryzalin was used to induce polyploidy in scales from *Lilium longiforum* (Takamura *et al.*, 2002) and from Nerine (Van Tuyl *et al.*, 1992). An alternative approach for creating triploid plants is regeneration of plants from endosperm found in seeds. Although the embryo in most angiosperm seeds is diploid, the adjoining endosperm (nutritive tissue) originates from the fusion of three haploid nuclei (one from the male gametophyte and two from the female) resulting in triploid tissue. This tissue can be excised from developing seeds and cultured *in vitro* (tissue culture) to eventually give rise to regenerated embryos

and plantlets. This approach has been successful for a range of plants including citrus, kiwifruit, loquat, passionflower, acacia, rice and pawpaw.

2.6.2 Factors influencing polyploid production

The basic factors which influence the induction of polyploids through chemical treatment are the concentration and duration of its exposure. Blakeslee and Avery, (1937) first reported the use of colchicine in polyploid formation in *Datura stramonium*. They found that a 4 day immersion in a 0.4 % solution of colchicine produced tetraploidy plants. They used different explants such as stem, terminal buds etc as well as application technique like agar application, spraying and dropping method. Seed and embryo treatments are often handled by employing immersion in a colchicine solution of 0.02 - 0.1 % for 2 - 48 hours. Germinating seedlings are generally treated with the same concentration for a longer period of time ranging from 12-48 hours (Sharma and Sharma, 1980). Seeds of *Portulaca grandiflora* were exposed to 0.1% colchicine in liquid MS medium for 48 hours then planted. Recovered seedlings showed a high percentage of polystomy. Many plants have been induced into tetraploid formation by treating meristematic regions such as buds or nodes with colchicine. *Ex vitro* seedlings can be treated by using soaked cotton plugs placed over the growing tip for 2-4 hours or by applying the colchicine in the form of a paste mixed with glycerin or lanoline (Sharma and Sharma, 1980). This method has been used to induce tetraploid formation of poinsettia lateral meristems. 1 % colchicine lanolin emulsion was applied to the buds *ex vivo*. Supplementing agarose medium with colchicine can also induce *in vitro* chromosome doubling. An effective method for doubling chromosome numbers in clover, *Trifolium spp.*, was developed by using axillary meristems on proliferation medium containing 0.1% colchicine for 48-72 hours. Chromosome doubling frequencies were 81% for initial root tips and 44% for mature shoots. A treatment of 0.05% colchicine to shoot apices and axillary shoot buds for 48 hours produced over 20% tetraploid plants in hop, *Humulus lupulus* L. (Roy *et al.*, 2001). Optimal tetraploid formation occurred with nodal explants placed in 0.05- 0.25 mM colchicine for a

period of 1-3 days. The tetraploids produced were distinguished by having slower growth, darker green leaves and shorter internodes (Rose *et al.*, 2000).

There are a number of other factors favouring polyploidy which includes the mode of reproduction, the mode of fertilization, the breeding system present, the growth habit of the plant, size of chromosomes etc. Polyploidy seems to be favoured in long lived/perennial plants possessing various vegetative means of propagation (eg: *Fragia, Rubus, Artemisia, Potamogeton* etc.) and in those with frequent occurrences of natural inter-specific hybridizations. Various possible reasons have been advanced by various workers (Lewis, 1980 ; Grant, 1981) to account for the above phenomenon; one of the widely accepted ones' being the enhanced chances of somatic doubling made possible in plants with enhanced lifespan and vegetative means of reproduction (Grant, 1981). Cross fertilization and allogamy were argued to be factors favouring polyploidy. Autogamy however was thought to restrict it (Stebbins, 1950).

The proportion of polyploids has been found to increase with latitude and altitude but this has not always been found to be true with respect to altitude (Hieter and Griffith, 1999). Various reasons which have been put forward to explain the above factor in the distribution of polyploids were the better adaptability of some polyploids to colder climates and also due to the changes that might have taken place in the Pleistocene period etc. Various ecological factors also have an effect on the distribution of polyploids eg: polyploids were found to be more frequently distributed in wet soils and meadows as opposed to more stable habitats with drier soils or forest communities respectively (Grant, 1981). With regard to the breeding system since the main mode of origin of allopolyploids in annuals is by the fusion of unreduced gametes, the presence of an outcrossing breeding system tends to reduce the chances of union of unreduced gametes (Grant, 1981). A perennial growth habit tends to favor polyploidy as opposed to an annual growth habit, probably due to the fact that having a long life span increases the chances that rare events will occur (e.g. polyploidization following hybridization), and allows for mating between polyploids and their offspring (Grant, 1981, Otto and Whitton, 2000).

A reciprocal relationship has been observed between cell size, chromosome size and chromosome numbers in polyploids (Grant, 1981).

2.6.3 Advantages of polyploids in breeding

Evidences of extensive gene duplication in organisms considered to be diploid suggest that polyploidy has contributed to evolutionary diversification in plants and animals through successive rounds of polyploidization and rediploidization. Understanding the mechanisms and consequence of such changes is undoubtedly the most topical area of polyploid research in plants and has been thoroughly reviewed (Osborn *et al.*, 2003; Blanc, 2003).

The introduction and movement of invasive species can be a significant threat to certain ecosystems. Development of sterile forms of important nursery crops is an ideal approach for addressing this problem. In doing so, plants can be grown and used for landscaping while minimizing the possibility that these plants could sexually reproduce and become invasive. There are a number of methods available for developing sterile plants. However, one of the most rapid and cost-effective approaches for inducing sterility in a plant is by creating polyploids. In most cases these plants function normally with the exception of reproduction, specifically meiosis. In some cases doubling the chromosomes of an individual plant (autotetrapoid) will result in sterility due to multiple homologous chromosomes and complications during meiosis (as discussed previously). Despite these complications, autotetraploids of some species can produce fertile seeds. In this case, tetraploids can then be hybridized with diploids to create sterile triploids. By doubling the chromosomes of a wide hybrid, each chromosome has an exact duplicate and chromosomal homology and fertility can be restored. This technique has been used successfully to restore fertility in *Rhododendron* 'Fragrans Affinity' and *Chitalpa tashkentensis* (Contreras, 2006; Olsen, 2006). However, in some cases this approach has been unsuccessful in restoring fertility, as was the case with tetraploid hybrids of *Alstroemeria aurea* & *A. caryophyllaea* (Lu and Bridgen, 1997). Certain aspects of plant physiology are sometimes altered by changes in cell size accompanying polyploidy

(e.g. polyploids might be more tolerant to water deficit (Wullschleger *et al.*, 1996) or have higher photosynthetic rates (Li *et al.*, 1996).

Triploids have more vigorous vegetative growth than their diploid counterparts. Hence, in plants where the vegetative parts are economically useful, triploids are of good use. For example, triploid *Populus tremuloides* contain superior pulp quality and is, therefore, preferred over its diploid counterparts (Bhojwani and Razdan, 1996). Triploids of mulberry (*Morus sp.*), which are under cultivation in the Northern part of Japan, are known for their superior quality of leaves and disease resistance (Hamada, 1963). The triploid plants of tomato produce larger and tastier fruits than natural diploids (Kagan-Zur *et al.*, 1990). The triploid plants of rice (*Oryza sativa*) produced from endosperm showed broader leaves, a faster growth rate, and more of tillering than the normal diploid plants (Bajaj *et al.* 1980). Triploid nature of endosperm is the characteristic feature of angiosperms. However, various ploidy levels are also observed in plants like *Butomopsis sp.* (diploid), *Fritillaria sp.* (pentaploid), *Acalypha indica* and *Peperomia sp.* (polyploid). In apomictic species of *Taraxacum* and *Erigeron* the endosperm develops autonomously, without fertilization of the secondary nucleus (Battaglia, 1963). In another apomictic species *Brachiaria setigera*, the endosperm gave rise to triploid embryos *in vivo* and seedlings where six out of 675 aposporous embryo sacs of postfertilization ovules contained endosperm-embryos (Muniyamma, 1977).

The increase in nuclear ploidy affects the structural and anatomical characteristics of the plant. In general, polyploidy results in increased leaf and flower size, stomatal density, cell size and chloroplast count (Dhawan and Lavania, 1996). These phenomena are collectively referred to as the gigas effect (Acquaah, 2007). For example, the volume of tetraploid cells usually is about twice that of their diploid progenitors (Acquaah, 2007; Emsweller and Ruttle, 1941; Schepper *et al.*, 2001). The increase in cell volume however is mainly attributed to increased water and not biomass. Therefore, its application is limited for breeding agronomically important crops such as cereals. Although chromosome doubling may result in significantly larger seeds and increased seed-

protein content in cereal crops, this advantage is offset by low seed set (Dhawan and Lavania, 1996). In contrast, the gigas effect has been explored in tree, ornamental, forage crop and fruit breeding (Acquaah, 2007; Schepper *et al.*, 2001). For example, through induced polyploidy, breeders have developed Bouschet tetraploid grapes with more yield and juice content than the diploid progenitor Alicante (Olmo, 1952). Ornamental crops such as snapdragons and marigolds have been bred through chromosome doubling to improve the quality and size of their blossoms (Emsweller and Ruttle, 1941). A strong inverse correlation between DNA content and development rates in plants has been reported by several authors (Levin, 1983; Smith and Bennett, 1975). It has been attributed to lower auxin levels, reduced surface to volume ratio and altered nuclear surface to cell volume ratio (Levin, 1983; Acquaah, 2007). The slower growth rate of polyploids allows them to flower later and for a longer period of time than their diploid progenitors (Levin, 1983). This quality may be of interest especially in ornamental breeding.

Apomixis provides another avenue for use of polyploids in breeding. In apomixis seeds are produced asexually through parthenogenesis. Most apomictic plants are polyploid but most polyploid plants are not apomictic (Otto and Whitton, 2000). In plants capable of both sexual and asexual reproduction, polyploidy promotes the latter (Dhawan and Lavania, 1996). Obligate apomicts are the most desired of hybrids but little gain has been realized towards their development. However, it has been suggested that obligate apomicts may be induced through development of very high ploidy plants (Levin, 1983). An example of an obligate apomict achieved at high ploidy level is the octoploid of the grass, *Themeda triandra* (Levin, 1983).

Protoplast fusion technology has been utilized in many crops to generate allotetraploid somatic hybrids, and sometimes autotetraploids as a byproduct of the process. The greatest level of success for fruit breeding has occurred in citrus, primarily due to the highly successful model of fusing embryogenic suspension derived protoplasts with leaf-derived protoplasts, resulting in the regeneration of somatic hybrid plants from nearly 500 different parental combinations

(previously reviewed by Grosser *et al.*, 2000; Grosser and Gmitter, 2005). Protoplast fusion is also being utilized to produce somatic hybrids that combine complementary diploid rootstocks, which have shown good potential for tree size control. Tree size control has gained importance as a means of reducing harvesting costs, maximizing the efficiency of modern cold protection methodology, and facilitating the adaptation of new fruit production systems (Grosser and Gmitter, 2011).

A more promising approach would be to create allopolyploids between plants with diverse endogenous secondary metabolites. A unique and valuable characteristic of allopolyploids is that the secondary metabolites from the parental species are typically additive. That is to say those allopolyploids often produce all the enzymes and metabolites (including defense chemicals) of both parents. This could be particularly effective for combining the pest resistant characteristics of two species, and potentially contributing to a much broader, more horizontal form of pest resistance. A similar approach may have utility for enhancing tolerance to certain environmental stresses. Chromosome doubling is reported to have an apparent effect on many physiological properties of a plant. Increasing the chromosome number and related gene dose can sometimes enhance the expression and concentration of certain secondary metabolites and defense chemicals. The most discernable of these has been the increase in secondary as well as primary metabolism (Levin, 1983). The resulting increase in secondary metabolites, in some cases by 100 %, after chromosome doubling has been widely exploited in the breeding of narcotic plants such as Cannabis, Datura and Atropa (Dhawan and Lavania, 1996 ; Gonzalez and Weathers, 2003). *In vitro* secondary metabolite production systems that exploit polyploidism have also been developed. The production of the antimalarial sesquiterpene artemisinin has been enhanced six fold by inducing tetraploids of the wild diploid *Artemisia annua* L. (Gonzalez and Weathers, 2003). In addition, commercial synthesis of sex hormones and corticosteroids has been improved significantly by artificial induction of tetraploids from diploid *Dioscorea zingiberensis,* native to China (Heping *et al.*, 2008). Attempts have been made to improve the production of

pyrethrin, a botanical insecticide, by chromosome doubling of *Chrysanthemum cinerariifolium* (Liu and Gao, 2007). Other plants whose production of terpenes has increased following artificial chromosome doubling include *Carum cari, Ocimum kilmandscharicum* and *Mentha arvensis* (Bose and Choudhury, 1962; Levin, 1983). The enhanced production of secondary metabolites such as alkaloids and terpenes in polyploids may concurrently offer resistance to pests and pathogens. Experiments with diploid *Glycine tabacina*, a forage legume, and its tetraploid forms to measure resistance to leaf rust, *Phakopsora pachyrhizi*, established that 42 % of the tetraploid plants were resistant compared to 14 % of the diploid plants (Levin, 1983). Similar results were observed while comparing resistance to insects and the clover eel nematode between *Trifolium pratense* (red clover) tetraploids and diploids (Mehta and Swaminathan, 1957).

High frequencies of chromosome mutations are desirable in modern breeding techniques, such as tilling, as they provide new sources of variation. The multiallelic nature of loci in polyploids has many advantages that are useful in breeding. The masking of deleterious alleles, that may arise from induced mutation, by their dominant forms cushions polyploids from lethal conditions often associated with inbred diploid crops (Gaul, 1958). This concept has been instrumental in the evolution of polyploids during bottlenecks where there is enforced inbreeding (Comai, 2005). Mutation breeding exploits the concept of gene redundancy and mutation tolerance in polyploid crop improvement in two ways. First, polyploids are able to tolerate deleterious allele modifications post-mutation, and secondly, they have increased mutation frequency because of their large genomes resulting from duplicated condition of their genes (Gaul, 1958). The high mutation frequencies observed with polyploids may be exploited when trying to induce mutations in diploid cultivars that do not produce enough genetic variation after a mutagenic treatment. This approach has been used in mutation breeding of *Achimenes sp.* (nut orchids) by first forming autotetraploids through colchicine treatment followed by the application of fast neutrons and X-rays. In this study, the autotetraploids were found to have 20-40 times higher mutation

frequency than the corresponding diploid cultivar due to the large genome (Broertjes, 1976).

2.7 Ploidy determination

Determination of the ploidy level is therefore a central aspect in many plant research fields, including taxonomy, plant development and plant breeding. Ploidy determination is used extensively on plants regenerated from tissue culture as ploidy variation is common. Plants can easily adapt to ploidy changes. Measuring ploidy level is of highest importance in the final stages of haploid, triploid and polyploid induction programs. For breeding purposes, a large number of haploid, triploid and polyploid usually need to be tested, so an efficient ploidy analysis is a prerequisite for successful application. Methods to determine ploidy level may be direct (chromosome counting) or indirect (flow cytometry, stomatal size, chloroplast number of the guard cells and morphological observations) (Saria *et al.*, 1999). The ploidy analysis can be performed either by chromosome counting or by flow cytometry.

Cytology is a scientific discipline that deals with the study of cells, their physiological and structural properties. Cytological staining is particularly used to predict the total content of DNA or ploidy of an individual cell within the mixed population of cells. In this technique, smears and squashes of the tissues are prepared which are then observed under the microscope for studies on chromosome counting, morphological differences and karyotyping. Although this technique retains the morphological information, it is tedious and requires longer time for analyzing. The sample taken should be of small quantity for better analysis. A flow cytometer is a device that measures light fluroresced or scattered from particles. For ploidy analysis, the particles used are interphase nuclei obtained from somatic tissues. Nuclei are stained with DNA specific flurochromes that after absorption of excitation light emit fluorescence of longer wavelenth. The emitted light passes through specific optical filters and dichroic mirrors enabling only emitted light to reach the appropriate photomultiplier. On photomultipliers signals are detected and measured for each particle (nucleus) separately. More than 1000 nuclei per second can be measured. Acquired data are

processed by computer and results are displayed on screen in real time distributed to linear or logarithmic scales usually divided into 1024 channels. After that acquisition data can be stored and further analyzed using specialized software. For ploidy analysis, only a single parameter i.e the fluorescence excited by the DNA specific fluorochrome is usually measured although a number of parameters can be measured for each particle simultaneously.

In most of the common chromosome counting protocols generally root tips are used. When roots tip unavailable, young buds and leaves but also callus and cell suspensions are also used. For chromosome preparation and staining applied may have to be modified according to the tissue source and, certainly, to the species studied. Three main basic operations for the handling of mitotic chromosomes are common to most species and include: material collection (Germana, 2011a) and pre-treatment, material fixation (Kasha and Maluszynsky, 2003) and preparation and staining of chromosomes (Bhojwani and Razdan, 1996). The cytological procedures for visualizing chromosomes in woody plants may not be the same as in herbaceous species. The crucial step for chromosome counting involves proper chromosome squash preparation. It is very important to obtain sufficient well spread metaphase plates and proper physical separation of the chromosomes. The chromosome number being established during mitotic cell division, although counting of mitotic chromosomes arrested in metaphase is generally easier and faster (Fukui and Nakayama, 1996; Sharma and Sharma, 1999). However, method of chromosome staining applied for ploidy level analysis depends on plant species and chromosome size (Maluszynska, 2003). The most commonly used staining methods for chromosome counting are aceto-orcein or aceto-carmine (Smith, 1947), Feulgen (Feulgen and Rossenbeck, 1924) and DAPI (Kapuscinski, 1995) staining, which stain only the chromosomes while the cytoplasm remains clear.

Recently a large number of articles on ploidy determination by flow cytometry approach for haplo-diploidization or chromosome doubling are available like Dolezel, *et al.*, 2007b, Ochatt, 2008; Ochatt *et al.*, 2009. Flow cytometry using DNA selective flourochromes has been considered to be a fast

and reliable method for the measurement of nuclear DNA content (Dolezel *et al.*, 2007a). Flow cytometry requires single cell suspension (Shapiro, 1985) and as plant cells are usually compact tissues, methods had to be developed for the preparation of such suspensions. Flow cytometry is an extremely efficient technique with high degree of accuracy.

DEVELOPMENT OF GYNOGENIC HAPLOIDS

Chapter 3

Development of Gynogenic Haploids

3.1 Introduction

In tree species like *Hevea*, where conventional breeding is complicated and time consuming due to the long reproductive cycle, high degree of heterozygosity and complex reproductive biology, the potential of gamete biotechnology offers attractive avenues for crop improvement. Presence of chromosomes in single copy in gametes (n) and the concept of totipotency have together led to the *in vitro* development of haploids in many plant species. Development of homozygous diploids by doubling the chromosome number of *in vitro* raised haploids is a viable approach for making homozygous diploids in a single step. Such doubled haploids are of great importance in *Hevea* breeding, especially because of their increased efficiency of selection and also beneficial in various genetic studies like linkage mapping, mutation breeding, genetic transformation etc.

In vitro androgenesis and gynogenesis are two approaches often used for haploid induction in plants. Culture of unfertilized ovaries to obtain haploid plants from egg cell or other haploid cells of the embryo sac is called ovary culture, and the process is termed as gynogenesis. The first report of gynogenesis was by San Noem in 1976 in case of barley. Gynogenesis has been successfully applied to several species in which androgenesis is generally ineffective, such as sugar beet, onion and gerbera. The gynogenesis approach includes the culture of unfertilized ovaries, ovules or female gametophytes (Bohanec, 2009). Isolation and culture of intact ovules/ovaries has been the usual practice for inducing gynogenic haploids in many crop species. However, by culturing intact organs, there is always an ambiguity of the results due to the presence of tissues with different ploidy levels and the chance of obtaining good percentage of haploids is meagre. A mature embryo sac, which represents the female gametophyte, consists of 7 cells viz. an egg cell, two synergids, one central cell and three antipodals (Li and Ma, 2002).

Out of these, all cells other than the central cell are haploids whereas the central cell is a natural homozygous diploid. Hence, culturing intact embryo sac as the initial explant is a viable approach for ensuring haploidy or homozygosity in the regenerants. Present study explores the feasibility of employing embryo sac for the development of gynogenic haploids in *Hevea*.

3.1.1 Objectives

- Anatomical study of female flower
- Standardisation of techniques for the isolation of
    a) Components of FGU
    b) Intact embryo sac
- Optimisation of culture conditions for callus induction from FGU components as well as from embryo sac
- Embryogenesis and plant regeneration from haploid callus
- Ploidy determination

3.2 Materials and Methods

3.2.1 Explants

Mature female flowers (Fig. 5a & b) of *Hevea,* clones RRII 105 and RRII 414, were collected and surface sterilized using 0.1 % of mercuric chloride for 5 min followed by several rinses with sterile distilled water.

Perianth was removed and ovaries were isolated with the help of forceps and surgical blade. Intact ovules were isolated from the ovaries in aseptic condition under a microscope.

Fig. 5 *Hevea* inflorescence

a Male (◄···) and female flowers (⟵)

b Magnified view of mature female flowers

3.2.2. Anatomical study of female flower

Mature female flowers of *Hevea* were used for detailed anatomical investigation in order to understand the nature of embryo sac and its development. Fresh flowers were collected and fixed in FAA (formalin 5 ml, acetic acid 5 ml and ethyl alcohol 90 ml) after removing the perianth. The fixed samples were subjected to various steps of tissue processing such as dehydration through ethyl alcohol series and infiltration of paraffin wax into the tissue. After infiltration of paraffin wax, the samples were subjected to tissue embedding using Histoembedder and paraffin blocks were prepared containing the processed tissue. Serial section of the embedded tissue were taken at 5µm thickness using Rechert multicut rotary microtome and the micro-slides were prepared after dewaxing in TBA-Xylene series. The dewaxed sections were stained with toluidine blue 'O' (O'Brien *et al.*, 1964) and mounted in DPX for microscopy. The microslides were observed under Leica Aristoplan trinocular research microscope and image analysis was done using Leica Qwin wetzlar Image analysis software.

3.2.3 Isolation and culture of Female Germ Unit (FGU) components

Isolated ovules were subjected to enzymatic digestion for the release of FGU components. Enzymes tried in this experiment were cellulase R-10, pectolyase y-23, macerozyme R-10 and rhozyme HP-150. These enzymes were tried individually as well as in different combinations at concentrations ranging from 0.2 % to 1.0 %. The enzyme solutions were prepared in an osmoticum consisting of mannitol (6 M), MES (0.5 mM), potassium sulphate (0.1 %) and dextrose (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 (2-24 h). Ten ovules were kept per treatment and each treatment was replicated five times. Observations were recorded on the number of FGU components released.

To collect the released FGU components without debris, remnants of ovules were removed from the enzyme solution through sieving and the filtrate was centrifuged at 100 rpm for 2 minutes. The supernatant was removed and the pelleted FGU components were suspended in minimum volume of liquid MS basal medium. This suspension was cultured as droplets over two basal media viz. modified MS and modified K&M fortified with different combinations of 2,4-D and BA (0.09 - 4.4 μM) and incubated in the dark.

3.2.4 Isolation of embryo sac

Different methods were attempted for the isolation of embryo sac in intact and undamaged condition.

a) Mechanical isolation

Direct isolation of embryo sac from intact ovule was attempted by carefully removing the outer integument followed by the inner integument. Utmost care was given while separating the integuments, to prevent any mechanical damage to the thin walled fragile embryo sac.

b) Partial enzymatic digestion

In this method the outer integument of the ovule was removed mechanically, followed by exposure of the remaining ovule to digestion enzymes. Combinations of the enzymes cellulase and pectolyase at different concentrations (0.2 - 0.6 %) and for different incubation periods (2-6 h) were tried. The embryo sacs were then separated from the partially digested inner integument.

c) Isolation after pre-treatment

The isolated ovules were subjected to pre-treatment by culturing in nutrient media for different time intervals before isolation of embryo sac. Solid and liquid pre-treatments using two different basal media viz. MS (Murashige and Skoog, 1962) and K&M (Kao and Michayluk, 1975), enriched with various levels of sucrose (2-15 %) for different time intervals (2 - 14 days) were experimented for the successful isolation of intact and viable embryo sacs.

3.2.5 Callus induction and proliferation

Embryo sacs isolated from the ovules were cultured for callus induction in four different basal media, modified MS*, modified K&M*, Nitsch (Nitsch and Nitsch, 1969) and WPM (Lloyd and McCown, 1980) fortified with different growth regulator combinations of 2, 4-D, Kin and BA (0.92 - 6.3 μM). All the cultures were incubated at 23°C in the dark.

Calli induced from the embryo sacs were transferred, for proliferation, to modified callus induction medium where the growth regulator concentrations were reduced to one fourth. The culture conditions were kept the same and the cultures were incubated in the dark.

3.2.6 Embryo induction

Various parameters influencing embryo induction were evaluated. Two different basal media viz. modified MS and K&M were experimented. A mixture of amino acids containing arginine (15 mgl^{-1}), proline (100 mgl^{-1}), glutamic acid (200 mgl^{-1}), glutamine (150 mgl^{-1}) and glycine (30 mgl^{-1}) which was found

* Composition of modified MS basal medium given in annexure I

* Composition of modified K&M basal medium given in annexure II

effective in promoting *Hevea* embryogenesis (unpublished data) was incorporated in the basal medium. Banana powder (500 mgl^{-1}) and coconut water (10 %) were also added as organic supplements. Effect of growth regulators on embryo induction was studied by supplementing different levels of GA_3 (0.57 - 2.89 μM) and BA (1.8 - 9.2 μM). Also different concentrations of the gelling agent phytagel (0.2 - 0.8 %) and the carbohydrate source sucrose (4 - 14 %) were incorporated at so as to evaluate their influence on embryo induction.

3.2.7 Embryo maturation and germination

Embryos emerging from the embryogenic callus were allowed to pass through globular and heart shaped stages in the same medium, after which they were transferred to maturation media. Two basal media MS and K& M with low concentrations of IBA (0.74 μM) and Kinetin (2.3 μM) were tried for embryo maturation. Role of ABA on embryo maturation was experimented by supplementing various levels (1.13 μM - 3.78 μM) of ABA. Half of the cultures were kept in the dark itself while the other half was kept under cool, white fluorescent light.

Mature embryos were transferred to germination media consisting of MS basal fortified with different concentrations of BA (2.2 μM – 8.8 μM) and GA_3 (0.57 μM - 1.73 μM), along with 0.49 μM IBA.

All media were adjusted to pH 5.7 with 1N KOH before autoclaving (15 min, 120°C).

3.2.8 Statistical analysis

All experiments were conducted in completely randomized design (CRD) and the data were analyzed using SPSS 16.0 software. The data were transformed into square root and analyzed using ANOVA with a significance of $p \leq 0.05$.

3.2.9 Confirmation of ploidy

3.2.9.1 Cytological analysis

The proliferating callus obtained from the embryo sac was subjected to cytological analysis (Rekha *et al.*, 1993). Callus with actively dividing cells were

pre-treated with 0.2 mM 8-hydroxyquinoline for 5 h at 4°C. After this pre-treatment 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).

3.2.9.2. Flow cytometric analysis

The ploidy analyzer I (Partec GmbH, Germany) was used to determine the ploidy level of the embryo sac 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 (Rashmi and Rakhi, 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.

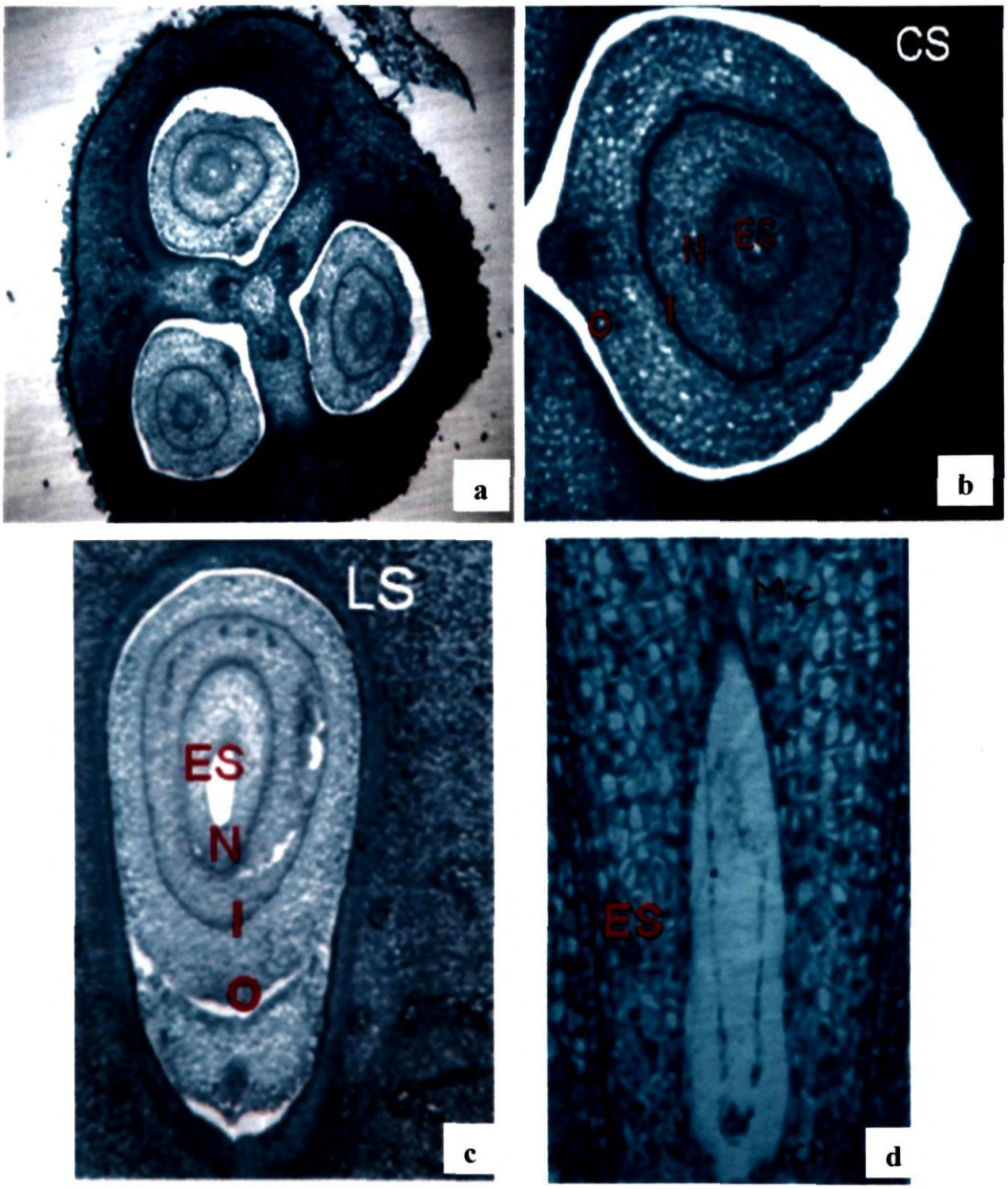
3.3 Results

3.3.1 Anatomy of female flower

Anatomical studies of *Hevea* ovule from mature flower showed the presence of embryo sac consisting of the egg apparatus (egg cell along with the synergids). The mature female flowers prior to anthesis (Fig. 8a) were found to be suitable for isolation of female gametes and gametophytes. Fully developed embryo sac with visible components could be observed in mature flower.

* Composition of galbriath's buffer given in annexure III

Hevea flower is tricapillary in nature, with an ovule in each locule arranged in axile placentation Figure 6a. The cross and longitudinal sectional views show that the fully developed nucellus enclose the embryo sac and covered with the outer and inner integuments (Fig. 6b&c). The embryo sac is clearly visible within the ovule (Fig. 6d). Egg apparatus consisting of the egg cell and the synergids is present as triangularly arranged at the micropylar end (Fig. 6e). In Fig. 6f egg cell is more distinguishable.



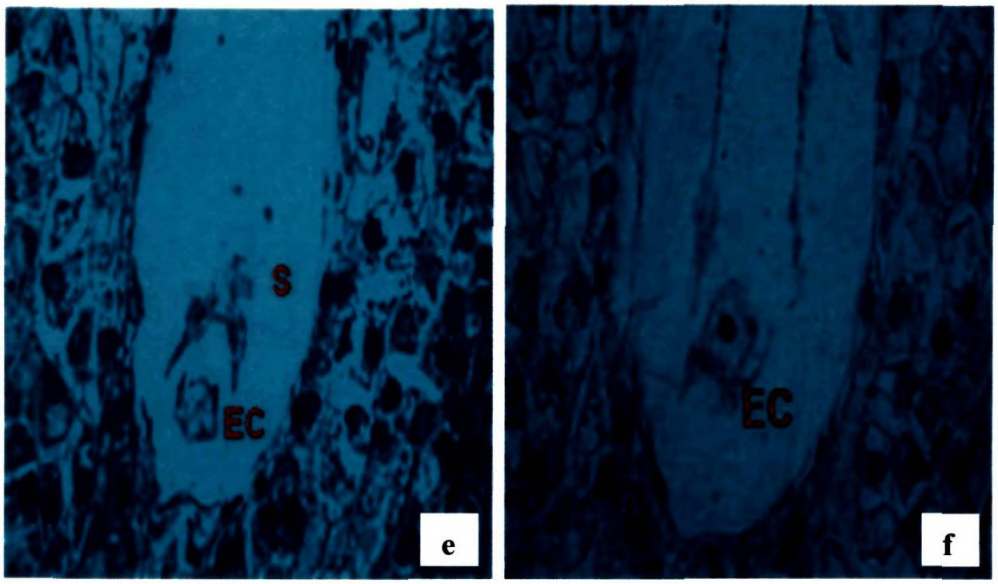


Fig. 6 Anatomical details of female flowers of *Hevea*

a Cross section of the Gynoecium of female flower showing axial placentation (x210)

b & c Cross & longitudinal sections of *Hevea* ovule (x530)

d Embryo sac (ES) showing the micropylar (Mic) and chalazal end (CH) (x850)

e Presence of egg apparatus consisting of egg cell (EC) and synergids (S) (x2100)

f Egg cell (EC) present in the micropylar end of the embryo sac (x2100)

3.3.2 Isolation and culture of female germ units

The isolated ovules were ivory in colour with reddish markings on the outer integument (Fig. 8b). In the case of *Hevea*, normally 3 ovules could be isolated from a single ovary. Among the different enzyme combinations tried, cellulase - macerozyme mixture was found to be effective for the release of FGU components. A combination of 0.6 % cellulase and 0.4 % macerozyme was found to be most suitable for the release of FGU components from the ovules (Table 2).

A maximum number of 24 FGU components were released from 20 ovules upon enzymatic maceration. Higher concentrations of both the enzymes resulted in

the damage of released FGU components. Figure 7 shows the effect of incubation period on the release of female germ unit components. Release of FGU components initiated after 4 h and the number of released FGU components steadily 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.

Table 2 Number of FGU components released from *Hevea* ovules in presence of different enzyme combinations

Cellulase (%) → / Macerozyme (%) ↓	0.2	0.4	**0.6**	0.8	1
0.2	2	5	8	9	7*
0.4	3	3	**24**	15	10
0.6	4	2	14	16	12
0.8	2	1	11	13	8
1.0	-	-	7	5	6

*** No. of FGU components released**

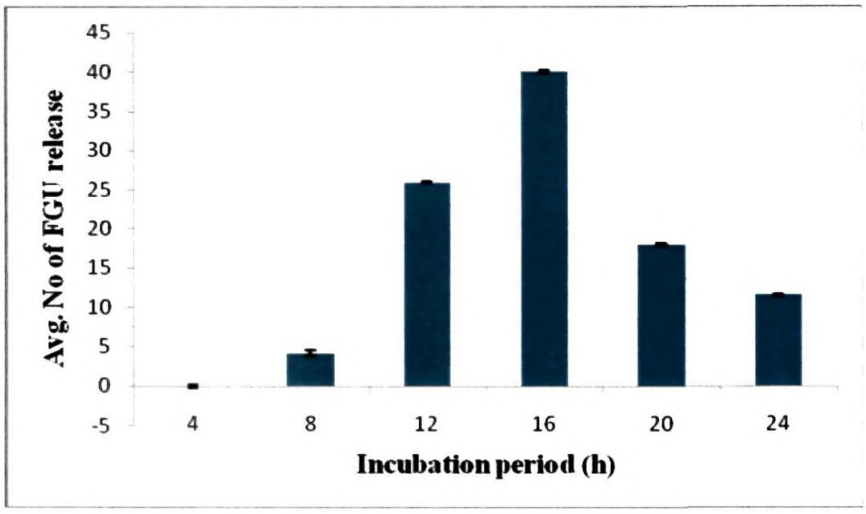


Fig. 7 Effect of incubation period on the release of FGU components from *Hevea* ovules

Sequential release of female germ unit can be observed from the ovule isolated from mature female flower when subjected to enzymatic digestion (Fig. 8c). Fig. 8d shows the initiation of egg cell release from the micropylar end, followed by the release of central cell (Fig. 8e). In Figure 8f various FGU components released within 16 h can be observed.

The liberated FGUs (egg cell, central cell, synergids and antipodals) 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 (Fig. 9b), followed by the egg cell which contains a large vacuole (Fig. 9d). Antipodals are smaller in size than the egg cells (Fig.9c). Synergids are initially visible as attached with the egg cell and later they get separated (Fig. 9a). Released FGUs could be pelleted upon centrifugation (Fig. 9e).

Observations were taken periodically on the pelleted FGUs cultured over different nutrient media for callus induction. Even though an increase in the size of the cells and initiation of cell division in a few cells were noticed, further development or callus initiation has not so far been obtained.

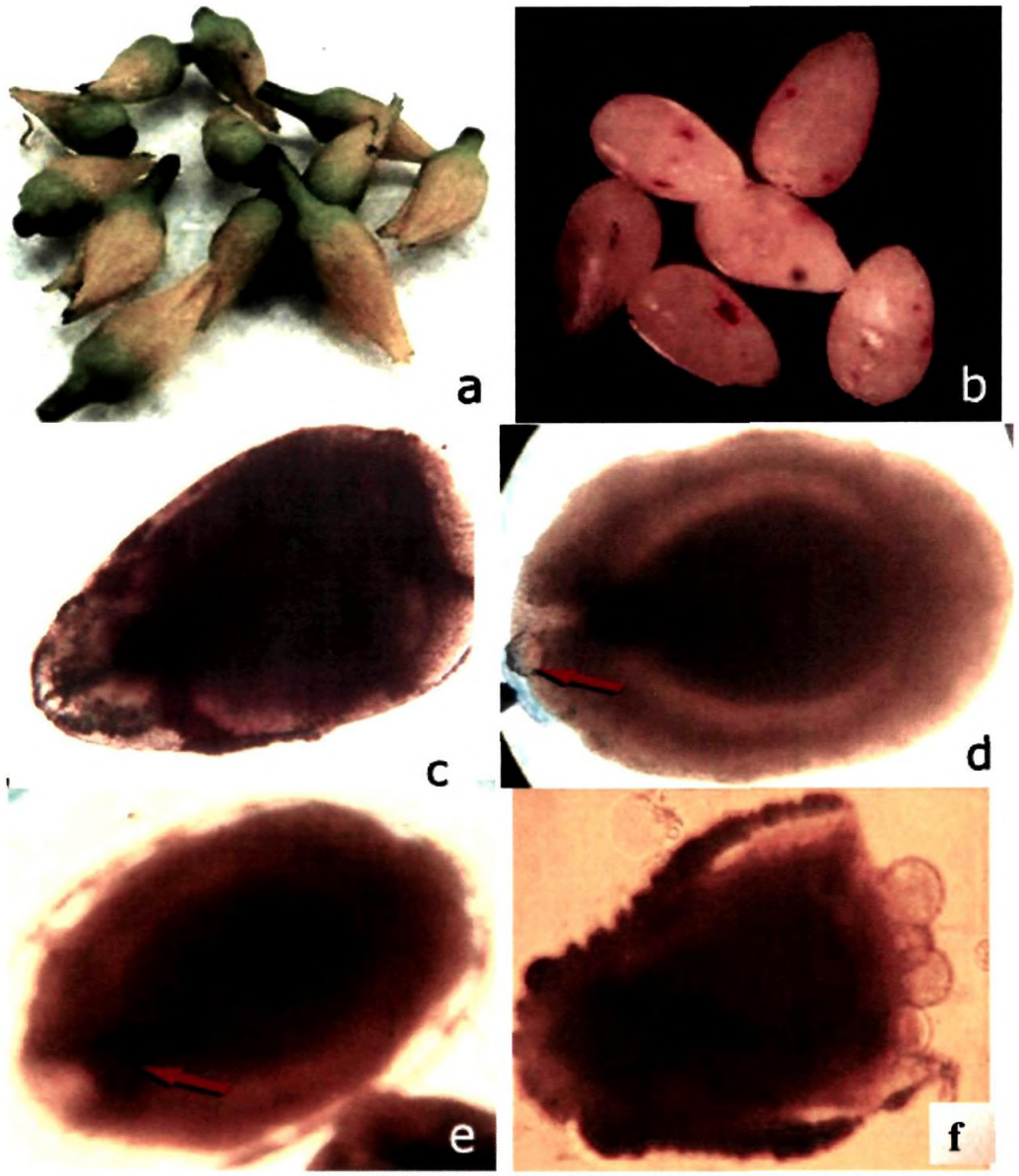


Fig. 8 Different stages of FGU releas

a Mature female flowers,

b Isolated ovules

c Enzymatic digestion (2h after incubation),

d Initiation of egg cell release from the micropylar region at 4 h,

e Release of central cell,

f Released FGU components (egg cell, synergids, central cell and antipodals) after 16 h

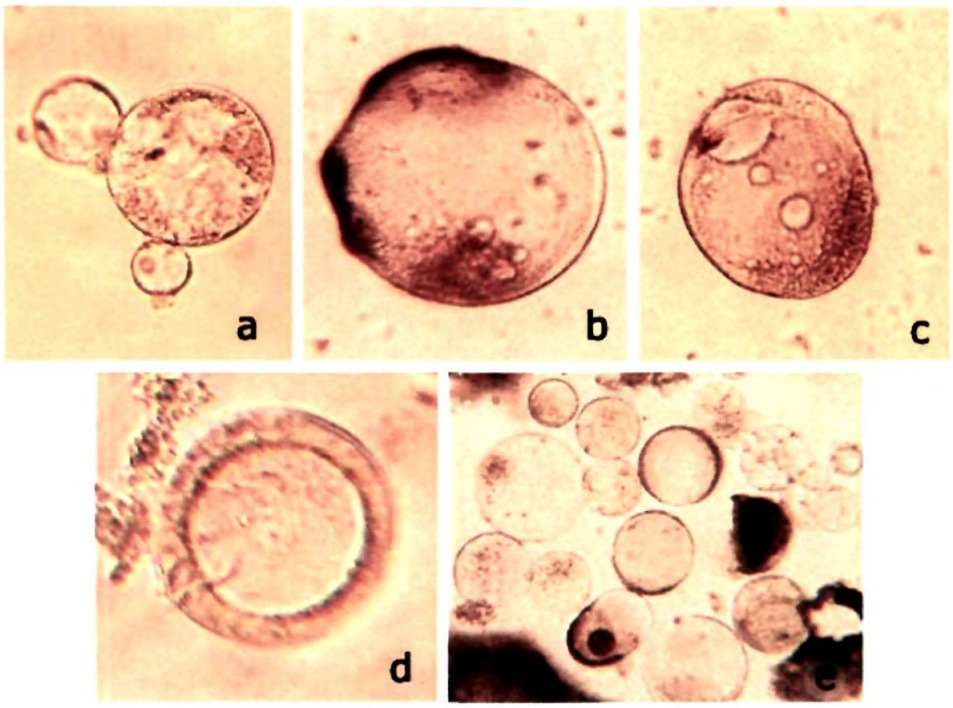


Fig. 9 Magnified view of the FGU components (x 100)

a Egg cell with synergids, b Central cell, c Antipodal cell

d Egg cell with large vacuole

e Pelleted FGU components used for culturing

3.3.3 Isolation of embryo sac

Successful isolation and culture of embryo sacs followed by callus induction, proliferation, embryo induction and germination has been achieved.

Embryo sacs could be isolated from the ovules in all the three methods attempted. However the number and the culture response of embryo sacs isolated through each technique showed considerable difference.

a) Mechanical isolation

Mechanical isolation of embryo sac from the intact ovule was found to be much laborious and tricky (Fig. 10 a&b). As a result, number of intact embryo sacs that could be recovered per unit time was quite low in this method. In order to overcome these difficulties, modifications such as partial enzymatic digestion of the inner integuments and inclusion of a pre-treatment phase before mechanical isolation were attempted.

b) Partial enzymatic digestion

Isolation of embryo sacs from partially digested ovule devoid of the outer integument was found to be easier compared to mechanical isolation from intact ovule (Fig. 10c&d). Percentage of intact embryo sacs that could be isolated was found to be much higher in this method. A combination of cellualse (0.5 %) and pectolyase (0.3 %) was effective in partially digesting and loosening the inner integument, thereby helping easy separation of the embryo sac. An incubation period of 2 to 3 h was optimum for the release of embryo sacs.

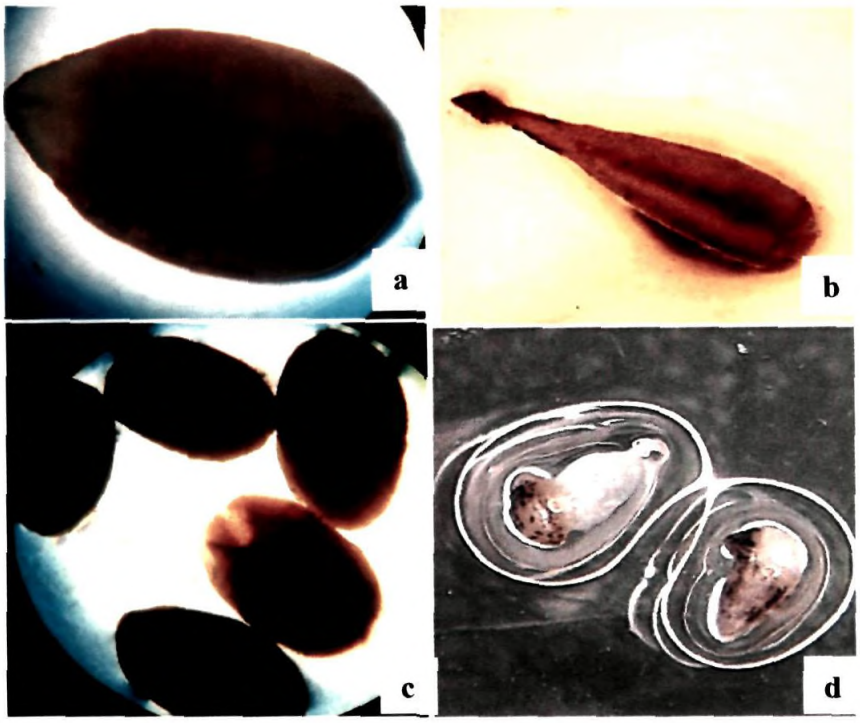


Fig. 10 Isolation of embryo sac

a & b Mechanical isolation

c & d Isolation after partial enzymatic digestion

c) Pre-treatment of the ovules

Since the number of embryo sacs that could be isolated through mechanical means was too low and embryo sacs isolated through partial enzymatic digestion process were not responsive in culture towards callus induction, pre-treatment of the ovules before mechanical isolation was tried in both liquid and solid media.

Of these two, liquid medium was found to be better when compared to solid medium. Ovules maintained in liquid medium were healthy and more in number whereas shrinking and browning of ovules was observed in the solid medium (Fig. 11 a&b). Also, it was observed that high sucrose concentrations (8-10 %) do have a favourable effect on ovule development as evidenced by the swelling of the ovule. In the liquid medium, swelling of the ovules was observed from the 3rd day onwards and maximum number of swollen ovules could be obtained within two weeks (Fig. 11c&d). Further increase in the incubation period beyond two weeks resulted in browning of the integuments followed by gradual decaying of the ovules as well as the embryo sacs inside.

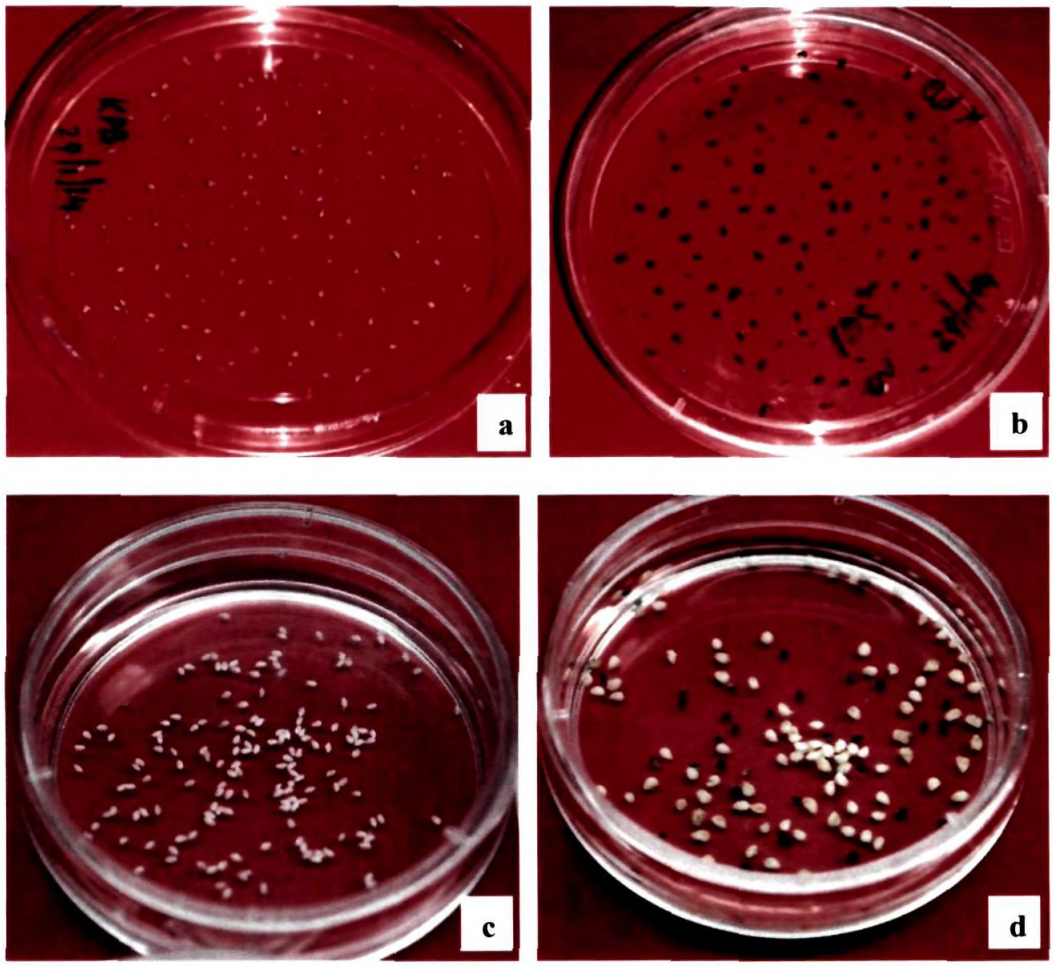


Fig. 11 Response of ovules in pre-treatment media

a Ovules in solid medium on the day of isolation

b Ovules in solid medium 2 weeks after isolation

c Ovules in liquid medium on the day of isolation

d Ovules in liquid medium 2 weeks after isolation

Swelling of ovules upon culturing in the pre- treatment medium was identified as a positive indication for easy isolation of intact and viable embryo sacs. It was observed that during swelling of the ovules, apart from the increase in size, both the integuments as well as the embryo sac wall became thicker, thereby helping in easy handling and removal of the integuments.

Among the different liquid pre-treatment media tried, MS basal medium enriched with 10 % sucrose was found to be the most suitable one for the isolation of embryo sacs, with around 63 % isolation frequency (Table 3). In K&M basal medium the percentage of intact embryo sacs that could be isolated was less when compared with the MS basal medium.

Thus, among the three methods employed for embryo sac isolation, pre treatment of the ovule followed by mechanical isolation has been proved to be the most efficient one enabling the separation of intact embryo sacs with regenerative potential.

Table 3 Effect of basal composition and sucrose in the pre-treatment media on embryo sac isolation

Basal medium	Sucrose concentration (%)					
	2	4	8	**10**	12	15
K&M	16.6	21.6	33.3	38.3	23.3	23.3*
MS	25	50	53.3	**63.3**	45.4	41.6

*** percentage of intact embryo sacs isolated**

3.3.4 Callus induction and proliferation

Callus induction could be observed in the embryo sacs isolated mechanically as well as through pretreatment method. In the case of embryo sacs isolated using partial enzymatic digestion method, only morphological changes like irregular swelling of the embryo sacs could be noticed when cultured in the callus induction medium (Fig. 12a&b). Among the different basal media and hormone combinations tried, callus initiation was observed in modified K&M medium supplemented with 2,4-D and Kinetin (Fig. 12c&d).

A combination of 4.6 μM 2, 4-D and 2.7 μM Kinetin was found to be the most suitable one giving a callus induction frequency around 36 % (Table 4). Callus proliferation occurred upon subculturing to medium with reduced growth regulators 0.53 μM NAA, 0.88 μM BA and 0.90 μM 2, 4-D (Fig. 12e).

Table 4 Effect of Kin and 2,4-D on callus induction from embryo sac

2,4-D (μM) → / Kin (μM) ↓	0.92	2.7	**4.6**	6.3
0.92	0(0.707)	3.3(1.95)	18.3(4.33)	13.3(3.68)*
2.7	0(0.707)	23.30(4.87)	**36.2(6.09)**	11.65(3.47)
4.6	6.6(2.6)	13.3(3.7)	33.3(5.81)	0(0.707)
6.3	11.6(3.47)	18.3(4.33)	23.3(4.87)	0(0.707)

CD= 0.47

***Callus induction frequency**

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

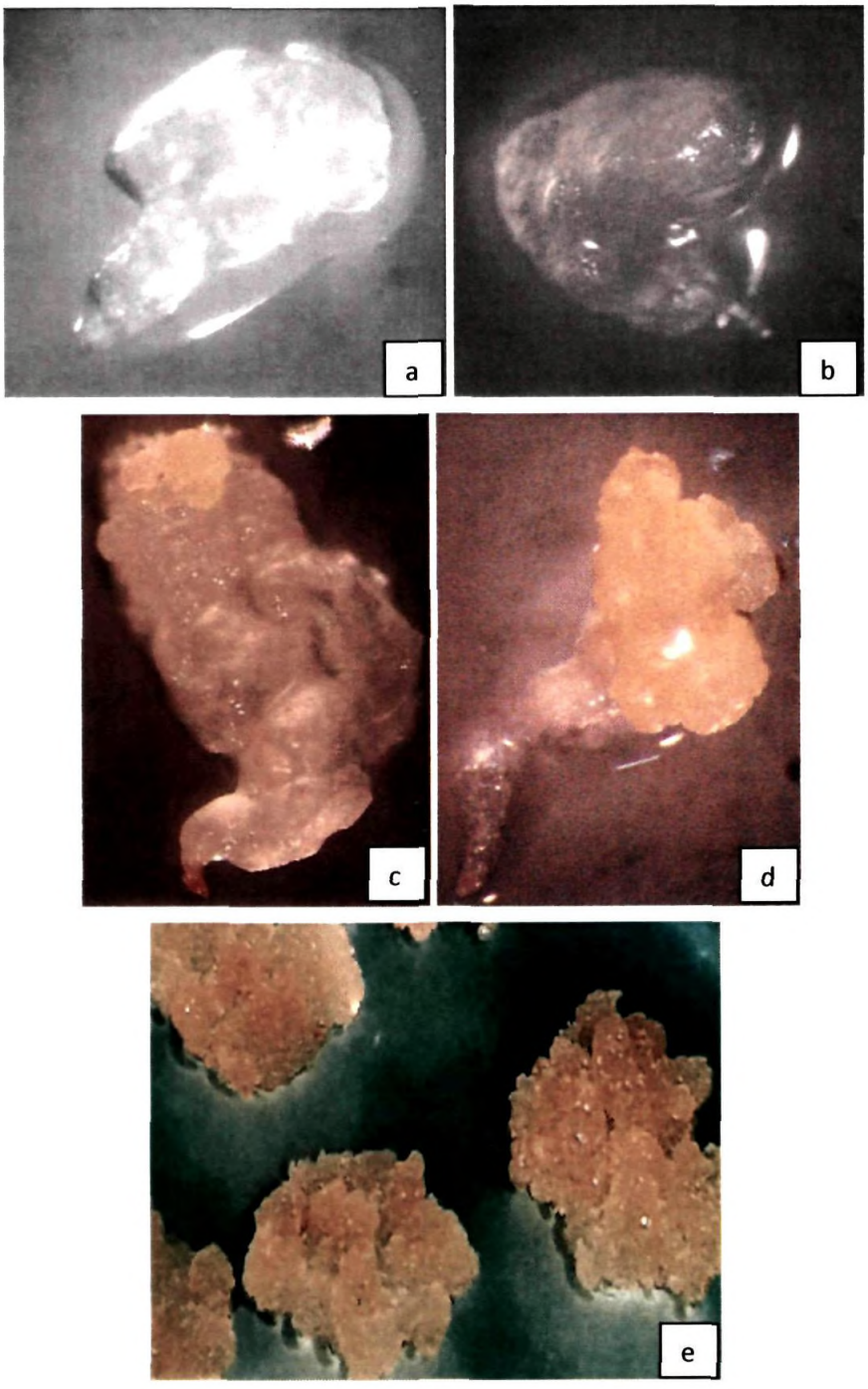


Fig. 12 Response of cultured embryo sacs isolated through different methods

a & b **Morphological changes observed in the cultured embryo sacs isolated from partially digested ovule (No callus initiation)**

c & d ***Callus initiation at the micropylar end of cultured embryo sacs*** **obtained from pretreated ovule**

e **Proliferated callus**

3.3.5 Embryo induction

Out of the different growth regulator combinations, one with GA_3 (1.73 μM) and BA (7.2 μM) was found to be most suitable for the emergence of embryogenic callus (Fig. 14a) from the proliferated calli. Embryogenic callus could be induced at a frequency of 18 % at this combination in both basal media tried (Table 5).

Table 5 Effect of GA_3 and BA on embryo induction from embryo sac derived callus

GA_3 (μM) → / BA (μM) ↓	0.57	1.15	**1.73**	2.31	2.89
1.8	0.0 (1.0)	0.0 (1.0)	0.0(1.0)	0.0(1.0)	0.0(1.0)*
3.6	0.0(1.0)	5.6 (2.6)	7.6(2.9)	7.00(2.8)	2.00(1.7)
5.4	5.3(2.5)	9.3(3.2)	14(3.9)	12.6(3.7)	11.3(3.5)
7.2	8.0(3.0)	14.3(3.9)	**18.0(4.4)**	15.3(4.0)	10.6(3.4)
9.2	9.3(3.2)	11.0(3.5)	11.6(3.6)	10.0(3.3)	7.6(2.9)

CD=0.10

***Embryo induction frequency**

Data were subjected to square root transformation and transformed means are given in parenthesis.

Experiments with sucrose and phytagel revealed that embryo induction frequency varied with different concentrations of these two components. Increase in sucrose concentration to 8 % resulted in a rise in the embryo induction frequency to 20 %. Further experiments with different levels of phytagel along with 8 % sucrose could enhance embryo induction frequency significantly. Maximum embryo induction frequency of 30 % was obtained in the presence of

0.5 % phytagel whereas it decreased at still higher concentrations of phytagel (Fig. 13). Embryos at different developmental stages *viz.* globular, heart and cotyledonary stages were obtained upon prolonged culture in the same medium (Fig. 14c&d).

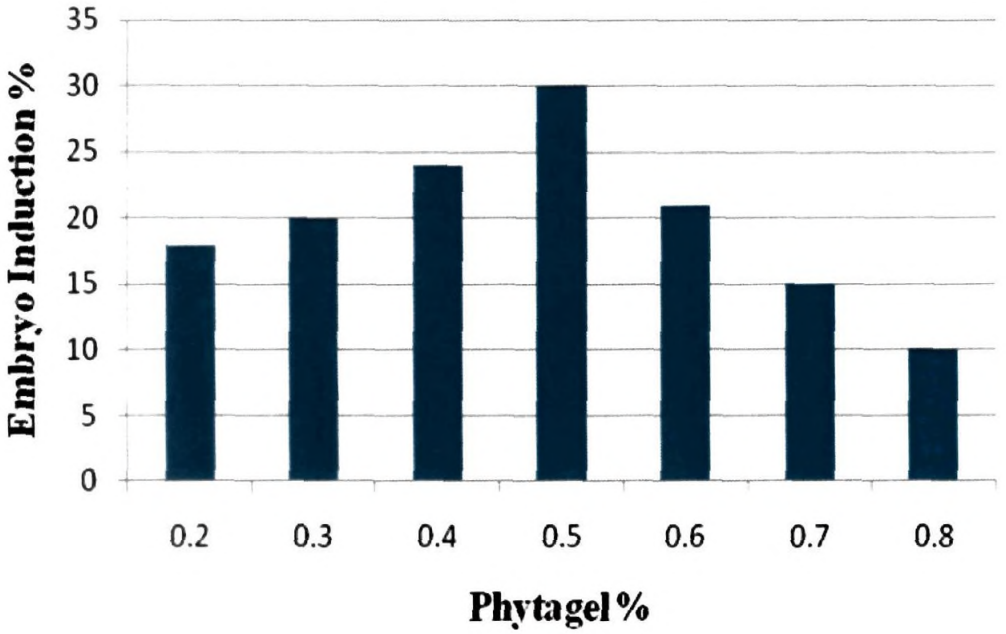


Fig. 13 Effect of phytagel on embryo induction

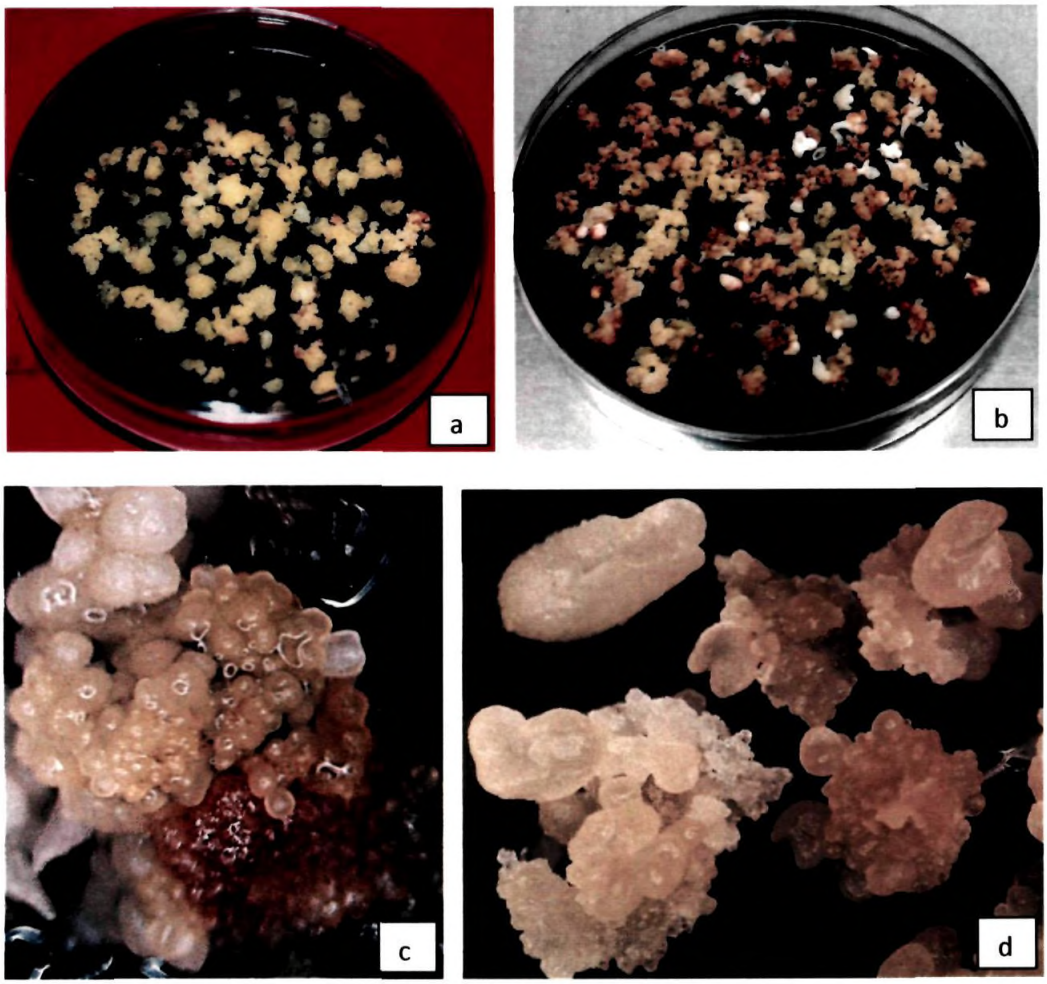


Fig.14 Different stages of embryogenesis

a Embryogenic callus

b Embryo induction

c & d Embryos at different developmental stages – globular, heart and cotyledonary

3.3.6 Embryo maturation and germination

Embryo maturation was obtained (Fig. 15a) in modified MS medium fortified with low levels of IBA (0.74 µM) and Kinetin (2.3 µM). Dark incubation for 1-2 weeks followed by transfer to light showed positive response towards maturation. Mature embryos with bipolar differentiation were obtained at a low frequency (2-3 %) after 3 weeks of culture in the maturation medium (Fig. 15a).

Inclusion of ABA (1.89 µM) in the maturation medium could enhance the maturation frequency to 8 %.

Embryo germination was observed in a few cultures where the medium was supplemented with 0.49 µM IBA, 1.15 µM GA_3 and 4.4 µM BA. A few of the embryos developed shoot and root (Fig. 15b). Plant regeneration from the germinating embryos is still awaited.

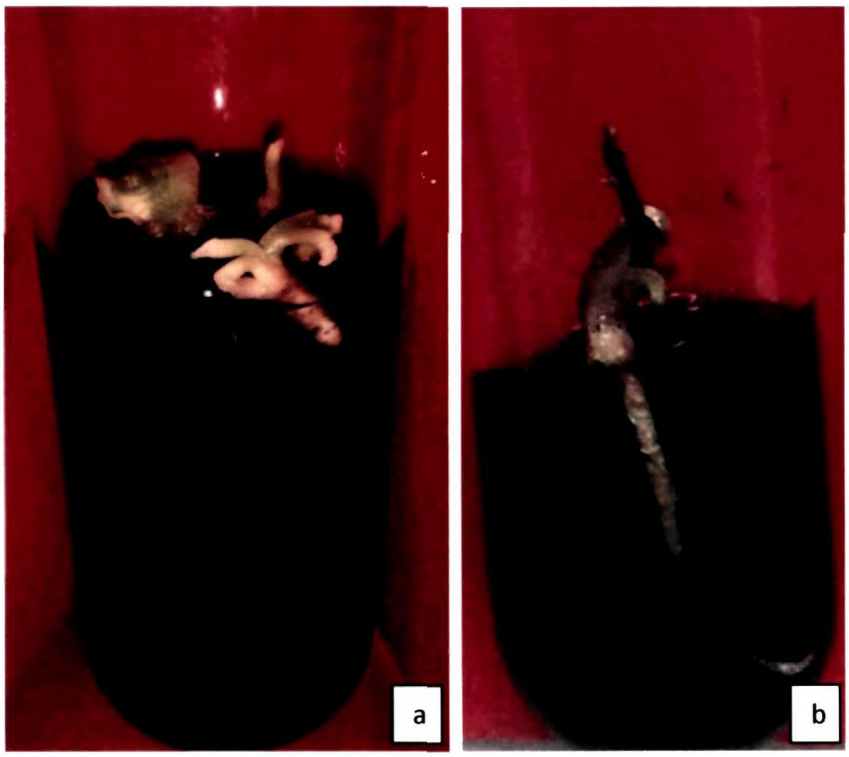


Fig. 15 Embryo maturation and germination

a Mature embryos

b Germinating embryo

3.3.7 Ploidy determination

3.3.7.1 Cytological analysis

From the cytological analysis of the proliferated callus derived from the embryo sac, a chromosome count of n=18 was obtained (Fig. 16), indicating that the callus from embryo sac contains only single set of chromosomes, which is half of the double set of the chromosomes present in the diploid tissue of *Hevea*.

Fig. 16 Chromosome count n=18 from embryo sac callus (x 400)

3.3.7.2 Flow cytometry analysis

This was performed as a confirmatory test for the haploid nature of callus obtained from the embryo sac. Nuclei content was compared between the control and the sample (Fig.17). The control diploid sample got the highest peak at channel 400 whereas for the test sample, peak was at channel 220, indicating the haploid nature of the callus with a DNA content less than the control.

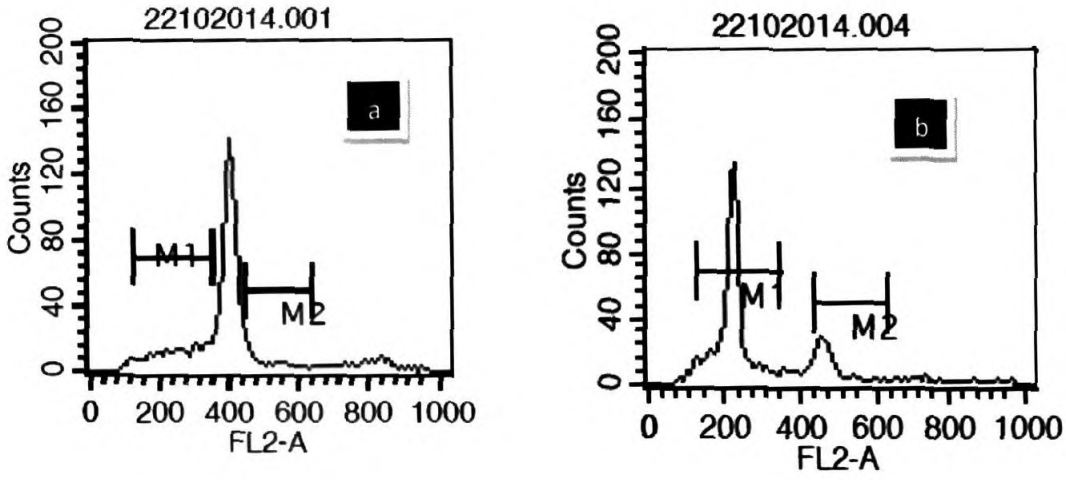


Fig. 17 Histogram showing peaks for

a Diploid (Immature anther derived callus)

b Haploid (Embryo sac derived callus)

3.4 Discussion

3.4.1 Explant

Earlier research on *in vitro* gynogenesis concentrated only on ovary/ovule culture in many crop species. However, culturing intact organs leads to an ambiguity of the results due to the presence of tissues with different ploidy levels. Also the chance of obtaining haploids is meagre due to the suppressing effect of somatic cells (ovule wall and the nucellus) on haploid cell development. San and Gelebart, in 1984, reported that the induction of excess callus from the ovary wall hindered haploid development. Hence embryo sac culture was followed in the present investigations. It is possible to induce division of the egg cell or some other cells of the embryo sac by external treatments and form an embryo (Bhojwani and Thomas, 2001). Since all the cells of the embryo sac are haploid, the plants derived from them are also haploid (Sant and Prem, 2013). *In-vitro* culture of embryo sac protoplasts were attempted by Zhao *et al,* however they could not make the cell mass in the embryo sac to divide (Zhao *et al.*, 2013).

3.4.2 Anatomy

Mature female flowers prior to anthesis were found to be suitable for isolation of female gametes and gametophytes in *Hevea* because fully developed embryo sac with visible components could be observed in the ovules of mature flowers. It has been reported that the developmental stage of the ovule is very important in the process of gynogenesis. Direct examination of the embryo sac before culturing to identify its stage of development is generally difficult. Hence anatomical and histological methods are used (Mukhambetzhanov, 1997). The structure of embryo sac is clear and can be well studied in mature flowers because in the fully developed stage, the embryo sac comprises the egg cell and other female germ units. As reported by San and Gelebart, (1986), the late staged ovary with mature embryo sacs gave good results in Barley (*Hordeum vulgare*). San and Demarly, (1984) reported that mature or nearly mature embryo sacs from barley, wheat, maize, sugarbeet and lettuce were generally more responsive *in-vitro.* In these species, all nuclei of the embryo sac (egg cell, synergids, polar nuclei, antipodal cells) were able to divide and yield embryos or callus. In contrast

to our results in the present study, in species like onion, sugar beet, squash, sunflower, gerbera, *Hyoscyamus muticus* and *Melandrium album* the flowers are inoculated at early stage of development (Bohanec, 2009).

Anatomical studies of flowers of Euphorbiaceae are scarce, but include notable studies of *Manihot utilissima Pohl* (*Manihot esculenta Crantz*) microsporogenesis and megasporogenesis (Graner, 1935). The embryo sacs of angiosperms are generally 8-nucleate, but all the cells may not be visible on the same plane in the same microtome section because each cell has its own different position. Moreover, the synergid cells are placed in such a manner that they surround the egg cell, where they provide nutrition to the egg and direct the sperm cells to the specific site for fertilization to occur (Russell, 1993). In our experiment also all the cells within the embryo sac were not visible in the same plane, even though the embryo sac could be clearly identified. We could observe only the egg apparatus, consisting of the egg cell and synergids, in the anatomical section of the mature female flower (Figs. 6e&f), in which the egg cell and synergids are arranged triangularly. Similar observations were made by Bittencourt and Mariath, (2002). According to them, in the recently formed egg apparatus, the synergids and egg cell are triangularly arranged and immediately after cellularization, the egg cell can be easily distinguished from the synergids.

3.4.3 Isolation and culture of FGU

In order to induce haplogenesis, the first attempt we have made was culturing of FGU's. Among the different enzyme combinations tried for the release of FGU components, cellulase - macerozyme mixture was found to be the most suitable one. Release of FGU components was observed from the ovules incubated for 12 to 16 h in a mixture of 0.6 % cellulase and 0.4 % macerozyme. It is already established that enzyme combinations and concentrations for effective release of cells varies with species. Yoichiro *et al.*, in 2006 reported the effectiveness of 2 % cellulase and 0.5 % macerozyme along with 0.05 % pectolyase combination in the isolation of viable egg cells from *Alstroemeria*. They also found that when whole ovules were treated with enzyme solutions for 2–3□h, the ovules could be dissected with glass needles under an inverted microscope since the outer

integument was digested by these commercial enzymes. In view of this, in our experiment, several procedures for excising a part of the ovule were tested in order to enhance enzyme solution permeability and thus render ovule tissues more amenable to dissection. Length of the incubation period was increased and effective release of various components of FGU was obtained within 12 – 16 h without using any process of dissection.

Increasing concentrations of both the enzymes resulted in the damage of released FGU components. Earlier researchers have reported that high enzyme concentrations adversely affected the survival (Huang and Russell, 1992; Ratchada *et al.*, 2009) and functions of isolated female gametophytic cells (Holm *et al.*, 1994; Leduc *et al.*, 1995). FGUs including central cell could be successfully isolated in *Torenia fournieri* (Mol, 1986) and *Nicotiana tabacum* (Fu *et al.*, 1997) by using enzyme solution containing macerozyme R-10. Van der *et al.*, 1993, reported that egg cells could be isolated only with a combination of enzymatic maceration and mechanical manipulation of the dissected ovules from perennial ryegrass.

In this study, even though the isolation of FGUs was standardised, on culturing the FGUs no callus induction could be achieved. Insufficient media requirements or culture conditions or the damage during enzymatic isolation may be the reason. Hence direct isolation and culture of intact embryo sac was attempted as the alternative step in our study.

3.4.4 Isolation of embryo sac

Embryo sacs could be isolated from *Hevea* ovules in all the three methods attempted. However the the efficiency of isolation and the response of embryo sacs isolated through each technique showed considerable difference.

Out of the three different technique used for the isolation of embryo sac from *Hevea* ovule, pre-treatment of the ovule was found effective in yielding good amount of intact embryo sac.

In mechanical method, number of intact embryo sacs that could be recovered per unit time was quite low, mainly due to the small size of the flowers

and fragile nature of the embryo sacs. Similar results were reported in Pearl Millet (Lakshmi *et al.*, 2002). They used mechanical techniques for the isolation of intact embryo sacs from the ovule, but only low percent of embryo sacs could be obtained.

In order to increase the recovery percentage of embryo sacs different methods were adopted by several workers. Laurie *et al.*, (1999) developed a novel technique involving mechanical sectioning of maize ovaries using vibrotome, in which serial sectioning of the ovaries resulted in the partial isolation of embryo sacs, making them accessible and easily manipulated. Forbes, (1960) and Solntseva and Levkovsky, (1978) had used enzymatic maceration of ovule followed by manual dissection with needles under a binocular microscope to isolate embryo sac.

We have tried partial enzymatic digestion whereby higher percentage of embryo sacs could be isolated from the ovules using a combination of cellulose (0.5 %) and pectolyase (0.3 %) for an incubation period of 2 to 3 h. This enzyme combination was effective in partially digesting and loosening the inner integument, thereby helping easy separation of the embryo sac. However the embryo sacs isolated through this technique did not respond positively when cultured for callus induction.

The third technique attempted in our study included different pre-treatments of the isolated ovules followed by mechanical isolation of the embryo sacs and was found to be the most efficient method for embryo sac isolation. Pre treatment of ovules in liquid MS medium, containing 10% sucrose, for 2-3 weeks followed by mechanical isolation was found to be most ideal for isolation of intact embryo sacs. Maintaining the ovules in liquid medium helped in the restoration of large number of healthy ovules whereas shrinking and browning of ovules was observed in the solid medium. The reason may be the availability of more nutrients in liquid medium due to continuous shaking where each ovule is better exposed to the nutrients. In wheat, similar results were observed by Gusakovskaya and Nadzher (1994). Swelling of ovules in liquid medium with high sucrose levels might be attributed to the presence of sucrose. In addition to its role as a carbon

source, sucrose content in the culture medium may also play additional role as an osmoticum. High sucrose concentration has been demonstrated to increase the percentage of zygotic embryogenesis in isolated embryo sacs of maize (Mol *et al.*, 1993). As indicated by Hayashi *et al.*, (1986), who applied ovary slice culture 40 days after self-pollination of *L. formosanum,* a high concentration of sucrose and a high pH are favourable for ovule development.

Isolation of embryo sacs after pre-treatment has been found to be much advantageous in *Hevea*. Firstly, this method is much easier compared to mechanical isolation from fresh ovules. This may be due to the enlarged size of the ovule and the integuments becoming much thicker and harder compared to the thin integuments in the fresh ovules. Consequently, handling and removal of the integuments becomes much easier and without damage to the embryo sac beneath. Secondly, the embryo sacs isolated through this technique are the most responsive ones in culture, capable of callus induction and further development. The reason may be that this method avoids exposure of the ovules/embryo sacs to the digestion enzymes which is suspected to cause damage to the embryo sac and making it recalcitrant.

3.4.5 Callus induction and proliferation

Mechanically isolated embryo sacs, both directly and after pre treatment, responded positively towards callus induction whereas no callus initiation, except some irregulr swelling, occurred in embryo sacs isolated through partial enzymatic digesstion method. Lack of callus initiation in these embryo sacs might be due to the damaging effect of the digestion enzymes to which the ovules were exposed. Vincent *et al.*, (1989) also have discussed the effect of enzyme solution which sometimes leads to the accumulation of lipid like molecules inside the embryo sac. Wagner *et al.*, (1988) used enzymatic digestion to isolate embryo sac from ovules of Zea mays. Exposure to enzymatic mixture and agitation did not cause any cellular damage to the isolated embryo sac, but had altered the nature of physical associations between the constitutive cells of the embryo sac. Enzymatic maceration technique was successful in the clearing of embryo sac elements as well as in the isolation of intact embryo sacs of *I. glandulifera*, however the

embryo sacs isolated by enzymatic maceration of ovules were not viable (Hussein, 2013). The reliability of such techniques often varies from plant to plant according to structural complexity as well as the size of ovary and ovule.

Among the different basal media and hormone combinations tried, callus initiation was observed in modified K&M medium supplemented with 2,4-D and Kinetin. MS medium did not favour callus induction. 36 % callus induction frequency was obtained when embryo sacs were cultured on K&M medium fortified with 4.6 µM 2, 4-D and 2.7 µM Kinetin. Similar growth hormone combinations were used for callus induction from immature inflorescence of *Hevea brasiliensis* (Sushamakumari *et al.*, 2000). Regarding the basal medium, in contrast to the present result, modified MS basal has been identified as effective for callus induction from different explants in *Hevea* (Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000). Requirement of a more complex medium (K&M) for callus induction from embryo sac while a simpler MS medium was sufficient for callus induction from inflorescence or anther tissue is justifiable based on the grounds that the single celled embryo sac needs much more nutrients/ complex medium for initiation of cell division and callus induction compared to the other tissue explants like inflorescence and anther.

Callus proliferation occurred upon subculturing to medium with reduced growth regulators i.e 0.53 µM NAA, 0.88 µM BA and 0.90 µM 2, 4-D in MS basal medium. In case of Mulberry, gynogenic haploids could only be produced on presence of cytokinin supplemented medium as reported by Lakshmi and Ravindran, 1991.

3.4.6 Embryo induction

MS basal medium supplemented with 8 % sucrose and 0.5 % phytagel along with the other additives like amino acids, banana powder and coconut water has been found to be suitable for embryogenesis. Emergence of embryogenic callus at a frequency of 30 % was obtained when the above medium was fortified with 1.73 µM GA_3 and 7.2 µM BA. Modified MS medium has been reported earlier to favour somatic embryogenesis in *Hevea* from anther and inflorescence

explants (Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000). Sushamakumari *et al.*, 2000 has also reported significant increase in embryo induction frequency in presence of GA_3.

Chang and Hong (1981) discussed that like pollen, embryogenesis can be induced in embryo sac *in vitro*. In species like rye grass and Lolium, exogenous cytokinins improved the frequency of somatic embryogenesis (Norstog, 1970; Dale, 1980) similar to our results.

High sucrose concentration (8%) has improved percentage of embryogenesis. Kobayashi *et al.*, (1993) have also reported that in sweet potato high sucrose concentration (8 - 10 %) in the culture medium was helpful in the induction of embryos. Similar results were reported by Campion *et al.*, (1992) in case of onion. Gerdakaneh *et al.*, (2009), tried to develop somatic embryos from immature flower buds of strawberry and found that higher number of embryos were induced in the presence 6 % sucrose but the number of embryos decreased as the percentage increased from 6 to 12 %. In another report (Blanc *et al,* 1999) found that somatic embryo production was significantly higher with maltose when they tested the effect of different carbohydrates on early somatic embryogenesis of *Hevea brasiliensis.*

3.4.7 Embryo maturation and germination

In the presence of low levels of IBA (0 .74 μM) and Kinetin (2.3 μM) in MS medium embryo maturation occurred at a low frequency (2 - 3 %) and the same could be augmented to nearly 8 % by the addition of ABA (1.89 μM). In 1995, Carron *et al.,* through a study of the zygotic model in *Hevea,* revealed the need to induce a maturation phase prior to embryo germination, primarily for the completion of cauline meristem formation, accumulation of starch / protein and desiccation. For somatic embryo maturation, the most frequently used PGRs is ABA, which is responsible for promoting maturation and inhibiting cell-cycle progress, growth and germination (Curaba *et al.*, 2003, Gazzarini *et al.*, 2004). Cardoza and D'Souza, (2002) reported the maturation of somatic embryos from globular to heart and cotyledon stages in *Anacardium occidental L.* on MS medium

containing 2.0□μM ABA. However it was reported, in contrast to our results, that a high ABA concentration of 10.0□μM combined with a high concentration of sucrose enhanced embryo maturation in *Hevea brasiliensis* (Cailloux *et al.*, 1996). One explanation is that the embryos employed in our study were raised from haploid cells of embryo sac, hence the response will be different as compared to somatic tissue.

The results on embryo germination indicate that more media manipulations are necessary for optimising a protocol for plant regeneration from haploid embryos. Among the media combinations tried in this experiment, embryo germination occurred in MS medium supplemented with 0.49 μM IBA, 1.15 μM GA_3 and 4.4 μM BA. However the percentage of germination was quite low. One possible explanation for this low germination frequency is the haploid nature of the embryos. Although haploid plants have been reported in many angiosperms species, there have been only a few reports on tree crops. Production of haploid plants of woody species appears to be much more difficult than herbaceous plants (Ahuja, 1993). The development and growth of haploids *in vitro* encounters considerable problems that make tissue maintenance and regeneration difficult and can even lead to tissue decay (Aleza *et al.*, 2009). The poor performance of haploid tissue may be related to the enhanced expression of recessive or sub-lethal genes in comparison with heterozygous diploids (Germana and Chiancone, 2001). In haploids, the lack of a homologous set of chromosomes also means that the plants are infertile (Ferrie and Caswell, 2011). It was reported in the case of sugar beet (Chodacka and Baranski, 2013) that the regeneration efficiency depends on both the genotype and ploidy level. Haploid individuals tend to be smaller in size, less vigorous, more sensitive to disease and environmental stress and most importantly are sterile (Segui-Simarro and Nuez, 2008b). Hence, for practical purposes, it is desirable to obtain doubled haploids through diploidisation of the haploid embryogenic callus through chemical treatment using antimitotic agents like colchicine, followed by embryo development and plant regeneration.

3.4.8 Ploidy determination

Callus derived from anther was used as the control in both cytological and flow cytometry analysis. Previous cytological observations in *Hevea* indicated that regenerated plantlets induced from another culture were diploid (Wang *et al.*, 1984; Jayasree *et al.*, 1999). Cytological analysis of the embryo sac derived callus showed a chromosome count of n=18. Previous cytological investigations in *Hevea brasiliensis* have confirmed the chromosome complement in the somatic cells as $2n = 2x = 36$ (Ramaer, 1935; Saraswathyamma *et al.*, 1984) indicating its diploid nature. Hence it can be confirmed that the callus obtained from embryo sac is haploid in nature.

In flow cytometry analysis, it can be inferred from the histogram that the test sample had peak at channel 220, just half of the control value, indicating the haploid nature of the callus. Similar assumptions were predicted by Rashmi and Rakhi, (2013) in case of dedifferentiated callus of haploid origin from unfertilized ovaries of tea through flow cytometry analysis. Flow cytometer has been used by Sirisom and Te-chato, (2013) and Srichuay *et al.*, (2014) to find the ploidy of the calli and the somatic embryos from cultured anther of *Hevea* and concluded that the regenerated plantlets from anther calli were diploid, same as of the mother plant.

DEVELOPMENT OF TRIPLOIDS

Chapter 4

Development of Triploids

4.1 Introduction

Triploidy, a genomic condition that is favourable for vigour and vegetative productivity has 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.

Traditionally triploids are produced by hybridization between tetraploids and diploids. *In vitro* regeneration of plants from 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 (Hoshino *et al.*, 2011). 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. Moreover, the endosperm exsists 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. 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.

4.1.1 Objectives

- Somatic embryogenesis from endosperm tissue using direct and indirect method
- Isolation and culture of protoplast from endosperm tissue

4.2 Materials and methods

4.2.1 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.

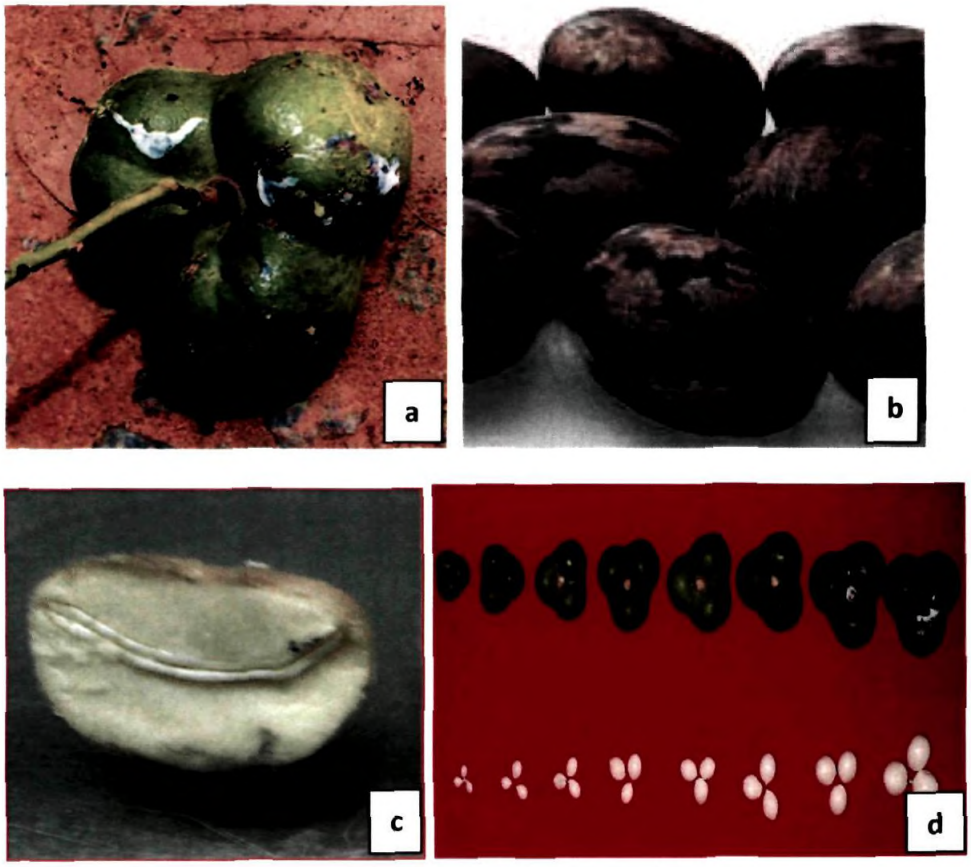
a) Mature seeds

Seeds (Fig. 18b) were collected from mature fruits (Fig. 18a) 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. The inner integuments as well as the embryos were removed and the remaining tissue, the endosperm (Fig. 18c), was cut into small pieces and used for further process.

b) Immature seeds

Young fruits at different maturity stages of 1-10 weeks (Fig. 18d) 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. 18e), 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. 18f).

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.



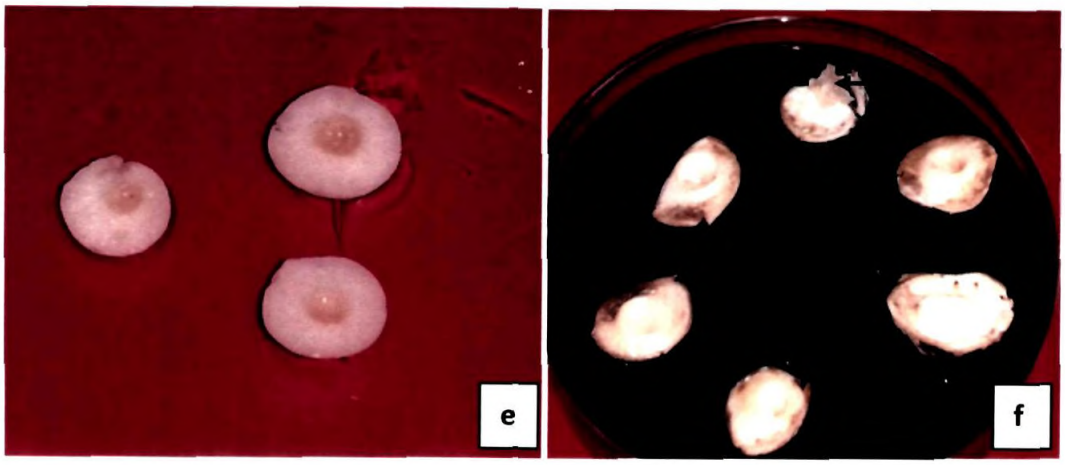


Fig. 18 Explants used for endosperm culture

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

4.2.2 Somatic embryogenesis from endosperm tissue

4.2.2.1 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.

4.2.2.2 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.

4.2.3 Protoplast culture

4.2.3.1 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.

4.2.3.2 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 optimisation of osmoticum. In addition, the osmotic stabilizer MES (5mM) was incorporated in all the above solutions and autoclaved at 120°C and 15lb/sq pressure for 15 min and stored at 25°C.

4.2.3.3 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 0.22μm), stored at 4°C and thawed to room temperature before use.

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* composition of CPW medium in Annexure IV

Thin slices of endosperm tissue were incubated in different enzyme solutions and incubated for different time intervals (2 - 6 h).

4.2.3.4 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.

Formulae:

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

4.2.3.5 Culturing of protoplasts

Three different basal media viz. MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980) and KM (Kao and Michayluk, 1975) 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 28°C. They were examined routinely to detect cell division, multiplication, growth and callusing.

4.2.4 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 $p \leq 0.05$.

4.2.5 Confirmation of ploidy

4.2.5.1 Cytological analysis

The callus obtained from endosperm tissue was subjected to cytological analysis following the procedure described in section 3.2.10.1

4.2.5.2 Flow cytometric analysis

Friable callus from endosperm tissue was used for flow cytometric analysis as per the protocol described in the section 3.2.10.2.

4.3 Result

4.3.1 Somatic embryogenesis from endosperm tissue

4.3.1.1 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. 19a&b). At higher concentrations of 2, 4-D and Kin there was no callus induction (Table 6). 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. 19c).

Table 6 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

***callus induction (%) from mature endosperm tissue**

Data were subjected to square root transformation and transformed means are given in Parenthesis

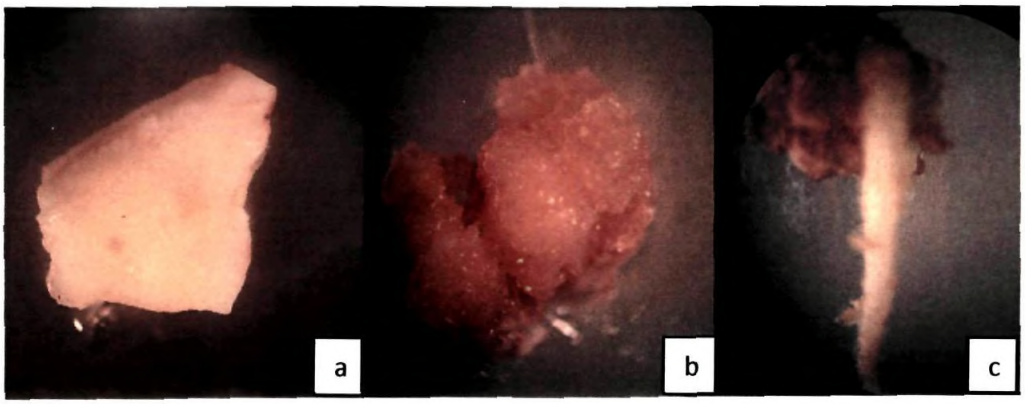

Fig. 19 Culture of mature endosperm

a & b Cultured mature endosperm isolated from mature seeds and induction of callus from this cultured endosperm.

c Root induction from mature endosperm

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) (Fig. 20a&b). At higher concentrations of these growth regulators, especially BA, the callus induction was nil (Table 7).

Table 7 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

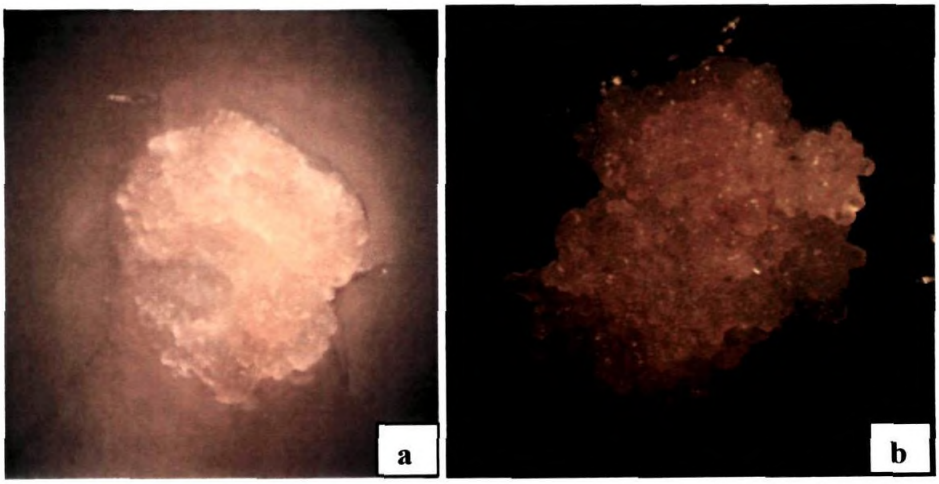

Fig. 20 Culture of immature endosperm

a &b Cultured immature endosperm and induction of callus from this cultured endosperm

4.3.1.2 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. 21a&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.

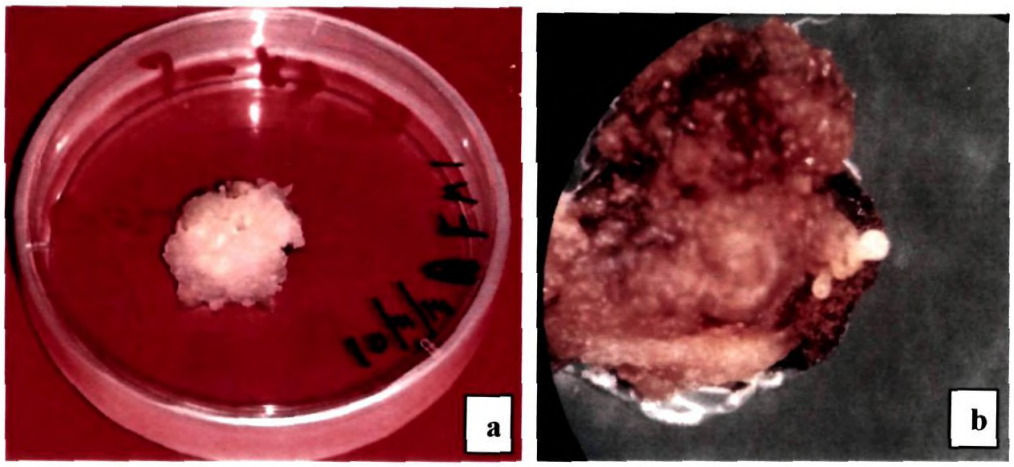

Fig. 21 Direct embryogenesis

a Cultured immature endosperm tissue (8 week old)

b Direct embryo induction

4.3.2 Protoplast isolation and culture

4.3.2.1 Protoplast isolation

Among the different stages of immature fruits cultured, endosperm development was observed in the 8 - 10 week old fruits (Fig. 18d). 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.

4.3.2.2 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 8). 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.

Table 8 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	0.2	0.4	0.6	0.8	1
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**

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. 22). 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.

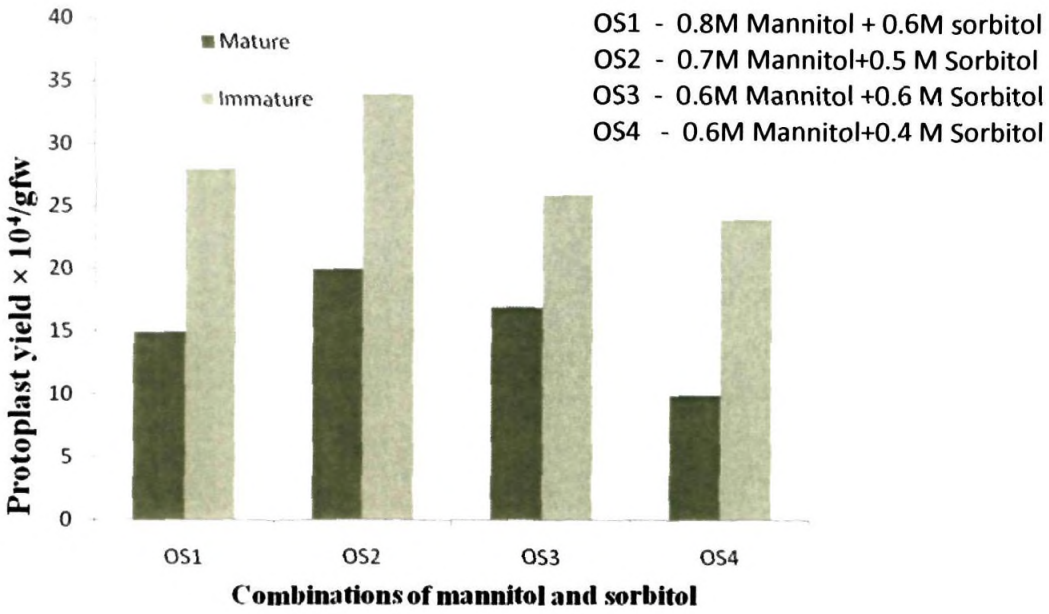

Fig. 22 Effect of osmoticum on protoplast release

4.3.2.3 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. 23).

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.

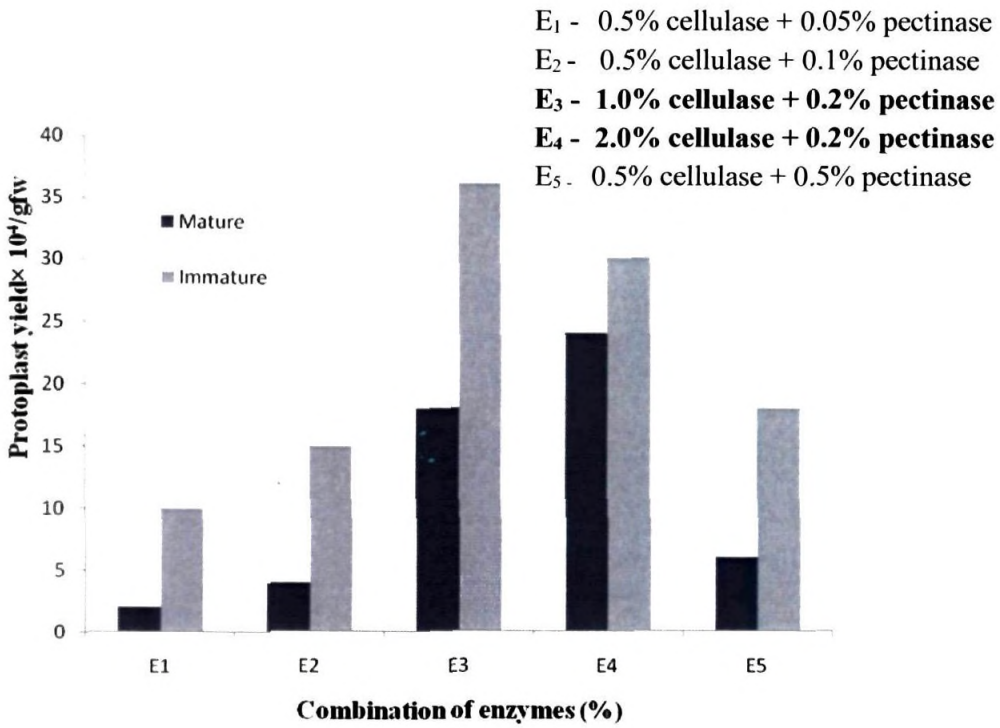

Fig. 23 Effect of digestion enzymes on protoplast release

With the optimized parameters of osmotica and enzymes, endosperm tissue isolated from immature seeds was found to be ideal for isolation of protoplasts (Fig. 24b) 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. 24a). Turbidity may be due to the release of oil granules from the mature endosperm tissue.

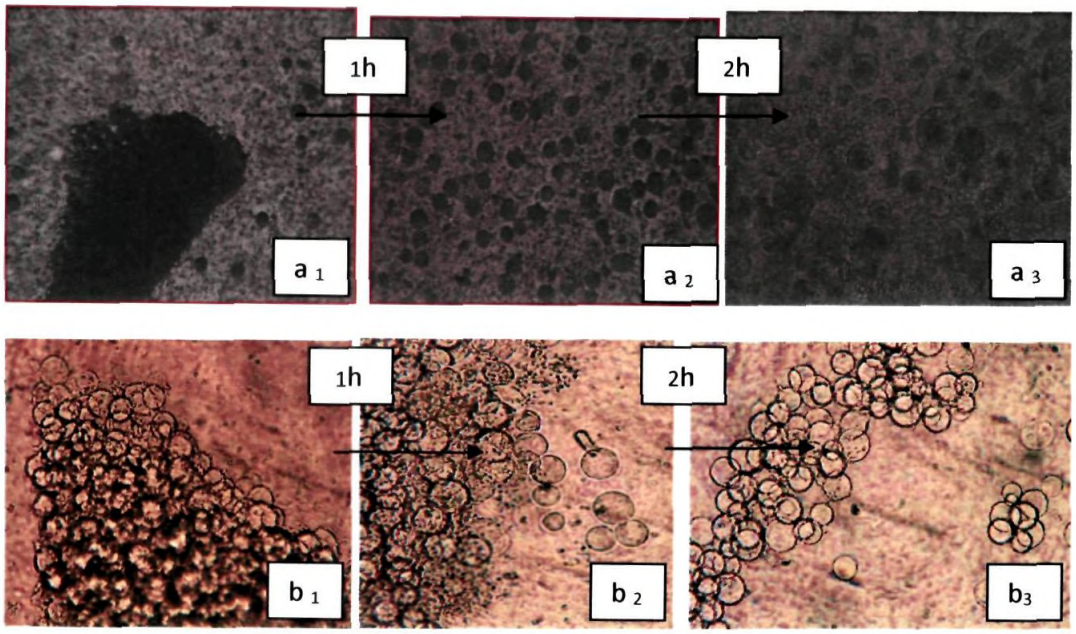

Fig. 24 Protoplast release from mature and immature endosperm

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

4.3.2.4 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. 25a&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. 25c&d). Further division and microcolony formation from these protoplasts is awaited.

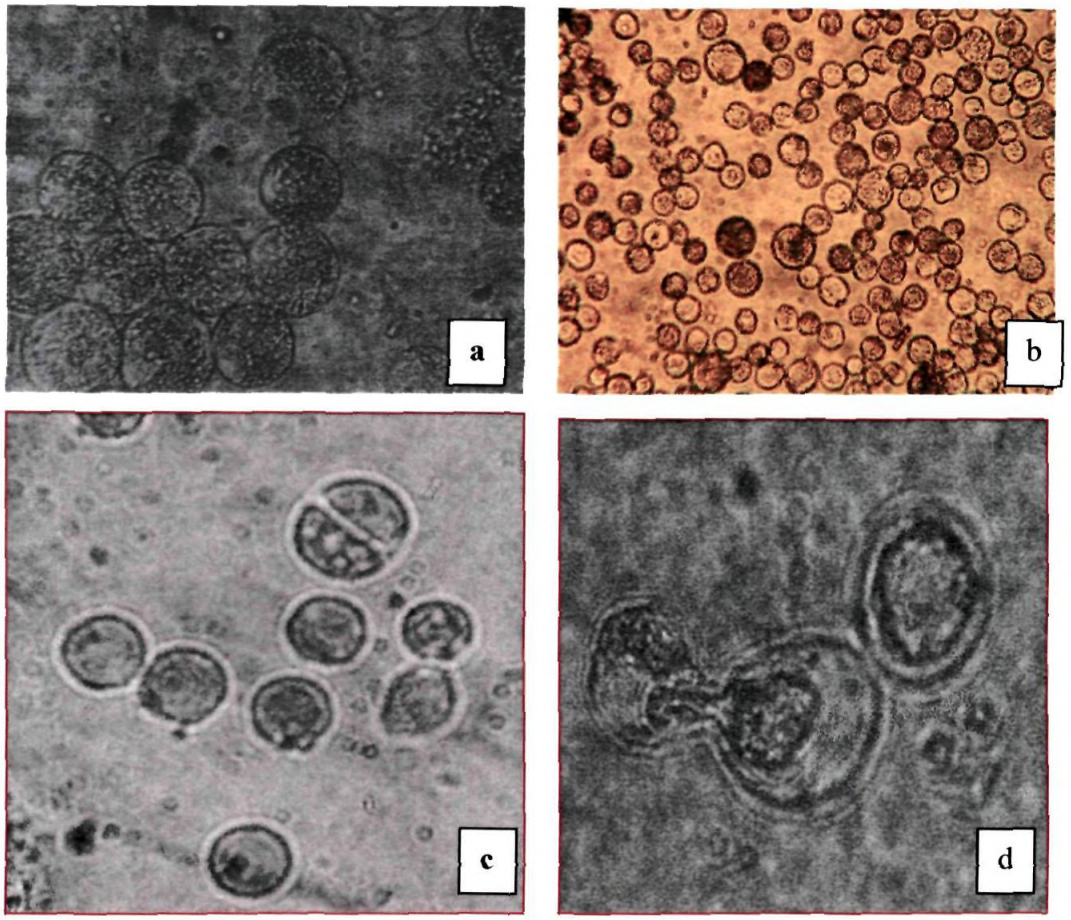

Fig. 25 Purification and culture of released protoplasts

a Purified protoplasts from mature endosperm tissue

b Purified protoplasts from immature endosperm tissue

c & d Protoplast division

4.3.3 Ploidy determination

4.3.3.1 Cytological analysis

Cytological studies showed a chromosome count of 3n=54 (Fig. 26) 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.

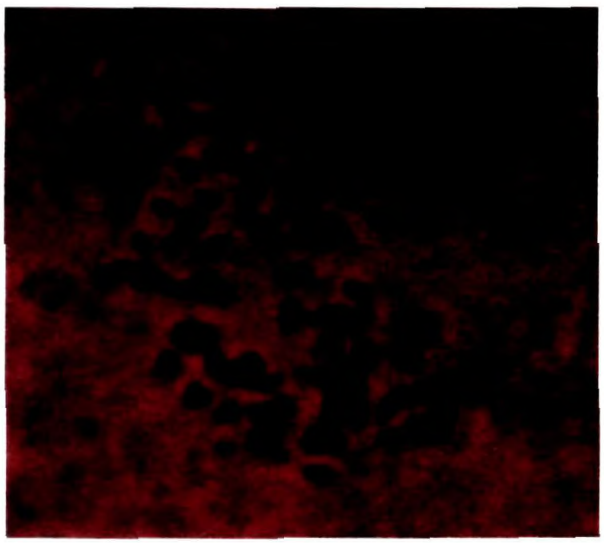

Fig. 26 Chromosome count from endosperm derived callus (3n=54)

4.3.3.2 Flow cytometer

Using flow cytometer, ploidy of the endosperm derived callus was determined (Fig. 27). 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.

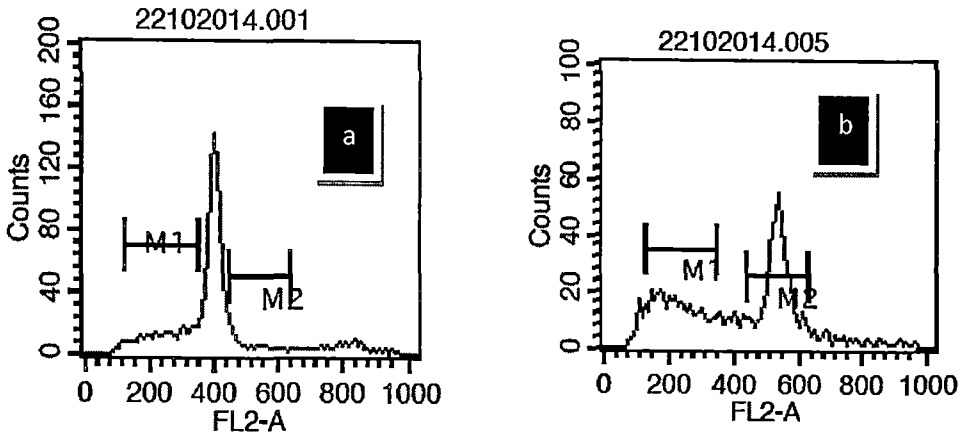

Fig. 27 Histogram showing peaks for

a Diploid (Immature anther derived callus)

b Triploid (Endosperm derived callus)

4.4 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.

4.4.1 Somatic embryogenesis from endosperm tissue

4.4.1.1 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 (Garg *et al.*, 1996; Bhojwani, 2004).

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. But 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

issue during the early stage of development and the late/mature stage. Sehgal & Khurana, (1985) had reported callus induction from endosperm of mature fruits in MS medium with IAA (1mgl^{-1}) + BAP (1mg/l) and also in 2,4-D (1 mgl^{-1}) + Kin (1 mgl^{-1}). Callus induction from mature endosperm of *Actinidia deliciosa* was obtained in MS basal medium with 2, 4-D (2 mgl^{-1}) + Kin (5 mgl^{-1}) (Goralski *et al.*, 2005). Also different growth regulator requirements for callus induction and subsequent callus proliferation from immature endosperm of neem (*Azadirachta indica*) have been reported by Thomas and Chaturvedi, (2008). 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, as reported by Tohri and Bhojwani, (1965) in the case of *Exocarpus cupressiformis*. Earlier attempts were made by Rekha *et al.*, (2007) for the development of triploids in *Hevea* using mature endosperm tissue, but due to low plant regeneration frequency, no further work was attempted.

In 1947 La Rue, for the first time, reported the possibility of obtaining continuously growing tissues from the cultured immature maize endosperm. 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 by Thomas *et al.*, (2000). The stages of immature endosperm at the time of culture were normally expressed as days after pollination (Thomas *et al.*, 2000). However, some researchers estimated the endosperm stage in relation to the stage of developing embryo (Walia *et al.*, 2007). 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 (Chaturvedi *et al.*, 2003).

4.4.1.2 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.

4.4.2 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 (Cocking, 1960). 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 (Bethke and Jones, 2001, Tena *et al.*, 2001).

Enzymatic isolation of protoplasts using cellulase was first reported in tomato from root tips (Cocking, 1960). 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 (Power and Cocking, 1970).

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 (Mukhtar *et al.*, 2012). Protoplasts can be isolated from a variety of tissues, young *in vitro*-grown plants (Bajaj, 1972), tissues like callus, cell suspension (Sushamakumari *et al.*, 2000a) and explants such as root tips (Xu *et al.*, 1982), hypocotyl, cotyledons (Hammatt *et al.*, 1987) and shoots (Russell and McCown, 1986) and leaves from old or mature plants (Sheen, 2001). In our experiment protoplast isolation was tried from both mature and immature endosperm tissue.

Similar combinations of enzymes were used by Takahashi *et al.*, (2004) 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 (Zafer *et al.*, 1995; Mizukami *et al.*, 2006). Pectinases interact with cells being in different phases of the cell cycle and act like biochemical sorters (Sinha & Caligari, 2004).

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 (Navratilova, 2004). Generally protoplast burst in hypotonic solution and collapse in hypertonic solution (Ohshima and Tyama, 1989). 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 (Vasil, 1976).

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 (Schwall and Feix, 1988) and wheat (Keeling *et al.*, 1989). 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. Helle *et al.*, (2010) studied the characterization of oil and starch accumulation in tubers of *Cyperus esculentus Var. Sativus* (Cyperaceae) where he 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. 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 10^4 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 (Sushamakumari *et al.*, 2000a). They also reported that the protoplasts, when cultured in KPR liquid medium, underwent division leading to callus formation and embryogenesis.

Similar results were obtained by Pongchawee, (2006) who reported first cell division of protoplast when cultured in KM8P medium. The isolated protoplasts from endosperm are generally metabolically active as discussed by Isabel, (1992) who reported stable transformation of protoplast from maize endosperm. Many useful genes expressing in barley are endosperm specific.

4.4.3 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 (Abraham and Mathew, 2011), kiwifruit (Goralski *et al.*, 2005).

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.

DEVELOPMENT OF TETRAPLOIDS

Chapter 5

Development of Tetraploids

5.1 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 and Ni, 2006). With the advent of *in vitro* techniques for chromosome doubling using antimitotic agents, polyploids have been produced in a large number of speciess. *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, 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 (Atichart, 2013).

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.

5.1.1 Objectives

- Induction of polyploidy in *Hevea* callus through colchicine application
- Embryo induction from the colchicine treated callus
- Plant regeneration from the embryos
- Confirmation of ploidy in the regenerated plants

5.2 Materials and Methods

5.2.1 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. 28).

Fig. 28 Embryogenic calli derived from immature Inflorescence

5.2.2 Colchicine treatment

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

1. 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.

2. 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.

5.2.3 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.

5.2.4 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.

5.2.5 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)

5.2.6 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).

5.2.7 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 $p \leq 0.05$.

5.2.8 Confirmation of ploidy

5.2.8.1 Cytological analysis

Cytological analysis of the proliferated calli from various colchicine treatments was carried out. After plant regeneration, developing root tips of the regenerated plants were also subjected to cytology in order to reassure ploidy of the regenerants. The procedure described in section 3.2.10.1 was followed for cytological investigation.

5.2.8.2 Flow cytometric analysis

The callus which showed good embryogenic response was subjected to flow cytometry analysis as per the standard protocol described in the section 3.2.10.2.

5.3 Results

5.3.1 Colchicine treatment

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. Here also 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. 29 a). However, emergence of new yellow embryogenic callus occurred, within 4-6 weeks, from the calli exposed to lower concentrations of colchicine (Fig. 29 b). It was observed that in this experiment concentration of colchicine is crucial for the emergence of new callus. As evident in Fig.30, 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.

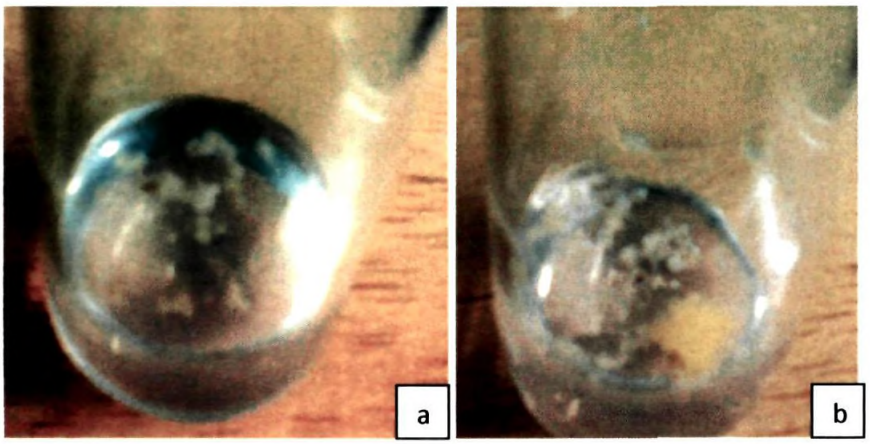

Fig. 29 Colchicine treatment of diploid callus

a Callus after colchicine treatment

b Emergence of friable yellow callus after colchicine treatment

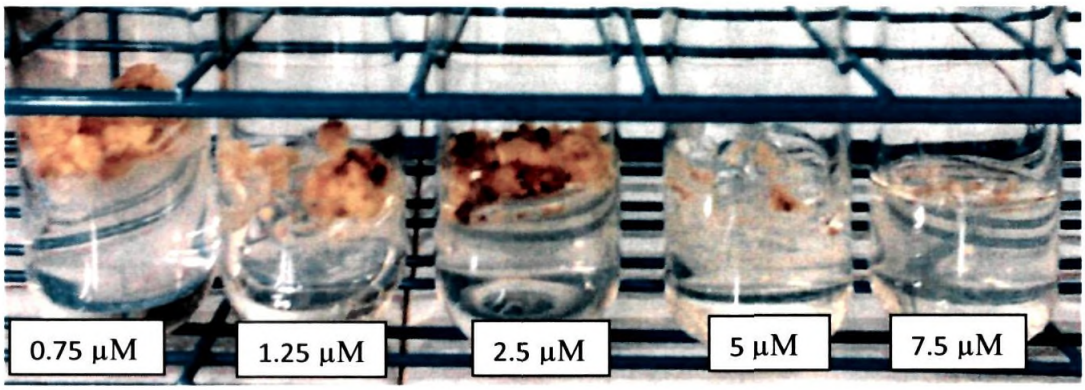

Fig. 30 Callus proliferation rate of calli exposed to different concentrations of colchicine

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 9).

Table 9 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

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

5.3.2 Embryo induction

Among the three basal media tried, MS medium was found to be most effective for induction of embryogenic callus and subsequent embryo formation. (Fig. 31a&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 10). Embryos at different developmental stages like globular, heart shaped and cotyledonary stages were obtained (Fig. 31c& 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.

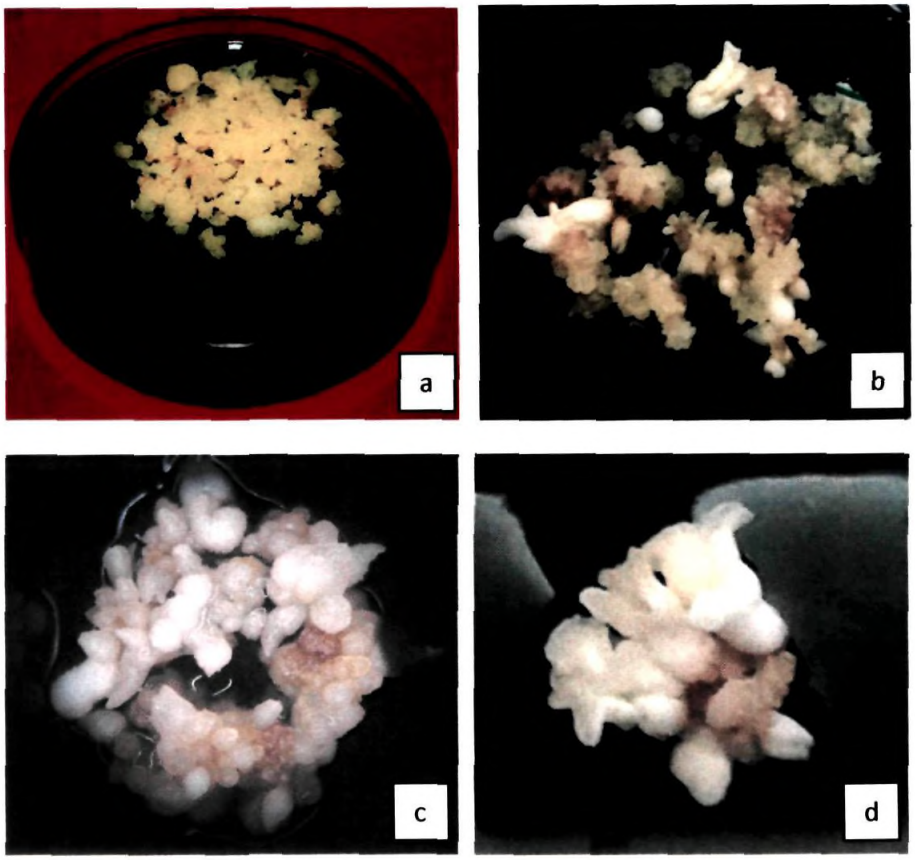

Fig. 31 Embryo induction from colchicine treated calli

a Embryogenic callus

b Induced embryos

c& d Embryos at different stages of development

Table 10 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

5.3.3 Embryo maturation

Out of the two basal media tried, embryo maturation was obtained in modified MS medium. A maturation frequency of 30 % could be obtained in MS medium fortified with 4.7 μM Kin and 1.9 μM ABA (Table 11). In the hormone free medium, maturation percentage was quite low.

Table 11 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)

CD= 0.25

*** Percentage of embryo maturation**

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

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 (3) 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.

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. 32). 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. 33).

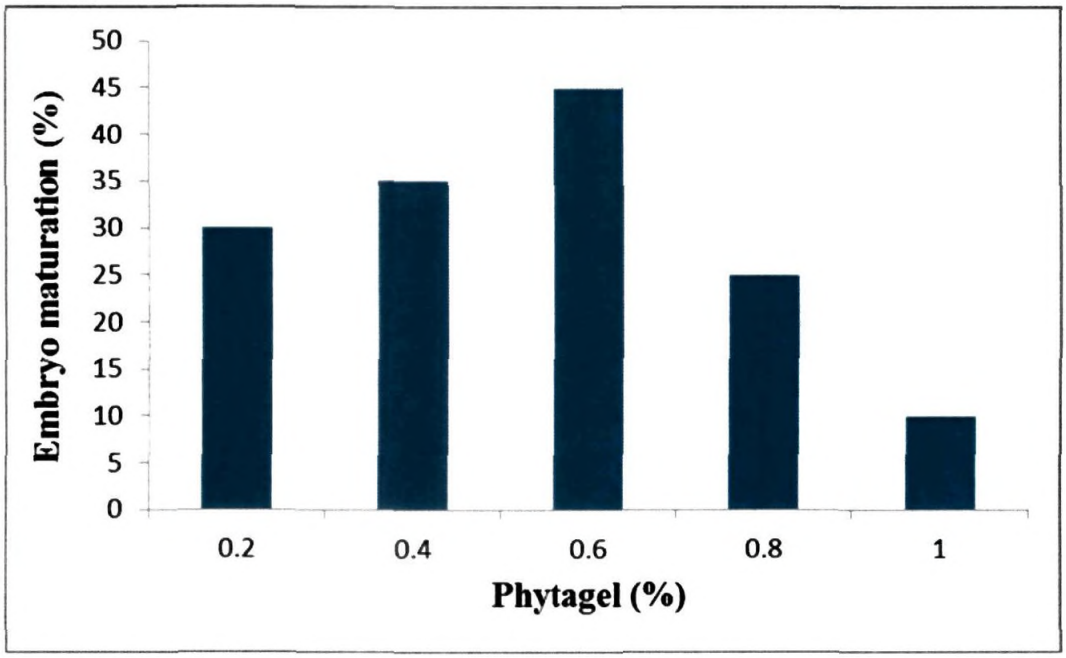

Fig. 32 Effect of phytagel concentration on embryo maturation

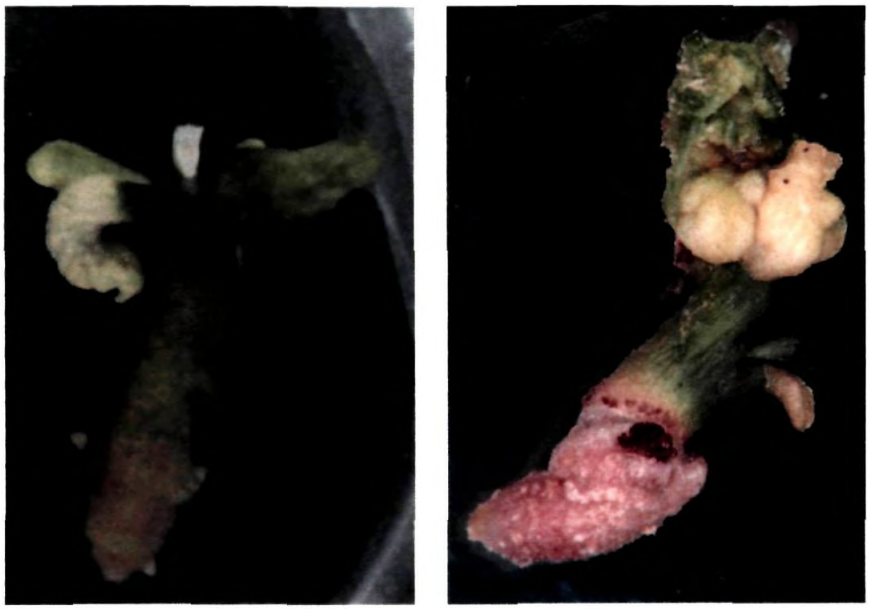

Fig. 33 Matured embryos from the colchicine treated calli

5.3.4 Embryo germination and plant regeneration

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. 34a&b) 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 12). Some of the embryos showed abnormalities in development. Some had multiple cotyledons (Fig. 34c), some became dormant and some showed only root development (Fig. 34d).

Table 12 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

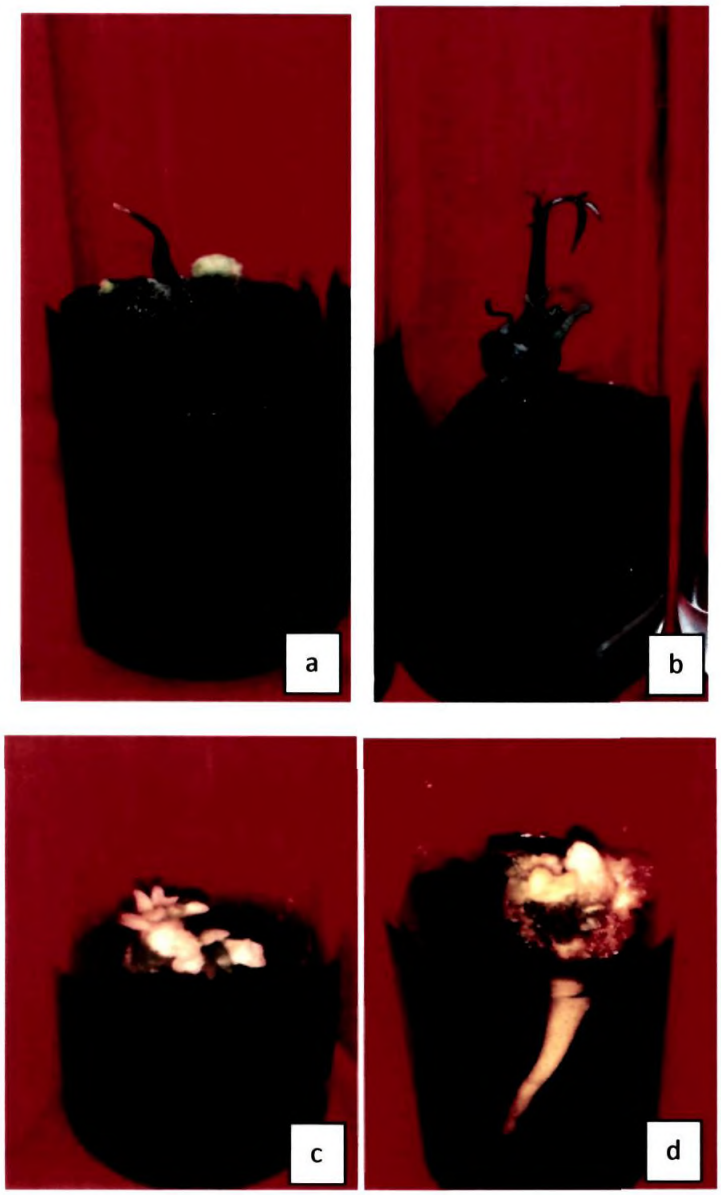

Fig. 34 Embryo germination

a & b Germinating embryos

c & d Abnormal embryos

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. 35). In other combinations, the regeneration frequencies were low (Table 13).

Table 13 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. 35 Plant regeneration

5.3.5 Ploidy determination

5.3.5.1 Cytological examination

Cytological studies have revealed a chromosome count of 4n=72 in the colchicine treated callus, observed at a magnification of X 400 (Fig. 36). 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.

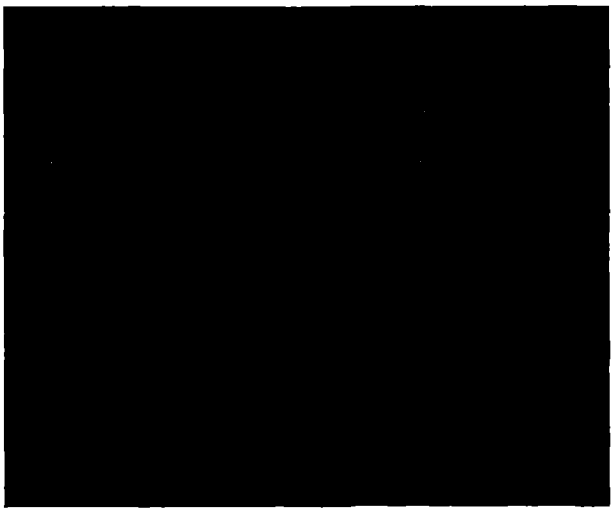

Fig. 36 Chromosome count of colchicine treated callus 4n=72 (X 400)

5.3.5.2 Flow cytometer analysis

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. 37). 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.

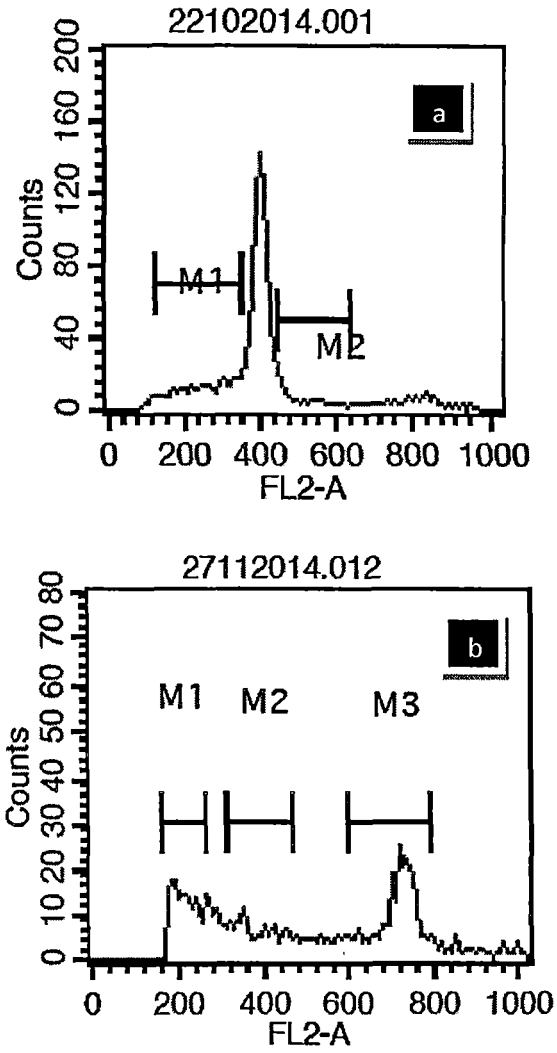

Fig. 37 Histogram showing peaks for

a Diploid (Immature anther derived callus)

b Tetraploid (Colchicine treated embryogenic callus)

5.4 Discussion

In-vitro induction of tetraploids from diploid callus of *Hevea* was achieved successfully through colchicine treatment. Among the different methods, colchicine concentrations and duration of treatments tried, culturing of diploid callus in medium containing 1.25 µM colchicine for 3 days, was found to be most effective treatment for inducing ploidy enhancement. Embryo induction (48 %), embryo maturation (45%) and embryo germination (30 %) could be achieved from

the calli subjected to the above treatment. Plant regeneration also could be obtained with 20% frequency. Now plants are in different stages of development. The tetraploid nature of the calli as well as the regenerants were confirmed by cytological and flow cytometric analyses. This is the first report on *in vitro* development of tetraploids of *Hevea* through chromosome doubling of diploid callus using colchicine.

Attempts to induce polyploidy in *Hevea* have been initiated by the various Rubber Research Institutes from 1969 onwards (Shepherd, 1969). Mendes and Mendes, (1963) reported that the tetraploid clones of *Hevea* showed increase in yield compared to the diploid in the immature stage based on the microtapping test. Markose, (1975) also made attempts to induce polyploids in *Hevea* and their initial results created great interest in this area because of the increased vigor of the resultant plants. Saraswathyamma *et al.*, (1984) developed synthetic polyploids of *Hevea brasiliensis* by applying colchicine on the axillary buds. Due to the chance of occurrence of chimeras and the difficulty in stabilizing the polyploids, success obtained through this method was very low. 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 of obtaining chimeras is remote. Also the clonal integrity can be maintained except for somaclonal variations. There are 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 (Chakarborty *et al.*, 1998), Chaicheron *et al.*, 1995).

5.4.1 Colchicine treatment

Among the different methods of colchicine application, direct exposure of the diploid callus to colchicine resulted in complete browning of the calli. No positive results were obtained in this experiment. 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. This explains the contradictory result obtained by Blakeslee and Avery, (1937), who were the first to treat *in vivo* seeds, axillary buds, and shoots with colchicine to produce tetraploid plants of *Datura stramonium* L. They immersed seeds in 0.4% colchicine solution for 4 days and could develop tetraploid plants.

Direct exposure of explants to colchicine has also been tried in other crops. Jala, (2014) attempted callus induction from leaf explants of *Dionaea muscipula* soaked in different concentration of colchicine. They observed that callus induction rate decreased when colchicine concentration was high.

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. As reported by Zeng *et al.*, (2006) that callus growth was repressed at higher concentrations and/or longer treatment duration, indicating the sensitivity of the callus to high levels of colchicine. Most of the cells after colchicine treatment died, owing to persistent lethality of colchicine.

In our study, we have obtained true tetraploids with chromosome number 4n=72 using a particular concentration of 1.25 µM colchicine for 3 days. 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 by Chaicharoen *et al.*, (1995) in mulberry, where the callus treated with 0.025 % colchicine for 3 days showed the highest percentage of survival (76 %). Sajjad *et al.*, (2013) reported that the mortality rate is very important while applying colchicine to explants for *in vitro* induction of polyploids. 0.05 % colchicine was found as lethal in marigold (Sajjad *et al.*, (2013). According to Sanguthai *et al.*, (1973) the fatality of colchicine is different for different plants depending upon its concentration.

In the case of *Echinacea peupurea*, higher concentration (120 mg/l) and longer exposure (28 days) to colchicine was needed to induce 23.5 % of tetraploids from petiole explant (Nilanthi *et al.*, 2009). Suzuki *et al.*, (2005) also demonstrated that colchicine treatment of callus at 0.05% for 72 hr was the best for chromosome doubling in *Gladiolus* species.

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.

5.4.2 Embryo induction

The culture conditions and basal media for somatic embryogenesis have already been standardized by many workers in *Hevea* (Montoro *et al.*, 2003; Sushamakumari *et al.*, 2000; Carron *et al.*, 1995; Jayasree *et al.*, 1999). Jayasree *et al.*, (1999) reported that 0.2 mg/l NAA was effective for embryo induction from immature anther. 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. Sushamakumari *et al.*,

(2006) reported 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.

5.4.3 Embryo maturation

According to Carron *et al.,* (1995) and Jayasree *et al.*, (1999), embryo maturation in *Hevea* is generally induced by a hormonal stress. 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. Vahdati *et al.*, (2006) reported that normal maturation of somatic embryos needs an ABA treatment in walnut. According to Kim *et al* (2007), maturation of somatic embryos of Fraser fir was not observed on medium lacking ABA for both precotyledonary and cotyledonary embryos. 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.* As discussed by Rekha *et al.*, (2007), a drastic increase in the embryo maturation frequency was noticed when phytagel was increased from 0.2 to 0.5 %. Teyssier *et al.*, (2011) also reported that raising the concentration of phytagel in the medium from 0.4 % to 0.8% improved the maturation of somatic embryos of *Larix eurolepsis*. The use of high concentrations of phytagel as gelling agent reduces water availability. Perera *et al.*, (2011) reported that 0.5 % phytagel was effective in the maturation of anther derived embryos of coconut.

5.4.4 Embryo germination

Earlier reports are there indicating the beneficial effect of GA_3 on germination in *Hevea* (Carron *et al.,* 1995; Sushamakumari *et al.,* 2000; Jayasree and Thulaseedharan, 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. Bhanumathi *et al.*, (2005) reported that BAP (0.2 mgl^{-1}) and IBA (0.2 mgl^{-1}) showed best response for somatic embryo regeneration in peanut.

Usman (2012), in his work of 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. But in our study such lethality was not observed in the embryos obtained from callus treated with 1.25 µM colchicine for 3 days. In an experiment where vegetative buds of mulberry were treated with colchicine for inducing ploidy variation, Ramesh *et al.*, (2011) reported that lower concentrations of colchicine (0.1%, 0.2% and 0.3%) did not affect the rooting behaviour. 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.

5.4.5 Plant regeneration

Plant regeneration frequency of 20 % was obtained in WPM medium fortified with IAA (2.8 µM), BA (8.8 µM) and GA_3 (1.4 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).

5.4.6 Ploidy determination

Ploidy determination of the callus and regenerated plants through cytological and flow cytometry analyses revealed tetraploidy (4n= 72).

Saraswathyamma *et al.*, (1984) used cytological analysis to determine the chromosome count in the induced tetraploids developed in *Hevea.*

The flow cytometer measures light fluoresced or scattered from particles. For ploidy analysis, only a single parameter i.e the fluorescence excited by the DNA specific fluorochrome is usually measured. In our result, highest peak was obtained at 780 channel in the histogram. From this, it can be assumed that the

DNA content in the colchicine treated callus has been doubled when compared with the control having the highest peak at 400 channel. Now a day's flow cytometer is used for ploidy determination since it is time saving and because of its easiness to predict result. Omidbaigi *et al.*, (2014) used different treatments for chromosome doubling in *Ocimum basilicum* through colchicine and identified polyploidization using flow cytometer.

SUMMARY & CONCLUSION

Chapter 6

Summary & Conclusion

Anatomical studies revealed that mature flowers prior to anthesis are ideal explants for the isolation of FGUs and embryo sacs. At this stage of flower egg cell along with synergids could be observed.

Successful isolation and purification of FGUs from the ovules of *Hevea* could be achieved. Isolated FGU's including the egg cells were cultured for inducing cell division and subsequent development.

Technique was standardized for the isolation of embryo sac from ovule. Pretreatment of the ovule followed by mechanical isolation was found effective in yielding appreciable amount of embryo sacs without any damage. Culture conditions for callus induction from the embryo sacs have been optimized. Embryo induction followed by embryo maturation and germination could be achieved. Experiments on plant regeneration using different media combinations are in progress. Cytological and flow cytometry studies of the callus proved its haploid nature (n=18). This is the first report of development of gynogenic haploids, with confirmed ploidy, in *Hevea brasiliensis*.

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

Development of tetraploids through chromosome doubling of diploid callus using colchicine has been achieved. Embryo induction, germination and plant regeneration have been obtained. Ploidy determination of the callus and regenerated plants through cytological and flow cytometry analyses revealed tetraploidy (4n= 72). Acclimatization and field establishment needs to be accomplished for further evaluation of the tetraploid plants.

In vitro induction of ploidy variation in *Hevea* has been accomplished through the development of haploids, triploids and tetraploids, using appropriate techniques. The ploidy of the different tissues has been confirmed using cytological and flow cytometric analyses. Triploid calli, haploid embryos and tetraploid plantlets were developed. All these can be further utilized in *Hevea* breeding for enhancing the vigor and productivity. The breakthrough achieved in the development of haploids, triploids and tetraploids could pave the way for developing future strategies for exploiting the benefits of ploidy variation towards crop improvement in *Hevea brasiliensis*.

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ANNEXURES

ANNEXURE-1

Modified MS Medium

INGREDIENTS	CONCENTRATION (mgl^{-1})
Macroelements	
NH_4NO_3	1000
KNO_3	1900
$Ca\ NO_3$	600
$MgSO_4$	370
KH_2PO_4	170
Microelements	
H_3BO_3	6.20
$MnSO_4.6H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
KI	0.083
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron Sodium EDTA	37.3
Myoinositol	100
Glutamate	150
Casein hydrolysate	400
Adenine sulphate	50
Sucrose	30
Phytagel	2

ANNEXURE II

Modified K&M medium

INGREDIENTS	CONCENTRATION (mgl^{-1})
Major Elements	
NH_4NO_3	600
CaCl2	453
MgSO4	146.6
MnSO4.H20	10
KCl	300
KNO3	1000
K3PO4	170
Minor Elements	
H_3BO_3	3
$CoCl_2$	0.25
$CuSO_4.7H_2O$	0.25
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
$MnSO_4.H_2O$	10
$Na_2 MoO_7.2H_2$	0.25
KI	0.25
$ZnSO_4.7H_2$	0 0.75
Vitamins	
L-Ascorbate	2.0
Biotin	0.01
D-Calcium Pantothenate	1.0
Folic acid	0.4
Nicotinamide	1.0
Pyridoxine HCL	1.0
Riboflavin	0.2
Thiamine HCL	1.0
Vitamin A	0.01
Vitamine B12	0.02

ANNEXURE III

Galbriath's buffer

INGREDIENTS	CONCENTRATION
MgCl2	45mM
MOPS	20mM
Sodium citrate	30mM
Triton X	100- 0.1% (vol/vol)

The pH was adjusted to 7 with 1M NaOH, filtered through a 0.22-mm filter and stored at -20°C as 10 ml aliquots.

ANNEXURE IV

CPW medium

INGREDIENTS	CONCENTRATION (mgl^{-1})
KH_2PO_4	27.4
KNO_3	101
$CaCl_2.2H_2O$	1480
$MgCl_2.7H_2O$	276
KI	0.16
$CuSO_4.5H_2O$	0.025

pH- 5.8